INHIBITION OF TUMOUR SPECIFIC ANGIOGENESIS BY NATURALLY OCCURRING SULFUR COMPOUNDS

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DECLARATION

I, Thejass.P hereby declare that this thesis has not previously formed the basis of the award of any degree or diploma or other titles of any other University.

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This is to certify that the present report is an authentic account of the work carried out by Mr. Thejass P, under my supervision and guidance and no part thereof has been presented before for any other degree.

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CONTENTS

INTRODUCTION

Cancer is one of the leading causes of death in India and around the world. It is responsible for about 20% of all deaths in industrialized countries and 10% of deaths in developing nations (Jones *et al.*, 2006). Epidemiology has revealed that certain cancers are more common among people of some cultures than others. Cancers of the lung, colon, prostate and breast are very common in Western countries; they are not as prevalent in Eastern countries. Similarly cancers of the head and neck and cervix are most common in India, whereas stomach cancer is most prevalent in Japan. Because human beings are 99.1% identical in their genetic sequence, these differences in cancer incidence cannot be attributed to the variation in their DNA sequence, but can be related to life style. The cancer burden in developing countries, including India, is expected to increase (Boutayeb and Boutayeb, 2005). Since 1985, the cancer registries in India, which are under the National Cancer Registry Programme, have reported a 12% increase in cancer cases, which is much higher than the rate of increase in the US (Pal and Mittal, 2004). The Indian rates for tobacco-related cancer sites such as oral and oesophageal cancer are among the highest in the world, whereas for other sites, such as prostate (most prevalent cancer in American men and those from other Western countries), they are among the lowest recorded (Jones *et al.*, 2006).

Despite significant improvements in diagnosis, surgical techniques, general patient care and local and adjuvant treatment therapies, 90% of human deaths from cancer are due to metastases that are resistant to conventional therapies. Metastasis-the spread of cells from the primary neoplasm to distant organs and their relentless growth-is the most fearsome aspect of cancer. Primary neoplasms and metastases consist of both tumour cells and host cells. Neoplasms are biologically heterogenous and contain genotypically and phenotypically diverse subpopulations of tumour cells, each of which have the potential to complete some steps in metastatic process, but not all. The outcome of metastasis depends on multiple interactions (cross-talk) of metastasizing cells with homeostatic mechanisms, which the tumour cells can upset. The main barrier to the treatment of metastasis is the biological heterogeneity of cancer cells in the primary neoplasm and in metastasis. Further more, the specific organ microenvironment can modify the response of a metastatic tumour cells to systemic therapy. Therapy of metastasis, therefore, should be targeted not only against the cancer

cells themselves, but also against the homeostatic factors that promote tumour cell growth, survival, invasion and metastasis (Steeg, 2006).

Another crucial step that is required to allow tumour propagation and progression is the induction of tumour vasculature, termed the "angiogenic switch". Tumour expansion is dependent on angiogenesis because tumour cells demand oxygen and nutrients to overcome hypoxia and starvation, and metastasize to distant organs through this angiogenic vasculature. When there is a hypoxic or starving condition in tumour tissue, hypoxia inducible factor-1 (HIF-1) become activated to promote angiogenic switch and induce angiogenesis. Tumours appears to activate the angiogenic switch by changing the balance of angiogenesis inducers including vascular endothelial growth factor (VEGF), angiopoietin, basic fibroblast growth factor (bFGF), placenta-like growth factor (PlGF), platelet derived growth factor (PDGF), Matrix metalloproteinases (MMPs) etc and countervailing inhibitors which include angiostatin, endostatin, thrombospondin, TIMP and so on. The process of angiogenesis consists of several steps, which include the stimulation of endothelial cells (ECs) by growth factors, the subsequent degradation of the extracellular matrix (ECM) by proteolytic enzymes followed by invasion of the ECM, migration and proliferation of ECs and finally the formation of new capillary tubes. Eventually the recruitment of periendothelial cells (pericytes) stabilizes the newly formed capillary network. An interruption to any of these steps leads to impair the process of angiogenesis, thereby delaying metastasis. Thus tumour angiogenesis offers a uniquely attractive therapeutic target (Nishida *et al.*, 2006).

Matrix metalloproteinases were classically thought to contribute to tumour angiogenesis and metastasis via their matrix degrading activity. In recent years studies have implicated MMPs at virtually all stages of tumour progression from initial development of the tumour, growth, angiogenesis, invasion and metastasis and growth at the secondary site. MMPs can favour new blood vessel sprouting by simply eliminating physical barriers through the degradation of ECM structural components or by the generation of proangiogenic factors. In fact, it has been demonstrated that the cleavage of collagen type IV exposes a cryptic binding site essential for endothelial cell

migration increasing the bioavailability of the proangiogenic VEGF (Egeblad and Werb, 2002; Yan and Boyd, 2007).

Apoptosis or programmed cell death- a series of genetically controlled events that result in removal of unwanted cells- seems to be a reliable marker for the evaluation of potential agents for cancer prevention. It is involved in maintaining homeostasis in multicellular organisms and any disruption of this process leads to abnormal growth. In cells undergoing apoptosis there is activation of a family of proteases called caspases which appears to be directly responsible for many of the molecular and structural changes in apoptosis. The p53 gene which is strongly implicated in animal and human carcinogenesis is a significant regulator of the process of apoptosis. While apoptotic pathway is related to the induction of p53, this pathway is held in check by the antiapoptotic gene Bcl-2. Induction of apoptosis in cancer cells or malignant tissues is recognized as an efficient strategy for cancer chemotherapy. Thus modulating apoptosis may be useful in the management and therapy or prevention of cancer (Cory and Cory, 2007).

Activation of transcription factor NF-κB has been linked to apoptosis as it can activate antiapoptotic genes. Upon activation by a variety of stimuli such as carcinogens, inflammatory agents and tumour promoters, NF-κB is translocated to the nucleus where it activate the transcription of target genes which are critical to the establishment of early and late stages of aggressive cancers, including expression of cyclin D1, apoptosis suppressor proteins such as Bcl-2 and Bcl-XL and those required for metastasis and angiogenesis, such as MMPs and VEGF. Activated protein-1 (AP-1) is another transcription factor that regulates the expression of several genes involved in cell differentiation and proliferation. Some of the target genes activated by the AP-1 transcription complex include those activated by NF-κB such as cyclin D1, Bcl-2, Bcl-XL, VEGF, MMPs and urokinase plasminogen activator (uPA). Expression of MMP and especially uPA promotes angiogenesis and invasive growth of cancer cells (Bonizzi and Karin, 2004).

Cancer cells escape innate and adaptive immune responses-cancer immunosurveillance- by immunoselection (that is, selection of non-immunogenic tumour cell variants, a process that is also known as immunoediting) or by

immunosubversion (that is, active suppression of the immune response). Chronic inflammation and presence of inflammatory cells, primarily macrophages, at tumour sites are highly associated with specific malignancies. The inflammatory components of tumours were characterized as key players in tumour promotion by virtue of their ability to release a large variety of factors that support angiogenesis, tumour growth, tissue remodeling and more. This is joined by the immune suppressive activities of the inflammatory mediators, giving rise to marked inhibition of potential immune reactions that could have been elicited by NK cells, cytotoxic T lymphocytes, macrophages and neutrophils against the tumour cells (Zitvogel *et al.*, 2006).

Many of the mediators, which are released in dysregulated chronic inflammation, were found to promote cell growth and invasion, to induce mutagenesis and to increase angiogenecity. The proinflammatory cytokines, which can be produced by the tumour cells and/or tumour associated leukocytes and platelets may contribute directly to malignant progression. The proinflammatory cytokines such as TNF-α, IL-1 and IL-6 can stimulate the production of angiogenic factors such as VEGF. Inflammatory cytokines may also affect genome integrity via inhibition of cytochrome p450 or glutathione-s-transferase isoenzymes (Balkwill and Mantovani, 2001).

Conventional cancer treatment modalities such as chemotherapy and radiation therapy have deleterious side effects. Both these methods have severe side effects such as nausea, vomiting, alopecia, mucosal ulceration, pulmonary fibrosis, cardiac and hepatic toxicity etc. One of the major drawbacks of both these modalities is immunosuppression. The modulation of immune response by using medicinal plant products as a possible therapeutic measure has become a subject of active scientific investigations. Of the 121 prescription drugs in use today for cancer treatment, 90 are derived from plants. Almost 74% of these, including taxol, were discovered by investigating a folklore claim. Between 1981 and 2002, 48 out of 65 drugs approved for cancer treatment were natural products, based on natural products or mimicked natural products in one form or another (Newman *et al.*, 2003; Craig, 1997). Several population based studies indicate that people in Southeast Asian countries have a much lower risk of developing colon, gastrointestinal, prostate, breast and other cancers when compared with their Western counterparts. It is likely that dietary constituents such as garlic,

ginger, soya, curcumin, onion, tomatoes, cruciferous vegetables, chillies and green tea, play an important role in protection from these cancers. These dietary agents are believed to suppress the transformative hyperproliferative and inflammatory processes that initiate carcinogenesis (Block *et al.*, 1992). Their inhibitory influences may ultimately suppress the final steps of carcinogenesis as well, namely angiogenesis and metastasis.

Natural dietary agents including fruits, vegetables and spices have drawn a great deal of attention from both the scientific community and general public owing to their demonstrated ability to suppress cancers. Dietary agents consist of a wide variety of biologically active compounds such as polyphenols, terpenes, alkaloids and phenolics that may provide substantial health benefits beyond basic nutrition. The active components of dietary phytochemicals that most often appear to be protective against cancer are curcumin, genistein, resveratrol, allicine, lycopene, capsaicin, diosgenin, ellagic acid, ursolic acid, silymarin, catechins, isoflavones, phytosterols etc (Newman and Cragg, 2007).

Chemoprevention is regarded as one of the most promising and realistic approaches in the prevention of human cancer. Among naturally occurring products, Sulfur containing compounds (OSCs), especially garlic compounds and isothiocyanates, represent two important and promising chemopreventive families because of their effects at multiple points of cancer development. OSCs may exert their protective effects at all main stages of the carcinogenic process and are especially efficient inhibitors of the initiation phase of chemically induced carcinogenesis in different organs in rats and mice.

In the present study we investigated the antiangiogenic activity of naturally occurring organosulfur compounds such as DAS, DADS, AITC, PITC and Sulforaphane using both *in vivo* and *in vitro* models. We also investigated the effect of these OSCs on the modulation of apoptosis and transcription factors in B16F-10 melanoma cells at molecular level. The immunomodulatory as well as antimetastatic activity of Sulforaphane was also analyzed.

CHAPTER 1

REVIEW OF LITERATURE

Cancer arises from the stepwise accumulation of genetic changes and progressive alterations in gene expression which disengage cells from homeostatic constraints that normally keep the tissue balance in the healthy adult. There are more than 100 distinct types of cancer, and subtypes of tumours can be found within specific organs. Several defined events have been described as common to cancer cells including self-sufficiency in proliferation signals, insensitivity to growth inhibitory signals, evasion from programmed cell death (apoptosis), limitless replicative potential, invasion and metastasis as well as sustained angiogenesis (Hanahan and Weinberg, 2000). Tumour–borne vascular connection and the acquisition of a migratory behavior of tumour cells are pre-requisites for the release of cells from organized tissue structures and invasion to more distant locations. The proliferating tumours activate the angiogenic switch by changing the balance of angiogenesis inducers and countervailing inhibitors in order to meet the increased demand of oxygen and nutrients and also expand to larger size (Hanahan and Folkman, 1996).

1) Tumour Immunology

The tight control of tumour development depends upon the intimate relationship between emerging tumour cells and the innate and adaptive immune systems. The immune system plays a key role in the elimination of cancer cells, before they can spread or form a tumour. On the other hand, most emerging tumours develop and escape immunosurveillance even though they are antigenic and immunogenic. Of the many mechanisms contributing to immune suppression, much emphasis was recently given to inflammatory cells, to soluble factors that are associated with their activities and to inflammatory mediators in general. Chronic inflammation may be one of the driving forces of transformation that together with other determinants, including the intrinsic properties of pre-malignant cells, support the initiation of cancer. Obviously, if inflammatory conditions prevail at the tumour site, they further support the progression of the tumour into more advanced stages and also promote metastasis. Many of the mediators, such as cytokines, chemokines, prostaglandins and reactive oxygen/nitrogen species, which are released in disregulated chronic inflammation, were found to promote cell growth and invasion, to induce mutagenesis and to increase angiogenecity

(Coussens and Werb, 2002; Schwartsburd, 2003). These inflammatory conditions are also characterized by recruitment of inflammatory cells. Premalignant and early tumour lesions are generally well infiltrated with immune cells, largely T-lymphocytes, macrophages and dendritic cells, although B-cell formations resembling lymphoid follicles are some times present (Von Kleist *et al.*, 1987). Tumour-infiltrating lymphocytes (TIL), which are enriched in tumour associated antigen (TAA)-specific memory T-cells, elaborate cytokines and growth factors necessary for tumour growth (Balkwill, 2004). The most prominent subpopulation of inflammatory cells at the tumour site is that of tumour associated macrophages (TAM). These cells are derived from circulating monocytes that infiltrate the tumour and differentiate to macrophages (Leek and Harris, 2002; Mantovani *et al.*, 1986). TAM and their products actually represent double-edged swords: in specific settings, these cells promote antitumour activity whereas in other cases, it promotes tumour growth. TAM products act in two manners to support tumour progression, on one hand supporting tumour growth, angiogenecity and extracellular matrix (ECM) degradation and on the other hand suppressing potential antitumour immune activities (Mantovani *et al.*, 2004). Moreover the presence of regulatory immune cells at tumour sites such as CD4+CD25+Foxp3+ regulatory T-cells (Tregs) promotes tumour growth by suppressing immune responses against non-self tumour antigens (Wang, 2006). Myeloid suppressor cells (MSCS) in the tumour microenvironment are potent inactivators of both CD4+ and CD8+ T-cells and are responsible for significant immune dysfunction seen in tumour related and unrelated settings (Bronte *et al.*, 2001; Serafini *et al.*, 2006).

1.1) Immune surveillance

The idea that the immune system plays a major role in the surveillance against malignant transformation stemmed from experimental work during the first part of the 20th century showing strong immune-mediated rejection of transplantable tumours in mice (Malmberg and Ljunggren, 2006; Burnet, 1970). The term immunosurveillance was coined to describe the concept of an immunological host resistance against the development of cancer. The concept of cancer immunosurveillance predicts that the immune system can recognize precursors of cancer and, in most cases, destroy these

precursors before they become clinically apparent. Both innate as well as adaptive immune systems play a pivotal role in immunosurveillance.

1.1.1) Antibodies and B-cells

B-cells appear to be necessary for efficient T-cell priming and tumour resistance. Human antisera and monoclonal antibodies reactive with autologous tumours have been isolated (Old, 1981). *In vitro* some tumour cells are killed by a process involving coating with antibody, opsonization, and subsequent phagocytosis by macrophages; this process may be enhanced by the presence of complement. Alternatively, antibody-coated tumour cells may be killed in the absence of phagocytosis by antibody dependent cell mediated cytotoxicity (ADCC) when co-cultured with macrophages, NK cells or neutrophils. Passive transfer of monoclonal antibodies against B-cell lineage-specific differentiation antigens can destroy even larger established B-cell lymphomas (Grillo-Lopez *et al.*, 2000).

1.1.2) T-lymphocytes

T-cells are lymphocytes equipped with heterodimeric cell surface receptors which are encoded in four somatically rearranging T-cell antigen receptor (TCR) loci: α and β or γ and δ. T-cell mediated immunity is of critical importance for the rejection of virally and chemically induced tumours by immunized mice or for the rejection of allogenic and UV light induced tumours by normal mice. For certain tumours, such as UV light induced tumours, CD8+cytolytic T-cell subset appears to be regularly required for rejection. $\gamma\delta$ T-cell may use their NKG2D receptor to counteract cutaneous carcinogenesis. Cytotoxic T-lymphocytes (CTL) can accomplish lysis of tumour cells through cell to cell contact. The antigen specific killer cells would recognize cells with specific tumour markers whereas those with broad specificity could lyse a variety of tumour cell targets.

1.1.3) Natural killer (NK) cells

Natural killer (NK) cells are specialized lymphocytes (large granular lymphocytes) comprising 5-20% total lymphocyte population that have been found in

the spleen, lymph nodes, bone marrow and blood. NK cells can kill cancer cells as well as non-malignant non-self cells without prior sensitization and without the requirement for MHC restriction. NK cells are the major effector cells of ADCC and kill cancer cells by cytolysis. NK cells that are stimulated by lymphokines such as IL-2 and IFN-γ display enhanced activity and are known as lymphokine-activated killer (LAK) cells (Yang *et al.*, 2006).

1.1.4) Macrophages

Among the earliest cell types to respond to invasion by pathogenic organisms are the macrophages which are key participants in the innate immune response (Janeway and Medzhitov, 2002). Macrophages can function as antigen presenting cells and interact with T-lymphocytes to modulate the adaptive immune response Furthermore, macrophages are involved in tissue remodeling during embryogenesis, wound repair, clearance of apoptotic cells and hematopoiesis (Klimp *et al.*, 2002).

1.2) Tumour escape mechanisms

Tumours develop a series of strategies to evade immunosurveillance. These strategies are known as immunoselection. One common strategy to elude a T-cell mediated immune response is the down regulation or loss of expression of HLA class I molecules. Tumour cells can also develop mechanisms to avoid being killed by CTLs. Immunoselection also includes down regulation or mutation of the gene that encodes caspase-8 and over expression of FLIP or decoy receptors for TRAIL, all of which cause resistance to CTL-induced killing of tumour cells (Ochsenbein, 2005).

Tumour cells also escape immunosurveillance by immunosubversion, which is the active suppression of the immune response. For example, some tumours (or tumour associated myeloid cells) overproduce nitric oxide and have increased arginase-1 activity, both of which can inhibit T-cell function (Bronte and Zanovello, 2005; Zitvogel *et al.*, 2006).

1.3) Immunotherapy

Immunotherapy refers to the use of cells, molecules and genes of the immune system for the therapy of infectious diseases, autoimmunity, neoplastic diseases and to prevent the rejection of organ transplants. Three different new approaches to the immunotherapy of malignant diseases have been adopted over the past two decades. These are use of recombinant cytokines, use of monoclonal antibodies and T-cell based cancer therapy. When used in cancer therapy, cytokines can bring about tumour rejection via direct inhibition of tumour growth and via indirect actions such as an antiangiogenic action, inhibition of growth factor production in the vascular connective tissue of tumours and enhancement of immune responses. More than a dozen recombinant cytokines have been developed to the stage of clinical trials over the past twenty years. IFN-α which is used in chronic myeloid leukemia and IL-2 which is used in the treatment of advanced myeloma and renal cell carcinoma are two recombinant cytokines which have become firmly established in the treatment of malignant diseases. Over the past 20 years, monoclonal antibodies have become indispensable tools in the immunohistochemical diagnosis of malignant diseases. T-cell therapies are based on the cytolytic activities of T-cells.

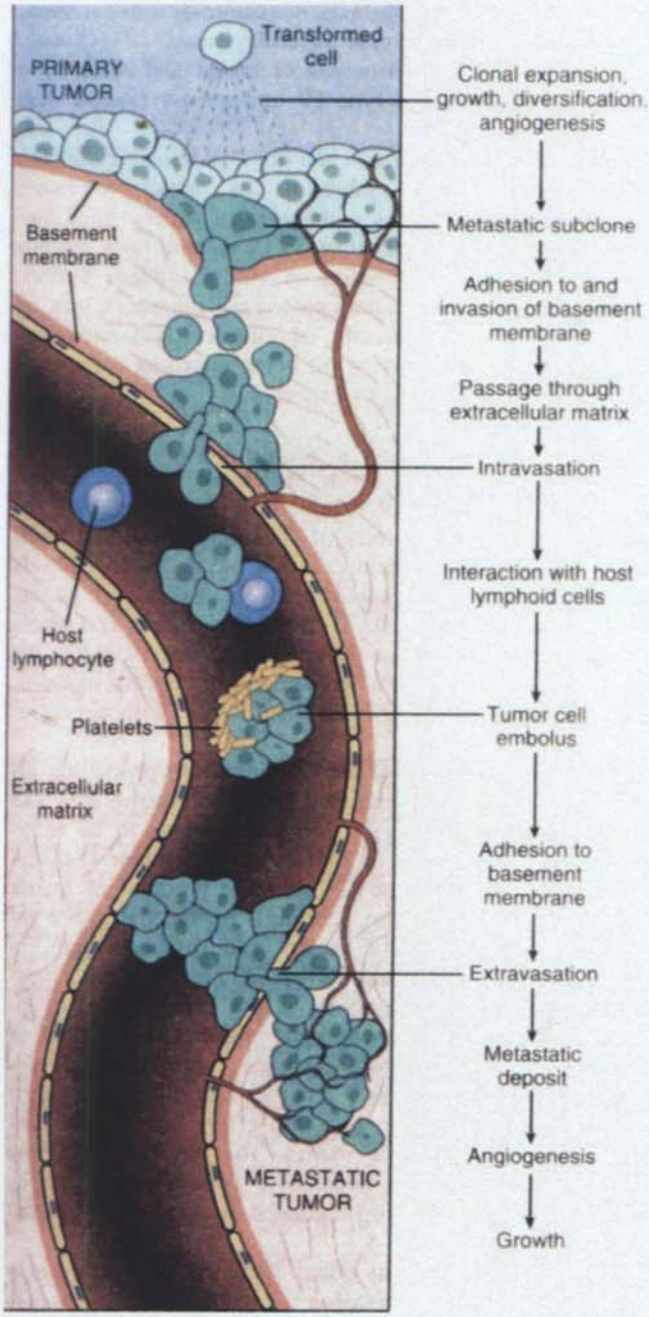
2) Tumour Metastasis

One of the major causes of cancer treatment failure is metastasis. Tumour metastasis consists of a discrete biological process that moves tumour cells from the primary neoplasm to a distant location. The metastatic cascade involves a series of sequential steps (Figure 1.1) (Steeg, 2006).

2.1) Disruption of the basement membrane

Basement membrane which contains Type IV collagen as a major component along with laminin, fibronectin, heparan sulphate proteoglycan and entactin have to be digested by proteolytic enzymes like matrix metalloproteinases (MMPs), collagenase, cathepsin B and enzymes of urokinase plasminogen activator system for the successful dissemination of tumour cells.

Figure 1.1. Metastatic Cascade



2.2) Cell detachment

Cancer cells exhibit deficiency in cohesiveness and are easily separated from one another. Cell-cell adhesions are mediated by cadherins. A 'switch' in tumour cell cadherin expression, such as from E-cadherin (which promotes tumour cell-tumour cell adherence) to N-cadherin (which promote tumour cell adhesion to extra cellular matrix-ECM) is really essential for metastasis.

2.3) Cell motility

Cancer cells have the capacity to become motile and help the cells to invade contiguous normal tissue and to gain access to and exit from blood and lymph vessels.

2.4) Invasion

The detached tumour cells which are motile invade through the ECM towards the capillaries in connective tissues which are biologically significant destination and involve the interplay between MMPs and growth factors.

2.5) Intravasation

Cancer cells penetrate the capillaries and disseminate via capillaries and lymphatic vessels.

2.6) Survival and arrest in blood stream

Blood stream is a harsh environment for metastasizing tumour cells because of velocity induced shear forces, lack of substratum and the presence of immune cells. Cancer cells in circulation adhere to each other and to lymphocytes and platelets, forming emboli that may adhere to the inner surface of capillaries. A fibrin-containing thrombus forms, which stabilizes the embolus. The fibrin enclosure may act as a barrier to undefined and unknown noxious factors and stabilize the trapped tumour cells, resulting in enhanced invasion. The endothelial cell E- and P-selectins also contribute to tumour cell arrest. Other potential mediators of tumour cell arrest are glycosylation patterns and integrins.

2.7) Extravasation and metastatic colonization

Extravasation of cancer cells requires cell motility and transgression of basement membrane of the capillary. By active migration, the cancer cells move between the retracted endothelial cells, through the breached basement membrane and into the intra cellular matrix of the connective tissue. Proliferation of tumour cells leads to metastatic colonization. The nidus of cancer cells is restricted in growth to a lump of 0.5 to 2 mm in diameter due to limited nutrient supply available by diffusion from blood. Therefore new capillaries are recruited from pre-existing capillaries, a process termed 'angiogenesis' (Cao and Liu, 2007).

3) Angiogenesis

Blood vessels are critical for the maintenance of cellular homeostasis of virtually all cells in the human body and therefore all cells must reside within 100μm of a capillary (Carmeliet and Jain, 2002). During embryonic vasculogenesis, blood vessels are formed de novo from endothelial cell precursors (angioblasts) that assemble into a primary capillary plexus. This primitive network then differentiates, and new blood vessels sprout and branch from pre-existing capillaries- the process of angiogenesis (Carmeliet, 2000). Normal tissue growth, such as embryonic development, wound healing and the menstrual cycle, is characterized by dependence on new vessel formation for the supply of oxygen and nutrients as well as removal of waste products. Also a large number of different and non-related diseases are associated with formation of new vasculature. In several diseases including cancer (both solid and hematologic tumours), cardiovascular diseases, chronic inflammation, diabetes, psoriasis, endometriosis and adiposity, excessive angiogenesis is part of the pathology (Folkman, 1995; Hanahan and Folkman, 1996; Nishida *et al.*, 2006).

The existence of a tumour-derived blood vessel growth stimulating factor, with possible responsibility for neovascularization induction, was first postulated in 1939 (Ide *et al.*, 1939). In 1945, Algire *et al* proposed that the rapid growth of tumour transplants is dependent upon the development of a rich vascular supply (Algire *et al.*, 1945).The first experiments to directly test the hypothesis that tumours produce angiogenic factors were conducted in 1968 (Greenblat and Shubik, 1968; Ehrmann and

Knoth, 1968). In 1971, Judah Folkman proposed that interfering with this angiogenic factor might be a way to kill tumours, by starving them of a blood supply and laid the foundation for therapeutic angiogenesis (Figure 1.2) (Folkman, 1971).

3.1) Differences between normal and pathological angiogenesis

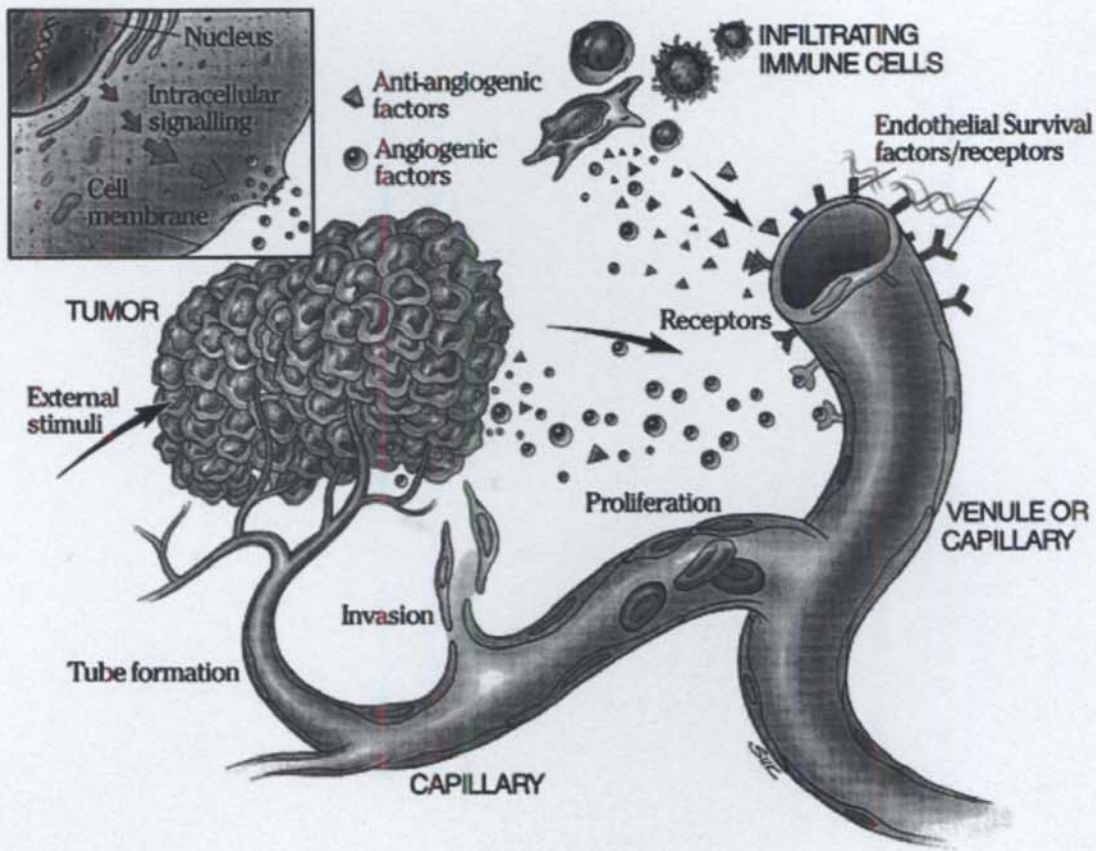
The vasculature is usually quiescent in the adult, and the balance between pro and antiangiogenic signals is tightly regulated. During normal physiological angiogenesis, new vessels rapidly mature and become stable. By contrast, tumours have lost the appropriate balance between positive and negative controls. Tumour blood vessels fail to become quiescent, enabling the constant growth of new tumour blood vessels. Tumour blood vessels are irregularly shaped, dilated, tortuous and can have dead ends. They are not organized into definitive venules, arterioles and capillaries. Vascular network in tumours is often leaky and hemorrhagic, partly due to overproduction of vascular endothelial growth factor (VEGF). Blood flows irregularly in tumour vessels, moving more slowly and sometimes even oscillating. The vessel density is irregularly controlled in tumours whereas those in normal tissues, is dynamically controlled by the metabolic needs of nutrients and oxygen (Bergers and Benjamin, 2003). The structure of the vessel wall is also abnormal in tumours. Vessel diameters are uneven, due in part to compression of the immature wall by proliferating tumour cells (Helmlinger *et al.*, 1997).

Further more, endothelial cells (ECs) form an imperfect lining with wide junctions at some locations and stacked layers of ECs at others. The ECs may contain a large number of fenestrations, vesicle vacuolar organelles (VVOs) or both. Some ECs do not express common endothelial markers (such as CD31) and may undergo apoptosis, thus exposing cancer cells to the lumen (so called mosaic vessels) (Chang *et al.*, 2000).

3.2) Role of endothelial cells (ECs) and extracellular matrix (ECM) in angiogenesis

Blood vessels consist of endothelial cells (ECs) that are in direct contact with the blood and subendothelially located pericytes, smooth muscle cells, fibroblasts, basement membrane (BM) and extracellular matrix (ECM). Depending on the location in the body, the organ microenvironment, the cellular constituents, BM and ECM of the

Figure 1.2. Tumour specific angiogenesis



vasculature, ECs differ in phenotype, composition and function (Rajotte *et al.*, 1998).The active vascular remodeling phase in tumours is reflected by the fact that tumour ECs proliferate 20 to 2000 times faster than normal tissue endothelium in the adult (Denekamp, 1984). When their integrity is maintained, ECs exert anticoagulative properties via the synthesis of thrombomodulin, tissue factor (TF) pathway inhibitor and tissue type plasminogen activator (t-PA). On activation or damage, ECs quickly release proteins like multimeric von Willebrand factor (vWF), which promotes platelet adhesion and aggregation and plasminogen activator inhibitor-1. In addition, TF expression by endothelium leads to initiation of the extrinsic blood coagulation pathway (Verstraete, 1995). ECs also direct cells of the immune system to specific sites in the body. Cellular adhesion molecules [eg:- E-selectin and intercellular adhesion molecule-1 (ICAM-1)] and soluble factors such as chemo attractants, cytokines and chemokines act in concert to recruit the immune cells to lymphoid organs or inflammatory sites (Carlos and Harlan, 1994). ECs are actively involved in vascular remodeling. ECs differentiate from angioblasts in the embryo and from endothelial progenitor cells (EPCs), mesangioblasts, multipotent adult progenitor cells or side population cells in the adult bone marrow (Luttun *et al.*, 2002; Mikkola and Orkin, 2002). ECs are elongated, thin and fragile cells, yet they build channels that do not collapse and that efficiently distribute blood to the various parts of the body. They also have long half-lives of several years, but when triggered are capable of rapidly sending out sprouts in a coordinated and directional manner. This is partly because cells within the vessel wall communicate with each other and with cells inside and outside the vessel lumen. They sense changes in blood flow and pressure and dynamically interact with the internal cytoskeleton and surrounding ECM, all in an integrated manner.

The ECM provides necessary contacts between ECs and surrounding tissue, and thus prevents vessels from collapsing. ECM plays a key role in tissue architecture and homeostasis. The principal proteinaceous components of the ECM are collagens that are secreted by a variety of stromal cells, of which fibroblasts are major contributors. The other proteins of the ECM include laminin, entactin and various growth factors and proteases (Rohrbach and Timple, 1993). A second class of molecules that play an essential role in the composition of the ECM are secreted proteoglycans whose protein

core is covalently bound to high molecular weight glycosaminoglycans, including chondroitin, heparin and keratin sulphate. Hyaluronan (HA), a major glucosaminoglycan, which is typically not sulphated or bound to a protein core, is also present in ECM. Hyaluronan participates in the regulation of numerous cellular functions, prominent among which are adhesion, trafficking and signaling (Iozzo, 1998; Laurent *et al.*, 1992). The ECM also regulates the formation of new vessel sprouts. When ECs migrate to form new sprouts, this matrix network is not only broken down, but its composition is also altered. Proteinases expose new cryptic epitopes in ECM proteins (such as in collagen IV) or change their structure (fibrillar versus monomer collagen), which induce EC and smooth muscle cell (SMC) migration (Hangai *et al*, 2002). In addition a provisional matrix of fibronectin, fibrin and other components provides a support scaffold, guiding ECs to their targets. Remodeling of the ECM during vessel sprouting requires breakdown by proteinases, including plasminogen activators [such as urokinase plasminogen activator (uPA) and its inhibitor, plasminogen activator inhibitor-1 (PAI-1)], matrix metalloproteinases (MMPs), tissue inhibitors of metalloproteinases (TIMPs), heparinases, chymases, tryptases and cathepsins (Pepper, 2001; Jackson, 2002; Zhu *et al.*, 2007).

3.3) Events during angiogenesis

The process of angiogenesis consists of several steps, which include the stimulation of ECs by growth factors, the subsequent degradation of ECM by proteolytic enzymes followed by invasion of the ECM, migration and proliferation of ECs and finally formation of new capillary tubes (Figure 1.3 & 1.4). Eventually recruitment of pericytes stabilizes the newly formed capillary network (Carmeliet, 2000).

3.3.1) Endothelial cell activation

In all types of angiogenesis, either under physiologic or pathologic conditions, endothelial cell activation is the first process. Vascular relaxation mediated by nitric oxide (NO) is a pre requisite for ECs to enter angiogenic cascade (Folkman, 1997). Angiogenesis is rapidly initiated in response to hypoxic or ischemic conditions.

Figure 1.3. Events during angiogenesis

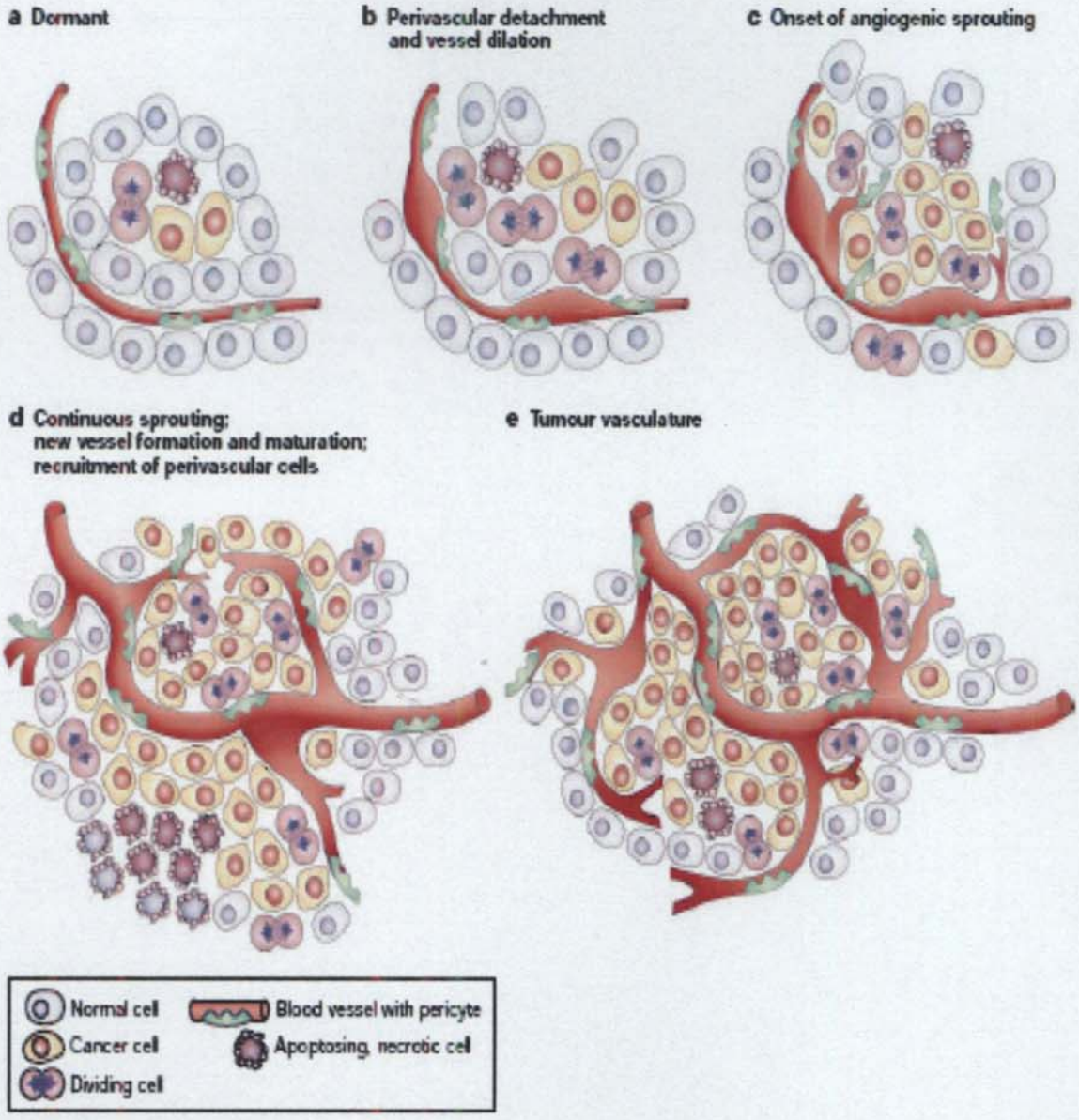
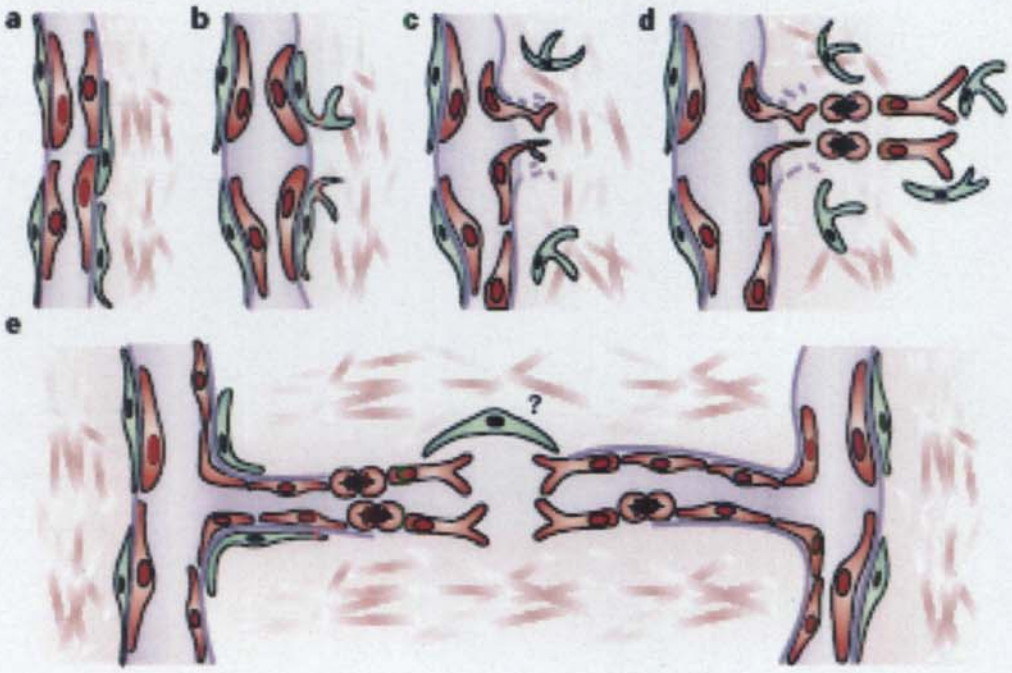


Figure 1.4. New blood vessel formation



a) Blood vessels arise from pre-existing capillaries or postcapillary venules in tumours.

b) Detachment of pericytes (green) and dilation of blood vessels before the basement membrane and extracellular matrix is degraded.

c) This allows endothelial cells (red) to migrate into the perivascular space towards angiogenic stimuli produced by the tumour cells or hostcells.

d) Endothelial cells proliferate, loosely following each other, and are presumably guided by pericytes.

e) Behind the migration columns, endothelial cells adhere to each other and create a lumen, which is accompanied by basement-membrane formation and pericyte attachment. Finally, blood vessel sprouts will fuse with other sprouts to build new circulatory systems.

Cytokines from various sources are released in response to hypoxia or ischemia. Among them vascular endothelial growth factor (VEGF) is a key factor in angiogenesis initiation with its ability to induce vasodilation via endothelial NO production and its endothelial cell permeability increasing effects (Ziche *et al.*, 1997). This allows plasma proteins to enter the tissue to form a fibrin rich provisional network on which activated ECs migrate. This is accompanied by loosening of pericyte covering (Dvorak, 1986). VEGF is abundantly produced by hypoxic tumour cells, macrophages and other cells of the immune system (Brown *et al.*, 1997). VEGF also induces the expression of proteases and receptors important in cellular invasion and tissue remodeling and is able to prevent endothelial cell apoptosis (Gupta *et al.*, 1999; Tonra and Hicklin, 2007).

3.3.2) Endothelial cell migration and proliferation

After proper activation of the endothelial cells, endothelial penetration into new areas of the body is achieved by degradation of the BM by MMPs, which are secreted as zymogens that become activated in the ECM compartment and subsequently degrade components of the ECM (Stetler-Stevenson, 1999). The vascular BM and ECM are locally degraded to allow underlying endothelial cells to migrate into the perivascular space towards chemotactic angiogenic stimuli. The ECs then multiply loosely following each other into the perivascular space and forming a migration column. During normal angiogenesis, migration columns lead to a differentiation zone, where endothelial cells change shape and adhere to each other to form a lumen. Continued proliferation within the vascular wall allows for the enlargement of the blood vessel diameter. Perivascular cells are attracted and a vascular basal lamina is produced around the newly formed blood vessels. In normal vasculature, pericytes association reduces EC proliferation and decreases their dependence on host tissue production of VEGF (Hirschi and D'Amore, 1996). Conversely, the improper or decreased vessel association with pericytes in tumours might partially explain the abnormal vessel diameters and sensitivity to VEGF inhibition (Benjamin *et al.*, 1999).

Plasmin, converted from plasma protein plasminogen by plasminogen activators uPA and t-PA, degrades fibronectin, laminin and protein core of protein glycans and is believed to be the most important protease for the mobilization of fibroblast growth

factor-α (FGF-α or basic FGF), a proangiogenic molecule, from the ECM pool. During angiogenesis low molecular weight FGF-α (18kDa) binding to endothelium induces FGF receptor (FGF-R) down regulation, increased motility, proliferation and proteinase activity and modulates integrin (a super family of cell adhesion molecules) levels. High molecular weight FGF-α (22-24kDa) may act on endothelial cell proliferation after nuclear translocation in the endothelial cells (Gleizes *et al.*, 1995). Besides its effect on angiogenesis initiation, VEGF also promotes endothelial cell proliferation through NO and cGMP mediated activation of the mitogen–activated protein kinase (MAPK) family.

3.3.3) Maturation of neovasculature

The nascent vessels are stabilized by recruiting mural cells (pericytes in small vessels and smooth muscle cells in large vessels) and by generating ECM. ECs facilitate the recruitment of mural cells via the synthesis and secretion of platelet derived growth factor (PDGF), a mitogen and chemo attractant for a variety of mesenchymal cells (Hellstrom *et al.*, 2001). Signaling through EDG1 receptor (endothelial differentiation sphingolipid G-protein-coupled receptor-1), which is expressed by mural cells, is another key pathway for mural cell recruitment. The lack of EDG1 may alter the ECM production or EC-mural cell interaction and interfere with vessel maturation (Kluk and Hla, 2002). Also critical for vessel formation and stabilization are the Tie receptors, Tie 1 and Tie 2 and two ligands for Tie 2, Ang 1 and Ang 2. Main sources of Ang 1 and Ang 2 are the mural cells and ECs respectively. Ang 1 is known to stabilize nascent vessels and make them leak resistant, presumably by facilitating communication between ECs and mural cells. On the other hand Ang 2 has a contextual function. In the absence of VEGF, Ang 2 acts as an antagonist of Ang 1 and destabilizes vessels, ultimately leading to vessel regression. In the presence of VEGF, Ang 2 facilitates vascular sprouting (Loughna and Sato, 2001). TGF-β1, a multifunctional cytokine, promotes vessel maturation by stimulating ECM production and by inducing differentiation of mesenchymal cells to mural cells (Pepper, 1997). Its function is also contextual. TGF- β1-ALK1 pathway induces ECs and fibroblasts to express Id 1, a protein required for proliferation and migration. On the other hand, the TGF- β1-ALK5

pathway induces the plasminogen activator inhibitor (PAI)1 in ECs which promotes vessel maturation by preventing degradation of the provisional matrix around the nascent vessel. Thus ratio of TGF- β signals through ALK1 versus ALK5 is likely to determine the pro or antiangiogenic effect of TGF-β (Goumans *et al.*, 2002).

3.3.4) Vessel specialization

This process includes arterio-venous determination, formation of homotypic and heterotypic junctions and EC differentiation to form organ specific capillary structures. Notch pathway determines the arterial-venous fate of ECs, possibly by committing angioblasts to the two lineages (Rossant and Howard, 2002). As the capillary plexus forms, bidirectional ephrin and ephrin receptor signaling repels the arterial and venous side and thus guides branching. Homotypic and heterotypic junctions, including, EC-EC, EC-mural cell and gap junctions, facilitate cell to cell communication and regulate vessel permeability. Vascular endothelial cadherin is an important component of EC-EC junctions, whereas neural (N)-cadherin facilitates EC-mural cell communication. Gap junctions made of connexins also facilitate communication between ECs and between ECs and perivascular cells. Tight junctions made of occludins, claudins and zona occludens contribute to the blood-tissue barrier in the brain and retinal capillaries (Jain, 2003).

3.4) The angiogenic switch

The induction of angiogenesis and subsequent tumour progression is dependent on the balance of a multitude of angiogenesis activators or inhibitors in the tumour microenvironment. Angiogenesis can be switched on at different stages of tumour progression depending on the nature of tumour and the microenvironment (Hanahan and Weinberg, 2000; Pang and Poon, 2006).

3.4.1) Activators of angiogenesis

3.4.1.1) Vascular endothelial growth factor (VEGF)

VEGF has been characterized as a heparin binding angiogenic growth factor displaying high specificity for endothelial cells (Ferrara and Henzel, 1989; Kiselyov *et*

al., 2007). Vascular permeability factor (VPF) has been characterized as a protein that promotes extravasation of proteins from tumour associated blood vessels (Senger *et al.*, 1983). Subsequently it is realized that the permeability inducing factor and the endothelial cell growth factor are encoded by a single VEGF gene. The VEGF family comprises six secreted glycoproteins that are designated VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and placenta growth factor (PlGF) (Ferrara *et al.*, 2003). The best characterized of the VEGF family members is VEGF-A (commonly referred to as VEGF, also known as VPF), a 34-45 kDa homodimeric glycoprotein. The VEGF-A gene undergoes alternative splicing to yield mature proteins of 121, 165, 189 and 206 amino acids (Houk, 1991). *In vivo*, the expression of VEGF-A was temporally and spatially associated with key events in physiologic vasculogenesis and angiogenesis (Jakeman *et al.*, 1993). VEGF-A also is linked to several pathologic conditions that are associated with increased angiogenesis including arthritis, psoriasis, nuclear degeneration and diabetic retinopathy. VEGF-B is associated with coronary artery development (Joukov *et al.*, 1997). VEGF-C has been implicated in lymphangiogenesis. VEGF-D is an endothelial cell mitogen. VEGF-E encoded by the ORF virus, induces angiogenesis through an interaction with VEGFR-2 (Meyer *et al*, 1999). PlGF is strongly expressed in placenta and is thought to have an accessory role in pathologic angiogenesis, serving to potentiate the activity of VEGF-A (Carmeliet *et al.*, 2001).

The members of VEGF family bind with different affinities to three related receptor kinases: VEGF receptor-1 (VEGFR-1; fms-like tyrosine kinase-1; flt-1), VEGF receptor-2 (VEGFR-2; kinase domain region, KDR; homologue to murine fetal liver kinase-1, FLK-1) and VEGFR-3 (Vaisman *et al.*, 1990; Shibuya, 2006). The various members of the VEGF family have different binding affinities for each of these receptors. All of the VEGF-A isoforms bind VEGFR-1 and VEGFR-2, whereas PlGF-1, PlGF-2 and VEGF-B are specific for VEGFR-1 binding and activation (Park *et al.*, 1994; Olofsson, 1998). VEGF-E interacts with VEGFR-2 whereas VEGF-C and VEGF-D interact with VEGFR-3 and VEGFR-2 (Achen *et al.*, 1998; Junok *et al.*, 1996). VEGFR-1 and VEGFR-2 are expressed on vascular endothelium except that in brain and to a lesser extend on monocytes / macrophages and certain tumour cell types. VEGFR-3 is mainly expressed on lymphatic endothelium (Neufeld *et al.*, 1999).

Interaction of VEGF with VEGFR-2 is a critical requirement to induce the full spectrum of VEGF biologic responses. VEGFR-2 mediates developmental angiogenesis and hematopoiesis by triggering EC proliferation, migration, differentiation and survival as well as by inducing vascular permeability and blood island formation (Shalaby *et al.*, 1995; Podar and Anderson, 2005). VEGFR-1 signaling is required in hematopoiesis, monocyte migration and paracrine release of growth factors (Gerber *et al.*, 2002; Barleon *et al.*, 1996). Neuropilin-1 (NRP-1) and neuropilin-2 (NRP-2) were shown to serve as co-receptors for VEGF.

NRP-1 differs from the tyrosine kinase VEGFRs (-1,-2 &-3) in that it does not have an intracellular signaling domain and its activity likely is mediated as a co-receptor for VEGFR-1 and VEGFR-2 by enhancing the binding affinity of ligands to the receptors. The binding of VEGF-A isoforms and other VEGF family members to NRP-1 is highly specific; NRP-1 binds to $VEGF_{165}$ and PlGF-2 but not $VEGF_{121}$. NRP-2 binds to $VEGF_{165}$ as well as a rarer isoform $VEGF_{145}$. Evidences also suggest that VEGF may signal directly through NRP without VEGFR-1 or VEGFR-2 serving as a co-receptor (Soker *et al.*, 1998; Miao and Klagsbrun, 2000; Gluzman-Poltorak *et al.*, 2000).

3.4.1.2) Fibroblast growth factors (FGF)

Members of the FGF family are also potent inducers of angiogenesis. FGFs promote cell migration, proliferation and differentiation. The FGF family consists of nine structurally related polypeptides, of which FGF-1 (acidic FGF) and FGF-2 (Basic FGF) are the most important ones. The cellular effects of FGFs are mediated via specific binding to high affinity tyrosine kinase receptors (Klein *et al.*, 1997). In addition, low affinity FGF receptors exist, that consist of polysaccharide components of heparan sulphate proteoglycans on cell surfaces and in ECM. Heparen sulphate on cell surfaces plays a more active role in displacing ECM bound FGF-2 and subsequent presentation to the high affinity signal transducing receptors (Miao *et al.*, 1996). Receptor dimerization by FGF is facilitated by heparin. It results in protein kinase activity and receptor autophosphorylation. As with VEGFR signaling, this autophosphorylation enables adaptor proteins such as Grb2, Shc and Nck to bind and

subsequently activate the Ras/Raf-MAPK pathway of endothelial cell proliferation activation (Klein *et al.*, 1997). P42 MAPK activation was also implicated in endothelial cell motility regulatory response to FGF (Sa *et al.*, 1995).

3.4.1.3) Platelet derived growth factor (PDGF)

Platelet derived growth factor (PDGF)-BB and its receptor, PDGFR-β have essential roles in the stabilization of nascent blood vessels by recruiting PDGFR-β-positive mesenchymal progenitors (Lindhal *et al.*, 1997). Inhibition of PDGFR-β signaling has been reported to result in a tumour microvasculature that is particularly dependent on VEGF mediated survival signals. PDGF-CC and PDGF-DD also promote angiogenesis, but their roles remain less well characterized (Cao *et al.*, 2002).

3.4.1.4) Angiopoietins

Angiopoietins and its receptor Tie-2 are important signaling molecules that have an established role in the development and differentiation of vessel wall. Unlike Ang-2, which activates Tie-2 on some cells but blocks Tie-2 on others, Ang-1 consistently activates Tie-2. Even though trapping angiopoietins suppresses pathological angiogenesis (Takagi *et al.*, 2003) their role is pleiotropic and context dependent. Ang-1 stimulates vessel growth in skin, ischemic limbs, gastric ulcers and in some tumours, presumably because it is an EC survival factor and mobilizes endothelial progenitor cells and HSCs (Shim *et al.*, 2002; Hattori *et al.*, 2001). But Ang-1 also suppresses angiogenesis in tumours and the heart (Ahmad *et al.*, 2001; Visconti *et al.*, 2002). Although it is still not clearly understood, the antiangiogenic effect of Ang-1 may relates to the fact that it tightens vessels by affecting junctional molecules and by promoting the interaction between ECs and mural cells as an adhesive protein and recruiting pericytes (Carlson *et al.*, 2001). Ang-2 has been proposed to stimulate the growth of immature tumour vessels by loosening endothelial–periendothelial cell interactions and degrading the ECM, thereby antagonizing Ang-1 (Ahmad *et al.*, 2001). The role of Ang-2 in angiogenesis has been found to be contextual. It synergizes with VEGF to stimulate angiogenesis in heart (Visconti *et al.*, 2002), but when insufficient

angiogenic signals are present, Ang-2 causes EC death and vessel regression (Maisonpierre *et al.*, 1997).

3.4.1.5) Transforming growth factor-β (TGF-β)

Members of the TGF-β super family contribute to the resolution and maturation phases of angiogenesis, but in a pleiotropic manner. TGF-β ligands stimulate type II receptors that in turn phosphorylate type I receptors (Activin receptor like kinase-ALK) and activate the down stream signaling smads. Endoglin is a type III receptor which facilitates binding of TGF-β 1 to the type II receptors. Both pro and antiangiogenic properties have been ascribed to TGF-β1. At low doses TGF-β1 promotes angiogenesis by up regulating angiogenic factors and proteinases, whereas at high doses, TGF-β1 inhibits EC growth, promotes basement membrane reformation and stimulates SMC differentiation and recruitment (Lamoville *et al.*, 2002; Goumans *et al.*, 2002). Id 1, a protein required for proliferation and migration is expressed by fibroblasts upon activation of TGF-β1-ALK 1 pathway. On the other hand TGF-β1-ALK 5 pathway promotes vessel maturation through the production of plasminogen activator inhibitor (PAI)1 in endothelial cells which prevents the degradation of the provisional matrix around the nascent vessel. Thus the pro and antiangiogenic effect of TGF-β depends on the ratio of TGF-β signals through ALK 1 versus ALK 5 pathways.

3.4.1.6) Integrins

Integrins are a class of adhesion molecules that is made up of transmembrane glycoproteins that involved in cell-cell and cell-ECM interactions. Integrin-α and -β subunits (18 α and 8β subunits in mammals) assemble into at least 24 possible α/β heterodimeric pairs. Each α β combination has specific ECM ligand binding and signaling properties (Plow *et al.*, 2000). Upon stimulation by growth factors or proangiogenic signals, ECs express high levels of the integrins, αVβ3 and αVβ1 (Brooks *et al.*, 1994). Along with integrin αVβ5, these integrins promote the migration of ECs and their adhesion to the provisional matrix proteins that are found in the angiogenic ECM. Two specific and distinct αV integrin mediated angiogenic pathways have been identified (Eliceiri and Cheresh, 1999). Integrin αVβ3 mediated angiogenesis

requires bFGF whereas VEGF mediated angiogenesis depends on integrin αVβ5. Integrin αVβ1 promotes angiogenesis that is induced by FGF, but not VEGF (Kim *et al.*, 2000). Ligation of integrins αVβ1 and αVβ3 by the ECM is required to promote EC survival. When cells are deprived of integrin mediated adhesion to the ECM, they rapidly undergo apoptosis (Meredith *et al.*, 1993).

3.4.1.7) Nitric oxide

During the past decade nitric oxide (NO) has emerged as an important mediator in physiological and pathological processes. In normal tissues, NO regulates neurotransmission, blood vessel tone, platelet aggregation, vascular permeability, elimination of certain free radicals and leukocyte endothelial interaction. On the other hand, NO and one of the associated reactive products peroxynitrite are cytostatic/cytotoxic and may contribute to the pathophysiology of many diseases including tumours (Moncada *et al.*, 1991). NO is the product of conversion of L-arginine to L-citrulline by nitric oxide synthase (NOS) which exist as 3 isoforms;the calcium dependent endothelial (eNOS) and neuronal (nNOS) nitric oxide synthases (both constitutes constitutive nitric oxide synthase - cNOS) and a calcium independent inducible (iNOS) nitric oxide synthase, which is induced by inflammatory cytokines and gram negative endotoxins. eNOS and nNOS produce relatively low amounts of NO whereas iNOS produces relatively high amounts of NO (Knowles and Moncada, 1994). Vasodilation by smooth muscle cell (SMC) relaxation by NO is a prerequisite for the induction of angiogenic switch. It took nearly seven years to establish that 'endothelium derived relaxing factor (EDRF)', an elusive factor responsible for SMC relaxation was nothing other than NO (Furchgott and Zawadeski, 1980; Palmer *et al.*, 1987). Experiments using NO donors established that NO promotes DNA synthesis, proliferation and migration of coronary venular endothelial cells where as inhibitors of NO synthesis attenuate endothelial cell proliferation and migration induced by VEGF (Ziche *et al.*, 1993; Morbidelli *et al.*, 1996). Studies to date have shown that inhibition of endogenous NO synthesis decreases tumour blood flow thereby derailing nutrient/oxygen supply to tumours (Andrade *et al.*, 1992). It would appear that induced production of NO by macrophages and other cells in the body, endothelium of tumour

vasculature or macrophages within the tumours may act as a double-edged sword against tumour or the host, depending on the circumstances. On one hand, NO can defend the host by arming host macrophages with antitumour activity. Furthermore, excess NO production by tumour cells may be detrimental to their own survival due to NO induced apoptosis (Stuehr and Nathan, 1989; Xie *et al.*, 1995). On the other hand, significant experimental as well as clinical data suggest that tumour derived NO is conducive to tumour progression and metastasis and thus detrimental to the host (Thomsen *et al.*, 1995). These apparently opposing roles of NO on the tumour–host balance make the study on the effect of NO on tumour biology more complex.

3.4.1.8) Matrix metalloproteinases (MMPs)

MMPs, also called matrixins, are a family of over 20 zinc-containing endopeptidases that are capable of degrading various components of the ECM. All MMPs are produced as zymogens containing a secretory signal sequence and a propeptide whose proteolytic cleavage is required for MMP activation. The propeptide is followed by the catalytic domain that contains the consensus zinc binding motif HEBXHXBGBXH, where X is a variable residue and B is a bulky hydrophobic residue. At least two MMPs (MMP-7 and MMP-26) are composed only of the signal peptide, propeptide and catalytic domain and are known as minimal domain MMPs. Most of the remaining MMPs contain a haemopexin-like domain, that is thought to confer some degree of substrate specificity and several have additional features, such as fibronectin-like repeats or serine protease recognition motifs. MMPs have been subdivided into at least five groups based on their structure and/or substrate specificities– matrilysins, collagenases, stromelysins, gelatinases and membrane-type MMPs (MT-MMPs). MMP activity is controlled at least at three levels: transcription, proteolytic activation of zymogen form and inhibition of the active enzyme by host natural inhibitors (Nagase and Woessner, 1999; Lee and Murphy, 2004; Yan and Boyd, 2007).

The prodomain serves to maintain pro-MMPs in an inactive state. The prodomain of all MMPs contain a conserved cysteine residue, called the "cysteine switch" (within the distinctive consensus PRCGXPDV motif) whose sulfhydryl group coordinates with zinc ion in the catalytic site to maintain latency. Disruption of this

interaction by physical or chemical means constitutes the first step in MMP activation, which is followed by proteolytic cleavage of the COOH terminal side of the PRCGXPDV site with irreversible loss of the cysteine residue (Nagase and Woessner, 1999; Nagase, 1997). Most MMPs can be activated by other MMPs and a variety of serine proteases *in vitro* (Nagase, 1997).

MMPs are very essential for angiogenesis, tumour cell invasion and metastasis. ECM remodeling (synthesis and deposition of ECM components and their proteolytic breakdown), an absolute requirement of angiogenesis, by MMPs open up an avenue for migrating endothelial cells. MMPs have been shown to have multiple effects on endothelial cells themselves. MMPs are necessary for endothelial cell migration and tube formation (Nguyen *et al.*, 2001). MMPs, but not the plasminogen activator/plasmin system, are involved in endothelial cell migration and invasion of fibrin barriers with MT1-MMP showing the greatest fibrinolytic activity (Hiraoka *et al.*, 1998). MMP-7 enhances endothelial cell proliferation, up regulates endothelial expression of MMP-1 and MMp-2 and induces angiogenesis *in vivo* (Huo *et al.*, 2002).

MMPs involved in angiogenesis can originate from infiltrating inflammatory cells, from tumour cells or from ECs themselves. Angiogenic factors can induce the expression of MMPs in endothelial cells. Membrane vesicles containing MMP-2, MMP-9 and MT1-MMp can be found in ECs, in some cases localized to invading pseudopodia and angiogenic stimulation of cells with bFGF or VEGF results in shedding of vesicles (Taraboletti *et al.*, 2002). In tumour angiogenesis, MMP-2 and MMP-9 have been shown to be critical for the angiogenic switch when tumours (or often preneoplastic lesions) first become vascularized (Iniesta *et al.*, 2007). Up regulation of MMP-9 expression results in the release of VEGF from ECM that is then responsible for induction of the angiogenic phenotype (Bergers *et al.*, 2000). In a rat chondrosarcoma model, angiogenic tumour nodules produced three times the amount of MMP-2 as compared to avascular nodules and down regulation of MMP-2 expression by antisense oligonucleotides resulted in loss of angiogenic potential and inhibited tumour growth. Thus in this model, MMP-2 appears to be the important regulator of the angiogenic switch (Fang *et al.*, 2000). MMPs are generally expressed in almost all

human cancers. Cancer cells can induce tumour stromal cells to express MMPs via paracrine secretion of cytokines and growth factors (Egeblad and Werb, 2002). 'Vascular mimicry', a mechanism where tumour cells act like endothelial cells, expressing endothelial cell associated genes and forming fluid conducting channels within tumours are also supported by MMPs. MMP-2, MT1-MMP and laminin 5γ2, which are over expressed in aggressive melanomas, are responsible for vascular mimicry (Hendrix *et al.*, 2003).

In addition, MMPs have been shown to generate endogenous angiogenesis inhibitors by proteolytic cleavage of plasma proteins and ECM components. MMP-2, MMP-7, MMP-9 and MMP-12 all have the capacity to hydrolyze plasminogen to form the potent angiogenesis inhibitor angiostatin (Patterson and Sang, 1997). Other angiogenesis inhibitors such as endostatin, arrestin and canstatin were also released by MMPs (Kalluri, 2003).

3.4.1.9) Hypoxia

Hypoxia is an important environmental factor that leads to neovascularization. The hypoxia inducible transcription factor-1 (HIF-1) enables the organisms to adapt to hypoxia. HIF-1 is an αβ heterodimer and both HIF-1α and HIF-1β subunits exist as a series of isoforms. HIF-1β subunits are constitutive nuclear proteins, whereas HIF-1α subunits are inducible by hypoxia. HIF-1α and HIF-2α isoforms interact with hypoxia response elements (HREs) to induce transcriptional activity. In addition to activation by hypoxia, the HIF system is also induced or amplified by a wide range of growth promoting stimuli and oncogenic pathways such as insulin, insulin like growth factor-1, epidermal growth factor and mutant Ras and Src kinase pathways. HIF-1 increases the transcription of several genes for proteins that promote blood flow and inflammation, including VEGF, endothelial and inducible NOS and cyclooxygenase-2 (COX-2) and thus play an important role in the activation of angiogenesis (Shweiki *et al.*, 1992; Pugh and Ratcliff, 2003; Hickey and Simon, 2006; Lungu *et al.*, 2007).

3.4.1.10) Proinflammatory cytokines

There exist a strong association between cancer and inflammation. About 15% of the gloabal cancer burden is attributed to infectious agents and inflammation is a major component of these chronic infections (Balkwill and Mantovani, 2001). The inflammatory microenvironment of tumours is characterized by the presence of host leukocytes, tumour associated macrophages, dendritic cells etc which secrete proinflammatory cytokines. Proinflammatory cytokines are a group of proteins with the ability to stimulate or inhibit cell growth, regulate cell differentiation, induce cell chemotaxis and modulate the expression of other cytokines. Altered levels of proinflammatory cytokines are observed in various forms of cancer (Chen *et al.*, 1999). The important among them are tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6) and granulocyte monocyte–colony stimulating factor (GM-CSF).

a) Tumour necrosis factor-α (TNF-α)

Tumour necrosis factor is a 17kDa polypeptide that was first isolated from macrophages. The major source of TNF-α is the cells of the monocyte/macrophage lineage, with T-lymphocytes, neutrophils, mast cells and under certain circumstances endothelium also. TNF-α is the most rapidly produced proinflammatory cytokine. TNF-α might be directly involved in iNOS activation in macrophages and acting as an additional signal for synergistic induction of NO formation which is essential for angiogenesis. TNF can be detected in malignant and /or stromal cells in human, ovarian, breast, prostate, bladder and colorectal cancer, lymphomas and leukemias, often in association with IL-1 and IL-6 (Naylor *et al.*, 1993). Moreover, TNF also stimulates the expression of VEGF.

b) Interleukin-1β (IL-1β)

Studies using human lung epithelial cells (line A549) indicate that IL-1β increases HIF-1α level in two ways. First, IL-1β stimulates HIF-1α production through activation of NF-κB in a pathway involving PI3K/akt/mTOR. Second, IL-1β inhibits

pVHL-dependent degradation of HIF-1α (Jung *et al.*, 2003). IL-1β also augments expression of adhesion molecules on endothelial cells (Mantovani *et al.*, 1992).

c) Interleukin-6 (IL-6)

IL-6 is a secreted, multifunctional glycoprotein. IL-6 expression in tumour tissues is high and correlates with the severity of cervical cancer (Wei *et al.*, 2001). IL-6 has been demonstrated to promote growth of multiple myeloma, kaposi's sarcoma and prostate cancer cells. It also promotes cervical tumour growth by enhancing angiogenesis through up regulation of VEGF via a STAT3 pathway (Wei *et al.*, 2003).

d) Granulocyte monocyte–colony stimulating factor (GM-CSF)

GM-CSF is a pleiotropic cytokine produced by fibroblasts, keratinocytes and endothelial cells. Previous reports on different tumour models indicated that GM-CSF may promote tumour progression by enhancing cell migration and thus favouring invasion and metastasis (Takeda *et al.*, 1991). In addition to their stimulation of growth and migration, GM-CSF may also contribute to the angiogenic reaction by stimulating endothelial cell proliferation and migration (Bussolino *et al.*, 1989).

3.4.2) Inhibitors of angiogenesis

3.4.2.1) Endogenous inhibitors

Endogenous inhibitors of angiogenesis are of two major classes- matrix derived inhibitors and non-matrix derived inhibitors.

a) Matrix derived inhibitors

a.i) Arresten

Arresten is a 26kDa molecule derived from the non-collagenous (NCI) domain of the α chain of type IV collagen. Arresten selectively inhibits endothelial cell tube formation, proliferation and migration (Colorado *et al.*, 2000). It also inhibits formation of new blood vessels in matrigel plug assay.

a.ii) Endorepellin

The COOH- terminal end of perlecan called endorepellin or perlecan domain V, potently inhibits endothelial cell migration, collagen induced endothelial tube morphogenesis and blood vessel growth in the chicken chorio allantoic membrane assay and in mouse matrigel plug assay (Mongiat *et al.*, 2003).

a.iii) Endostatin

Endostatin is a 20kDa fragment derived from the COOH-terminal NCI domain of type XVIII collagen. It has been shown that endostatin interferes with FGF-α induced signal transduction, blocking endothelial cell motility, inducing apoptosis, causing G1 arrest of endothelial cells through inhibition of cyclin D1, blocking VEGF mediated signaling via the VEGFR-2/KDR/Flk-1 receptor tyrosine kinase in human umbilical vein endothelial cells and blocking TNF-induced activation of c-Jun NH_2-terminal kinase dependent proangiogenic gene expression (Shichiri and Hirata, 2001).

a.iv) Thrombospondins

Thrombospondin-1 (TSP-1) was the first identified naturally occurring antiangiogenic protein (Good *et al.*, 1990). The antiangiogenic activity of TSP-1 has been mapped to the type-1 repeats and within the NH_2-terminal portion of the molecule within the procollagen like domain and it blocks both FGF-α and VEGF angiogenic signals (Iruela-Arispe *et al.*, 1999). Thrombospondin-2 (TSP-2) also shows antiangiogenic activity and the antiangiogenic region of TSP-2 lies approximately within the 80kDa fragment of the NH_2-terminal globular region (Noh *et al.*, 2003; Varner, 2006).

a.v) Tumstatin

Tumstatin is the entire 28kDa fragment of α3 chain of NC1 domain of type IV collagen. Tumstatin has two binding sites for αVβ3 integrin, one in the NH_2-terminal end of the molecule that is associated with the antiangiogenic properties and the other in the COOH-terminal end, that is associated with antitumour activity. Tumstatin inhibits

the formation of new blood vessels in matrigel plug assays. The antiangiogenic and proapoptotic activity of tumstatin is specific for endothelial cells (Maeshima *et al.*, 2000).

b) Non-matrix derived inhibitors

b.i) Angiostatin

Thirty eight-45kDa peptides containing homologous triple-disulfide bridged kringle domains, resulting from the cleavage of plasminogen by proteases are collectively called angiostatin. Angiostatin is a cryptic fragment of plasminogen that possesses antiangiogenic properties. It inhibits endothelial cell proliferation and migration. It binds directly to the ATP synthase on the surface of endothelial cells, which might play a role in allowing the intracellular pH to drop, thus triggering apoptotic events in endothelial cells (O'Reilly *et al.*, 1994; Moser *et al.*, 1999).

b.ii) Interferons

Interferons (IFNs) are pleiotropic cytokines that regulate antiviral, antitumour, apoptotic and cellular immune responses. IFN-α and IFN-β inhibit angiogenesis in tumour bearing nude mice. IFN-α reduces urokinase type plasminogen activator and plasminogen activator inhibitor-1 activity (Lingen *et al.*, 1998). Both IFN-β and IFN-γ significantly inhibit MMP-9 enzymatic activity and protein expression (Ma *et al.*, 2001).

b.iii) Pigment epithelium derived factor (PEDF)

Pigment epithelium derived factor (PEDF) is the most potent inhibitor of angiogenesis in the mammalian eye and is involved in the pathogenesis of angiogenic eye diseases, such as proliferative diabetic retinopathy (Bouck, 2002).

b.iv) 2-methoxy estradiol

2-methoxy estradiol, an endogenous estradiol metabolite, is an inhibitor of angiogenesis with direct effect on cancer cells and it also induces apoptosis in endothelial cells (Yue *et al.*, 1997).

b.v) Platelet factor-4

Platelet factor-4 is a protein released from platelet-α granules during platelet aggregation that has been shown to have antiangiogenic properties both *in vitro* and *in vivo*. Recombinant human PF-4 inhibits blood vessel proliferation in the chicken chorio allantoic membrane assay in a dose dependent manner by blocking the binding of FGF-α to endothelial cells (Maione *et al.*, 1990).

b.vi) Vasostatin

Vasostatin, a NH_2-terminal domain of human calreticulin, selectively inhibits endothelial cell proliferation and angiogenesis in response to stimulation from growth factors and suppresses tumour growth. It specifically inhibits endothelial cell attachment to laminin and reduces subsequent endothelial cell growth induced by bFGF (Pike *et al.*, 1998).

b.vii) Troponin-1

Troponin-1 is a novel cartilage derived angiogenesis inhibitor which inhibits both bFGF-stimulated and basal levels of endothelial cell proliferation probably via an interaction of troponin-1 with the cell surface bFGF receptor on capillary endothelial cells (Feldman and Rouleau, 2002).

b.viii) Tissue inhibitor of metalloproteinases (TIMP)

Tissue inhibitor of metalloproteinases (TIMP) suppress MMP activity and ECM turnover. The four TIMP members (TIMP-1,-2, -3 and -4) inhibit MMP activity when inserted into the zinc activity pocket (Brew *et al.*, 2000). Although all of the TIMPs bind tightly to most MMPs, they have differential inhibitory activity against different MMPs. TIMP-2 and TIMP-3, but not TIMP-1, are efficient inhibitors of the MT-MMPs. TIMP-1 inhibits MMP-9 activation by directly binding to the PEX domain of MMP-9 while TIMP-3 complexes with both MMP-2 and MMP-9 (Brew *et al.*, 2000). TIMP-2 inhibits MMP-2 and also blocks the bFGF induced EC proliferation by binding integrin α3β1 and down regulates VEGF induced proangiogenic signals (Murphy *et al.*, 1993).

3.4.2.2) Synthetic angiogenesis inhibitors

a) TNP-470

TNP-470, an analogue of the fungus derived antibiotic fumagillin, is one of the first compounds identified to exhibit inhibitory effects on endothelial cell growth and was used in the first formal clinical trial of antiangiogenic therapy. It prevents the EC to enter the G1 phase of the cell cycle, resulting in a decrease in proliferation (Ingber *et al.*, 1990).

b) Bavacizumab (Avastin)

Avastin is a humanized monoclonal antibody against VEGF which was approved for use as a first line therapy for metastatic colorectal cancer in February 2004 by United States Food and Drug Administration (USFDA) (Ferrara *et al.*, 2004; Finger, 2007).

c) Vitaxin (LM609)

Vitaxin is an antibody against integrin αVβ3 which inhibits bFGF induced angiogenesis and induces endothelial cell apoptosis (Gutheil *et al.*, 2000).

d) Thalidomide

Thalidomide and its analogue inhibit tube formation and angiogenesis and suppress tumour growth effectively. Its usage is restricted because of dangerous teratogenesis and phocomelia (stunted limb growth) (Ng *et al.*, 2003).

e) Celicoxib

Celicoxib is an inhibitor of inducible prostaglandin G/H synthase COX2 and it down regulates the expression of FGF and VEGF (Inguez *et al.*, 2003).

4) Apoptosis

Apoptosis is an evolutionary conserved, intrinsic programme of cell death that occurs in various physiological and pathological situations (Hengartner, 2000). The term 'apoptosis' which means 'falling of petals from a flower' or 'of leaves from a tree in autumn' was first coined by John Kerr (Kerr *et al.*, 1972). Apoptosis is associated with a distinct set of biochemical and physiological changes involving the cytoplasm, nucleus and plasma membrane. Early in apoptosis, the cells round up and shrink; the endoplasmic reticulum dilates and the cisternae swell to form vesicles and vacuoles. In the nucleus, chromatin condenses and aggregates into dense compact masses and is fragmented intranucleosomally by endonucleases, which can be analyzed by the typical 'DNA ladder' formation in apoptosis (Johnson *et al.*, 1996). The nucleus becomes convoluted and buds off into several fragments, which are encapsulated within the apoptotic bodies. Due to disintegration of cell junctions, the plasmamembrane becomes convoluted and eventually results in blebbing. The cell breaks up in a florid manner leading to the falling away of several membrane bound packaged cellular contents identified as apoptotic bodies of various sizes (Kerr *et al.*, 1994).

4.1) Pathways of apoptosis

Although understanding of the detailed signaling pathways that trigger apoptosis is incomplete, this process is controlled by a number of complex proteins, which are activated by various triggers and arranged in sequential signaling modules. Apoptosis occurs through two main pathways-extrinsic as well as intrinsic pathways (Cory and Cory, 2007). The extrinsic or cytoplasmic pathway is triggered through Fas death receptor, a member of the TNF receptor super family (Zapata *et al.*, 2001). The intrinsic or mitochondrial pathway is characterized by the release of cytochrome-c from the mitochondria and activation of the death signal (Hockenbery *et al.*, 1990). Both pathways converge to a final common pathway involving the activation of a cascade of proteases called caspases that cleave regulatory and structural molecules, culminating in the death of the cell. The pathways are linked. Over expression of Bcl-2 in the intrinsic pathway may lead to the inhibition of extrinsic mediated apoptosis (Scaffidi *et al.*,

1998). Conversely, TNF-α may increase the expression of NF-κB and stimulate antiapoptotic members of the Bcl-2 family proteins.

4.1.1) The extrinsic pathway: Fas

Activation of the extrinsic pathway is initiated with ligation of cell suface receptors called death receptors (DRs). Fas is a member of the TNF receptor superfamily and is also called Apo-1 or CD95. Other TNF receptors include TNFR1, DR3 (Apo-2), DR4 (TNF-related apoptosis inducing ligand receptor 1- TRAILR1), DR5 (TRAILR2) and DR6 (Zapata *et al.*, 2001; Li *et al.*, 2006). The Fas ligand (Fas L) - Fas system is mainly recognized for its death related functions, but it is also involved in several proliferative and inflammatory signaling pathways that are not well defined (Krammer, 2000). When a death stimulus trigger the pathway, the membrane bound Fas L interacts with the inactive Fas complexes and forms the death inducing signaling complex. The Fas death inducing signaling complex contains the adaptor protein Fas-associated death domain protein and caspases-8 and -10 and leads to activation of caspase-8, which in turn can activates the rest of the downstream caspases. In some cells, activated caspase-8 itself can execute death, while in other cell types, caspase-8 interacts with the intrinsic apoptotic pathway by cleaving Bid, leading to the subsequent release of cytochrome-c (Wajant, 2002). NF-κB, FAP-1 and Fas-associated death-domain protein like interleukin-1β-converting enzyme-like inhibitory protein are the major inhibitors of this pathway.

4.1.2) The intrinsic pathway

One of the most important regulators of this pathway is the Bcl-2 family of proteins, which include both antiapoptotic members, eg: Bcl-2, Bcl-X_L, Mcl-1 as well as proapoptotic molecules such as Bax, Bak, Bad and BH3 domain only molecules that link the death receptor pathway to the mitochondrial pathway (Cory and Adams, 2002). Bcl-2 or Bcl-X_L exert their antiapoptotic function, at least in part, by sequestering BH3 domain only proteins in stable mitochondrial complexes, thereby preventing activation and translocation of Bax or Bak to mitochondria (Cory and Adams, 2002; Campas *et al.*, 2006). In addition, Bcl-2 and Bcl-X_L prevent cytochrome-c release through a direct

effect on mitochondrial channels such as voltage-dependent anion channel (VDAC) or the permeability transition pore complex (PTPC) (Cheng *et al.*, 2003). The shift of equilibrium between anti and proapoptotic Bcl-2 proteins determine the fate of the cell. Following a death signal, proapoptotic proteins undergo post translational modifications that include dephosphorylation and cleavage resulting in their activation and translocation to the mitochondria leading to apoptosis. In response to apoptotic stimuli, the outer mitochondrial membrane becomes permeable, leading to the release of cytochrome-c and second mitochondria-derived activator of caspase. Cytochrome-c, interacts with Apaf-1 (Apoptotic protease activating factor-1) leading to the activation of caspase-9 proenzymes. Active caspase-9 then activates caspase-3, which subsequently activates the rest of the caspases and leads to apoptosis (Scorrano and Korsmeyer, 2003).

4.2) Caspases

Caspases (which are so called as they are cysteine proteases that cleave after an aspartate residue in their substrates) are a conserved family of enzymes that irreversibly commit a cell to die. Not all caspases are involved in apoptosis. At least 7 of the 14 known mammalian caspases have important roles in apoptosis (Earnshaw *et al.*, 1999). The apoptotic caspases are generally divided into two classes: the initiator caspases, which include caspase-2, -8, -9 and -10 and the effector caspases, which include caspase-3, -6 and -7. All caspases are produced in cells as catalytically inactive zymogens and must undergo proteolytic activation during apoptosis. The activation of an effector caspase (caspase-3) is carried out by an initiator caspase (caspase-9) through cleavage at specific internal Asp residues. The initiator caspases are auto activated, which is tightly regulated and often requires the assembly of a multicomponent complex under apoptotic conditions. For example, activation of procaspase-9 is facilitated by the apoptosome, an ~1.4MDa complex that includes Apaf-1 and cytochrome (Adams and Cory, 2002). The intrinsic (caspase-9) and extrinsic (caspase-8) apoptotic pathways converge to caspase-3, which cleaves the inhibitor of the caspase-activated deoxyribonuclease and the caspase activated deoxyribonuclease becomes active leading to nuclear apoptosis (Qi *et al.*, 2007; Druskovic *et al.*, 2006).

4.3) Inhibitor of apoptosis proteins (IAPs)

Inhibitor of apoptosis proteins (IAPs) are the family of endogenous caspase inhibitors which potently inhibit the enzymatic activity of caspases and can permanently remove caspases through the ubiquitylation-mediated proteosome pathway (Salvesen and Duckett, 2002). There are 8 mammalian IAPs which include XIAP, c-IAP1, c-IAP2, ML-IAP, ILP-2 (IAP-like protein-2), NAIP (neuronal apoptosis-inhibitory protein), Bruce/Apollon and surviving. Caspase-3,-7 and -9 are subject to inhibition by IAps (Shi, 2002).

5) p53

The p53 tumour suppressor is a nuclear phosphoprotein that functions as a DNA-damage inducible sequence specific transcription factor. In the absence of genetic damage, p53 transcriptional activity is inert (Prives and Hall, 1999). Depending on the conditions of cell growth, the type and duration of stress or DNA damage, p53 selectively activates a different subset of target genes which can cause either apoptosis, growth arrest, altered DNA repair or altered differentiation. p53 regulates the G1-S check point in response to relatively low doses of DNA damage, heat shock, hyperoxia, hypoxia and other forms of stress. Among the multiple targets for the transcriptionally active p53 which mediates efficient G1 arrest are cyclin-dependent kinase inhibitor $p21^{waf-1}$, 14-3-3 and reprimo, which enable a sufficient pause to allow for the repair of damaged DNA to take place (Mc Kay *et al.*, 1999). Under extreme stress and severe DNA damage, p53 triggers the activation of genes implicated in the apoptotic cascade (Miyashita and Reed, 1995; Zhao *et al.*, 2006). p53 appears to activate the apoptotic machinery by triggering translocation of Bax to mitochondria, with a concomitant release of cytochrome-c from mitochondria into the cytosol (Schuler *et al.*, 2000). This causes activation of several caspases including caspase-8, which might amplify the apoptotic signal by activating caspase-9 and its downstream caspases (Gao *et al.*, 2001). p53 can also directly transactivate the transcription of caspase-1 and caspase-6 (Gupta *et al.*, 2002). During apoptosis, p53 can also inhibit the transcription of antiapoptotic genes such as Bcl-2, which protects cells against programmed cell death (Reed, 1994).

6) Nuclear factor-κB (NF-κB)

Nuclear factor-κB (NF-κB), an ubiquitously expressed transcription factor, was first identified in 1986 by Sen and Baltimore as a nuclear factor bound to an enhancer element of the immunoglobulin kappa light chain gene in B-cells (Sen and Baltimore, 1986). It controls apoptosis, cell proliferation and differentiation and is a major player in the control of immune response and inflammation. The mammalian NF-κB family contains 5 members: NF-κB-1(p105 and p50), NF-κB-2 (p100 and p52), c-Rel, RelB and RelA (p65). These proteins share a Rel homology domain (RHD), which mediates DNA binding, dimerization and interaction with specific inhibitory factors, the IκBs (inhibitory κB), which retain NF-κB dimers in the cytoplasm. Many stimuli activate NF-κB, mostly through IκB kinase-dependent (IKK-dependent) phosphorylation and subsequent degradation of IκB proteins. The liberated NF-κB dimers enter the nucleus, where they regulate transcription of diverse genes encoding cytokines, growth factors, cell adhesion molecules and pro and antiapoptotic proteins (Ghosh and Karin, 2002). The IKK complex consists of two highly homologuous kinase subunits, IKKα and IKKβ and a non-enzymatic regulatory component, IKKγ/NEMO (Karin and Delhase, 2000).

There are two distinct NF-κB activation pathways: the classical pathway and the alternative pathway (Bonizzi and Karin, 2004). The classical pathway is normally triggered in response to microbial and viral infections or exposure to proinflammatory cytokines that activate the 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 alternative pathway leads to selective activation of p52: RelB dimers by inducing processing of the NF-κB2/p100 precursor protein. This pathway is triggered by certain members of the TNF cytokine family, through selective activation of IKKα homodimers by the upstream kinase NF-κB-inducing kinase (NIK). The classical pathway is responsible for inhibition of programmed cell death under most conditions. The alternative pathway is important for survival of premature B-cells and development of secondary lymphoid organs

The first clear evidence for NF-κB as a programmed cell death inhibitor was provided by RelA knockout mice that die mid-gestation by massive liver apoptosis (Beg *et al.*, 1995). Several gene products that negatively regulate apoptosis in tumour cells, including IAP-1 and -2, X-linked IAP, cellular Fas-associated death domain–like interleukin-1β-converting enzyme (FLICE)-like inhibitory protein (cFLIP), were shown to be controlled by NF-κB activation (Shishodia and Aggarwal, 2002). An antiapoptotic role of NF-κB has been alleged in T-cell lymphoma, osteoclasts, melanoma, pancreatic cancer, bladder cancer and breast cancer. Cell types that display an antiapoptotic role for NF-κB includes B-cells, T-cells, granulocytes, macrophages, neuronal cells and smooth muscle cells (Garg and Aggarwal, 2002). Although rare, there are systems in which NF-κB plays a proapoptotic role, which is seen in B-cells, T-cells, neuronal cells and endothelial cells. These outwardly paradoxical effects of NF-κB on apoptosis may result from the different activation pathways of NF-κB that cause the expression of proteins that promote (eg: Fas, c-myc, p53 and IB) or inhibit (eg: TRAF2, IAP proteins and Bcl-2 like proteins) apoptosis. In addition NF-κB activation variably regulates cell cycle proteins and their interaction with various cellular components that promote or induce apoptosis (Shishodia and Aggarwal, 2002).

Highly metastatic melanoma cells were found to express high levels of constitutive NF-κB activity and suppression of constitutive NF-κB activity inhibited tumour growth, prevented lung metastasis and decreased angiogenesis (Huang *et al.*, 2000; Vilimas *et al.*, 2007). NF-κB mediates the invasion of tumour cells through the transcriptional activation of MMP-9 which is essential for the ECM remodeling. NF-κB activation also induces the over expression of uPA, the promoter of which contains an NF-κB binding site that directly mediates the induction of uPA expression by RelA (Wang *et al.*, 1999). The constitutive activation of NF-κB also appears to have a role in cell proliferation (Libermann and Baltimore, 1990). NF-κB has been also shown to mediate the up regulation of angiogenic factors including VEGF (Yu *et al.*, 2003). Thus the design of NF-κB inhibitors that are pharmacologically safe will be critical for the treatment of cancer.

7) Conventional cancer treatment modalities

There are three established strategies to eradicate cancer cells in a patient's body- surgery, radiotherapy and chemotherapy.

7.1) Surgery

Surgical procedures are used to physically remove malignant tissue. Surgery can be a simple, safe method to cure patients with solid tumours when the tumour is confined to the anatomic site of the origin. Advances in surgical techniques and a better understanding of the patterns of spread of individual cancers have allowed surgeons to perform successful resections for an increased number of patients. Cancer surgery carries the risk of surgery in general: dangers of anesthesia, loss of hemostasis and infection. Since larger tumours are more likely to be associated with tumour extensions into critical or fragile normal tissue, there is always greater risk of lethal hemorrhage during resection and suppressed immune functions with increased risk of infection.

7.2) Radiation therapy

During radiotherapy, malignant cells are exposed to ionizing radiation from either an external or implanted radiation source and the resulting damage causes the death of the cell during division, which leads to a gradual reduction in tumour mass. In radiotherapy, radiation is focused like a beam of light on the treated area called a radiation field, but cancer cells that reside outside of the irradiated area will not be damaged.

Radiation is most toxic to proliferating cells and higher doses are required to kill cells that are capable of proliferation but are not actively dividing at the time of exposure. Cellular damage produced by radiotherapy is an indirect result of ionization of chemicals in the cells to very reactive compounds such as H_2O_2, super oxide anion (O_2^-) and hydroxyl radicals (OH).

Radiation treatment causes cytoreduction not only in tumour tissue but also in healthy normal tissues that lie within the radiation field. Death of rapidly dividing

normal cells in renewing tissues causes the acute side effects of radiotherapy and the mostly affected ones are cells of the hematopoietic tissues in bone marrow, hair follicles and oral-gastro intestinal epithelium.

7.3) Chemotherapy

Chemotherapy is based on the toxicity of chemicals on rapidly proliferating tissues of the body. Chemotherapy is effective against cells actively progressing through the cell cycle and these chemicals are generally less effective against the same cells in a quiescent state. In contrast to surgery and radiotherapy, which are local and regional therapies respectively, chemotherapy is a form of systemic therapy because the dose is distributed throughout the body. A great variety of cytotoxic drugs have been developed and all cell cycle phases can be targeted.

Chemotherapeutical drugs cause many side effects like bone marrow suppression, mucositis and hair loss. Some of them cause cumulative dose-dependent toxicities to slowly-proliferating or non-proliferating normal tissues like kidney, liver, nervous system and heart. Cytotoxicity results when exposed cells attempt cell division before repairing the critical damage. Moreover, high cost of chemotherapy reduces the accessibility of this treatment to poor patients.

8) Immunomodulation by Natural products

Immunomodulatory agents can enhance or inhibit the immunological responsiveness of an organism by interfering with its regulatory mechanisms. The modulation of immune response by using medicinal plant products as possible therapeutic measures has become a subject of active scientific investigations (Newman and Cragg, 2007). Ayurveda, a traditional Indian medicine of plant drugs has been successful from very early times in using many plant extracts called 'rasayanas' as potential immunomodulators (Balachandran and Govindarajan, 2005). Immunomodulation, especially using rasayana drugs, could provide an alternative to conventional chemotherapy and radiotherapy under the conditions of impaired immune responsiveness (Praveenkumar *et al.*, 1999). Indian medicinal plants are rich source of

substances that are claimed to induce paraimmunity, the non-specific immunomodulation of granulocytes, macrophages, natural killer cells and complement functions (Davis and Kuttan, 2002a). *Allium sativum* (garlic) was found to enhance immune functions, by stimulating peripheral blood mononucleus cells and antibody producing cells. The aquous extract of whole plant of *Asparagus racemosus* administered orally to experimental animals, protected against exposure to a variety of biological, physical and chemical stresses (Kamat *et al.*, 2000). *Andrographis paniculata* stimulated both humoral as well as cell mediated immune responses in mice models (Sheeja and Kuttan, 2007). *Azadirachta indica* (neem) is a plant with general immunopotentiating ability and its aquous extract was shown to enhance the immune response of BALB/c mice to sheep red blood cells *in vivo*. In another study neem oil enhanced the phagocytic activity of peritoneal macrophages and expression of MHC class–II antigens (Upadhyay *et al.*, 1992).

Curcuma longa (Turmeric) contains several small molecular weight components with antioxidant, medicinal and immunomodulatory activities (Ruby *et al.*, 1995). Curcumin, an antioxidant present in turmeric, has been shown to stimulate immune system in BALB/c mice (Antony *et al.*, 1999). Fruits of *Emblica officinalis* with rich vitamin C have been used in ayurveda as a potent rasayana and also possess potent antioxidant activities (Bhattacharya *et al.*, 1999). Glabridin, an isoflavon isolated from *Glycyrrhiza glabra* root and its derivatives have been shown to inhibit the oxidation of LDL induced by copper ions or mediated by macrophages (Belinky *et al.*, 1998). *Nyctanthes arbor-tristis* was found to increase humoral as well as delayed type hyper sensitivity responses and maximum activity was found in the seeds in which the active principle(s) appear to be mainly associated with lipids. *Piper longum* is a potent immunostimulator, which enhances stem cell proliferation and number of antibody producing cells in spleen (Sunila and Kuttan, 2004). It was also shown to possess radioprotective activity (Sunila and Kuttan, 2005). The active principles from *Tinospora cordifolia* stimulate host immune system by increasing immunoglobulin and blood leukocyte levels and by promoting stem cell proliferation (Mathew and Kuttan, 1999). Tinospora extract may be useful in reducing the chemotoxicity induced by free radical forming chemicals like cyclophosphamide (Mathew and Kuttan, 1997). *Withania*

somnifera has been proved to stimulate the host immune system through enhanced stem cell proliferation. It also stimulates cell mediated immune responses through enhanced NK cell as well as CTL activity (Davis and Kuttan, 2002b). It also reduces cyclophosphamide induced myelosuppression and leucopenia (Davis and Kuttan, 2000a). Orientin and Vicenin, two water soluble flavonoids isolated from the leaves of *Ocimum sanctum* have shown significant protection to the human lymphocyte against the clastogenic effects of radiation, radiation lethality and chromosomal aberrations (Vrinda and Umadevi, 2001). Since these immunomodulators are capable of influencing the biological systems in a multidimensional manner, a systematic approach is needed to employ them as possible therapeutic agents.

9) Chemoprevention by natural products

Cancer chemoprevention, a term coined by Sporn for the protective effects of retinoids (Sporn and Newton, 1979), is defined today as the blocking or suppressing of the carcinogenic process by one or several compounds (Wattenberg, 1985). Several natural products and dietary components have been shown to function as cancer chemopreventive agents. These natural products may disrupt many signaling pathways, including transduction of cell surface (epidermal growth factor) or nuclear (estrogen) receptors via inhibition of their associated tyrosine kinase activities that regulate mitogenic signaling cascades. Alternatively, cytoprotective signal transduction pathways may be activated in a concentration and time dependent manner. People, who have diet consisting of fruits and green-yellow vegetables, have lower risk of many kinds of cancer (Block *et al.*, 1992).

Vinblastin and vincristine were first introduced in the late 1960s and have contributed to long-term remissions and cures with child hood leukemia, testicular teratoma, Hodgkin's disease and many other cancers. Guggulsteron obtained from the gum resin of *Commiphora mukul* was found to suppress inflammation by inhibiting iNOS expression (Meselhy, 2003). Guggulsteron also suppressed the constitutive NF-κB activation expressed in most tumour cells (Shishodia and Aggarwal, 2004).

Curcumin (diferulolyl methane), has been shown to suppress carcinogenesis of the skin, liver, lung, colon, stomach and breast. It has also been shown to inhibit the proliferation of a wide variety of tumour cells in culture and to promote apoptosis through Bid cleavage, cytochrome-c release, caspase-9 activation and then caspase-3 activation. Curcumin mediates this wide variety of therapeutic effects by regulating the transcription factors NF-κB and activator protein, suppressing IκBα kinase and c-Jun N-terminal kinase and inhibiting expression of COX-2, cyclinD1, adhesion molecules MMPs, iNOS, Bcl-2, Bcl-X_L and TNF (Anto *et al.*, 2002). Some synthetic curcuminoid derivatives have also been shown to inhibit tumour specific angiogenesis (Leyon and Kuttan, 2003).

Resveratrol from grapes, berries and peanuts was found to suppress proliferation of a wide variety of tumour cells. It also activates caspases and down regulates the activation of several transcription factors including NF-κB, AP-1 and Egr-1 (Manna *et al.*, 2000). Capsaicin from *Capsicum annum* and Piperine from *Piper longum* are two potent anticancer agents which activate apoptosis in tumour cells (Wolvetang *et al.*, 1996; Pradeep and Kuttan, 2004; Sunila and Kuttan, 2004). [6]-Gingerol and [6]-paradol from ginger (*Zingiber officinalis*) show cytotoxic activity through apoptosis (Lee and Surh, 1998). [6]-Gingerol also inhibits Cox and lipoxygenase activities (Kiuchi *et al.*, 1992).

Intake of certain kinds of polyhydroxy phenols such as flavanoids or lignans in the diet has been correlated with low incidence of colon cancer and breast cancer (Adlercreutz, 1984). Genistein (a hydroxyl isoflavon) is reported to inhibit tyrosine kinase, angiogenesis and cell cycle progression and promote apoptosis (Fotsis *et al.*, 1993). Tangeritin occurring in tangerine peel was found to inhibit leukemia HL-60 cell growth partially through induction of apoptosis (Hirano *et al.*, 1995). Epigallocatechin gallate (EGCG), a polyphenol from green tea, has been shown to stimulate apoptosis of various cancer cell lines such as prostate, lymphoma, colon and lung (Yang *et al.*, 1998, Katiyar, 2006). It also strongly induced DNA fragmentation in PC-9 cells and inhibited TNF-α gene expression (Suganuma *et al.*, 1996).

The medicinal plant *Withania somnifera* is widely known for its anti-inflammatory, cardio active and CNS effects. It is a potent inhibitor of angiogenesis, inflammation, tumour development, metastasis and oxidative stress. Its active principle withanolide (Leyon and Kuttan, 2004) suppressed NF-κB activation induced by a wide variety of inflammatory and carcinogenic agents including TNF, IL-1β, doxorubicin and sigaratte smoke condensate. It also enhanced apoptosis and suppressed invasion. *Viscum album*, a phytopreparation is being used in adjuvant cancer therapy for several years and is reported to stimulate the immune system specifically increasing the number and activity of NK cells and neutrophils (Lee *et al.*, 1998).

A variety of tannins (tellimagrandin II, remurin B, nobotanin A) and lignin-related compounds induce DNA fragmentation in different human myelogenous leukemia cell lines and HL-60 cells. Monoterpenes such as D-limonene and perillyl alcohol have been shown to possess chemopreventive properties against mammary, liver and lung carcinogenesis (Yano *et al.*, 1999). Naturally occurring monoterpenes such as carvone, limonene and perillic acid have been shown to inhibit experimental lung metastasis (Raphael and Kuttan, 2003). Shikonin, a quinine ingredient of *Lithospermum erythrorhizon* induced apoptosis in HL-60 leukemia cell line through the activation of caspase-3 (Yoon *et al.*, 1999). *Thuja occidentalis* has been reported to inhibit experimental lung metastasis (Sunila and Kuttan, 2006). Solamrgine, an alkaloid purified from the Chinese herb, *Solanum incanum* has been observed to induce apoptosis in human hepatocyte (Hep-3B) and normal skin fibroblast cells in culture (Hsu *et al.*, 1996).

Higher intake of vegetables and fruits protects against cancers of the stomach, oesophagus, lung, oral cavity and pharynx, endometrium, pancreas and colon. The types of vegetables and fruits that most often appear to be protective against cancer are raw vegetables, followed by cooked allium vegetables, carrots, green vegetables, cruciferous vegetables and tomatoes. The substances in vegetables and fruits that may help protect against cancer include dithiolethiones, isothiocyanates, indole-3-carbinol, allium compounds, isoflavons, protease inhibitors, saponins, leutine, β-carotene, lycopene etc (Aggarwal *et al.*, 2006).

10) Chemoprevention by naturally occurring Sulfur compounds

Among naturally occurring products, Sulfur containing compounds (OSCs), especially garlic compounds (GCs) and isothiocyanates (ITCs) represent two important and promising chemopreventive families because of their potent chemopreventive effects in various *in vivo* and *in vitro* models.

10.1) Garlic constituents

Garlic, *Allium sativum*, has been well known for its medicinal properties since time immemorial. Egyptian records dating to about 1550 B.C mention garlic as a remedy for a variety of diseases (Block, 1985). Experimentally garlic and its associated Sulfur components are reported to suppress the incidence of tumours of rodent models in breast, colon, skin, uterus, oesophagus and lung (Amagase and Milner, 1993; Wargovich *et al.*, 1988). Epidemiological findings also demonstrated an inverse relationship between garlic consumption and the incidence of stomach cancer, colorectal cancer and prostate cancer (You *et al.*, 1989). Two pathways are involved in the conversion of natural garlic to Sulfur compounds. The first pathway is natural aging bioconversion, which leads to the formation of mainly water soluble Sulfur compounds such as S-allyl cysteine (SAC), and S-allyl mercapto cysteine (SMAC). The second pathway is cell decomposition to allicin, which again breaks down rapidly under uncontrollable chemical reactions to produce odorous oil soluble Sulfur compounds namely Diallyl sulfide (DAS), Diallyl disulfide (DADS), Diallyl trisulfide (DATS) and Ajoene.

Chemopreventive effects of garlic constituents are based on: i) enhancement of the activity of specific mixed–function oxidases that depress the activation of carcinogens (Chen *et al.*, 2003); ii) induction of phase II enzymes which enhance detoxification and excretion of potential carcinogens and reduction of the formation of DNA adducts (Ameen *et al.*, 2003); iii) increased synthesis of GSH, an endogenous tripeptide thiol that directly protects cells from damage by free radicals (Kweon *et al.*, 2003) and iv) induction of apoptosis (Wu *et al.*, 2005).

10.2) Isothiocyanates (ITCs)

Isothiocyanates (ITCs) are hydrolysis products of a group of naturally occurring thioglucoside compounds, glucosinolates, found in cruciferous vegetables such as watercress, brussels sprouts, broccoli, cabbage, horseradish, radish and turnip (Conaway *et al.*, 2002). ITCs inhibit cancer formation or reduced cancer growth in various tissues such as rat lung (Hecht *et al.*, 2002); oesophagus (Stoner and Morse, 1997); liver (Sidransky *et al.*, 1996) and small intestine (Hecht, 1995).

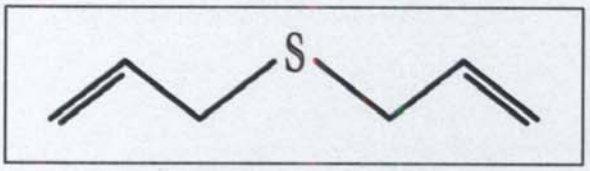
ITCs pertube several steps in carcinogenic process by: i) blocking DNA damage by both inhibition of carcinogen activation through inhibition of Phase 1 enzymes (mainly cytochrome p450) and detoxification of reactive carcinogens through induction of phase II enzymes (glutathione-s-transferase); ii) inhibiting cell growth by cell cycle arrest and iii) removing premalignant and malignant cells through activation of apoptosis (Zhang and Talalay, 1994; Hasegawa *et al.*, 1993; Conaway *et al.*, 2002). Benzyl isothiocyanate, Allyl isothiocyanate (AITC), Phenethyl isothiocyanate and Sulforaphane are examples of ITCs that induce GST and block carcinogenesis.

In the present study, the garlic compounds Diallyl sulfide (DAS) and Diallyl disulfide (DADS), and ITCs Allyl isothiocyanate (AITC), Phenyl isothiocyanate (PITC) and Sulforaphane (Figure 1.5) have been analyzed for their antiangiogenic and apoptosis inducing properties. The immunomodulatory as well as antimetastatic activity of Sulforaphane also has been studied in detail.

Diallyl sulfide (DAS), a flavour component derived from fresh garlic, has been shown to protect against chemically induced toxicity and carcinogenesis in animals. Modulation of the metabolism of the carcinogen by DAS is considered as one of the possible mechanisms for protection against cancers. Several studies showed that DAS could modulate hepatic drug-metabolizing enzymes (Wargovich *et al.*, 1992). DAS has been shown to inhibit N-nitroso dimethyl-amine induced immunosuppression in BALB/c mice (Jeong and Lee, 1998).

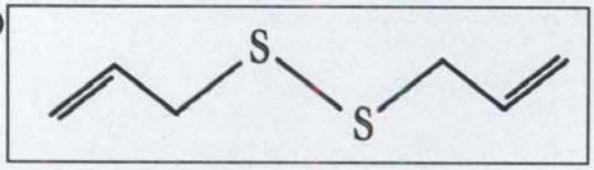
Figure 1.5. Structure of Sulfur compounds

a)



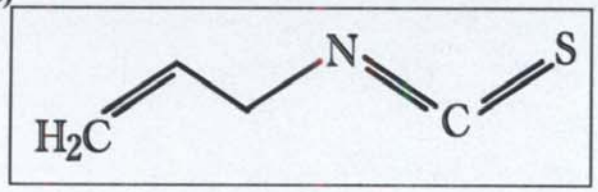
Diallyl sulfide (DAS)

b)



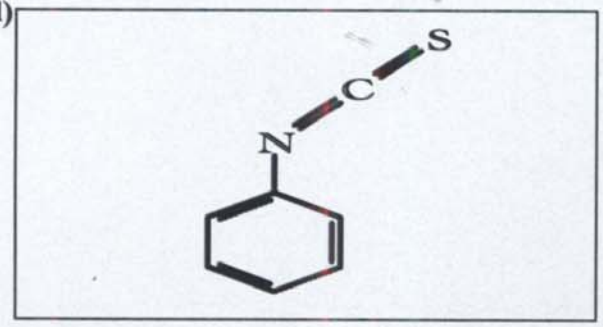
Diallyl disulfide (DADS)

c)



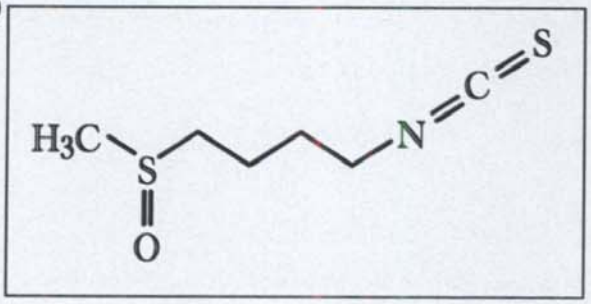
Allyl isothiocyanate (AITC)

d)



Phenyl isothiocyanate (PITC)

e)



Sulforaphane

Diallyl disulfide (DADS) is an oil soluble Sulfur compound from garlic that is produced as a result of decomposition of allium. About 60% of garlic oil was reported to be DADS, indicating that it is the most prevalent oil soluble garlic constituent. Studies conducted by Sundaram and Milner proved that the inhibition of proliferation of human tumour cells by DADS is through alteration in calcium homeostasis (Sundaram and Milner, 1996). Studies also revealed that oil soluble organosulfur compounds in garlic were more effective in inhibiting *in vitro* growth of tumour cells while water soluble compounds had little or no inhibitory effect (Sundaram and Milner, 1993). Earlier studies in our laboratory clearly demonstrated the immunomodulatory and antimetastatic activity of DAS and DADS (Kuttan, 2000; Kuttan and Kuttan, 1999). It has been shown that metabolism of DADS leads to the formation of allyl mercaptan, which contributes to the anticarcinogenic effect against N-nitroso diethylamine-induced fore stomach cancer in mice (Sundaram and Milner, 1996).

Allyl isothiocyanate (AITC) which exist in nature as the glucosinolate precursor, sinigrin, has been reported to induce GST activity in the liver and small intestine of the rat. Studies conducted earlier in our laboratory proved the immunopotentiating activity of AITC and Phenyl isothiocyanate (PITC) and found that both these compounds enhanced stem cell proliferation (Manesh and Kuttan, 2003a). These compounds also inhibited pulmonary lung metastasis (Manesh and Kuttan, 2003b). DAS, DADS, AITC and PITC have been reported to reduce cyclophosphamide induced urotoxicity (Manesh and Kuttan, 2003c; Manesh and Kuttan, 2005) and leucopoenia associated with radiation (Manesh and Kuttan, 2006).

Sulforaphane is present in several cruciferous vegetables, but predominantly in broccoli. Previous studies demonstrated that Sulforaphane induces high levels of mammalian phase II enzymes via an antioxidant response element (ARE)-mediated transcriptional activation (Zhang *et al.*, 1992; Talalay *et al.*, 1995; Fahey *et al.*, 1997). In addition, Sulforaphane reduced breast cancer incidence and minimized the size of the tumour in a rat model (Fahey *et al.*, 1997).

Overall it is well established that ITCs and garlic constituents (GCs) affect xenobiotic metabolizing enzymes in such a way that carcinogens are less activated or detoxified and excreted rapidly or that DNA damage is circumvented. Knowledge of mechanism of cancer chemopreventive effect of ITCs and GCs sheds more light not only on the beneficial effects to humans of garlic and cruciferous vegetables, but may also pave the way for the development of GCs and ITCs for dietary supplementation or even cancer therapeutic drugs.

CHAPTER 2

MATERIALS AND METHODS

A. MATERIALS

1) Test compounds

1. Sulforaphane	- Sigma Chemicals, St.Louis, USA
2. Diallyl disulfide (DADS)	- -do-
3. Allyl isothiocyanate (AITC)	- Merck- Schuchardt, Munchen
4. Diallyl sulfide (DAS)	- -do-
5. Phenyl isothiocyanates (PITC)	- Lancaster, Morecambe, England

2) Animals

BALB/c mice and Swiss albino mice were taken from small animal breeding station of Amala Cancer Research Centre. C57BL/6 mice were purchased from National institute of Nutrition, Hyderabad, India. The animals were kept in well-ventilated cages in air-controlled rooms. They were fed with normal mouse chow (Sai Durga Feeds, Bangalore, India) and water *ad libitum*. All the animal experiments were conducted according to the rules and regulations of Animal Ethics Committee, Govt. of India.

3) Cell lines

L929 mouse lung fibroblast cells; B16F-10, a highly metastatic mouse melanoma cells and K-562 leukemic cells were obtained from National Centre for Cell sciences, Pune, India. Ehrlich ascites carcinoma (EAC) cells were obtained from Cancer Research Institute, Mumbai and maintained as ascites tumour in Swiss albino mice. Human umbilical vein endothelial cells (HUVECs) were isolated from the umbilical cord of neonatal according to the protocol of Jaffe *et al* (Jaffe *et al.*, 1973).

4) Chemicals

Casein
Dulbecco's Modified Eagles Medium
Fluid Thioglycollate Medium
L-Glutamine

} Hi Media Laboratories, Mumbai, India

Medium 199 Minimum Essential Medium Trypsin	}	Hi Media Laboratories, Mumbai, India
Foetal Calf Serum	:	Biological Industries, Kibbutz, Israel.
Aprotinin Benzamidine Bromophenol blue Concanavalin – A Collagenase Type VIII (From *Clostridium histolyticum*) Collagen solution Type I (From calf skin) DEPC (Diethylpyrocarbonate) DTT (Dithiotheritrol) EGTA Ethidium bromide ECM-Gel (from Engelbreth Holrn-Swarm mouse sarcoma) Gelatin Type A (from Porcine skin) Guanidinium thiocyanate γ-GT substrate (r-glutamic acid γ-ρ nitroanilide) L-γ Glutamyl p-nitroanilide Glycyl glycine 4, 6 Glucuronic acid lactone		Sigma chemicals, St. Louis, USA

Hydroxyproline	Sigma chemicals, St. Louis, USA
Leupeptin	
MTT (3-[4,5- Dimethyl-2- thiazol]-2,5-diphenyl tetrazolium bromide)	
2-mercaptoethanol	
PMSF	
Sodium acetate	Sisco Research Laboratories, Mumbai, India
Sodium citrate	
Triton X-100	
Acetyl acetone	
Acrylamide	
1, 4- Bis(phenyloxazol-2yl) Benzene (POPOP)	
2,5 Diphenyl oxazole (PPO)	
Folins reagent	
Glucosamine hydrochloride	
HEPES buffer	
N,N-Methylene Bisacrylamide	
Papain (Extracted from Papaya latex)	
Lipopolysaccharide	: Difco Laboratories, USA
^{3}H-Thymidine	BARC, Mumbai, India.
Sodium Chromate ($Na_2Cr^{51}O_4$) (Specific Activity > 50mci/mg)	
Human recombinant FGF-basic	: Pepro Tech, USA.
Human recombinant VEGF	: -do-

Diffquick stain set : Dade behring, USA.

Crystal violet : Romali, Mumbai, India

Trypan blue

DNase, RNase free water

dNTP mixture } Genei, Bangalore, India

Taq DNA polymerase

5) Reagent Kits

BD Mercury Transfactor Kit : BD Biosciences, USA

Cells to cDNA Kit : Ambion Inc, USA

Gelcode Blue Stain Reagent : Pierce Inc, USA

Mouse ELISA Kits of IL-1β, IL-6, TNF-α, GM-CSF : Endogen Inc, USA

Mouse ELISA Kits of VEGF and TIMP-1 : R&D Systems,USA

Mouse Pro-Inflammatory Cytokine Multiplex PCR Kit : Biosource Inc, USA

Mouse quantikine m-RNA kit for VEGF : -do-

6) Tissue Culture Wares

Medium filtering assembly : Millipore, USA

Polycarbonate membrane filter : Whatman, USA

(13 μm, 8 mm, PVP free)

Cellulose syringe filtering apparatus : Sartorius, Germany

Tissue culture flask (25 cm^2)

Tissue culture petri dish (90mm). } Tarson, India

96-well flat bottom culture plate

Blind well chamber (Modified boyden Chamber) : Nucleopore Cambridge, USA

7) Instruments

Automatic Gamma Counter	: PerkinElmer, USA
Rack Beta Liquid Scintillation Counter	: Wallac, Finland
CO_2 Incubator	: Napco, Canada
Deep Freezer	: Remi, Chennai, India
Disc electrophoresis unit	: Balaji Scientific Service Chennai, India
Electronic Balance	: Schimadzu, Japan
ELISA-Reader	: AwarenessTechnology, Gujarat, India
Gel Documentation system	: Vilber Lourmat, France
High speed cooling centrifuge	: Remi, Chennai, India
Inverted Microscope	: Leica, Germany
Lyophilizer	: Labconco Inc, USA
Minicycler – Thermocycler	: MJ Research, USA
Spectrophotometer	: Elico, India
Spinwin Microcentrifuge	: Tarson, India
Submerged electrophoresis unit	: Bangalore, Genei, India
Tissue homogenizer	: Yorco Scientific Industrues, Delhi
Transilluminator	: Vilber Lourmat, France

8) Reagents

a) Phosphate Buffered Saline (PBS)

NaCl	- 8.00 g
KCl	- 0.20 g
$Na_2HPO_4.2H_2O$	- 1.44g

KH_2PO_4 - 0.20g

Distilled Water - 1000ml

pH was adjusted to 7.2 with I N HCl or NaOH

b) Cord buffer

NaCl - 8.176g

KCl - 0.298g

Dextrose - 2.028g

Dissolved in one litre 0.001M phosphate buffer. pH-7.2. Sterilized by autoclaving at 15lbs for 15minutes.

c) PBS-EDTA solution

EDTA - 20mg

PBS - 100ml

Sterilized by autoclave

d) Trypsin solution

Trypsin - 200mg

Glucose - 20mg

PBS-EDTA - 100ml

Sterilized by filtration

e) Alsevier's solution

Dextrose - 2.05g

Sodium citrate - 0.80g

NaCl - 0.42g

Distilled water - 100ml

pH adjusted to 6.1 with 10% citric acid.

f) Griess Reagent

A. 0.1% N- (1-Naphthylethylene diamino dihydrochloride) (NNED)

B. 1% Sulfanilic acid in 5% H_3PO_4

A+B= 1:1

g) Scintillation Fluid

PPO - 2.5g

POPOP - 0.25g

Naphthalein - 100g

Dioxan - 1000ml

Stains

h) Trypan blue - 100mg

Saline (0.9%) - 100ml

Trypan blue was dissolved in saline by overnight stirring. Any suspended particles were removed by filtration

i) Eosine - 500mg

Ethanol - 100ml (Final volume)

j) Harris haematoxylin

Haematoxylin - 5g

Ethyl alcohol - 50ml

Potassium alum - 50mg

Potassium iodide - 50mg

Distilled water - 950ml

Haematoxylin was dissolved in alcohol using gentle heat. The alum was dissolved in distilled water by heating with frequent stirring and keep overnight at 4^0C. Alcoholic haematoxylin was added to the alum solution. The mixture was cooled and potassium was added and filtered.

k) Crystal violet

Crystal violet - 50mg

Methanol - 20ml

Distilled water - 80ml

B. Methods

1) Tissue culture

I.1) Sterilization of glass wares

All glass wares and filtration apparatus used for tissue culture purposes were soaked in a solution of Extran (1%) overnight, cleaned using brush and washed

thoroughly under running water. All the glass wares were rinsed in distilled water and dried in a hot air oven. These were then autoclaved at a pressure of 15 pounds/square inch for 15 minutes, dried and used for experiments.

I.2) Preparation of culture media

DMEM (9.98g/l), MEM (10.3g/l), Medium 199 (12.45g/l) and RPMI (10.3g/l) were prepared in autoclaved double distilled water; pH adjusted to 7.2 using sodium bicarbonate; supplemented with L-glutamine (2mM) and filtered under negative pressure using a 0.22μm cellulose filter. For culture of normal cells such as endothelial cells, an antioxidant – HEPES (10mM) was also supplemented in the medium. Sterility of the medium was tested using fluid thyoglycollate medium. For this 1 ml of the filtered medium was inoculated into 10 ml of sterile thioglycollate (29.96g/l) and incubated at 37°C for 7 days and checked for visible contamination. Broad spectrum antibiotics such as penicillin (100units/ml) and streptomycin (100μg/ml) and foetal calf serum (10%) were added to the medium prior to use.

1.3) Maintenance of L929 cell line in tissue culture

The spent medium was removed from the confluent bottles and the cells were washed thrice with 2ml of PBS-EDTA. 1 ml of trypsin solution containing 0.02% EDTA was added and incubated for 3-4 minutes at 37^0C and the bottles were tapped to dislodge the cells. MEM (5ml) containing 10% goat serum and antibiotics (complete medium) was added. Cells were dispersed to single cell suspension by repeated pipetting and an aliquot of cell suspension was added to fresh tissue culture bottles containing 10ml of complete medium and incubated at 37^0C. Cells were subcultured every week.

1.4) Maintenance of K-562 cell line in Tissue culture

The cell suspension was mixed well, dispersed the clumps by repeated pipetting. The cells were counted and $1x10^6$ cells were seeded to fresh bottles containing 10 ml of RPMI-1640 medium with 10% FCS and antibiotics and incubated at 37^0C and subcultured every third day.

1.5) Maintenance of B16F-10 cell line in tissue culture

The spent medium was removed from confluent bottles and the cells were washed three times with PBS. 1ml trypsin solution free of EDTA was added and incubated for 3-4 minutes at 37^0C. Bottles were then tapped to dislodge the cells. DMEM containing 10% FCS and antibiotics (complete medium) was added and the cells were dispersed to single cell suspension by repeated pipetting. An aliquot of the cell suspension was added to fresh bottles containing 10ml of complete medium and incubated at 37^0C. The cells were subcultured every week.

1.6) Isolation and maintenance of Human Umbilical Vein Endothelial Cells (HUVECs)

Endothelial cells were collected from human umbilical cord vein, by the protocol proposed by Jaffe *et al* (Jaffe *et al.*, 1973). A sterile technique was followed in all manipulations of the cord. The cord was served from the placenta soon after birth, placed in a sterile container filled with cord buffer, and held at 4^0C until processing. Storage time averaged about 1h, and cords were discarded if held more than 3h. The cord was inspected and all areas with clamp marks were cut off. The umbilical vein was cannulated with a blunt needle, and the needle was secured by clamping the cord over the needle with an umbilical cord tie. The vein was perfused with 100ml of cord buffer to wash out any blood and allowed to drain, and the other end was also tied. 1-2ml of 0.2% collagenase in cord buffer was then infused into the umbilical vein through the needle and the cord was placed in a water bath containing cord buffer and incubated at 37^0C for 15minutes. After incubation, the collagenase solution containing the endothelial cells was flushed from the cord by perfusion with 30ml of cord buffer. The effluent was collected in a sterile centrifugal tube containing medium 199 with 20% FCS. The cells sedimented at 250g for 10minutes and the cell pellet were suspended by trituration in 5ml of fresh medium. The cell suspension was then added to gelatin pre-coated tissue culture flask. The flasks were then incubated at 37^0C under 5% CO_2.

The cells were fed twice a week with a complete change of fresh culture medium. Endothelial cells were cultured in medium 199 containing FCS (20%), penicillin (100U/ml), streptomycin (100µg/ml), L-glutamine (2mM), HEPES (10mM) and VEGF

(2ng/ml). On reaching confluence the cells were subcultured by conventional trypsinisation. These cells were used for experiments from 3rd passage to 6th passage.

1.7) Preparation of cells for *in vitro* studies

For experiments 70-80% confluent cultures were used .Monolayer cells were washed three times with PBS and the cells were harvested by mechanical dislocation using a cell scraper. Cell number was adjusted and the viability was checked by trypan blue exclusion method (Kuttan *et al.*, 1985). Cell suspension with more than 95% viability was used for experiments.

1.8) Determination of cell viability

Cell viability was determined by trypan blue dye exclusion method (Kuttan *et al.*, 1985). 0.1 ml of cell suspension was mixed with 0.1ml of 1% trypan blue, kept for 2-3 minutes and loaded on a haemocytometer. Viable cells exclude trypan blue dye, while non-viable cells take up the dye and thus appeared blue in colour. The number of stained and unstained cells was counted separately.

$$\% \text{ Dead cells} = \frac{\text{Number of dead cells}}{\text{Number of viable cells} + \text{Number of dead cells}} \times 100$$

1.9) Long term *in vitro* cytotoxicity studies in tissue culture

Cells growing in log-phase were used for this experiment. Cells were seeded in 96-well flat bottom tissue culture plate (5000cells/well) containing 200µl complete medium and incubated for 24h at 37°C with 5% CO_2 atmosphere. After incubation, various concentrations of the test compounds were added to the wells and the incubation was continued for 48h. Before 4h of the completion of incubation, 20µl of MTT (5mg/ml) was added to each well. After the incubation period the plates were centrifuged, supernatant was removed and 100µl of DMSO was added to each well. The plates were then incubated at room temperature for 15 minutes and the optical density was measured at 570nm with reference of 690nm (Cole, 1986; Campling *et al*, 1991). The percentage of dead cells was determined using the formula,

$$\% \text{ Viable cells} = \frac{\text{OD of drugs treated}}{\text{OD of control}} \times 100.$$

2) Maintenance of experimental animals

C57BL/6 mice, BALB/c mice and Swiss albino mice were used for the experiments. They were housed in ventilated cages and fed with pelleted mouse chow (Sai feeds, Bangalore) and water *ad libitum*.

3) Maintenance of tumours in animals

B16F-10 melanoma cells were propagated in C57BL/6 mice as transplantable solid tumours. $1x10^6$ cells were injected subcutaneously to the hind limb of the mouse. After 10-15 days, the animal was sacrificed, tumour mass mashed and processed in PBS. $1x10^6$ viable cells were injected to another set of animals.

4) Hematological parameters

4.1) Determination of Haemoglobin (Cheesbrough and McArthur, 1976)

Principle

Ferricyanide forms methaemoglobin with haemoglobin, which is converted to cyanmethaemoglobin by cyanide, which has absorption at 540nm.

Procedure

0.02 ml of blood was mixed with 5ml of Drabkin's reagent and allowed to stand for 5minutes at room temperature. Optical density (OD) was measured against reagent blank. Haemoglobin content was calculated using the formula,

$$\text{gm \% of Hb} = \frac{\text{OD of the test}}{\text{OD of Std.}} \times \frac{251 \times \text{conc. of Std.}}{1000}$$

4.2) Determination of total count of leukocytes (Cheesbrough and McArthur, 1976)

Principle

The cells were diluted in Turk's fluid, which contains a weak acid (acetic acid) to lyse RBC and a stain (crystal violet), for staining the leukocytes. The number of cells in the large four corner squares was counted.

Procedure

Blood (0.02ml) was mixed with 0.38ml of Turk's fluid and kept at room temperature for 2-3 minutes. The cells were mixed gently and loaded on to the haemocytometer, allowed to settle at the bottom of the chamber for 2 minutes and counted under a microscope using 10X objective. The total WBC counts was determined using the formula,

$$\text{Total leukocyte counts/mm}^3 = \frac{\text{No. of cells counted x dilution factor x depth factor}}{\text{Area counted}}$$

where,

Dilution factor = 1/20

Depth = 1/10mm

Area counted = 4sq.mm

$$\text{Therefore, Total leukocyte counts/mm}^3 = \frac{\text{N x 20 x 10}}{4} = \text{N x 50}$$

4.3) Differential count of leucocytes (Cheesbrough and McArthur, 1976)

Procedure

A thin film of blood was made by spreading a drop of blood evenly across a clean glass slide using a glass spreader and air dried. Few drops of Leishman's stain was poured over the smear and kept for 3minutes. The stain was diluted with distilled water and kept for minutes, washed with tap water and allowed to air dry. Various types of cells were scored using the morphology under oil immersion with 100X objective and a total of 100 cells were counted.

5) Immunological parameters

5.1) General techniques

a) Collection and preparation of SRBC (Mehera and Vaidya, 1993)

Sheep blood was freshly collected from the slaughter house in equal volumes of sterile Alsever's solution and stored at 4^0C for not more than one week. Cells were

washed three times with PBS (pH 7.2). Supernatant was discarded and the pellet was suspended in Hanks balanced salt solution (HBSS).

b) Trypsinization of SRBC

10 parts of 4% SRBC and one part of 1%trypsin solution were incubated at 37^0C for 30 minutes. After incubation cells were washed twice in PBS (pH 7.2) and resuspended at a concentration of 2%.

c) Preparation of anti SRBC antibody

A young healthy rabbit was injected intradermally with 2% SRBC in saline which was mixed with Freunds complete adjuvant in a ratio of 1%. A booster dose was given four weeks after the initial dose. Next day after the booster dose, blood was collected and serum separated; heat inactivated and checked the antibody titre by the haemagglutination method (Singh *et al.*, 1984). According to the antibody titre value, serum was diluted and used for the experiments.

d) Preparation of spleen cells

All the procedures were done under sterile condition. The animals were sacrificed, an incision was made on the left side just below the rib and spleen was removed with out any adherent tissue. Spleen was cut into small pieces and teased over a stainless steel mesh in cold PBS or HBSS. Clumps were allowed to settle in a centrifuge tube, kept in ice bath for 2minutes. Supernatant was collected, washed three times with HBSS and resuspended in RPMI-1640 medium at required concentrations.

e) Preparation of bone marrow cells

All the procedures were done under sterile conditions. Mice were sacrificed by cervical dislocation and fixed on a board with fore and hind limbs fully stretched. The skin and flesh overlying the limbs were removed and the femur was exposed. The shaft of the femur was separated from both ends and the bone marrow was flushed out of the cavity by passing a jet of medium with 2% FCS through the ends of the shaft using a 26G needle and syringe. Flushed bone marrow was made into a single cell suspension

by repeated pipetting. It was then centrifuged and suspended at required cell concentrations in RPMI-1640 medium.

f) Preparation of thymus cells

All the procedures were done under sterile conditions. Animals were sacrificed by cervical dislocation. The skin was cleaned and body was incised at the upper part above the heart. Bilobed thymus was detached, suspended in HBSS, and processed the same way for spleen and the thymocytes were suspended in RPMI-1640 medium containing 10%foetal calf serum.

g) Preparation of peritoneal macrophages

Peritoneal macrophages were elicited by injecting 0.2ml of 5% sodium caesinate solution. After five days animals were sacrificed by cervical dislocation. All the procedures were done under sterile conditions. Mice were fixed on a board, skin was removed and the peritoneum was exposed. The peritoneal cavity was distended by injecting 5ml of PBS or HBSS. The peritoneal cavity was gently prodded and the peritoneal fluid containing macrophages was aspirated. The cells were washed and suspended in RPMI-1640 to the desired cell concentrations.

5.2) Determination of circulating antibody titre (Singh *et al.*, 1984).

Principle

The non-agglutinated SRBC will settle to the bottom of the well as a clear 'button' while agglutinated cells settle as a diffused 'mat'. The maximum dilution of anti-sera at which clear agglutination observed gives the titre of the antibody

Procedure

Anti-sera (0.1ml) were serially diluted in round bottom 96-well tissue culture plates containing 0.1ml PBS/well (pH 7.2). 0.1ml of trypsinized SRBC was added to each well, mixed gently and incubated at room temperature for 3hours. The dilution at which clear agglutination seen was noted.

5.3) Determination of antibody forming cells (Jerne and Nordin, 1963)

Principle

Antibody produced by the lymphoid cells from animals immunized with SRBC cause lysis of red cells in its vicinity (plaques) in a semi-solid support in the presence of complement

Procedure

0.5 ml of Agarose (0.5%) was distributed into tubes kept at 45^0C and 0.05ml SRBC (7%) and 0.05ml spleen cells ($8x10^6$ cells/ml) were added and mixed well. The contents were poured over a glass slide, spread in an area of $10cm^2$ and the gel was allowed to solidify. The slides were kept in an incubation rack filled with fresh rabbit serum (1:10 diluted with PBS, pH 7.2) as a source for complement and incubated for 1hour at 37^0C. The number of plaques were counted using a colony counter and represented as plaque forming cells/10^6 spleen cells.

5.4) Assay for lymphocyte, thymocyte and bone marrow cell proliferation

Principle

Mitogens can stimulate resting lymphocytes to undergo a series of biochemical and physical changes and are converted to blast-like cells. This process leads to cell division and can be quantitated by ^{3}H thymidine incorporation assay.

Procedure

All the techniques were sterile during the experiment. Spleen cells/thymus cells/bone marrow cells ($5x10^4$) were incubated with and with out mitogens in a final volume of 200µl of RPMI-1640 medium in 96-well flat bottom titre plates supplemented with 10% FCS and antibiotics in a humidified atmosphere containing 5% CO_2 at 37^0C for 48 h. The concentrations of the mitogens added were; PHA-2.5µg/ml; Con-A-10µg/ml; PWM- 10µg/ml and LPS-10µg/ml. 1µCi of ^{3}H thymidine was added to each well and incubated further for 18 h under the same conditions. After incubation, DNA was precipitated using 10% perchloric acid and pellets were dissolved in 0.5 ml of 0.5N NaOH. Then the contents were transferred to 5ml scintillation fluid, kept

overnight in dark. Counts per minutes (CPM) was measured in a liquid scintillation counter.

5.5) ^{51}Cr-release assay (Kim *et al.*, 1980)

^{51}Cr-release assay was used to determine the cytotoxicity mediated by immune effector cells such as natural killer cells and cells expressing Fc receptors (ADCC) and was performed in round bottom titre plates.

Principle

^{51}Cr binds to cytoplasmic proteins after diffusing through the cell membrane and is released only when the cell membrane is sufficiently damaged.

Labeling of target cells

The target cells, K-562 (10^6) and SRBC (10^7) were washed twice in RPMI-1640 and were resuspended in few drops of FCS. 100μCi of $Na_2{}^{51}CrO_4$ was added and incubated at 37^0C for I h on a shaker. The cells were washed in medium twice and allowed to incubate in large volumes (5ml) of medium for 1h at 4^0C. Cells were washed twice in medium and resuspended in complete medium at a concentration of $1x10^5$ cells/ml.

a) Determination of Natural Killer cell- mediated cytotoxicity

Labeled target cells (K-562, 0.1ml) and equal volumes of various dilutions of spleen cells (to yield effector: target ratios of 100:1, 50:1, and 25:1) were added to 96-well round bottom titre plates. Final volume was adjusted to 0.2ml with RPMI-1640 supplemented with 10% FCS and incubated at 37^0C for 4 h. Titre plates were centrifuged for 15 minutes, supernatant (100μl) was collected and radioactivity measured in a gamma ray spectrometer.

The following control tubes were kept along with each experiment.

Spontaneous release (SR) - wells contained only target cells and medium.

Total release (TR) - wells contained target cells, medium and 0.1 ml of 1N HCl.

Calculations

$$\% \text{ Lysis} = \frac{\text{Experimental release- Spontaneous release}}{\text{Total release - Spontaneous release}} \times 100$$

b. Determination of antibody-dependent cellular cytotoxicity (ADCC)

0.1ml of labeled SRBC (target cells) and 0.1ml of spleen cells (effector cells) were added to get effector-target ratios of 100:1, 50:1 and 25:1.

0.05ml of anti-sera against SRBC was added and incubated at 37^0C for 4h. The final volume was made up to 0.2ml with complete medium and the 4 h ^{51}Cr release assay was performed as explained above.

c. Determination of antibody-dependent complement-mediated cytotoxicity

Principle

When tumour cells are incubated with specific antibodies in presence of complement, the classical pathway will be activated leading to the lysis of target cells

Procedure

Antiserum was diluted in RPMI-1640 to get 1:1, 1:2 and 1:4 dilutions of the antibody and 0.1 ml of the serum was mixed with 10^6 EAC cells. 0.05ml of 1:10 diluted fresh rabbit serum as a source of complement was added and the final volume was made up to 2ml and incubated at 37^0C for 3h. The cells were centrifuged and 1 ml of the supernatant was discarded and cytotoxicity was assessed by trypan blue exclusion method (Kuttan *et al.*, 1985).

5.6) Determination of α-naphthyl acetate esterase activity (Bancroft and Cook, 1984)

The enzyme hydrolyses the substrate to form an invisible primary reaction product. The complex is coupled with the diazonium salt to produce coloured final reaction product.

Procedure

Animals were sacrificed by cervical dislocation. Skin and flesh removed from the thigh of the animal and femur bone was taken out. Bone marrow cells were flushed out from femur into PBS containing 10% goat serum using a syringe. Cells were counted and thin smears prepared on glass slides. The smear was air dried and fixed using 37% formaldehyde. Slides were incubated in a reaction buffer containing pararosaniline, sodium nitrate and α-naphthyl acetate at room temperature. Smear slides were counterstained with haematoxylin for 2 minutes. α–esterase positive cells take up a yellowish brown colour and cells were counted under microscope using oil immersion.

6) Antiangiogenic studies

6.1) Antiangiogenic studies *in vivo*

The antiangiogenic activities of the test materials were studied in C57BL/6 mice. Angiogenesis was induced by injecting $1x10^6$ B16F-10 melanoma cells intradermaly on the shaven ventral skin of the mice. The angiogenesis induced animals were sacrificed on 9^{th} day after tumour inoculation. The ventral skin was cut removed, washed in PBS and the number of tumour directed capillaries per cm^2 around the tumour was counted using dissection microscope (Kishi *et al.*, 2000).

Blood of these angiogenesis induced animals were collected at two time points viz. day 1 and day 9, from the caudal vein in an aseptic manner. Serum was separated by centrifugation and used for cytokine profiling. Cytokines such as IL-1β, IL-2, IL-6, TNF-α, GM-CSF, VEGF and an endogenous inhibitor of MMPs- TIMP-1 were assayed using respective ELISA kits.

6.2) Antiangiogenic studies *in vitro*

6.2.1) Rat aortic ring assay

Rat aortic ring assay was used as the *in vitro* angiogenesis study model. Dorsal aorta from a freshly sacrificed Sprague Dawley rat was cut removed in a sterile manner, rinsed in ice cold PBS to remove blood and any membranous tissue. It was then cut into ~1mm thick transverse sections using surgical blades. Each segment was placed in a collagen pre-coated 96-well tissue culture plate. The rings were incubated for 24 h at

37°C in medium, afterwards exchanged for conditioned medium from B16F-10 melanoma cells. The rings were further incubated for six days and then analyzed by phase contrast microscopy for any microvessel out growth from the aorta and photographs were taken.

6.2.2) Quantitation of gene specific mRNA of VEGF in B16F-10 melanoma cells

Quantikine mRNA is a novel method, which can be used to quantitate gene specific mRNA at lower levels. Briefly, B16F-10 cells (10^6 cells) were plated in 30mm petridish in DMEM with 10% FCS at 37^0C in 5% CO_2 atmosphere. Cells were then treated with test compounds (5μg/ml) for 4 h. After incubation, the cells were washed and mRNA preparations were made according to the manufacturer's procedures. mRNAs were hybridized with gene specific biotin labeled detection probes and digoxigenin alkaline labeled detection probes in a microplate. Hybridization solution was transferred to a streptavidin coated microplate and the mRNA probe hybrid was captured. Following wash to remove the unbound conjugate, a substrate solution was added. An amplifier solution was then added and the developed colour was measured spectrophotometrically at 490nm.

6.2.3) *In vitro* studies using HUVECs

a) Endothelial cell motility assay (Guo *et al.*, 2002)

Endothelial cells (HUVECs) were grown to confluence in 96-well tissue culture plates coated with gelatin. A clear area was then scraped in the monolayer with a 20μl yellow tip by applying suction and fresh medium along with or without the test compounds were added. The culture were further incubated for 24h and then fixed using methanol and stained with crystal violet. Migration of cells into the wounded area was evaluated with an inverted microscope and photographed.

b)Tube formation assay (Gupta *et al.*, 2002)

ECM gel (25μl) was added to 96-well plate and incubated at 37°C for 30 minutes. Endothelial cells were added to the solidified gel and further incubated for 48h in 5% CO_2 atmosphere in 199 medium supplemented with 2ng/ml VEGF and 2ng/ml

FGF. Various concentrations of test compounds were added and along with this three wells were kept without any treatment and was the control. Supernatant was then removed, cells were fixed and stained using Diff-Quick stain set. Tube formation was examined and the area of the capillary –like structure formed was photographed using an inverted microscope.

7) Antimetastatic studies

7.1) *In vivo* antimetastatic studies

7.1.1) Determination of the metastatic potential of B16F-10 melanoma cells in animal model

Studies on the metastatic ability of tumour cells (*in vivo*) were done in C57BL/6 mice. Pulmonary colony forming ability of B16F-10 cells was carried out as described by Fidler *et al* (Fidler *et al.*, 1978). C57BL/6 mice were injected with B16F-10 cells ($1x10^6$) through the lateral tail vein. Animals were sacrificed on 21st day. Metastasis of the lungs were determined by counting the metastatic foci on the surface of the lungs; measuring biochemical parameters such as lung collagen hydroxyproline, lung uronic acid, lung hexosamine, serum sialic acid, and serum γ-glutamyl transpeptidase ; histopathological analysis of lungs and determining the rate of survival.

a) Biochemical parameters

a.1) Estimation of protein (Lowry *et al.*, 1951)

Principle

This assay relies on the formation of protein copper complex and reduction of Phosphomolybdate-Phosphotungstate reagent (Folin Ciocaltaeu reagent) by tyrosine and tryptophan residues of protein

Reagents

Solution A

Sodium potassium tartarate - 1ml (2%)

$CuSO_4$ - 1ml (1%)

Na_2CO_3 - 98ml (2% in 0.1N NaOH)

Solution B

Folin's phenol reagent - 1N, diluted 1:1 with distilled water

Procedure

20µl sample and different concentrations of standard BSA (150µg, 100µg, 50µg and 25µg) were made up to 1.2ml with distilled water. To this, 6ml of solution A was added and then incubated at room temperature for 10minutes. 300µl solution B was then added to the vortex mixed reaction mixture, incubated at room temperature for 30minutes. Optical density read at 660nm.

a.2) Estimation of Hydroxyproline (Bergman and Loxley, 1970)

Principle

Hydroxyproline present in samples were oxidized by chloramines T. The coloured product is more stable in the presence of high concentrations of isopropanol.

Reagents

1. Oxidant solution

Sodium acetate	- 5.7g
Trisodium citrate	- 3.75g
Citric acid	- 0.55g
Isopropanol	- 38.5ml
Distilled water	- 61.5ml

2.Ehrlisch's reagent

p-dimethyl amino benzaldehyde	- 4.4g
Perchloric acid	- 10.2g (60%)
Isopropanol	- 25ml (Final volume)
3. Chloramine T	- 1.75g/25ml oxidant solution prepared on the day of use

Procedure

Lung tissue (1g) was homogenized using 4ml isotonic saline and hydrolyzed in 6 N HCl. The tubes were sealed and incubated at 110^0C for 24 h. 1ml hydrolysate was

neutralized with KOH then make up to 5ml with H_2O. To 0.5ml neutralized sample 2.5ml isopropanol and 1ml oxidant solution was added by mixing and kept at room temperature for 4 minutes. 2ml Ehrlisch's reagent was added to the tubes and incubated at 60^0C in water bath for 21minutes. Then the tubes were kept at room temperature for 1h. The absorbance was taken at 560nm.

a.3) Extraction and estimation of uronic acid

Extraction of uronic acid from the tissue was carried out according to the method of Schiller *et al* (Schiller *et al.*, 1961). Digestion of the tissue was carried out with crude papain (10mg/g dry weight of tissue) in 5ml of 0.5M acetate buffer of pH 5.5 containing 0.005 M cysteine and 0.005 M disodium salt of EDTA at 65^0C for 24 h. An aliquot of the sample containing approximately 5-15 mg uronic acid was taken for estimation. Uronic acid was estimated by the method of Bitter and Muir (Bitter and Muir, 1962).

Procedure

Aliquote (5ml) of sulphuric acid reagent (prepared with 0.025M sodium tetraborate in conc. H_2SO_4) was taken in tubes and cooled at 4^0C for some time. 1ml of sample or standard glucaronolactone solution containing 5-20mg was layered on the acid. Tubes were closed with ground glass stoppers and the rack was shaken first gently and then vigorously. Tubes were kept in a boiling shaking-water bath for 10minutes and cooled at room temperature. 0.2ml of carbazole reagent (0.125% carbazole in absolute alcohol) was added and the tubes were shaken heated in a boiling water bath for 15minutes and cooled. The pink colour thus developed was read at 530nm.

Uronic acid content of the tissues were expressed as μg/100mg wet weight.

Reagents

Sulphuric acid reagent	- 0.952g sodium tetraborate in 100ml of Con.H_2SO_4 (0.025M)
Carbazole reagent	- 0.125g carbazole in 100g absolute alcohol.
Acetate Buffer (0.1M)	- Solution A. 0.2M solution of acetic

acid + Solution B. 0.2M solution of sodium acetate.

a.4) Estimation of Hexosamine (Elson and Morgan, 1933)

Lyophilized tissue samples (5mg) were hydrolyzed with 2N HCl (5ml) at 100^0C for 6h. HCl was then removed by evaporation; the residue was dissolved in water and made up to a known volume.

Procedure

Aliquots containing 10-15µg hexosamine were treated with 1ml of freshly prepared 2% acetyl acetone in 0.5N Na_2CO_3 in capped tubes and kept in boiling water bath for 15minutes. After cooling in tap water, 5ml of 95% ethanol and 1ml of Ehrlisch's reagent (1.33% p-dimethylaminobenzaldehyde in 1:1 ethanol: Conc.HCl mixture) were added and mixed thoroughly. The purple red colour developed was read after 30minutes at 530nm. Water blank and standard glucosamine solutions of various concentrations were also treated similarly to get a standard curve.

Hexosamine contents of tissues were expressed as µg/100mg dry weight.

Preparation of 2% acetyl acetone in 0.5M Na_2CO_3.

2ml of acetyl acetone in 100ml of 0.5M Na_2CO_3.

Preparation of Ehrlisch's reagent

Dissolve 1.33g of p-dimethyl aminobenzaldehyde (PDAB) in 100ml of 1:1 ethanol: conc. HCl.

a.5) Estimation of protein bound serum sialic acid (Skoza and Mohos, 1976)

Principle

Acid hydrolysis of serum for liberation of sialic acid forms a coloured compound with thiobarbituric acid.

Reagents

1. H_2SO_4 - 0.2N

2. Periodic acid - 25μM in 62.5 mM H_2SO_4

3. Sodium arsenite - 0.2% in 0.5M HCl

4. Thiobarbituric acid - 6% (pH 9.0)

5. Dimethyl sulphoxide

Procedure

200μl of sample was mixed with equal volume of 0.2N H_2SO_4 and hydrolyzed for 1h at 80^0C. To this hydrolysate 50μl periodic acid (25μM) was added and incubated for 30minutes at 37^0C. To this reaction mixture 50μl of sodium arsenite was added, followed by 100μl of thiobarbituric acid and was heated in a boiling water bath for 7.5minutes. After heating, 400μl of DMSO was added to intensify the colour and read at 549nm and 532nm

a.6) Estimation of γ-glutamyl transpeptidase (Szasz, 1976)

Principle

γ-glutamyl transpeptidase catalyses the transfer of γ-glutamyl moiety of a γ-glutamyl donor to a variety of acceptors.

2γ- (glutamyl-p-nitroanilide) ⟶ γ-glutamyl- γ-glutamyl-p-nitroanilide + acceptor ⟶ γ- glutamyl acceptor + p-nitroaniline

Reagents

L- γ-glutamyl-p-nitroanilide - 2.5mM

Glycyl glycine - 20mM

Tris-HCl (pH 8.0) - 0.05M

NaCl - 75mM

Procedure

The standard assay mixture contained (1ml) 0.05M Tris HCl, 75mM NaCl, 2.5mM/L- γ-glutamyl-p-nitroanilide and 20mM glycyl glycine along with 25μl sample.

The rate of release of p-nitroaniline was measured at an optical density of 410 nm using a spectrophotometer.

b) Histopathological Analysis

The tissue was fixed in 10% neutral formalin for at least 4h. The tisues were dehydrated in alcohol series, cleaned in xylene and embedded in paraffin. About 5-6μm thick sections were taken on a glass slide and stained with haematoxylin and eosin and visualized under the microscope for histological changes (Culling, 1976)

c) Determination of the rate of survival

Animals were injected with B16F-10 melanoma cells ($1x10^6$) intravenously. The mortality of the animals was noted and the percentage increase in life span (% ILS) was calculated from the formula,

$$\%ILS = \frac{T - C}{C} \times 100$$

where, 'T' is the number of days the drug treated animals survived and 'C' is the number of days the control animals survived.

7.2) *In vitro* antimetastatic studies

7.2.1) Collagen matrix invasion assay (Albini *et al.*, 1987)

Invasion of collagen matrix by tumour cells was carried out using modified Boyden chambers (Blind well chambers) as described by Albini *et al* (Albini *et al.*, 1987).The lower compartment of the chamber was filled with serum free DMEM and a polycarbonate filter coated with 25μg Type I collagen was placed above this. B16F-10 melanoma cells (10^5 cells/150 μl DMEM) were then seeded on to the upper chamber in the presence and absence of different concentrations of test compounds and incubated at 37^0C in 5% CO_2 atmosphere for 10 h. After the incubation period medium from the upper chamber was removed and the cells on the upper side of the filter was removed by a cotton swab. The filter was then fixed in methanol for 1minute and stained for 3minutes with crystal violet. Cells migrating to the lower surface of the polycarbonate

filters were counted in 10 fields under a microscope. Results were calculated as % inhibition of invasion using the formula,

$$\% \text{ inhibition of invasion} = 100 - \left(\frac{\text{mean no. of migratory cells in test}}{\text{mean no. of migratory cells in control}} \right) \times 100$$

7.2.2) Tumour cell motility assay

B16F-10 cells ($1x10^5$/0.15ml) were seeded on the upper compartment of blind well chamber, containing polycarbonate filter without collagen coating. Chambers were incubated at 37^0C for 24 h. Migrated cells were collected from the lower chamber and counted using a haemocytometer. Results were calculated as,

$$\% \text{ Motility} = \frac{\text{Mean no.of migrating cells in test}}{\text{Mean no.of migrating cells in control}} \times 100$$

7.2.3) Tumour cell adhesion assay (Inokuchi *et al.*, 1991)

Principle

Transformed cells have higher adhesive attachment rates to a variety of homotypic or heterotypic cell susbstrates. Metastatic cells are always found to have higher rates of homotypic attachment (Nicolson *et al.*, 1978).

Procedure

B16F-10 melanoma cells ($5x10^3$ cells/well) were added to 96-well flat bottom titre plates, pre coated with collagen type I (25µg/well) and incubated for 5 h at 37^0C in 5% CO_2 atmosphere. After incubation, medium was removed and the wells were washed with PBS. Adhering cells were fixed with 5% formaldehyde and stained using crystal violet for 20minutes each. The cells were counted under an inverted microscope.

7.2.4) Gelatin Zymography (Billings *et al.*, 1991)

Principle

Proteases of tumour cell lysate were initially resolved on SDS- poly acrylamide gels, which were incorporated with gelatin. Following incubation of the gel in the

activation buffer, protease separated on the gel will breakdown the gelatin and appears as transparent zones or clearings against a dark back ground (upon staining)

Reagents

a) **0.25M sucrose- 0.01M Tris-HCl buffer, pH 7.4**

Sucrose - 85.87g

Tris-HCl - 1.21g

Distilled water - 1000ml (Final volume)

b) **0.1M Tris-HCl, pH-8.0, 10mM $CaCl_2$**

$CaCl_2$. $2H_2O$ - 1.47g

Tris - 12.1g

Distilled water - 1000ml (Final volume)

pH adjusted with Conc.HCl

c) **Trypsin solution**

Trypsin -75µg/ml in 0.1M Tris-HCl, with 10mM $CaCl_2$, pH 8.

d) **Activation buffer (0.1M Tris-HCl, 10mM $CaCl_2$, pH 7.8)**

Tris HCl - 12.1g

$CaCl_2.2H_2O$ - 1.47g

Distilled water - 1000ml (Final volume)

e) **Preparation of gels**

Resolving Gel

11% Polyacrylamide gels with 0.1%SDS and 0.6% gelatin

29.2% acrylamide + 0.5% bisacrylamide - 11ml

0.1M Tris-HCl, pH 8.8 - 1.2ml

20% SDS - 0.15ml

20% Ammonium per sulphate - 0.10ml

Gelatin (180mg/2ml distilled water, heated to dissolve) - 2ml

Distilled water - 6.505ml

TEMED - 0.045ml

Mix and pour at room temperature.

5% Stacking gel

29.2% acrylamide +0.5% bis acrylamide	- 1.67ml
0.1M Tris-HCl, pH 8.8	- 1.75ml
20%SDS	- 0.10ml
20% Ammonium per sulphate	- 0.10ml
Distilled water	- 6.36ml
TEMED	- 0.02ml

Mix and pour above the resolving gel at room temperature

f) **Sample buffer (2x)**

Glycerol	- 1ml
1M Tris-HCl, pH 6.8	- 0.25ml
20% SDS	- 1ml
Bromophenol blue	- 1.65mg
(Tracking dye)	

Made up to 5ml with distilled water

g) **Running buffer**

Tris base	- 3g
SDS	- 2g
Glycine	- 14.2g

Made up to 1L with Distilled water

h) **2% Triton X-100**

Triton X-100	- 2ml
0.1M Tris HCl, pH 7.8	- 100ml (Final volume)

i) **10mM EDTA solution**

EDTA- Na_2	- 372.24mg
0.1M Tris-HCl, pH 7.8	- 1000ml (Final volume)

Procedure

Gelatin Zymography was followed according to the procedure of Billings *et al* (Billings *et al.*, 1991) with some modification. The medium from sub-confluent (70%)

bottles of B16F-10 tumour cells were removed, cells were then washed with serum free medium and incubated in serum free medium (DMEM) at 37^0C for 24 h.

After the incubation, medium was collected, centrifuged, and supernatant was used for zymographic analysis. After determining the protein concentration, supernatant (equvalent to 50μg protein) containing the proteases were activated with trypsin (75μg/ml, 5μl trypsin solution for 100μg protein) in the presence and absence of test compounds in 0.1 M Tris-HCl, 10mM $CaCl_2$ buffer (pH-8.0) and incubated for 1 h at room temperature.. Trypsin treated and untreated samples (equivalent to 50μg protein) were mixed with an equal volume of 2X sample buffer and loaded on to 0.1%SDS - 11% polyacrylamide gels containing 0.1% gelatin. Electrophoresis was carried out at 4^0C with constant current of 2mA/tube until the tracking dye (Bromophenol blue) reached the periphery of the gels. The gels were then washed with 2% Triton X-100 on a shaker at 20-25^0C for three changes of 30 minutes each, to remove the SDS which could interfere with proteolytic activity. This was followed by 2 h washing with activation buffer and the gels were finally incubated in the same buffer at 37^0C for 18 h. Gels were then fixed and stained with Gelcode Blue stain reagent for 2 h and clear bands were visualized against a dark back ground.

8) *In Vitro* Apoptotic Studies

8.1) Morphological Analysis

To detect the morphological changes, one million B16F-10 melanoma cells were incubated in the presence and absence of test compounds at 37^0C in 5% CO_2 atmosphere for 48 h. The cells were washed thrice with PBS, centrifuged and the cell pellets were suspended in PBS and cell smear was prepared and stained with haematoxylin and eosin. Apoptosis was characterized by the morphological changes such as chromatin condensation, nuclear condensation, cellular membrane blebbing and formation of apoptotic bodies.

8.2) Extraction of DNA for DNA-ladder Analysis

Reagents

a) Cytoplasm Extraction Buffer

Tris-HCl pH 7.5	- 10mM
NaCl	- 150mM
$MgCl_2$	- 5mM
Triton X-100	- 0.5 %

b) DNA Lysis Buffer

Tris-HCl pH 7.5	- 10mM
NaCl	- 400mM
EDTA	- 1mM
$MgCl_2$	- 5mM
Triton X-100	- 1%

c) Tris-EDTA Buffer

Tris-HCl, pH 8.0	- 10mM
EDTA	- 1mM

One million B16F-10 cells were incubated in the presence and absence of test compounds at 37^0C in 5% CO_2 atmosphere for 48 h. The cells were washed thrice with PBS, centrifuged and the cell pellets were incubated with 1 ml of cytoplasm extraction buffer on ice for 20 minutes, and pelleted by centrifugation. The pellet was resuspended in DNA lysis buffer for 20 minutes on ice and then centrifuged at 10,000 rpm for 10min at 4^oC. The supernatant obtained was incubated overnight with RNase (2μg/ml) at room temperature and then with proteinase K (50μg/ml) for 2 h at 37^0C. DNA was extracted using phenol-chloroform (1:1) and precipitated with ice-cold 100% ethanol. The DNA precipitate was centrifuged at 10,000 rpm for 15 minutes and the pellet was air dried and dissolved in 50μl of Tris-EDTA buffer. Purity and quantification of the DNA was carried out by monitoring the ODs at 260 and 280nm. The extracted DNA was resolved on 1.5% agarose gels.

9) Gene Expression Studies

9.1) Cell to cDNA Synthesis

Gene expression analyses were carried out by RT-PCR method. Cells to cDNA^{TM} II kit from Ambion Inc, U.S.A., were used for producing cDNA from mammalian cells in culture without isolating mRNA. The cDNA produced is specifically intended for use in the polymerase chain reaction (PCR). RT-PCR is one of the main methods used for measuring mRNA levels from a small number of cells.

Materials provided in the kit

1 x PBS	- 40ml
Cell Lysis Buffer	- 10ml
DNase I (2 U/µl)	- 200µl
10 X RT Buffer	- 500µl
M-MLV Reverse Transcriptase	- 100µl
RNase inhibitor (10 U/µl)	- 100µl
dNTP (2.5 mM each d NTP)	- 400µl
Oligo $(dT)_{18}$ Primers	- 50µM
Nuclease Free water	- 3.5ml.

Principle

In the cells to cDNA II kit, a crude cell lysate is subjected to RT-PCR without purifying the RNA. Cells from tissue culture were washed in PBS and then heated in cell lysis buffer. This treatment has two important effects. First it raptures the cells, releasing the RNA into the cells lysis buffer. The heating step also inactivates endogenous RNases, protecting the RNA from degradation. Next the crude cell lysate is treated with DNase I to degrade genomic DNA and the mixture is heated a second time to inactivate the DNase-I. At this point the cell lysate is ready for reverse transcription and PCR were carried out using two step RT- PCR strategy.

Procedure

B16F-10 melanoma cells ($2x10^4$cells/well) were seeded in the 96-well flat bottom titre plate using serum free DMEM supplemented with antibiotics (penicillin 100 units/ml and streptomycin 100µg/ml) and incubated for 4 h at 37°C in 5% CO_2 atmosphere. After incubation medium was removed and the cells were washed with ice cold PBS. Ice cold cell lysis buffer (100µl) was added to the cells and incubated for 15 minutes at 75°C in a water bath. The cell lysate was then transferred to 200µl nuclease free microcentrifuge tubes. To this 2 µl DNase-1 was added and incubated for 15 minutes at 37°C. DNase was inactivated by heating at 75°C for 5 minutes.

Following reagents were assembled in nuclease free microcentrifuge tubes

Components	Amount
Cell lysate containing total RNA	10µl
dNTP Mix	4µl
Oligo $(dT)_{18}$	2µl
Nuclease free water	16µl

Reagents were mixed by vortexing, centrifuged briefly and placed on crushed ice. The remaining Reverse transcription reagents to the same microcentrifuge tubes as follows.

Components	Amount
10X RT Buffer	2µl
M-MLV Reverse Transcriptase	1µl
RNase Inhibitor	1 µl

The reagents were mixed by vortexing and centrifuged briefly.

Reverse Transcription Reaction

The reaction mixture was incubated at the following temperatures and time by using a thermocycler.

Temperature	Time
42^0C	60 minutes
92^0C	5 minutes
4^0C	5 minutes

9.2) Polymerase Chain Reaction (PCR) Analysis of Proinflammatory

Cytokine gene expression.

cDNA synthesized above were directly used for PCR amplification (5µl for each reaction). PCR was performed with Message Screen Mouse Inflammatory Cytokine Set Multiplex PCR kit from Biosource International, USA.

Biosource's message Screen Mouse Inflammatory Cytokine Multiplex PCR kit have been designed to detect the expression of Mouse GAPDH, IL-6, IL-1β, GM-CSF, TNF-α and IL-12p40 genes. The PCR primers have similar melting temperature (Tm's) and no obvious 3'end overlap, which would enhance the amplification of multiple targets. The target PCR products generated from positive control cDNA, is included in this kit. This kit provides a quick and simple method for analyzing IL-6, IL-1β, GM-CSF, TNF-α and IL-12p40 gene expression and to normalize the expression of these genes against GAPDH.

Materials provided in the kit

10X dNTPs	- 250µl
2 X PCR Buffer	- 1250µl
Dnase free Water	- 1500µl
10 X Positive Control	- 50µl
10 X PCR Primers	- 250µl
DNA. M.W. Marker	- 100µl

List of primers and the expected length of the amplified products

Target	Expected product size
Mouse GAPDH	557 bp
Mouse IL-6	484 bp

Mouse IL-1β	430 bp
Mouse GM-CSF	375 bp
Mouse TNF-α	290 bp
Mouse IL-12p40	239 bp

Procedure

All the reagents were thawed before starting experiments, mixed them thoroughly and centrifuged briefly. All the reagents were assembled in nuclease free microcentrifuge tubes as follows.

Components	**Amount**
2 X PCR Buffer	25µl
10 X MPCR Primer pairs	5µl
Taq DNA polymerase	5µl
Nuclease free water	9µl
cDNA sample or +ve Control	5µl

Reaction mixture was vortexed and centrifuged briefly before and after adding the cDNA sample. PCR thermal cycling was performed according to the protocol

Steps	Conditions	Temperature	Duration (minutes)
i	Initial Denaturation	95^0C	1
ii	Denaturation	94^0C	1
iii	Annealing	60^0C	4
iv	Denaturation	94^0C	1
v	Annealing	60^0C	2.5
vi	Extension	72^0C	1
vii	Go to step iv and repeat 40 cycles		
viii	Final extension	72^0C	10
ix	Store at	4^0C	

9.3) Polymerase Chain Reaction (PCR) analysis of p53, Bcl –2, Caspase-3 and iNOS gene expression

cDNA synthesized from B16F-10 melanoma cells ($2X10^4$ cells/well) through the above method was directly used for PCR amplification. PCR was performed with primers obtained from Maxim Biotech, Inc, USA.

Materials provided in the kit

Pre Mixed forward and reverse primer	- 1000µl
Optimized PCR buffer	- 750µl X 4 tubes
(Chemicals, Enhancer, Stabilizer, dNTPs)	
Positive Control	- 100µl
Nuclease free water	- 1000µl

Procedure

All the reagents were thawed before starting the experiments, mixed them thoroughly and centrifuged briefly. All the reagents were assembled in nuclease free microcentrifuge tubes according to the protocol of primer kit as follows.

Master mixture preparation

250µl each of pre-mixed primers were added to each tube of optimized PCR buffer. This master mixture was aliquoted and used for preparation of reaction mixture as follows for further PCR amplification.

Components	Amount (µl)
Master Mixture	40
Taq DNA polymerase	0.2
cDNA sample	10

Reaction mixture was vortexed and centrifuged briefly before and after adding the cDNA sample. PCR thermal cycling was performed according to the protocol of Maxim Biotech, Inc at the following conditions.

Steps	Conditions	Temperature	Duration (minutes)
i	Initial Denaturation	96^0C	1
ii	Denaturation	94^0C	1
iii	Annealing	58^0C	1
iv	Extension	72^0C	1
v	Go to step ii and repeat 35 cycles		
vi	Final extension	72^0C	10
vii	Store at	4^0C	

9.4) Tissue to cDNA synthesis

9.4.1) Isolation of RNA from the tissue

Isolation of RNA from tissue follows the method of Chomczynski and Sacchi (1987; 2006).100mg of the lung from treated and untreated metastasis bearing C57BL/6 mice was minced and homogenized in 1mL of denaturing solution (Guanidinium thiocyanate 4M, 25mM sodium citrate, pH 7.0, 0.1M β-mercaptoethanol, prepared in DEPC treated de-ionized water). The contents were transferred into a polypropylene tube and 0.1ml of 2M sodium acetate (pH 4.0) was added and mixed well by inverting the tube. After the mixing 1ml of water saturated phenol was added to each tube and the contents were mixed well. This was followed by the addition of 0.2ml of chloroform-isoamyl alcohol (49:1) and the mixture was kept at -20^0C for 5 minutes. The contents in the each tube were centrifuged at 12000 rpm for 30 min at 4^0C. The aqueous phase was transferred into a new vial and mixed with 1ml of isopropanol and placed again at -20^0C for 1 h. The vials were centrifuged again at 12000 rpm for 20 minutes at 4^0C and the supernatant was discarded from each vial. The pellet so obtained was dissolved in 300µl of denaturing solution and mixed with 500µl of isopropanol again. Further the mixture was kept at -20^0C for 30 minutes. The vials were centrifuged at 12000 rpm for 30 minutes at 4^0C and the supernatant was discarded. The pellet was resuspended in 1ml of 75% ethanol, the contents were vortexed for few seconds and incubated at room temperature for 15 minutes. The mixture was centrifuged again at 12000 rpm for 10

minutes at 4^0C and the supernatant was discarded. The pellet was air dried. Finally dissolved the pellet in 100µl of DEPC water and stored at-70^0C. The RNA isolated was used for the preparation of cDNA.

9.4.2) Preparation of cDNA

The cDNA was prepared from RNA by RT-PCR as described below. 10µl of the RNA sample was taken and it was denatured at 95^0C for 5minutes.
Preparation of the reaction mixture for RT-PCR:

Reagents	Quantity per sample
10X PCR buffer	2 µl
25mM MgCl2	2 µl
10mM dNTPs	2 µl
Oligo dT	1 µl
RNase inhibitor	1 µl
AMV RT	1 µl
DEPC water	7 µl
Total	16 µl

The contents were centrifuged and 5µl of denatured RNA sample was added. The mixture was vortexed and centrifuged and cDNA was synthesized using a mini thermocycler.

9.4.3) Reverse transcription reaction

The reaction mixture was incubated at the following temperatures and time by using a thermocycler.

Temperature	**Time** (minutes)
42^0C	60
92^0C	5
4^0C	5

After the reaction contents vials were stored at -70^0C.

The cDNA prepared by the above described protocol was used for the analysis of expression of the following genes. Their primer sequence and expected product size is given below.

Primer sequence and product size of genes:

Name of the gene with primer sequence	**Product size (bp)**
k-ras	
Forward 5'-TGTGGATGAGTACGACC-3'	338
Reverse 5'-ACGGAATCCCGTAACTC-3'	
Prolyl hydroxylase	
Forward 5'-CGGGATCCTAGACCGGCTAACAAGTA-3'	317
Reverse 5'-GGAATTCCAAGCAGTCCTCAGCTGT-3'	
Lysyl oxidase	
Forward 5'-CTACATCCAGGCTTCCACG-3'	283
Reverse 5'-TCTCCTCTGTGTGTTGGCAT-3'	
nm-23	
Forward 5'-CTCAGCCTTAATTTTTTCCCCC-3'	310
Reverse 5'-TTAACTTCCGACACTGGGTGT-3'	
MMP-2	
Forward 5'-GAGTTGGCAGTGCAATACCT-3'	354
Reverse 5'-GCCGTCCTTCTCAAAGTTGT-3'	

MMP-9	
Forward 5'-AGTTTGGTGTCGCGGAGCAC-3'	327
Reverse 5'-TACATGAGCGCTTCCGGCAC-3'	
TIMP-1	
Forward 5'-CTGGCATCCTCTTGTTGCTA-3'	414
Reverse 5'-AGGGATCTCCAGGTGCACAA-3'	
TIMP-2	
Forward 5'-AGACGTAGTGATCAGGGCCA-3'	525
Reverse 5'-GTACCACGCGCAAGAACCAT-3'	
ERK1	
Forward 5'-GCACGACCACACTGGCTTTC-3'	512
Reverse 5'-GATCAACTCCTTCAGCCGCTC-3'	
ERK2	
Forward 5'-ACAGGACCTCATGGAGACGG-3'	216
Reverse 5'-GATCTGCAACACGGGCAAGG-3'	
GAPDH (Glyceraldehyde 3 phosphate dehydrogenase)	
Forward 5'-TGCTGGCGCTGAGTACGTCGT-3'	527
Reverse 5-'GTGGAGGAGTGGGTGTCGCTG-3'	
VEGF	
Forward 5'-TGCTCACTTCCAGAAACACG-3'	453
Reverse 5'-GGAAGGGTAAGCCACTCACA-3'	

Amplification of cDNA by RT-PCR for the analysis of the expression of genes during the progression of metastasis

Reagents	**Quantity per sample**
10X PCR buffer	2.0 µl
25mM MgCl2	0.4 µl
10mM dNTPs	0.4 µl
Primer (forward and reverse)	2.0+2.0 µl
Taq DNA polymerase	0.1µl
Molecular biology grade water	11.1 µl
Total	18µl

The contents were vortexed and centrifuged and 2µl of the cDNA was added. (The cDNA was diluted 1:1 and from the diluted cDNA preparation 2µl was taken for the RT-PCR analysis).

The following PCR reaction profile was followed in the minicycler

94^0C	-	2 minutes	
94^0C	-	1 minute	
55^0C	-	1 minute	39 cycles
72^0C	-	1 minute	
72^0C	-	10 minutes	
10^0C	-	Hold for 10 minutes	

8µl of the amplified sample was subjected to electrophoresis in an agarose gel (1.5%) containing 0.5µg/mL ethidium bromide at 70V for 2 h in TE buffer (10mM Tris HCl and 1mM EDTA, pH 10.0).

9.5) Detection of PCR products

10 µl of each PCR product resolved on 1.5% agarose gel electrophoresis.

Reagents

10 X TEB

Tris-HCl, pH 8.3 - 21.6g

EDTA - 0.372g

Boric acid - 11g

Made upto 200ml using double distilled water.

10X Loading dye

Bromophenol blue - 0.05%

TEB 10X pH 8.3 - 1ml

Glycerol - 100µl

i. Sufficient electrophoresis buffer (1X TEB buffer 0.08m Tris-phosphate and 0.002M EDTA) used to fill the electrophoresis tank.

ii. Edges of a the clean, dry, plastic gel tray was sealed with sealing tape and the comb was placed.

iii. Agarose powder (2%) added to the electrophoresis buffer and heated the slurry in a boiling water bath or gas flame until the agarose dissolved and the slurry became visible.

iv. Ethidium bromide was added to a final concentration of 5µg/ml to the slurry, then poured to the sealed gel tray and allowed to cool for 30-45 minutes and the gel tray placed in the electrophoresis tank after removing the sealing tape.

v. 10µl of PCR products mixed with 2µl of 10X gel loading buffer and loaded into the wells.

vi. The samples were resolved at 100v until the dye has migrated up to the ¾th length of the gel.

vii. The gels were examined using a gel-documentation system.

10) Transcription factor profiling

Transcription Factor profiling was done with BD Mercury TransFactor kit obtained from BD Biosciences. This kit provides rapid, high throughput detection of specific transcription factor activities in cell extracts. Using an enzyme-linked immunosorbent assay (ELISA)-based format, the Transfactor Kits detect DNA binding by specific transcription factors. This method is faster, easier and more sensitive than electrophoretic mobility shift assays (EMSA) and does not require the use of radioactivity.

Principle

Each Transfactor kit is provided in a 96-well format with oligonucleotides containing the consensus binding sequences for each transcription factor coated on the wells. When cell extracts containing the transcription factors are incubated in the wells, the DNA bind to their consensus sequences. A specific primary antibody was then used to detect bound transcription factors. A horse radish peroxidase conjugated secondary antibody was used to detect the bound primary antibody. The enzymatic product was measured with standard micro titre plate reader.

Materials provided in the kit

Transfactor plate

Transfactor Rack

Primary antibody

Secondary antibody

Wild-Type competitor Oligos

TMB substrate

10 X Transfactor Buffer

Blocking Reagent

Stop solution (Na azide)

Transfactor ELISA procedure

Cytosolic and Nuclear Extract Preparation

The following procedure was used to prepare cytosolic or nuclear extracts from cell line culture (Lee *et al.*, 1998). This procedure is designed for extraction from 1 × 10^7 cells, which is roughly equivalent to three 15 cm tissue culture plates at 80% confluency. All steps were performed at 4°C unless otherwise specified. Reagents were kept at 4°C during the procedure, and should not be used until fully defrosted. Tubes and reagents were kept on ice when not centrifuging. All reagents were centrifuged at 4°C in a pre-cooled rotor.

Materials Required

10 X Pre Lysis - Buffer

100mM HEPES (pH 7.9)

15mM $MgCl_2$

100mM KCl

Pre -Extraction Buffer

20mM HEPES (pH 7.9)

1.5mM $MgCl_2$

0.42M NaCl

0.2mM EDTA

25% (v/v) glycerol

Protease Inhibitor Cocktail

Aprotinin (1mg/ml)	– 5µl
Leupeptin (1mg/ml)	– 5µl
PMSF (100mM in Isopropanol)	– 5µl
DTT (100mM in D.W.)	– 10µl
Benzamidine (1mg/ml)	– 5µl
Triton × 100 (10% in D.W)	– 10µl
Tris pH 7.4 (1M)	– 20µl
NaCl (5M)	– 50µl

EDTA (0.5M)	– 5µl
EGTA (10mg/ml)	– 5µl

Make up this cocktail into 1ml and store at – 22^0C.

1 X Lysis Buffer

10 X Pre-Lysis buffer	– 150µl
0.1 M DTT	– 15µl
Protease inhibitor cocktail	– 15µl
DD H_2O	– 1.32µl

a. Cell Lysis

1. Cells were collected after incubation using a cell scraper, and transferred to a clean centrifuge tube , centrifuged for 5 minutes at 450 x g. Supernatant was decanted.
2. The cell pellet were rinsed with ice cold PBS and centrifuged for 5 minutes at 450 x g and supernatant were decanted.
3. Cell pellet volume was estimated using a micropipette and this volume was used in subsequent step of this procedure.
4. Five times volume of lysis buffer to the cell pellet volume was added to the cell pellets.
5. The cell pellets were resuspended gently with out the formation of foam and incubated for 15 minutes on ice.
6. The cell suspension was centrifuged for 5 minutes at 420 × g and the supernatants were discarded and the cell pellet resuspended in a volume of lysis Buffer equal to twice the cell pellet volume.

b. Cell Disruption

1. Cell suspension in the lysis buffer was filled in a syringe with narrow gauge needle (No. 27).
2. All air from the syringe removed as fully as possible.
3. The cell suspension ejected with a rapid stroke. The steps 1-3 were repeated for 10 times.

4. The disrupted cell suspensions were centrifuged at 10000-11000 × g for 20 minutes.
5. Transfer the supernatant was transferred to a fresh tube and this fraction is known as the cytosolic fraction.

c. Nuclear Extraction

1. Nuclear extraction buffer by mixing the following reagents as follows,

   Pre- extraction Buffer – 147µl

   0.1 M DTT – 1.5µl

   Protease inhibitor cock-tail – 1.5µl
2. The crude nuclear pellet obtained in Step b.5 (above) suspended in a volume of nuclear extraction buffer equal to two-third (2/3) of the cell pellet volume
3. The nuclei were disrupted by using a fresh syringe as step b.1 - b.3.
4. Nuclear suspension were allowed to shake gently for 30 min at $4^{O}C$
5. The nuclear suspension was centrifuged at 20000-21000 × g for 5 min.
6. The supernatant were transferred to a clean, chilled test tube and this fraction is the nuclear extract.
7. The protein concentration of the nuclear extract was measured by Bradford method.
8. The supernatant was aliquoted in to small vials and stored at $^{-}70^{0}C$.

Determination of protein concentration of the nuclear extract by Bradford Method

This is a rapid, simple and sensitive method for the estimation of proteins in a sample extract. The colour development is virtually complete in 2 minutes and the colour is stable for 1 h. The procedure is based on the interaction of a dye, Coomassie Brilliant Blue with proteins. The unbound dye has an absorbance maximum at 465 nm. However on interaction with proteins the dye turns blue and its absorbance at 595 nm the amount of protein in a sample solution can be quantitatively estimated.

Materials and Reagents

Bradford Reagent.

Dissolve 100mg Coomassie Brilliant Blue G250 in 50ml of ethanol, add 100ml of 85% phosphoric acid make the volume to 1L with water.

Procedure

0.1ml of sample solution was made up to the volume of 1ml with 0.1M phosphate buffer (pH 7.5). 5ml of Bradford reagent was added to the sample and mixed thoroughly. Absorbance was recorded at 595ηm against the blank and the protein concentrations were determined from the standard curve.

Trans factor ELISA Procedure

Assay wells were incubated with 150µl of 1X Transfactor buffer for 15 minutes at room temperature. Nuclear extracts (6µl) were made up to 50µl with 1X Transfactor buffer and added to the assay wells and incubated 60 minutes at room temperature. Wells were washed with 1X Transfactor buffer and incubated with 100µl primary antibody at room temperature for 60 minutes, then washed with 1X Transfactor buffer and incubated with 100µl of secondary antibody at room temperature for 30 minutes. Wells are washed with 1X Transfactor buffer and incubated with 100µl of TMB substrate to each well and incubated at room temperature for 10 minutes. After seeing the blue colour development the reaction were stopped by adding 100ml of sodium azide stop solution. Absorbance was measured at 655 nm.

11) Statistical Data Analysis

All data were expressed as mean ± S.D. The statistical analysis was done by one way ANOVA or Student's t-test using Graphpad InStat version 3.00 for Windows 95, GraphPad Software, San Diego, California, USA.

CHAPTER 3

IMMUNOMODULATORY ACTIVITY OF SULFORAPHANE

3.1. INTRODUCTION

Modulation of immune system by cytotoxic agents is emerging as a major area in pharmacology, especially in cases where undesired immunosuppression is the result of therapy (Geetha *et al.*, 2005). A major drawback of current cancer therapeutic practices such as chemotherapy and radiation therapy is bone marrow suppression resulting in cytopoenia and subsequent suppression of humoral and cellular as well as non-specific and specific cellular responses (Devasagayam and Sainis, 2002). For advanced tumours developed from epithelial tissues such as lung, colon, breast, prostate and pancreas, these therapies are less successful. Drug resistance and dose limiting toxicities are the major obstacles for the success of cancer chemotherapy (Ratain and Relling, 2001). Therefore, standard chemotherapy and radiotherapy might negate or reduce the therapeutic benefits obtained by the increased tumour killing of the treatment. Thus combination of chemotherapy or radiotherapy with immunomodulating agents may provide a strategy for overcoming the immunosuppressive effects of chemotherapy or radiotherapy.

The modulation of immune response using medicinal plant products as a possible therapeutic measure has become a subject of active scientific investigations (Bhat *et al.*, 2005). Some of the plants with known immunomodulatory activities are *Tinospora cordifolia* (Sonel and Kuttan, 1999), *Withania somnifera* (Davis and Kuttan, 2000b), *Piper longum* (Sunila and Kuttan, 2004) etc. Components such as polysaccharides, lectins (Haijto *et al.*, 1989), proteins and peptides (Kuttan and Kuttan, 1992) present in plants have been shown to stimulate the immune system.

In this chapter, the immunomodulatory activity of Sulforaphane has been investigated by analyzing its effects on hematological parameters, production of bone marrow cells, specific antibody and antibody producing cells in spleen, phagocytosis, proliferation of spleen, thymus and bone marrow cells.

3.2. MATERIALS AND METHODS

3.2.1. Animals

Male BALB/c mice (4-6 weeks old) were used for this study.

3.2.2. Chemicals

Pararosaniline hydrochloride and α-naphthyl acetate were used for this study. All other chemicals and reagents were of analytical grade.

3.2.3. Toxicity study

A detailed toxicological study was undertaken before the determination of the non-toxic dose of Sulforaphane. Toxicity of single as well as repeated doses was evaluated. We administered different doses of Sulforaphane (100μg - 5mg/dose) and analyzed the toxicity by determining the parameters such as relative organ weights and by assaying renal and hepatic function tests (Table 3.1a, & b). Based on these results a non- toxic concentration of 500μg/dose was used for *in vivo* studies.

3.2.4. Administration of Sulforaphane

Sulforaphane was suspended in phosphate buffered saline (PBS, pH-7.4) and administered intraperitoneally at a dosage of 500μg/dose/animal

3.2.5. Determination of the effect of Sulforaphane on the haematological parameters

Two groups of BALB/c mice (6 mice/group) were used in this study. Group I animals were given the vehicle, PBS (i.p) and kept as untreated vehicle control. Group II animals were treated with Sulforaphane at a concentration of 500μg/ dose/ animal (i.p)/day for 5 consecutive days. Blood was collected from the tail vein and various parameters such as total WBC count (Hemocytometer), differential count (Leishman's stain) and haemoglobin (Hb) content (Cyanmethaemoglobin method) were recorded prior to the administration of Sulforaphane and continued every 3rd day for 30 days. The change in body weight was also recorded.

3.2.6. Determination of the effect of Sulforaphane on the organ weights

BALB/c mice (6 mice/group) were divided into two groups as described above. Body weight of animals was recorded before sacrifice. Animals were sacrificed 24 h

Table 3.1. Toxicological analysis of Sulforaphane

a. Effect of Sulforaphane on the relative organ weight

	Relative organ weight (g/100g body weight)				
Treatment	Spleen	Thymus	Liver	Kidney	Lungs
Vehicle control	0.37 ± 0.08	0.11 ± 0. 10	5.25 ± 0.39	1.31 ± 0.31	0.65 ± 0.12
Sulforaphane (μg/ml)					
100	0.39 ± 0.10	0.14 ± 0.12	5.26 ± 0.21	1.31 ± 0.82	0.66 ± 0.15
500	0.46 ± 0.13	0.16 ± 0.05	5.33 ± 0.06	1.32 ± 0.03	0.64 ± 0.03
1000	0.46 ± 0.12	0.17 ± 0.04	5.36 ± 0.45	1.33 ± 0.25	0.65 ± 0.22
5000	0.47 ± 0.16	0.17 ± 0.06	5.34 ± 0.38	1.32 ± 0.62	0.64 ± 0.19

BALB/c mice were given single dose of Sulforaphane (100- 5000μg/ml). On 14th day all animals were sacrificed and relative organ weights were determined.

Table 3.1. Toxicological analysis of Sulforaphane

b. Effect of Sulforaphane on liver and kidney function tests

Treatment	Liver Function Tests			Kidney Function Tests	
	GPT U/ml	GOT U/ml	ALP KA Units	Creatinine mg/100ml	Urea mg/dl
Normal	59.58 ± 2.36	61.72 ± 2.53	21.08 ± 1.35	0.89 ± 0.04	42.78 ± 2.46
Sulforaphane (µg/ml)					
100	58.75 ± 2.78	61.51 ± 2.71	20.86 ± 1.24	0.88 ± 0.02	42.35 ± 2.07
500	59.63 ± 2.32	61.83 ± 2.69	21.34 ± 1.52	0.89 ± 0.06	42.64 ± 2.55
1000	61.21 ± 2.48	62.44 ± 2.47	22.40 ± 1.37	0.91 ± 0.04	43.77 ± 2.96
5000	63.55 ± 2.92	65.09 ± 2.73	22.96 ± 1.46	0.93 ± 0.03	45.43 ± 3.12

BALB/c mice were given single dose of Sulforaphane (100- 5000µg/ml). On 14th day all animals were sacrificed, blood was collected, serum separated and used for the estimation of hepatic and renal function tests.

after the last dose of Sulforaphane and weight of the vital organs such as liver, spleen, thymus, lungs and kidneys were recorded and expressed as relative organ weights.

3.2.7. Determination of the effect of Sulforaphane on the bone marrow cellularity and α-esterase positive cells

Bone marrow cellularity was determined by the method of Sredni *et al* (Sredni *et al.*, 1992). BALB/c mice (6 mice/group) were divided into two groups as described above. The animals were sacrificed 24 h after the last dose and bone marrow cells from femur was collected into the medium containing 2% fetal calf serum (FCS). Bone marrow cell number was determined using a haemocytometer and expressed as total live cells/femur.

The number of α-esterase positive cells was determined by the azodye coupling method (Bancroft and Cook, 1984). A smear of bone marrow cells from the above preparation was made on clean glass slides, stained with α-naphthyl acetate and pararosaniline hydrochloride and counter stained with haematoxylin. The numbers of α-esterase positive cells were expressed out of 4000 cells.

3.2.8. Determination of the effect of Sulforaphane on the circulating antibody titre

BALB/c mice (6 mice/group) were divided into two groups as described early. Along with the 5^{th} dose of Sulforaphane, Group II animals were immunized with SRBC (2.5×10^8 cells/ animal, i.p). Group I animals were also immunized with SRBC. Blood was collected from tail vein every 3^{rd} day after antigen administration and continued for a period of 30 days. Serum was separated and heat inactivated at 56^0C for 30 minutes. Antibody titre was conducted by haemagglutination assay using SRBC as antigen (Singh *et al.*, 1984).

3.2.9. Determination of the effect of Sulforaphane on plaque forming cells (PFC) in spleen

BALB/c mice were divided into 2 groups (7 mice/group) as described in the previous experiment. Along with the last dose of Sulforaphane, Group II animals were immunized with SRBC (2.5×10^8 cells/ animal, i.p). Group I animals were also

immunized with SRBC. The animals were sacrificed on different days starting from the 3rd day after immunization up to 9th day; spleen was processed to single cell suspension and used for the determination of the antibody producing cells by Jerne's plaque assay (Jerne and Nordin, 1963).

3.2.10. Determination of the effect of Sulforaphane on the phagocytic activity of macrophages

Peritoneal macrophages were elicited with sodium caseinate in BALB/c mice (6 mice/group) treated with 5 doses (500µg/ dose/ animal (i.p)) of Sulforaphane. Macrophages were harvested after 5 days and the phagocytic activity was determined using opsonized SRBC (Mehera and Vaidya, 1993) and compared with the macrophages isolated from the untreated vehicle control.

3.2.11. Determination of the effect of Sulforaphane on the proliferation of lymphoid cells- spleen cells, bone marrow cells and thymocytes.

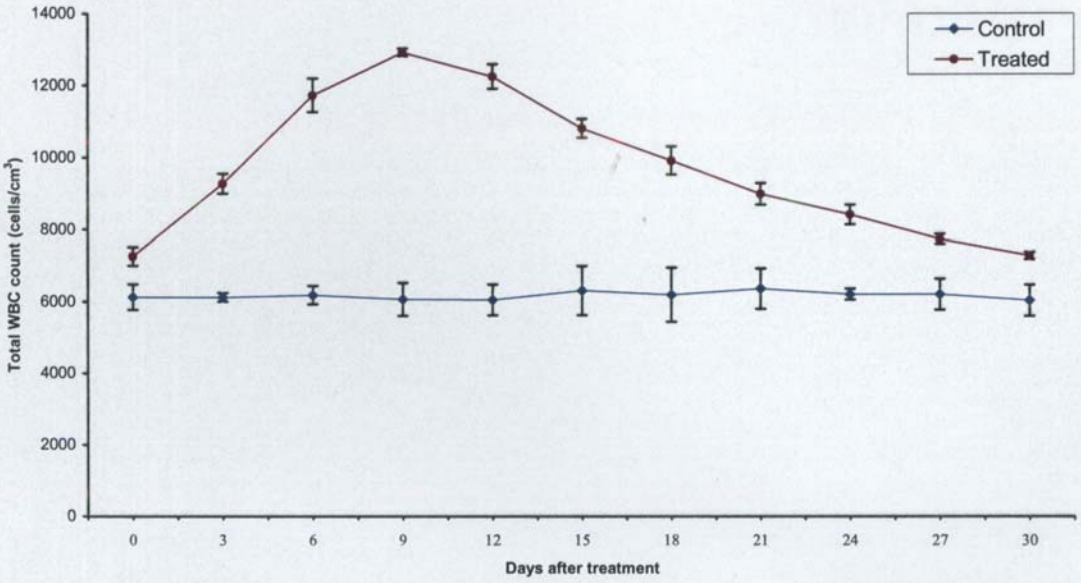
BALB/c mice were divided into two groups (4 mice/group). Group I animals were kept as normal control and Group II animals were treated with Sulforaphane (500µg/dose/animal/day, i.p) for 5 consecutive days. Animals were sacrificed 24 h after last dose of Sulforaphane administration and spleen, thymus and bone marrow from femur were collected aseptically and processed to single cell suspensions (Chapter 2). Triplicate cultures were set to determine the proliferation in the presence and absence of various mitogens such as ConA-10µg/ml, PHA-2.5µg/ml, LPS-10µg/ml and PWM-10µg/ml by thymidine incorporation assay (chapter 2).

3.3. RESULTS

3.3.1. Effect of Sulforaphane on haematological parameters

The total WBC count in normal BALB/c mice was increased by the administration of Sulforaphane (Figure 3.1). The maximum WBC count obtained in the Sulforaphane treated animals was 12950cells/ mm^3 on 9th day. There was no significant difference in the ratio of lymphocytes to neutrophils (data not shown) as well as hemoglobin level

Figure 3.1. Effect of Sulforaphane on total WBC count



(data not shown) after treatment with Sulforaphane. There was a slight enhancement in the body weight of Sulforaphane treated group (29.3 ± 1.68g) compared to vehicle control group (27.9 ± 1.73g) at the end of the experiment which were initially 23.41 ± 1.46g and 23.82 ± 1.86g respectively.

3.3.2. Effect of Sulforaphane on organ weight

Effect of Sulforaphane on organ weight is given in Table 3.2. There was an increase in the weight of thymus (0.17 ± 0.004g/100g body weight) in Sulforaphane treated group when compared to vehicle control (0.11 ± 0.01g/100g body weight). The size and weight of spleen was also enhanced significantly by the administration of Sulforaphane (0.47 ± 0.03g/100g body weight) compared to vehicle control (0.37 ± 0.03g/100g body weight). There was no significant change in the weight of other vital organs such as liver, lungs and kidney.

3.3.3. Effect of Sulforaphane on bone marrow cellularity and α-esterase positive cells

The effect of Sulforaphane on the bone marrow cellularity and α-esterase positive cells is given in Table 3.3. Administration of Sulforaphane significantly increased the bone marrow cellularity and number of α-esterase positive cells. In animals treated with Sulforaphane, bone marrow cellularity was increased to 23×10^6 cells/ femur compared to 14.38×10^6 cells/femur of vehicle control group. The number of α-esterase positive cells was also found to be increased in Sulforaphane treated animals (1346.66 cells/4000 cells) compared to untreated control animals (817 cells/4000 cells).

3.3.4. Effect of Sulforaphane on the circulating antibody titre

The aim of this experiment is to check specific immune response by using SRBC as the antigen. There was a significant increase in the production of specific antibody in animals treated with Sulforaphane (Figure 3.2). The maximum titre value of 1024 was observed on 12^{th} day in Sulforaphane treated animals, while the control animals showed a maximum titre of only 128 on the same day.

Figure 3.2. Effect of Sulforaphane on antibody titre

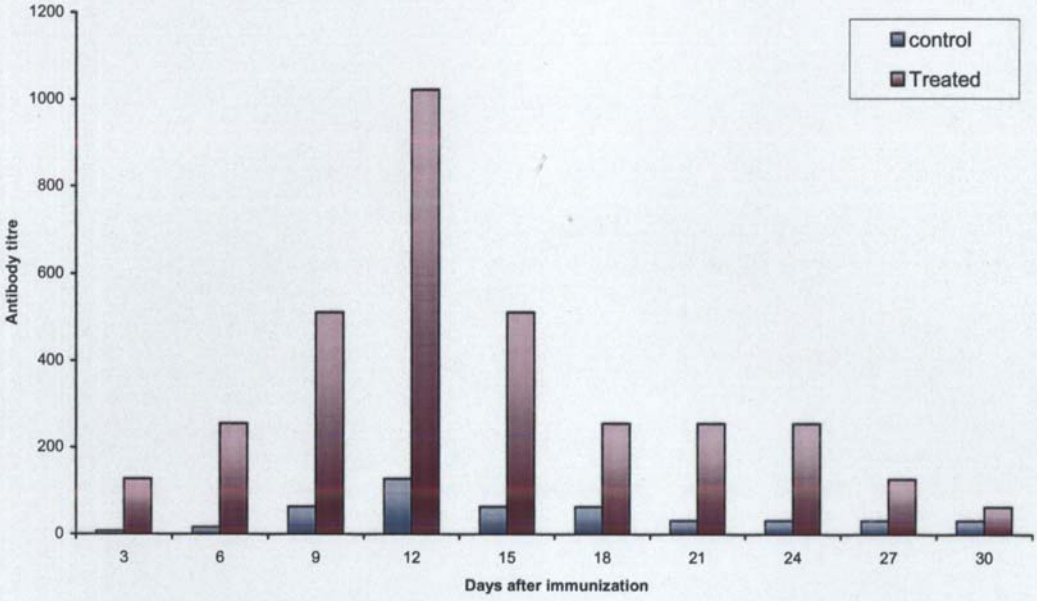


Table 3.2. Effect of Sulforaphane on the relative organ weight

Treatment	Relative organ weight (g/100g body weight)				
	Spleen	Thymus	Liver	Kidney	Lungs
Normal	0.37 ± 0.08	0.11 ± 0.10	5.25 ± 0.39	1.31 ± 0.31	0.65 ± 0.12
Vehicle control	0.37 ± 0.03	0.11 ± 0.01	5.08 ± 0.21	1.32 ± 0.33	0.64 ± 0.04
Sulforaphane	0.47 ± 0.03*	0.17 ± 0.004*	5.33 ± 0.06*	1.32 ± 0.03*	0.64 ± 0.03*

BALB/c mice (6 mice /group) were treated with 5 consecutive doses of Sulforaphane (500μg/ dose/ animal (i.p)/day).Vehicle control group received only PBS. Animals were sacrificed after 24 h of last dose and organ weights were taken. Values are mean ± S.D. *$p<0.01$

Table 3.3. Effect of Sulforaphane on bone marrow cellularity and α-esterase activity

Treatment	Bone marrow cellularity (cells/femur x 10^6)	α-esterase (No.of esterase positive cells/4000 cells)
Normal	14.95 ± 0.72	824.66 ± 27.35
Vehicle control	14.38 ± 0.81	817.00 ± 32
Sulforaphane	23.00 ± 0.83*	1346.66 ± 32.65*

BALB/c mice (6 mice /group) were treated with 5 consecutive doses of Sulforaphane (500μg/ dose/ animal (i.p)/day).Vehicle control group received only PBS. Bone marrow cells were collected from femur and made into single cell suspension. The bone marrow cellularity was determined using hemocytometer. The α-esterase positive cells were determined by the azodye coupling method. Values are mean ±S.D. *$p<0.001$

3.3.5. Effect of Sulforaphane on plaque forming cells (PFC) in spleen

Administration of Sulforaphane significantly enhanced the number of antibody producing cells in spleen (Figure 3.3). The maximum number of plaque forming cells was observed in Sulforaphane treated animals (314.83 PFC/10^6 spleen cells) on 6^{th} day after immunization while the vehicle control animals had only 161.67 PFC/10^6 spleen cells on the same day.

3.3.6. Effect of Sulforaphane on the phagocytic activity of macrophages

Administration of Sulforaphane significantly enhanced the phagocytic activity of peritoneal macrophages (Table 3.4). Number of macrophages with engulfed SRBCs was significantly increased in Sulforaphane treated group (72 ± 3.16/ 200 cells) compared to untreated vehicle control group (31 ± 1.82 cell/200 cells).

3.3.7. Effect of Sulforaphane on blastogenesis of lymphoid cells

(a) Spleen blastogenesis

Effect of Sulforaphane on spleen cell blastogenesis is given in Table 3.5. Significant enhancement in the proliferation of spleen cells (6531 ± 31.04cpm) from the Sulforaphane treated group by the mitogen Con A was observed compared to normal group (1494 ± 33.26cpm) as well as group treated with Con A alone (4592 ± 26.31cpm). Administration of Sulforaphane also enhanced the mitogenic potential of PWM (5845 ± 46.13 cpm), PHA (5809 ± 47.6cpm) and LPS (5526 ± 30.1cpm) on spleen cell proliferation.

(b) Bone marrow blastogenesis

Effect of Sulforaphane on Bone marrow proliferation is given in Table 3.6. Administration of Sulforaphane significantly enhanced bone marrow proliferation. Mitogenic activity of PHA (3726 ± 54.44cpm) and LPS (3829 ± 60.47cpm) on bone marrow proliferation was significantly increased in Sulforaphane treated animals compared to normal group (1166 ± 81.83cpm) as well as groups treated with PHA (3197 ± 30.85cpm) and LPS (3334 ± 67.76cpm) alone. Treatment of Sulforaphane also

Figure 3.3. Effect of Sulforaphane on plaque forming cells (PFC) in spleen

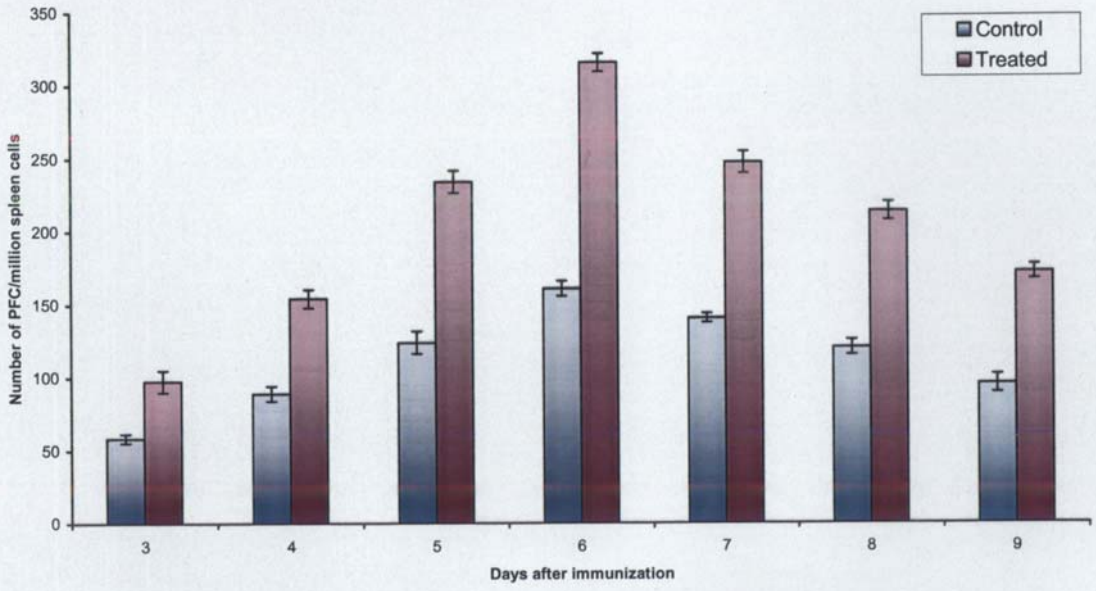


Table 3.4. Effect of Sulforaphane on phagocytic activity of peritoneal macrophages

Treatment	Number of pigmented macrophages /200 cells
Normal	32 ± 2.11
Vehicle control	31 ± 1.82
Sulforaphane	72 ± 3.16*

Macrophages were elicited by injecting 5% sodium caseinate intraperitoneally in BALB/c mice (6 mice /group), of which one group was treated with 5 consecutive doses of Sulforaphane (500μg/ dose/ animal (i.p)/day) whlile the other, the Vehicle control group received only PBS. The phagocytic activity was determined using opsonized SRBC. Values are mean ± S.D. *P<0.001

Table 3.5. Effect of Sulforaphane on splenocyte proliferation

Treatment	Rate of Proliferation (cpm)				
	No Mitogen	PHA (5μg/ml)	CON A (10μg/ml)	PWM (10μg/ml)	LPS (10μg/ml)
Normal	1494±33.26	4173±46.19	4592±26.31	4119±43.50	4070±54.37
Sulforaphane	4003±25.8*	5809±47.6*	6531±31.04*	5845±46.13*	5526±30.1*

Treated animals received Sulforaphane (500μg/dose/animal/day, i.p) for 5 consecutive days. Spleen cells were cultured in the presence and absence of various mitogens and rate of proliferation was checked by H^3-thymidine incorporation assay.

*P< 0.01 compared to Normal

Table 3.6. Effect of Sulforaphane on bone marrow proliferation

Treatment	Rate of Proliferation (cpm)				
	No Mitogen	PHA (5μg/ml)	CON A (10μg/ml)	PWM (10μg/ml)	LPS (10μg/ml)
Normal	1166±81.83	3197±30.85	1587±73.18	2051±70.03	3334±67.76
Sulforaphane	1837±44.52*	3726±54.44*	2119±37.32*	2441±58.14*	3829±60.47*

Treated animals received Sulforaphane (500μg/dose/animal/day, i.p) for 5 consecutive days. Bone marrow cells were cultured in the presence and absence of various mitogens and rate of proliferation was checked by H^3-thymidine incorporation assay. *P< 0.01 compared to Normal

enhanced the bone marrow proliferation by the mitogens PWM (2441 ± 58.14cpm) and Con A (2119 ± 37.32cpm).

(c) Thymocyte blastogenesis

Effect of Sulforaphane on thymocyte proliferation is presented in Table 3.7. Stimulation by the mitogens such as PHA and Con A was significantly increased by the administration of Sulforaphane and a corresponding enhancement in the proliferation of thymus cells by the mitogens Con A (6278 ± 43.43cpm) and PHA (4060 ± 65.57cpm) was observed in Sulforaphane treated animals compared to normal group without mitogen (1563 ± 69.52cpm).

3.4. DISCUSSION

Control of disease by immunological means has two aspects, namely the development and improvement of protective immunity and the avoidance of undesired immunological side reactions. Modulation of the immune system by cytostatic agents is emerging as a major area in pharmacology, especially in cases where undesired immunosuppression is the result of therapy. Immunomodulatory agents can enhance or inhibit the immunological responsiveness of an organism by interfering with its regulatory mechanisms. They may selectively activate either cell mediated or humoral immunity by stimulating either T_H1 or T_H2 type cell response respectively. Immunomodulatory agents that are free from side effects and which can be administered for long duration to obtain a continuous immune activation are highly desirable for the prevention of diseases. There are a variety of naturally and chemically derived compounds discovered with immunomodulatory activity such as levamosole, glucan, IL-2, interferon etc, which are used in combination with cisplatin, adiramycin, 5-flurouracil etc. against many types of carcinomas. But most of these immunomodulatory compounds have side effects namely fever, myaligias fatigue etc. (Herberman, 1985). Modulation of immune response using medicinal plant products as a possible therapeutic measure has become a subject of active scientific investigations (Bhat *et al.*, 2005). Our laboratory has reported earlier that an extract from plants such as *Withania somnifera* (Davis and Kuttan, 2000b), *Piper longum* (Sunila and Kuttan,

Table 3.7. Effect of Sulforaphane on thymocyte proliferation

Treatment	Rate of Proliferation (cpm)		
	No Mitogen	PHA (5μg/ml)	Con A (10μg/ml)
Normal	1563 ± 69.52	2909 ± 49.36	3943 ± 54.68
Sulforaphane	2761 ± 113.8*	4060 ± 65.57*	6278 ± 43.43*

Treated animals received Sulforaphane (500μg/dose/animal/day, i.p) for 5 consecutive days. Thymus cells were cultured in the presence and absence of various mitogens and rate of proliferation was checked by H^3-thymidine incorporation assay.

*P< 0.01 compared to Normal.

2004) etc could stimulate the immunity in normal mice. The present study demonstrate that unlike other chemically defined compounds, Sulforaphane modulate the immune system without undesired side effects.

The common hemopoietic stem cells of bone marrow are the source of major cell types involved in the immune system. Bone marrow also provides microenvironment for the antigen independent differentiation of B-cells. It has been reported that *Piper longum*, an immunopotentiating plant, enhances the total bone marrow cells (Sunila and Kuttan, 2004). The present study shows significant enhancement in bone marrow cellularity and total WBC count by the administration of Sulforaphane, thereby clearly indicating its potentiating effect on the hematopoiesis, especially the cell mediated immune system. Both innate and adaptive immunity depends upon the activities of WBCs. Innate immunity largely involves granulocytes and macrophages. Administration of Sulforaphane significantly enhanced the phagocytic activity of macrophages. Among the earliest cell types to respond invasion by pathogenic organisms are the phagocytes, which are key participants in the innate immune response (Janeway and Medzhitov, 2002). Macrophages together with neutrophils represent the first line of host defense after the epithelial barrier. In addition, Macrophages can function as antigen presenting cells and interact with T lymphocytes to modulate the adaptive immune response (Schepetkin and Quinn, 2006).

The mononuclear phagocyte system is composed of bone marrow monocyte precursors, circulating monocytes and macrophages in all tissues including lung, liver, spleen, adrenal glands, intestine and bone marrow. These cells are highly phagocytic and play an active role in antigen processing. Like proliferating bone marrow cells, bone marrow monocytes show non-specific esterase activity. Administration of Sulforaphane stimulated stem cell proliferation and differentiation as there was significant enhancement in the number of α-esterase positive bone marrow cells.

Sulforaphane also stimulated the humoral immune response, as there was significant enhancement in the circulating antibody titre as well as antibody producing cells in the spleen (PFC). The increased titre remained for several days indicating that there is a sustained immunological activity even after the treatment. The enhancement

of spleen and thymus after Sulforaphane administration also support its stimulatory effect on the immune system.

Sulforaphane significantly promoted the proliferation of spleen, thymus and bone marrow cells as is evident from blastogenesis assays and is a potent stimulator of lymphocytes, especially T cells as it can enhance the mitogenic potential of mitogens such as PHA and Con A. PHA and Con A are two T cell proliferators, PWM is a B cell mediated T cell proliferator and LPS is a B cell proliferator. When spleen and thymus cells are stimulated by Con A which bind to T cell receptor CD3 complex, T lymphocytes are activated resulting in a cascade of biochemical events that leads to elevated expression of IL-2 and finally cell proliferation (Licastro *et al.*, 1993). From our data it is clear that administration of Sulforaphane stimulates immune cell system and immune cell proliferation.

In conclusion, immunomodulation is the regulation of immune responses by stimulating them to prevent infectious diseases or by suppressing them in the undesired conditions. In the present study we found that Sulforaphane is a potent immunomodulator as it stimulated humoral as well as cell mediated immune system with enhanced stem cell proliferation and differentiation.

CHAPTER 4

ANTIANGIOGENIC ACTIVITY OF NATURALLY OCCURRING SULFUR COMPOUNDS

4.1. Introduction

Angiogenesis, the development of new blood vessels from the endothelium of a pre-existing vasculature, is a critical process required by most solid tumours to support their localized growth and metastatic dissemination with in the host (Griffioen and Molema, 2000; Carmeliet and Jain, 2002). The earliest stages of angiogenesis are defined by vasodilation mediated by nitric oxide (NO) and an increased vascular permeability of pre-existing capillaries or post capillary venules in response to vascular endothelial growth factor (VEGF) (Griffioen and Molema, 2000). Apart from NO and VEGF, the proinflammatory cytokines play paramount and indispensable roles in regulating the multiple facets of the angiogenic and lymphangiogenic processes (Hanahan, 1997; Korpelainen and Alitalo, 1998; Neufeld *et al.*, 1999). The proinflammatory cytokines such as IL-1β, IL-6, TNF-α and GM-CSF act as autocrine growth factors for tumour angiogenesis and their deregulated expression directly correlate with the metastatic potential of several human carcinomas (Isner and Asahara, 1993). Recent studies clearly demonstrated that matrix metalloproteinases (MMPs), a family of Zn dependent endopeptidases that are able to degrade ECM, mediate the release and accumulation of VEGF from the cell matrix (Hiratsuka *et al.*, 2002) and triggers angiogenic switch by rendering VEGF bioavailable to its receptors (Bergers *et al.*, 2000). These factors together contribute to the activation of endothelial cells, which are normally quiescent in healthy adult vasculature, followed by new blood vessel formation. Since pathological angiogenesis have an important role in tumour progression, the antiangiogenic therapy is a promising diversion in the treatment of cancer. Thus, the identification of agents, which could inhibit tumour specific angiogenesis, has much importance in the prevention of metastasis. Unfortunately, clinical trials in cancer patients with synthetic antiangiogenic agents showed lack of therapeutic efficacy and unacceptable side effects.

In this study we tried to evaluate the antiangiogenic activity of DAS, DADS, AITC, PITC and Sulforaphane using *in vivo* as well as *in vitro* models.

4.2. Materials and Methods

4.2.1. Animals

Four to six weeks old male C57BL/6 mice were used for this study

4.2.2. Cells

B16F-10 melanoma cells were used to induce angiogenesis. Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cord vein by collagenase treatment as described in Chapter 2.

4.2.3. TNP-470

Takeda Neoplastic Product–470 (TNP-470) was a kind gift from Dr. Ravi Varma, NCI, USA.

4.2.4. Kit, Medium and Reagents

Quantikine mouse mRNA estimation kit, DMEM with 10% FCS and 199 with 20% FCS. HEPES, MTT, recombinant mouse VEGF, recombinant mouse bFGF, ^{3}H-thymidine, Matrigel (ECM), Collagen type-1, Scintillation fluid, cells to cDNA kit, mouse iNOS kit and GAPDH primer sets were used for this study.

4.2.5. ELISA kits

The quantitative ELISA kits used were mouse IL-1β, IL-2, IL-6, GM-CSF, TNF-α, VEGF and TIMP-1. The manufacturer's protocol was followed to estimate the level of the respective cytokine in the serum sample of angiogenesis induced animals.

4.2.6. Administration of Sulfur compounds

DAS, DADS, AITC and PITC were suspended in light liquid paraffin and administered intraperitoneally. DAS and DADS were administered at a dosage of 250μg/dose/animal and AITC and PITC were administered at a dosage of 25μg/dose/animal. Sulforaphane was suspended in phosphate buffered saline (PBS, pH-7.4) and administered intraperitoneally at a dosage of 500μg/dose/animal.

4.2.7. *In vivo* angiogenesis experiments

4.2.7.1. Determination of the effect of Sulfur compounds on tumour specific capillary formation

Angiogenesis was induced in seven groups of C57BL/6 mice (8 mice /group) by injecting B16F-10 melanoma cells (10^6 cells/animal) intradermally on the shaven ventral skin of each mouse. Group I animals were kept as control animals treated with vehicle (paraffin oil, i.p) only. Group II animals were treated with 5 consecutive doses of standard reference compound TNP-470 (30mg/kg body wt of animal /day, i.p; as recommended by NCI). Group III, IV, V, VI and VII animals were treated with 5 consecutive doses of DAS, DADS, AITC, PITC and Sulforaphane respectively starting simultaneously with the tumour challenge. The animals were sacrificed on 9^{th} day after the induction of angiogenesis. The skin from the ventral side was dissected out, washed with PBS and the number of tumour directed capillaries were counted using a dissection microscope at 20X magnification (Leyon and Kuttan, 2004).

4.2.7.2. Determination of the effect of Sulfur compounds on the production of IL-1β, IL-6, TNF-α, GM-CSF, IL-2, VEGF and TIMP-1 during angiogenesis

Angiogenesis was induced in six groups of C57BL/6 mice (8 mice /group) as in the previous experiment. Group I animals were given the vehicle (paraffin oil, i.p) and kept as untreated control animals. Group II, III, IV, V and VI animals were treated with 5 consecutive doses of DAS, DADS, AITC, PITC and Sulforaphane respectively starting simultaneously with the tumour challenge. Blood was collected from all groups of animals at two times intervals-24^{th} hour and 9^{th} day after the induction of angiogenesis and the serum was separated and used for the estimation of IL-1β, IL-6, TNF-α, GM-CSF, IL-2, VEGF and TIMP-1 using ELISA kits according to manufacturer's protocol (Leyon and Kuttan, 2004).

4.2.7.3. Determination of the effect of Sulfur compounds on serum nitrite levels during angiogenesis

Angiogenesis was induced in six groups of C57BL/6 mice (8 mice /group) as in the previous experiment. Group I animals were given the vehicle (paraffin oil, i.p) and kept as untreated control animals. Group II, III, IV, V and VI animals were treated with 5 consecutive doses of DAS, DADS, AITC, PITC and Sulforaphane respectively starting simultaneously with the tumour challenge. Blood was collected from all groups of animals at two times intervals-24th hour and 9th day after the induction of angiogenesis and the serum was separated and used for the estimation of nitrite level by Griess reaction (Green *et al.*, 1982).

4.2.8. *In vitro* angiogenesis experiments

4.2.8.1. Determination of the effect of Sulfur compounds on the microvessel outgrowth from the rat aortic ring (Rat aortic ring assay)

Dorsal aorta from a freshly sacrificed Sprague-Dawley rat was cut into ~1mm long pieces and each ring was placed in a collagen pre-coated 96-well plate. The rings were incubated for 24 h at 37^{0}C in complete medium and then replaced with conditioned medium from B16F-10 melanoma cells and incubated with or without different concentrations of Sulfur compounds (1, 2 & 5μg/ml). In a second set of experiment, the aortic rings were incubated for 24 h in complete medium initially and then replaced with conditioned medium from Sulfur compounds-treated (pre-treated conditioned medium) and non-treated B16F-10 melanoma cells. On day 6, the rings were analyzed by phase-contrast microscopy for microvessel outgrowth and photographed.

4.2.8.2. Determination of the effect of Sulfur compounds on quantitation of gene specific mRNA of VEGF in B16F-10 melanoma cells

B16F-10 cells (10^6 cells) were plated in 30mm petridish in DMEM with 10% FCS at 37^{0}C in 5% CO_2 atmosphere. Cells were then treated with Sulfur compounds

(5μg/ml) for 4 h. After incubation these cells were used for quantification of VEGF mRNA using the quantikine mRNA kit according to the manufacturer's procedure.

4.2.8.3. Determination of the effect of Sulfur compounds on endothelial cell viability by MTT assay

HUVECs were seeded (5000 cells/well) into 96-well flat bottomed titre plate and incubated for 24 h at 37^0C in 5% CO_2 atmosphere. Different concentrations of Sulfur compounds (1-50μg/ml) were added and incubated further for 48 h. The cell viability was assessed by MTT assay and percentage viability was calculated (Chapter 2).

4.2.8.4. Determination of the effect of Sulfur compounds on endothelial cell proliferation (^{3}H-thymidine incorporation assay)

Endothelial cell proliferation assay was carried out as described in Chapter 2. Briefly, HUVECs (5000 cells/well) were incubated with or without different concentrations of Sulfur compounds (1, 2 & 5μg/ml) along with 2ng/ml VEGF. 18 h prior to the termination of the assay 1μCi of ^{3}H- thymidine was added to each well. After completing incubation, the cells were washed with PBS and then treated with ice cold PCA. The resulting precipitate was dissolved in 0.5N NaOH and transferred to the scintillation fluid and kept overnight in dark. The radioactivity was counted using a Rack Beta liquid scintillation counter.

4.2.8.5. Determination of the effect of Sulfur compounds on endothelial cell motility

HUVECs were seeded into wells of collagen coated 96-well plates at a density of 2×10^5 cells/well and incubated for 24 h at 37^0C in 5% CO_2 atmosphere. A clear area was scraped in the monolayer with a narrow tip by applying suction and washed with serum free medium. Different concentrations of Sulfur compounds (1, 2 & 5μg/ml) were added along with 2ng/ml VEGF and further incubated for 24 h. After incubation

the cells were fixed in formalin and stained with crystal violet and photographed (10X) (Guo *et al.*, 2002).

4.2.8.6. Determination of the effect of Sulfur compounds on endothelial cell invasion

The invasion assay was carried out in Boyden chambers as described by Albini *et al* (Albini *et al.*, 1987) (Chapter 2). Briefly, the lower compartment of the chamber was filled with serum free medium 199 and a polycarbonate filter coated with 25μg Type I collagen was placed above this. HUVECs (10^5 cells/150μl medium 199) were then seeded on to the upper chamber in the presence and absence of Sulfur compounds (1, 2 & 5μg/ml) along with 2ng/ml VEGF and FGF and incubated at 37^0C in 5% CO_2 atmosphere for 10 h. After incubation, the filters were removed, fixed with methanol and stained with crystal violet. Cells migrating to the lower surface of the polycarbonate filters were counted under a microscope. The results were expressed as percentage inhibition of invasion.

4.2.8.7. Determination of the effect of Sulfur compounds on endothelial cell tube formation

Tube formation was carried out according to the protocol of Gupta *et al* (Gupta *et al.*, 2002). Thirty microlitre ice cold matrigel was pipetted into a 96-well flat bottomed titre plate and kept for 30 minutes at 37^0C to allow gelling of matrigel. HUVECs in medium 199 were seeded into the layer of matrigel at a density of 1×10^3 cells/well along with 2ng/ml VEGF and FGF. Various concentrations of Sulfur compounds (1, 2 & 5μg/ml) were added into the wells and incubated for 48 h at 37^0C in 5% CO_2 atmosphere. After incubation, the cells were fixed with Diff Quick fixative and stained with Diff Quick stain solution. Tube formation was examined and photographed using an inverted microscope (20X).

4.2.8.8. Gelatin zymography

Gelatin Zymography was followed according to the procedure of Billings *et al* (Billings *et al.*, 1991) with some modification as described in Chapter 2. HUVECs of

subconfluent cultures were incubated with serum free medium for 24 h at 37^0C in 5% CO_2 atmosphere. Fifty microlitres of sample (conditioned medium- equivalent to 100µg protein) was activated with 5µl trypsin solution (75µg/ml) in the presence and absence of Sulfur compounds (2 & 5µg/ml) and subjected to zymographic analysis. Gels were fixed, stained and clear bands were visualized against a dark background.

4.2.8.9. Determination of the effect of Sulfur compounds on iNOS gene expression

cDNA was synthesized using B16F-10 cells (2X 10^4 cells/well) grown in 96-well titre plate in the presence of Sulfur compounds (5µg/ml) for 4 h at 37^0C in 5% CO_2 in serum free medium. cDNA was synthesized using cells to cDNA^{TM} II kit (Ambion Inc,USA). Gene expression analysis was done by PCR analysis. The mouse iNOS gene was amplified against GAPDH primers obtained from Maxim biotech, USA. The target PCR products generated from a positive control cDNA was also included in this kit. PCR products were analyzed by agarose gel electrophoresis and visualized using gel documentation system (Chapter 2).

4.3. Results

4.3.1. *In vivo* angiogenesis experiments

4.3.1.1. Effect of Sulfur compounds on tumour specific capillary formation

All the B16F-10 melanoma induced animals developed a palpable tumour and visible tumour directed blood vessels (Figure 4.1). The number of tumour directed capillaries were significantly reduced in the Sulforaphane (62.6%) and DADS (60%) treated animals compared to the untreated control animals. The groups, which received Sulforaphane and DADS, had an average of 10.75 ± 1.6 (Figure 4.1g) and 11.5 ± 1.9 (Figure 4.1d) capillaries respectively whereas control group had an average of 28.75 ± 1.7 (Figure 4.1a) capillaries around 1cm^2 area around the tumour. Administration of DAS and AITC produced 42.60% (16.5 ± 1.3 capillaries; Figure 4.1c) and 40.43% (17.12 ± 1.8 capillaries; Figure 4.1e) reduction of tumour directed capillary formation respectively whereas PITC treatment produced only 30% (20.12 ± 1.9 capillaries; Figure 4.1f) reduction of neovascularization. The reference compound (TNP-470)

Figure 4.1. Effect of Sulfur compounds on tumour specific capillary formation

The animals were induced tumour directed neoangiogenesis by injecting 10^6 B16F-10 melanoma cells intradermally.

a) Vehicle treated control

b) Treatment with TNP-470

c) Treatment with DAS

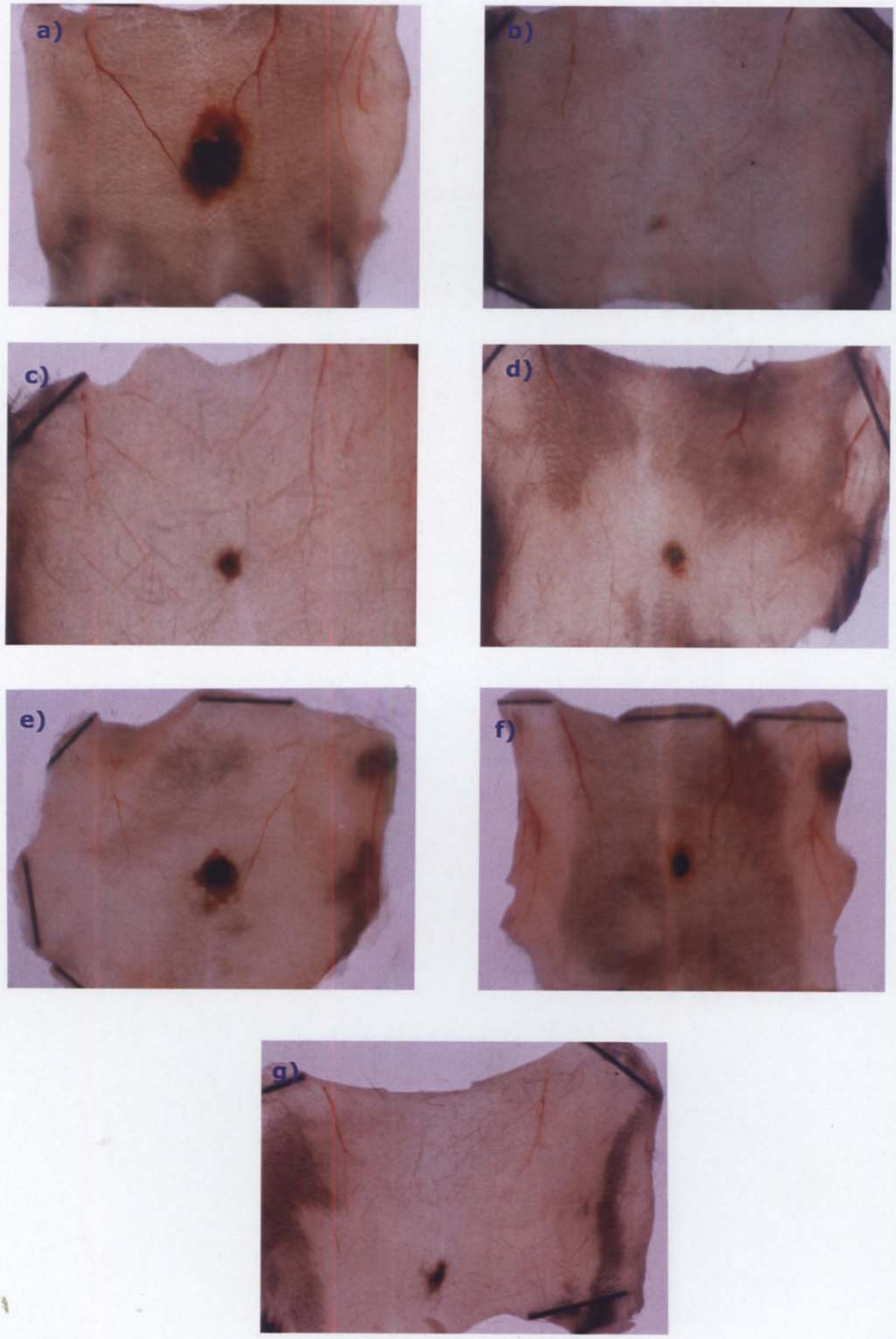
d) Treatment with DADS

e) Treatment with AITC

f) Treatment with PITC

g) Treatment with Sulforaphane

Figure 4.1



treated animals showed 85.66% reduction in the neovessel formation and had an average number of 4.12 ± 1.3 capillaries (Figure 4.1b) (Table 4.1).

4.3.1.2. Effect of Sulfur compounds on the production of IL-1β, IL-6, TNF-α, GM-CSF and VEGF during angiogenesis

The level of proinflammatory cytokines such as IL-1β, IL-6, TNF-α and GM-CSF is presented in Table 4.2a & b. In control animals, the level of IL-1β in the serum was drastically enhanced 24 h after tumour challenge (31.74 ± 1.04pg/ml) which was double when compared to normal (16 ± 3.5pg/ml) and maintained the same level even after 9 days. Treatment of DADS (28.97 ± 1.26pg/ml) and Sulforaphane (28.35 ± 1.51pg/ml) slightly checked this initial elevation of serum IL-1β level after 24 h of tumour challenge and by day 9 both these compounds effectively reduced the IL-1β level to normal (15.93 ± 0.93pg/ml and 15.06 ± 1.05pg/ml respectively for DADS and Sulforaphane). Even though administration of DAS (30.5 ± 0.57pg/ml), AITC (30.2 ± 1pg/ml) and PITC (30.6 ± 0.69pg/ml) did not affect this initial elevation in the serum level of this cytokine on the 24th h, it effectively reduced the IL-1β level almost to normal (18.05 ± 0.8pg/ml, 17.59 ± 1.03pg/ml and 18.21 ± 0.84pg/ml respectively for DAS, AITC and PITC)) by day 9 after angiogenesis induction.

IL-6 level was found to be elevated in the serum of untreated angiogenesis induced control animals 24 h after tumour induction (41.21 ± 3.2pg/ml) compared to normal level of 35 ± 6.5pg/ml. On day 9, the level of IL-6 in serum of control animals was drastically elevated to 326.30 ± 5.58pg/ml. Treatment of DADS (60.89 ± 3.59pg/ml) and Sulforaphane (62.76 ± 5.6pg/ml) significantly reduced the drastic enhancement of IL-6 level on 9th day of tumour inoculation, even though these two compounds did not produced much effect on the initial elevation after 24 h of angiogenesis induction. Administration of AITC (72.33 ± 3.22pg/ml), DAS (81.64 ± 4.8pg/ml) and PITC (86.28 ± 2.73pg/ml) was also found to be effective in checking the drastic enhancement of IL-6 level on 9th day of tumour inoculation.

Drastic enhancement in the level of serum TNF-α (188.08 ± 7.3pg/ml) was observed in untreated control animals after 24 h of angiogenesis induction compared to normal serum TNF-α level (20 ± 3.2pg/ml) and was much more elevated on day 9 of

Table 4.1. Effect of Sulfur compounds on tumour directed capillaries

Treatment	No. of capillaries/ cm^2	% inhibition
Tumour control	28.75 ± 1.7	
DAS	16.50 ± 1.3*	42.60
DADS	11.50 ± 1.9*	60.00
AITC	17.12 ± 1.8*	40.43
PITC	20.12 ± 1.9*	30.00
Sulforaphane	10.75 ± 1.6*	62.60

Animals were induced angiogenesis by injecting 10^6 B16F-10 melanoma cells intradermally. Animals were treated with Sulfur compounds for 5 consecutive days. Number of capillaries per 1 cm^2 around the tumour was counted using a dissection microscope (X 20). The values are mean ± SD. * $P < 0.001$ compared to control.

Table 4.2a. Effect of DAS and DADS on the VEGF and proinflammatory cytokine profile of angiogenesis induced animals

Cytokines (pg/ml)	Normal	Tumour control		DAS		DADS	
		Day 1	Day 9	Day 1	Day 9	Day1	Day 9
VEGF	16 ± 8	63.97 ± 2.12	159.27 ± 4.28	38.99 ± 0.65*	64.88 ± 1.72*	36.47 ± 3.40 *	60.44 ± 1.32*
IL-1β	16 ± 3.5	31.74 ± 1.04	32.58 ± 0.95	30.50 ± 0.57*	18.05 ± 0.80*	28.97 ± 1.26*	15.93 ± 0.93*
IL-6	35 ± 6.5	41.21 ± 3.2	326.30 ± 5.58	39.62 ± 2.56*	81.64 ± 4.80*	35.9 ± 2.87*	60.89 ± 3.59*
TNF-α	20 ± 3.2	188.08 ± 7.3	609.50 ± 8.8	173.49 ± 5.06*	155.17 ± 6.76*	166.39±8.42*	122.64 ± 7.29*
GM-CSF	18 ± 3.1	65.42 ± 1.06	28.62 ± 1.89	36.23 ± 0.34*	21.95 ± 0.47*	28.46 ± 2.12 *	18.79 ± 1.42*

Blood was collected from the angiogenesis induced animals at the indicated time points after tumour challenge. Serum was separated by centrifugation and the cytokine level was estimated by ELISA method. All the values are mean ± SD. *p<0.001 compared to control.

Table 4.2b. Effect of Sulforaphane, AITC and PITC on the VEGF and proinflammatory cytokine profile of angiogenesis induced animals

Cytokines (pg/ml)	Sulforaphane		AITC		PITC	
	Day 1	Day 9	Day 1	Day 9	Day1	Day 9
VEGF	34.32 ± 2.78*	57.68 ± 1.95*	37.47 ± 0.86*	66.14 ± 1.7*	41.50 ± 0.78*	71.72 ± 0.82*
IL-1β	28.35 ± 1.51*	15.06 ± 1.05*	30.20 ± 1.0*	17.59 ± 1.03*	30.60 ± 0.69*	18.21 ± 0.84*
IL-6	38.55 ± 2.79*	62.76 ± 5.6*	39.09 ± 2.87*	72.33 ± 3.22*	40.1 ± 0.32*	86.28 ± 2.73*
TNF-α	145.83 ± 6.73*	111.42 ± 4.66*	170.50 ± 8.09*	144.7 ± 7.85*	181.02 ± 7.56*	156.55± 6.45*
GM-CSF	27.84 ± 1.67*	18.09 ± 1.13*	34.59 ± 1.27*	23.71 ± 0.39*	38.40 ± 2.0*	26.53 ± 1.05*

Blood was collected from the angiogenesis induced animals at the indicated time points after tumour challenge. Serum was separated by centrifugation and the cytokine level was estimated by ELISA method. All the values are mean ± SD.

*$p<0.001$ compared to control.

tumour challenge (609.5 ± 8.8pg/ml). Sulfur compounds, especially Sulforaphane and DADS, were found to be good regulators of serum TNF-α level during angiogenesis. Even though initial enhancement of TNF-α level was observed on 24th h after tumour inoculation (145.83 ± 6.73pg/ml, 166.39±8.42pg/ml, 170.5 ± 8.09pg/ml, 173.49 ± 5.06pg/ml and 181.02 ± 7.56pg/ml respectively for Sulforaphane, DADS, AITC, DAS and PITC), administration of Sulforaphane, DADS, AITC, DAS and PITC significantly reduced the elevated serum TNF-α level to 111.42 ± 4.66pg/ml, 122.64 ± 7.29pg/ml, 144.7 ± 7.85pg/ml, 155.17 ± 6.76pg/ml and 156.55 ± 6.45pg/ml respectively after 9th day of tumour challenge.

Injection of B16F-10 melanoma cells to the intradermal region of mice showed a tendency to immediately elevate the GM-CSF level in the serum as estimated from the 24th h serum sample. Control animals had a highly elevated level of GM-CSF (65.42 ± 1.06 pg/ml), which was significantly inhibited by the treatment with Sulforaphane (27.84 ± 1.67 pg/ml) and DADS (28.46 ± 2.12 pg/ml). The other Sulfur compounds such as AITC (34.59 ± 1.27pg/ml), DAS (36.23 ± 0.34 pg/ml) and PITC (38.40 ± 2pg/ml) were also had a reduced level compared to the control. By 9th day of angiogenesis induction this elevated level was normalized (18 ± 3.1pg/ml) in Sulforaphane (18.09 ± 1.13pg/ml) and DADS (18.79 ± 1.42pg/ml) treated groups. Treatment with DAS (21.95 ± 0.47pg/ml) and AITC (23.71 ± 0.39pg/ml) also reduced the elevated level of GM-CSF more close to normal values after 9th day of tumour inoculation.

Serum VEGF levels also showed an immediate elevation in control (63.97 ± 2.12pg/ml) and all treated animals compared to the normal levels (16 ± 8 pg/ml), on the 24th h serum sample. For Sulforaphane treated animals VEGF level was 34.32 ± 2.78pg/ml and the treatment with DADS (36.47 ± 3.40 pg/ml), AITC (37.47 ± 0.86pg/ml), DAS (38.99 ± 0.65pg/ml) and PITC (41.50 ± 0.78pg/ml) also had higher levels of VEGF, but lower than the untreated control. This increase was continued in control animals (159.27 ± 4.28pg/ml) on 9th day as well whereas in Sulforaphane and DADS treated animals, it was only 57.68 ± 1.95pg/ml and 60.44 ± 1.32pg/ml

respectively. Also for DAS (64.88 ± 1.72pg/ml), AITC (66.14 ± 1.70pg/ml), and PITC (71.72 ± 0.82pg/ml) treated animals the VEGF level was much lower than the control of the same day.

4.3.1.3. Effect of Sulfur compounds on IL-2 and TIMP-1 levels of angiogenesis induced animals

The IL-2 levels showed a decrease in the serum samples of untreated control animals on the 24th h (16.60 ± 0.67pg/ml) and 9th day (18.20 ± 0.55 pg/ml) of angiogenesis induction as compared to the normal levels (23 ± 3.2 pg/ml). But, interestingly all Sulfur compounds especially Sulforaphane (27.01 ± 0.34pg/ml) and DADS (25.24 ± 1.73pg/ml) significantly inhibited this depression in IL-2 levels in the 24th h serum sample. The IL-2 level in the 24th h serum samples of AITC, DAS and PITC treated animals were 20.23 ± 0.96pg/ml, 19.9 ± 1.01pg/ml and 18.86 ± 1.37pg/ml respectively. Similar results were also observed in the 9th day serum sample. In control animals, the serum IL-2 level was 18.20 ± 0.55pg/ml whereas treatment with Sulforaphane (63.63 ± 4.63pg/ml), DADS (56.33 ± 2.32pg/ml), AITC (47.14 ± 1.99pg/ml), DAS (43.91 ± 1.6pg/ml) and PITC (40.58 ± 2.06pg/ml) significantly enhanced the serum level of IL-2 (Table 4.3).

Serum profile for TIMP–1 of the angiogenesis induced animals showed a similar pattern as that of IL-2. The TIMP level in the serum of normal mouse was 600 ± 36pg/ml which was reduced by the induction of tumour cells to 353.48 ± 1.25pg/ml in untreated control animals. But administration of Sulforaphane (752.37 ± 3.42pg/ml), DADS (731.08 ± 2.56pg/ml), AITC (686.83 ± 2.77pg/ml), DAS (673.15 ± 4.71pg/ml) and PITC (664.72 ± 3.53pg/ml) significantly elevated the level of this MMP inhibitor. Ninth day serum sample also showed a similar TIMP-1 profile in angiogenesis induced animals. In control it was 359.75 ± 1.58pg/ml and Sulforaphane (971.32 ± 4.34pg/ml), DADS (923.35 ± 4.89pg/ml), AITC (804.07 ± 2.54pg/ml), DAS (788.08 ± 5.99pg/ml) and PITC (777.58 ± 3.44pg/ml) treatment maintained the initial elevation thereby checking MMPs (Table 4.3).

Table 4.3. Effect of Sulfur compounds on the IL-2 and TIMP-1 profiles of angiogenesis induced animals.

Treatment	IL-2 (pg/ml)		TIMP (pg/ml)	
	Day 1	Day 9	Day 1	Day 9
Normal	23 ± 3.2		600 ± 36	
T. Control	16.60 ± 0.67	18.20 ± 0.55	353.48 ± 1.25	359.75 ± 1.58
DAS	19.90 ± 1.01*	43.91 ± 1.6*	673.15 ± 4.71*	788.08 ± 5.99*
DADS	25.24 ± 1.73*	56.33 ± 2.32*	731.08 ± 2.56*	923.35 ± 4.89*
AITC	20.23 ± 0.96*	47.14 ± 1.99*	686.83 ± 2.77*	804.07 ± 2.54*
PITC	18.86 ± 1.37*	40.58 ± 2.06*	664.72 ± 3.53*	777.58 ± 3.44*
Sulforaphane	27.01 ± 0.34*	63.63 ± 4.63*	752.37 ± 3.42*	971.32 ± 4.34*

Blood was collected from the angiogenesis induced animals at the indicated time points after tumour challenge. Serum was separated by centrifugation, and levels of TIMP and IL-2 were estimated by ELISA method. All the values are mean ± SD (Mean of triplicate). *$p<0.001$ compared to control.

T. Control – Tumour Control

4.3.1.4. Effect of Sulfur compounds on serum nitrite levels during angiogenesis

The effect of Sulfur compounds on serum nitrite level is shown in Table 4.4. The serum nitrite level of control animals was highly elevated (38.86 ± 0.81μ mols) after 9th day of tumour challenge whereas administration of Sulfur compounds significantly reduced the elevated serum nitrite level and it was only 21.96 ± 0.97μ mols with 43.48% reduction in DADS treated group, 22.24 ± 0.84μ mols with 42.76% reduction in Sulforaphane treated group, 22.98 ± 1.02μ mols with 40.86% reduction in AITC treated group, 23.58 ± 1.1μ mols with 39.83% reduction in DAS treated group and 24.23 ± 0.05μ mols with 37.64% reduction in PITC treated group on the same day which were nearer to normal serum nitrite level (21.76 ± 0.52μ mols).

4.3.2. *In vitro* angiogenesis experiments.

4.3.2.1. Effect of Sulfur compounds on the microvessel outgrowth from the rat aortic ring (Rat aortic ring assay)

Conditioned medium from B16F-10 melanoma cells induced microvessel out growth from rat aorta ring (Figure 4.2a). Treatment with Sulforaphane (Figure 4.2f) and DADS (Figure 4.2c) at 5μg/ml significantly inhibited microvessel outgrowths from rat aortic rings induced by the conditioned medium from the B16F-10 melanoma cells. Administration of AITC (Figure 4.2d), DAS (Figure 4.2b) and PITC (Figure 4.2e) at a concentration of 5μg/ml also checked microvessel outgrowths from rat aortic rings. The aortic rings incubated with conditioned medium from Sulforaphane (Figure 4.2k), DADS (Figure 4.2h), AITC (Figure 4.2i), DAS (Figure 4.2g) and PITC (Figure 4.2j) treated B16F-10 melanoma cells (pre-treated conditioned medium) were also showed significant reduction in microvessel outgrowth when compared to aortic rings incubated with conditioned medium from untreated B16F-10 melanoma cells.

4.3.2.2. Effect of Sulfur compounds on VEGF mRNA expression

Highly elevated level of VEGF mRNA expression was observed in untreated B16F-10 melanoma cells (27.65 ± 0.21attomoles). Administration of Sulforaphane (8.87 ± 0.14 attomoles), DADS (8.96 ± 0.12 attomoles), AITC (10.01 ± 0.15 attomoles*), DAS (9.44 ±0.18 attomoles) and PITC (11.77 ± 0.16 attomoles*) at 5μg/ml significantly reduced the elevated VEGF mRNA level (*$p<0.001$).

Table 4.4. Effect of Sulfur compounds on serum nitrite level of angiogenesis induced animals

Treatment	Amount of nitrite (μ mols)	% inhibition
Normal	21.76 ± 0.52	
T. Control	38.86 ± 0.81	
DAS	23.58 ± 1.10*	39.83
DADS	21.96 ± 0.97*	43.48
AITC	22.98 ±1.02*	40.86
PITC	24.23 ± 0.05*	37.64
Sulforaphane	22.24 ± 0.84*	42.76

Animals were induced angiogenesis by injecting 10^6 B16F-10 melanoma cells intradermally. Animals were treated with Sulfur compounds for 5 consecutive days. Blood was collected 9^{th} day after tumour challenge, serum was separated and used for NO estimation by Griess reaction and is expressed as amount of nitrite. Values are mean ± SD.* $p<0.001$ compared to control.

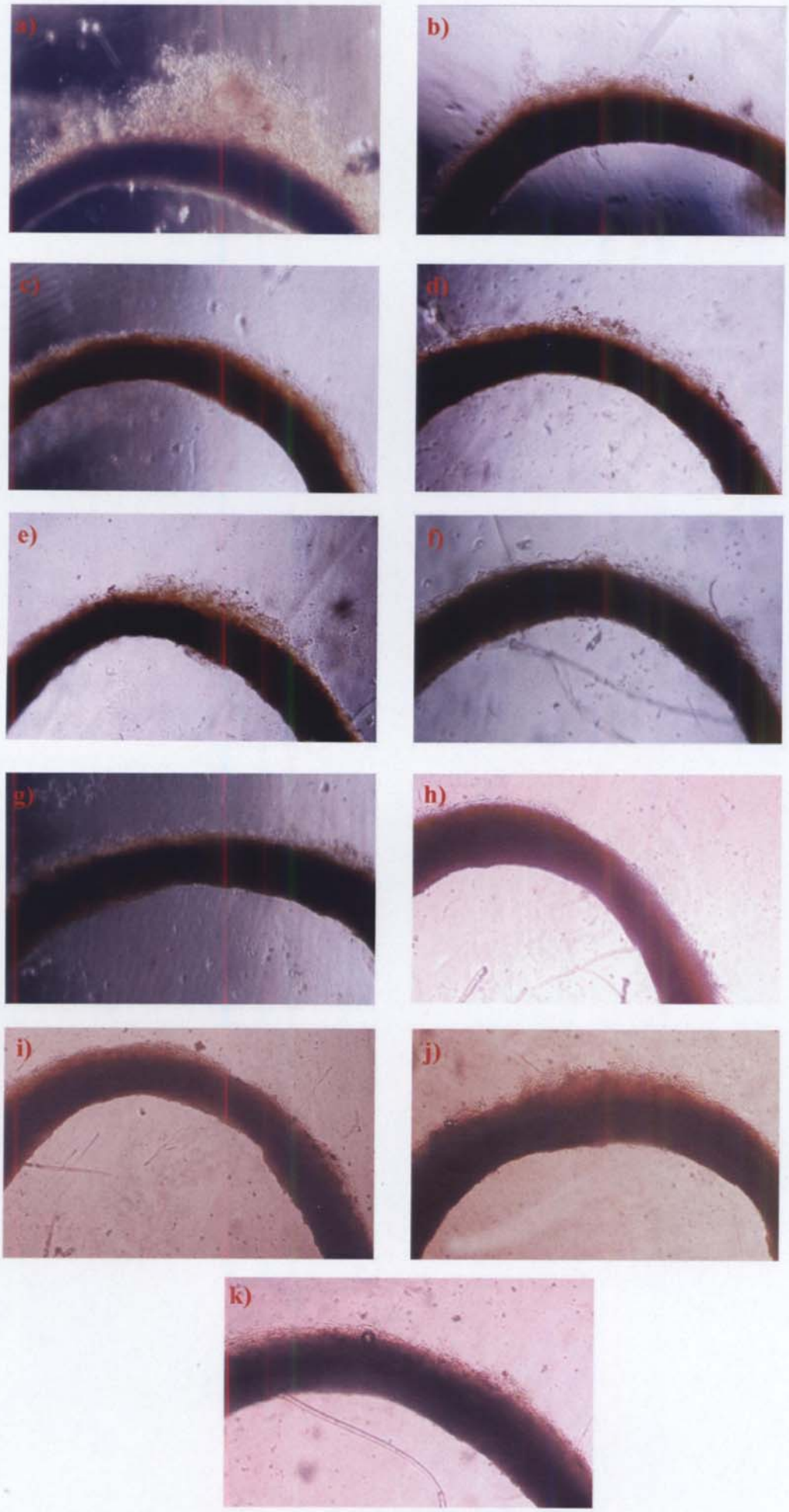
T. Control – Tumour Control

Figure 4.2. Effect of Sulfur compounds on the microvessel outgrowth from the rat aortic ring

Conditioned medium from normal semi confluent bottles of B16F-10 cells act as the control. Treatment along with conditioned medium or the conditioned medium of pretreated B16F-10 reduced the microvessel out growth from the aorta.

a) Control – Conditioned medium alone
b) Conditioned medium + Treatment with DAS (5μg/ml)
c) Conditioned medium + Treatment with DADS (5μg/ml)
d) Conditioned medium + Treatment with AITC (5μg/ml)
e) Conditioned medium + Treatment with PITC (5μg/ml)
f) Conditioned medium + Treatment with Sulforaphane (5μg/ml)
g) DAS (5μg/ml) pre treated conditioned medium
h) DADS (5μg/ml) pre treated conditioned medium
i) AITC (5μg/ml) pre treated conditioned medium
j) PITC (5μg/ml) pre treated conditioned medium
k) Sulforaphane (5μg/ml) pre treated conditioned medium

Figure 4.2



4.3.2.3. Effect of Sulfur compounds on endothelial cell viability by MTT assay

To determine the non-toxic doses of Sulfur compounds to HUVECs, MTT assay was performed. Up to concentrations of 1-5μg/ml all Sulfur compounds were found to be non-toxic to HUVECs as there was more than 95% cell viability and these concentrations were used for further *in vitro* experiments (Table 4.5).

4.3.2.4. Effect of Sulfur compounds on endothelial cell proliferation (^{3}H-thymidine incorporation assay)

Rate of proliferation was determined by ^{3}H-thymidine incorporation by the DNA of HUVECs. Thymidine incorporation is proportional to the potential of the cells to synthesize DNA. Proliferation was expressed as radioactive count per minute (cpm). HUVECs showed very high rate of proliferation (4463.33 ± 112.61cpm) when stimulated with VEGF. Administration of Sulforaphane (63.96%, 1608.32 ± 42.79 cpm) and DADS (61.38%, 1723.66 ± 58.56 cpm) at a concentration of 5μg/ml significantly inhibited VEGF induced proliferation of HUVECs. Considerable inhibition of proliferation was also observed when Sulforaphane (39.30%, 2708.94 ± 52.63 cpm) and DADS (37.63%, 2783.66 ± 35.72 cpm) were administered at a lower concentration of 2μg/ml. Administration of other Sulfur compounds such as DAS (59.39%, 1812.55 ± 29.61cpm), AITC (53.29%, 2084.82 ± 38.89 cpm) and PITC (45.43%, 2435.33 ± 54.72 cpm) were also found to effectively inhibit VEGF induced proliferation of HUVECs at a concentration of 5μg/ml. Lower concentration (2μg/ml) of DAS (36.06%, 2853.85 ± 54.99 cpm), AITC (34.02%, 2944.90 ± 42.43 cpm) and PITC (27.55%, 3233.33 ± 41.06 cpm) also produced considerable inhibition of endothelial cell proliferation (Table 4.6).

4.3.2.5. Effect of Sulfur compounds on endothelial cell migration/ motility

Effect of Sulfur compounds on the motility of HUVECs is shown in Figure 4.3. HUVECs migrated into the clear area when stimulated with VEGF (Figure 4.3b). All Sulfur compounds, especially Sulforaphane and DADS significantly inhibited the VEGF induced migration of endothelial cells in a concentration dependent manner and maximum inhibition of endothelial cell migration was observed at 5μg/ml (Figure 4.3c-

Table 4.5. Effect of Sulfur compounds towards viability of HUVECs in culture

Concentration (μg/ml)	% of viable cells				
	DAS	DADS	AITC	PITC	Sulforaphane
1	99.58	99.15	99.20	99.53	99.64
2	99.15	98.73	98.58	99.06	99.10
5	97.45	96.18	97.16	98.11	97.25
10	95.75	93.70	89.58	94.32	92.68
20	87.66	84.26	82.47	85.80	81.34
25	68.52	65.54	62.56	75.83	67.56
50	57.45	53.62	49.80	55.93	51.38

HUVECS were incubated with different concentrations (1-50μg/ml) of Sulfur compounds. Percentage of cell viability was determined using MTT assay.

Table 4.6. Effect of Sulfur compounds on proliferation of HUVECs

Concentration	% inhibition				
(μg/ml)	DAS	DADS	AITC	PITC	Sulforaphane
1	16.15	18.1	12.20	11.74	17.50
2	36.06	37.63	34.02	27.55	39.30
5	59.39	61.38	53.29	45.43	63.96

HUVECs ($5X10^3$ cells/ well) were grown in 96- well flat bottom plate. After 24 h various concentrations of Sulfur compounds were added and incubation was continued for 48 h. After incubation, ^3H –thymidine was added to each well (1μCi /well) and further incubated for 18 h. Cells were lysed and radioactivity was counted by using Rack Beta liquid scintillation counter.

Figure 4.3. Effect of Sulfur compounds on the motility of HUVECs

a) Control - '0' hour incubation

b) Control after 24h incubation in medium without treatment

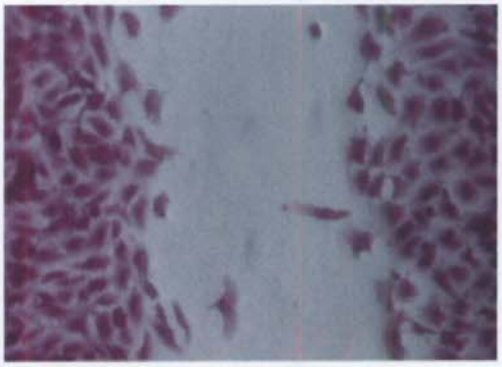
c) Treatment with DAS (2μg/ml)

d) Treatment with DAS (5μg/ml)

e) Treatment with DADS (2μg/ml)

f) Treatment with DADS (5μg/ml)

Figure 4.3



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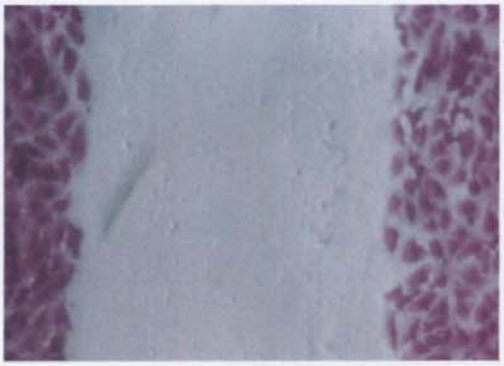

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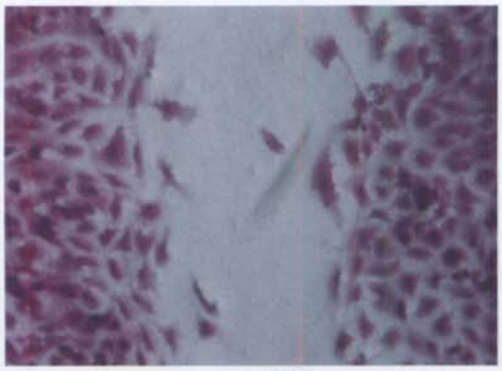

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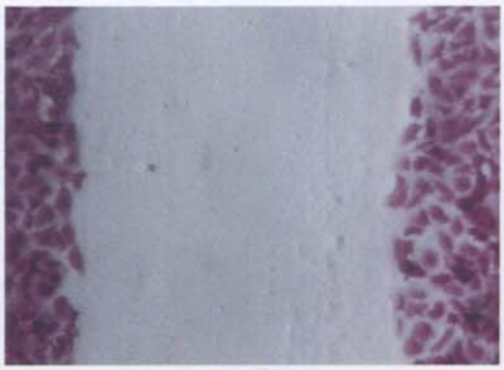

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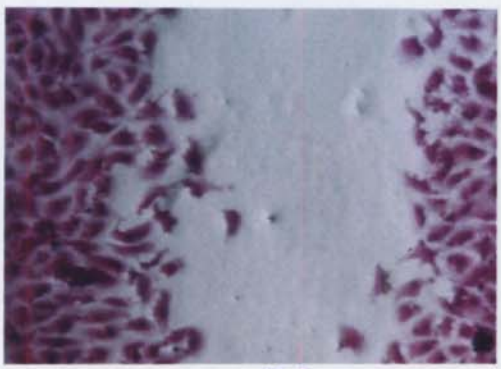

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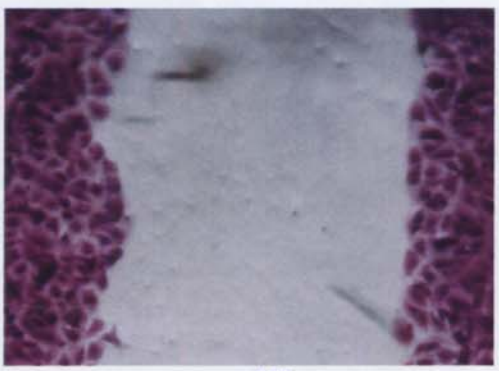


(l)

Figure 4.3. Effect of Sulfur compounds on the motility of HUVECs

g) Treatment with AITC (2μg/ml)

h) Treatment with AITC (5μg/ml)

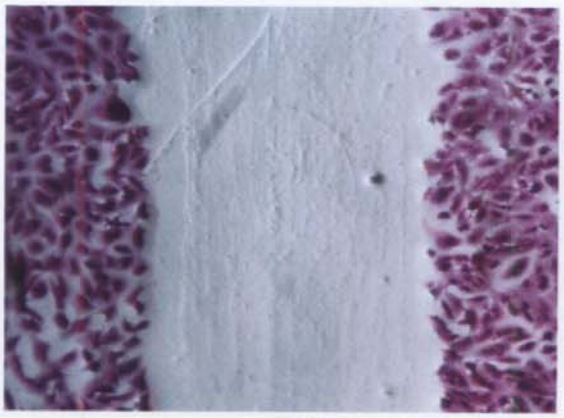
i) Treatment with PITC (2μg/ml)

j) Treatment with PITC (5μg/ml)

k) Treatment with Sulforaphane (2μg/ml)

l) Treatment with Sulforaphane (5μg/ml)

Figure 4.3



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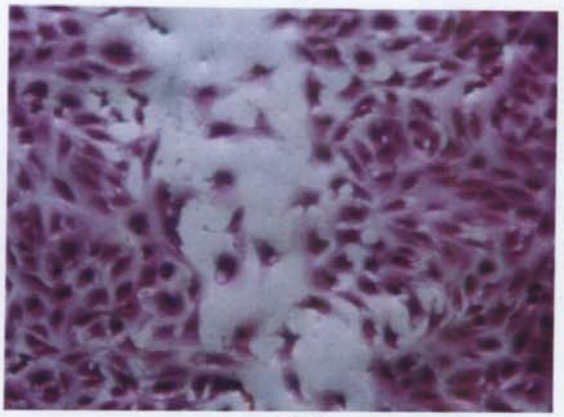

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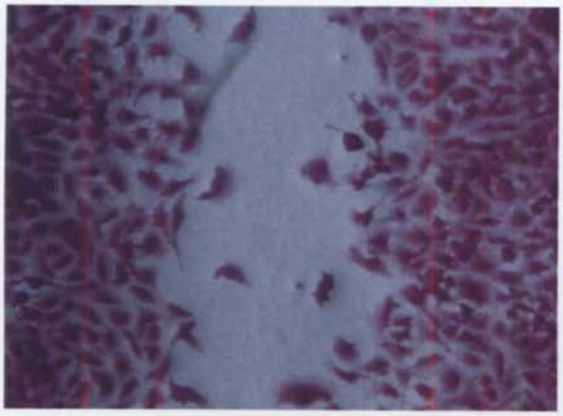

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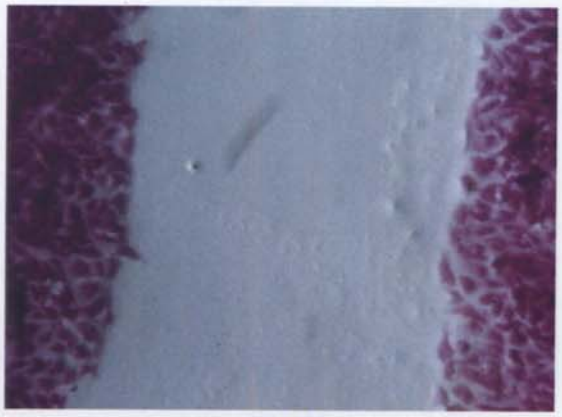

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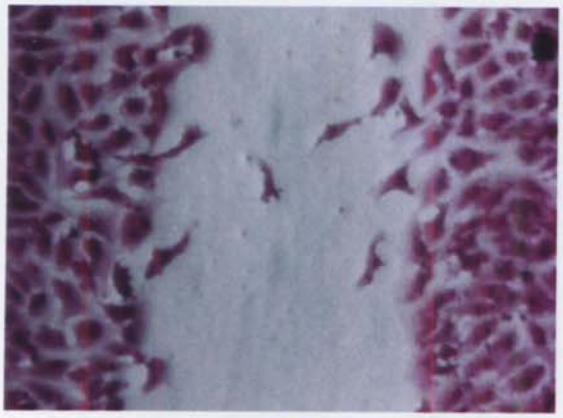

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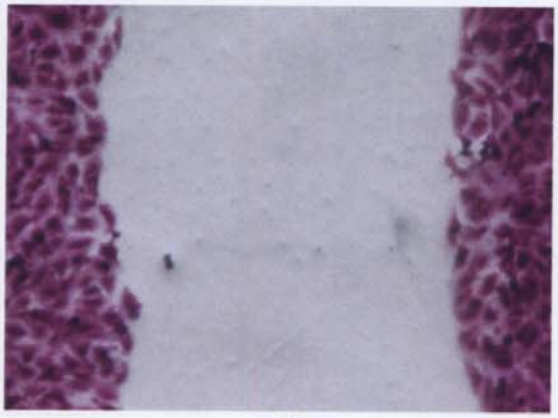


(f)

l) and was similar to that of zero hour incubation (Figure 4.3a). This concentration is non-toxic as is evident from MTT assay and hence the inhibitory effect could not be attributed to cytotoxic activity.

4.3.2.6. Effect of Sulfur compounds on endothelial cell invasion

HUVECs showed high invasive property through the collagen matrix when stimulated with VEGF and FGF. Large numbers of cells were found on the lower surface of the polycarbonate, but administration of test compounds produced significant inhibition in the invasion of the collagen matrix by HUVECs in a concentration dependent manner. Sulforaphane was found to be most effective with 95.32% inhibition of invasion at a concentration of 5μg/ml, followed by DADS (93.70%), AITC (86.14%), DAS (80.53%) and PITC (68.99%). At 2μg/ml also considerable inhibition of invasion was observed. At this concentration Sulforaphane produced 70.58% inhibition of invasion followed by DADS (62.4%), AITC (53.77%), DAS (51.94%) and PITC (43.46%) (Figure 4.4).

4.3.2.7. Effect of Sulfur compounds on endothelial cell tube formation

Treatment of HUVECs with Sulfur compounds significantly inhibited tube formation (Figure 4.5). Incubation of HUVECs on matrigel with VEGF resulted in the formation of elongated and tube like structures (Figure 4.5a). Sulfur compounds, especially Sulforaphane and DADS, effectively reduced the width and length of endothelial tubes in a concentration dependent manner and maximum inhibition was observed at 5μg/ml. These results demonstrate that Sulforaphane, DADS, AITC, DAS and PITC have the ability to block VEGF-induced *in vitro* angiogenesis.

4.3.2.8. Gelatin zymography

The major forms of proteases detected by gelatin zymography are type IV collagenases, MMP-2 and MMP-9 (Figure 4.6). Since these proteases secreted by the cells into the conditioned medium are mainly proenzymes (latent form requiring activation) an activation step (trypsin activation) is necessary to get the MMPs activated. Conditioned medium after trypsin activation showed digested clear areas at

Figure 4.4. Effect of Sulfur compounds on invasion of HUVECs

a) Untreated control

b) Treatment with DAS (2μg/ml)

c) Treatment with DAS (5μg/ml)

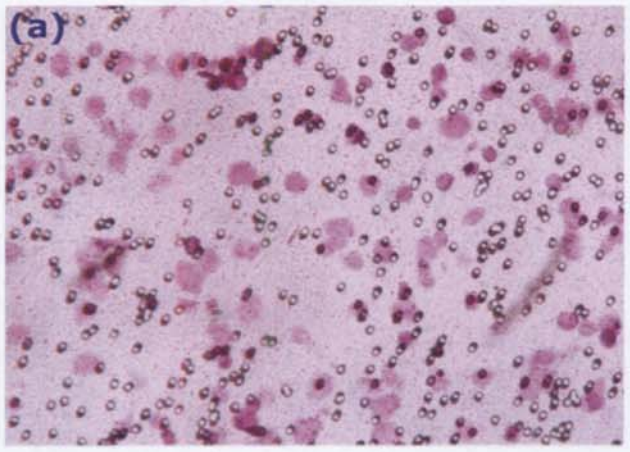
d) Treatment with DADS (2μg/ml)

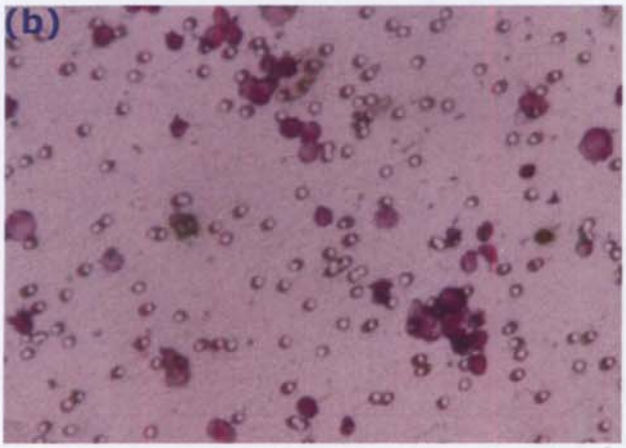
e) Treatment with DADS (5μg/ml)

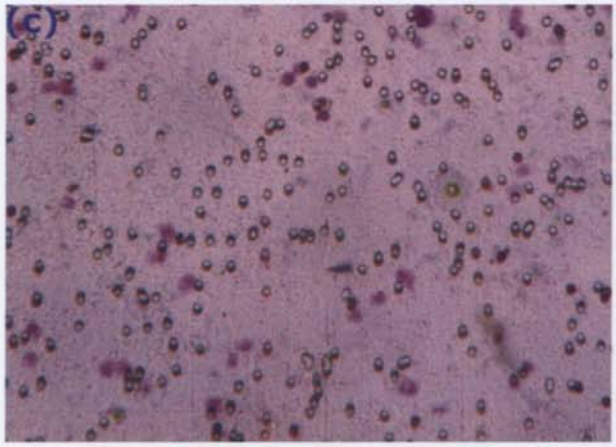
f) Treatment with AITC (2μg/ml)

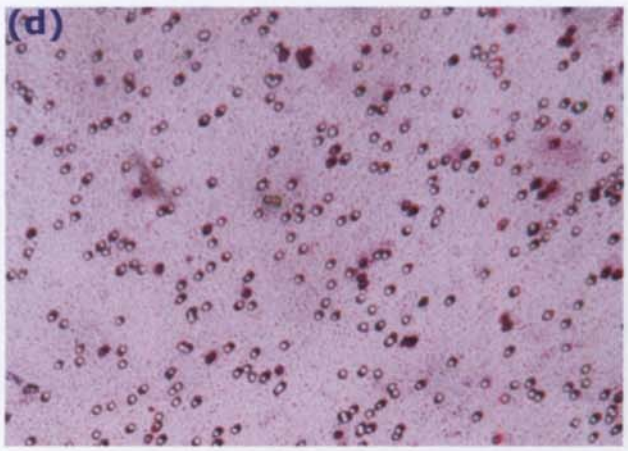
g) Treatment with AITC (5μg/ml)

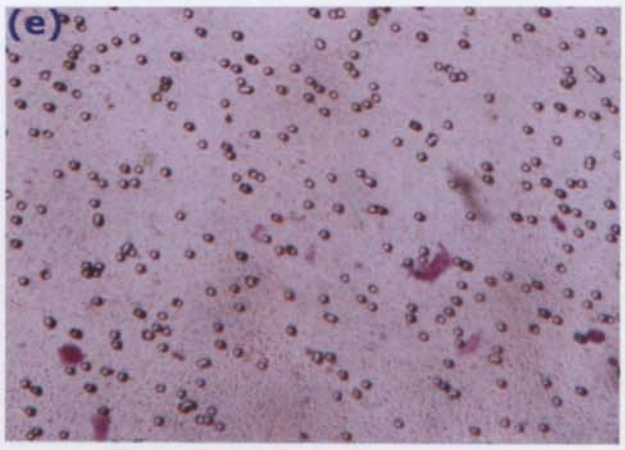
Figure 4.4

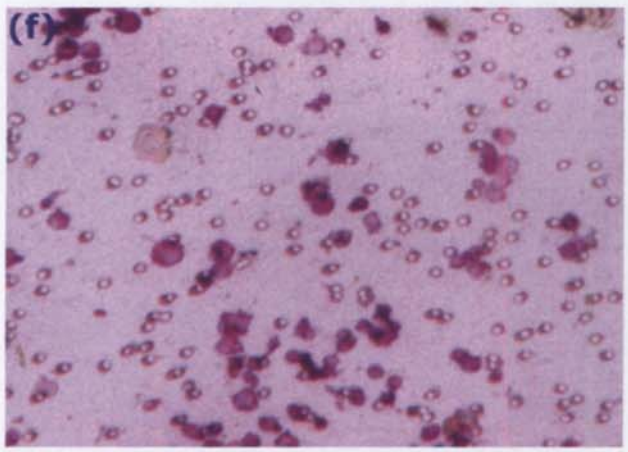












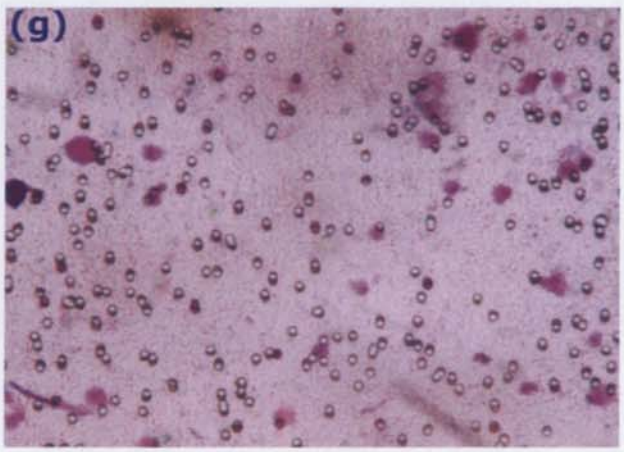


Figure 4.4. Effect of Sulfur compounds on invasion of HUVECs

h) Treatment with PITC (2μg/ml)

i) Treatment with PITC (5μg/ml)

j) Treatment with Sulforaphane (2μg/ml)

k) Treatment with Sulforaphane (5μg/ml)

Figure 4.4

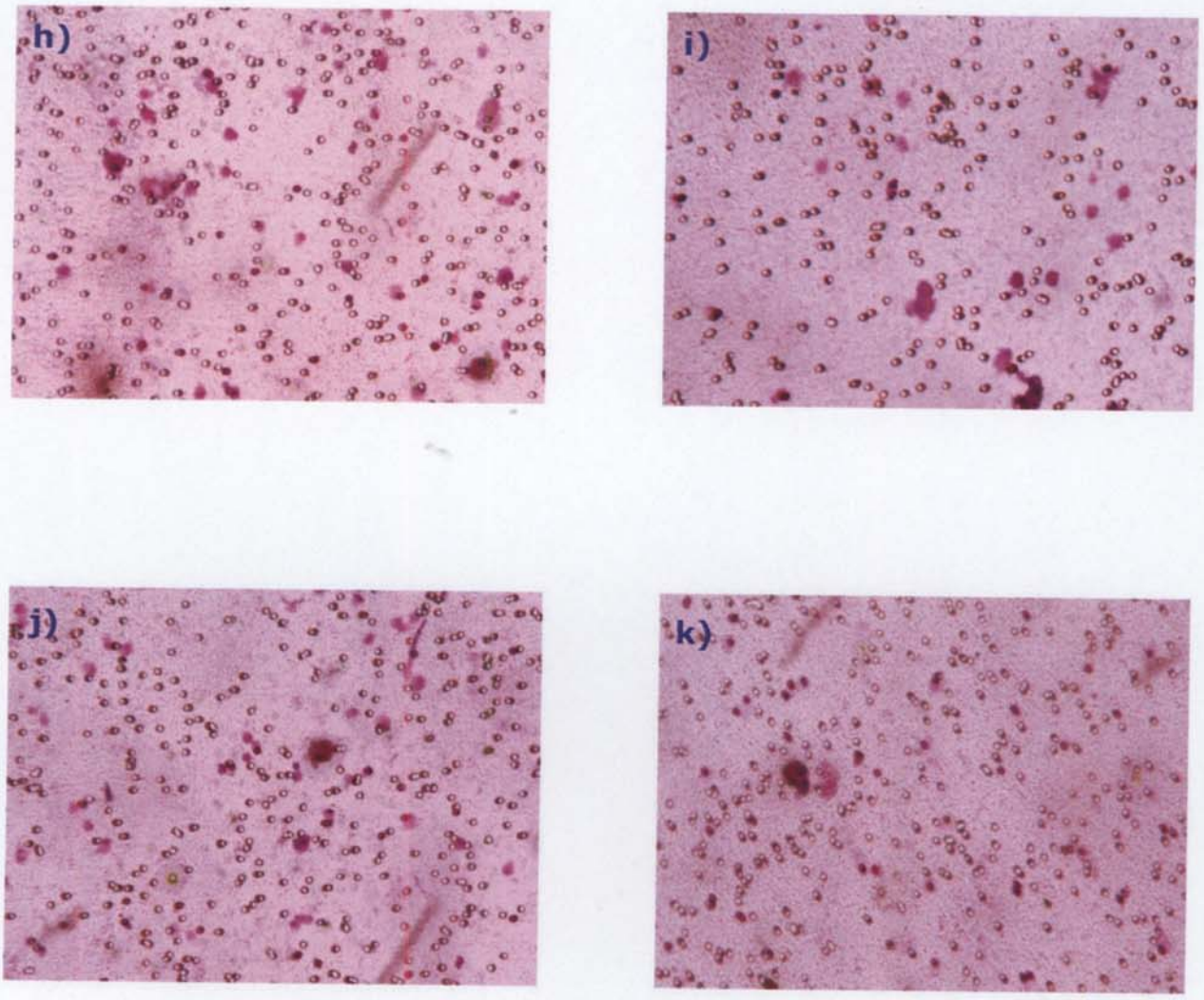


Figure 4.5. Effect of Sulfur compounds on endothelial cell tube formation

a) Untreated control

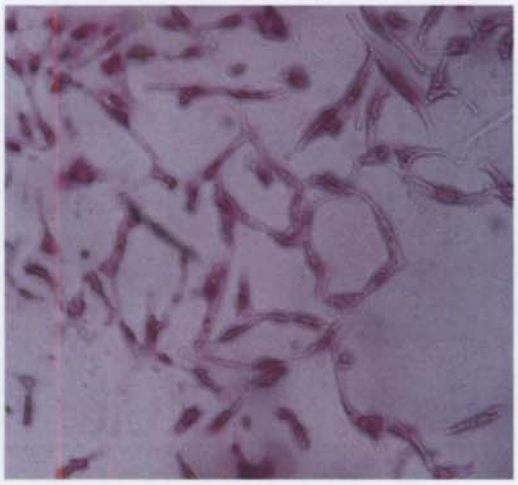
b) Treatment with DAS (2μg/ml)

c) Treatment with DAS (5μg/ml)

d) Treatment with DADS (2μg/ml)

e) Treatment with DADS (5μg/ml)

Figure 4.5



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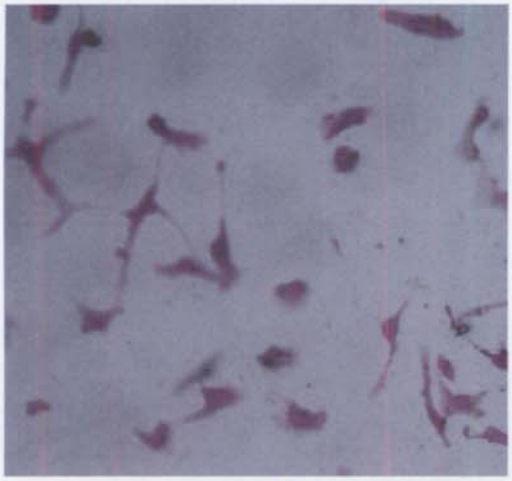

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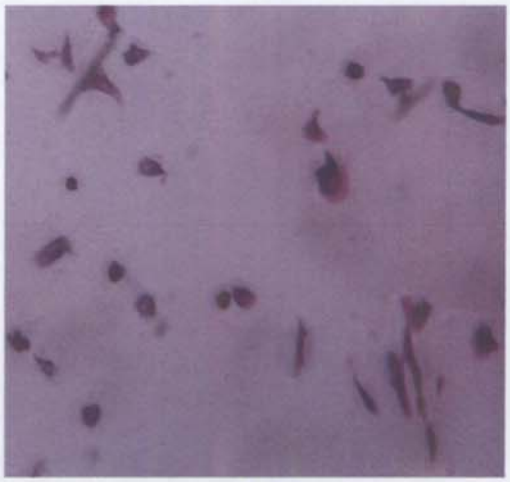

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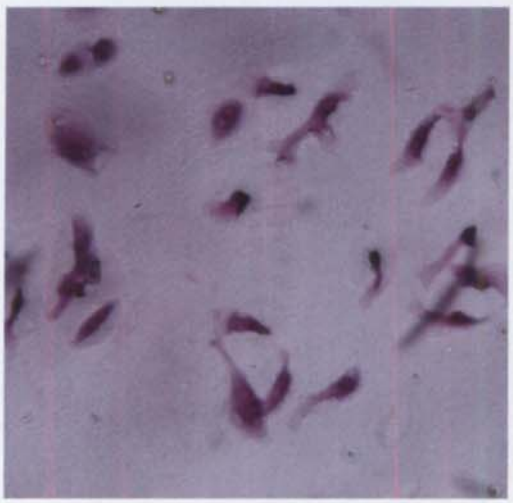

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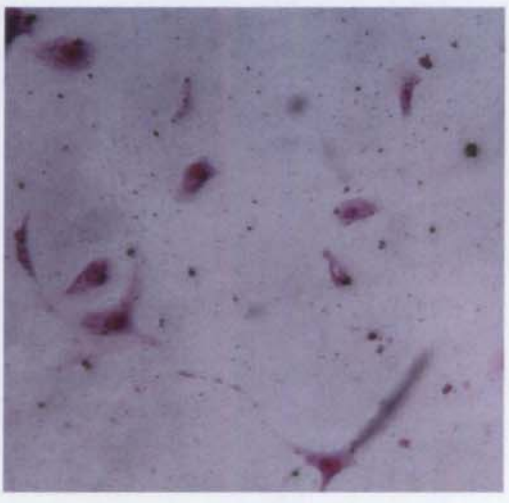


(e)

Figure 4.5. Effect of Sulfur compounds on endothelial cell tube formation

f) Treatment with AITC (2μg/ml)

g) Treatment with AITC (5μg/ml)

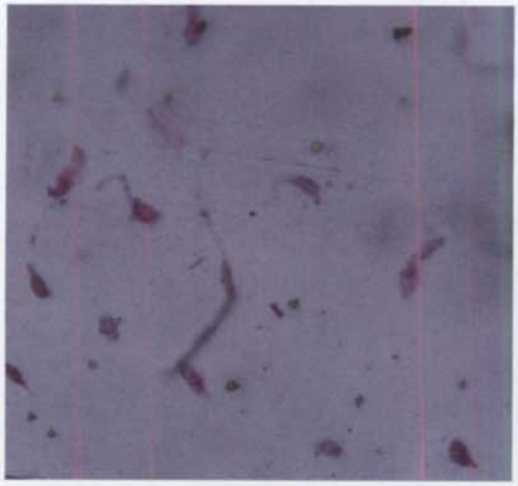
h) Treatment with PITC (2μg/ml)

i) Treatment with PITC (5μg/ml)

j) Treatment with Sulforaphane (2μg/ml)

k) Treatment with Sulforaphane (5μg/ml)

Figure 4.5



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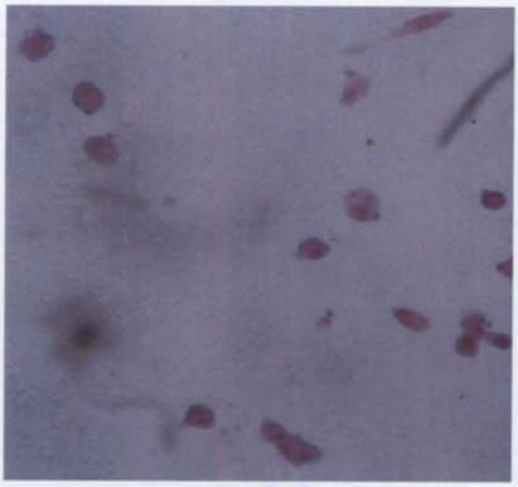

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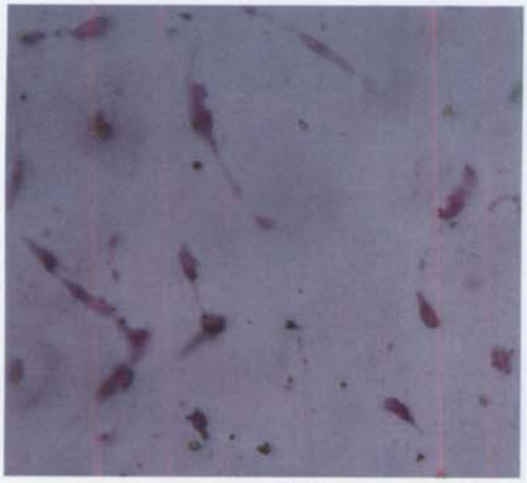

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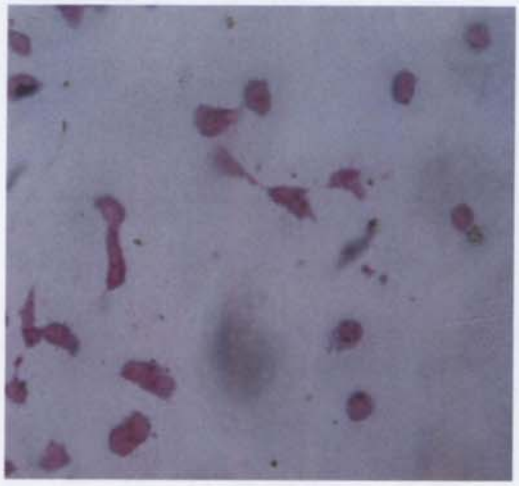
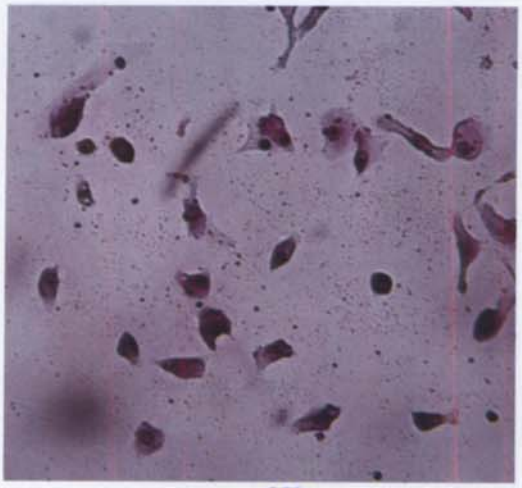
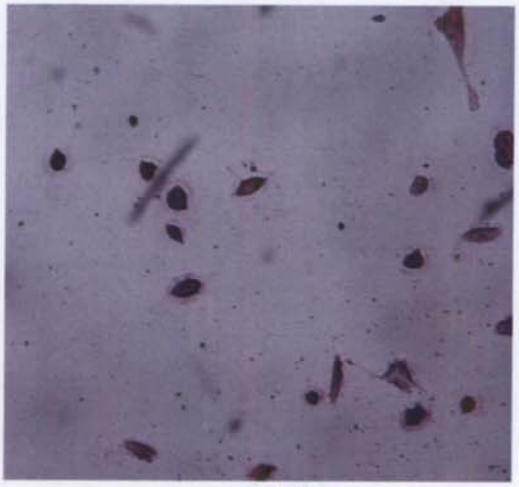


(i)



(j)



(k)

92 kD and 72 kD which was identical to MMP-9 and MMP-2 activity (Figure 4.6c). Gels loaded with conditioned medium without trypsin activation, did not show clear degradative areas, indicating the inactive form of the enzyme (Figure 4.6a). Trypsin activated conditioned medium loaded gels, when incubated with 10 mM EDTA (EDTA inhibits the proteolytic activity by chelating Ca^{++}) did not show clear degradative areas which indicate that the enzyme responsible for degradation is metalloproteinase (Figure 4.6b). When conditioned medium was treated with test compounds (5µg/ml) during trypsin activation, no clear bands were observed with Sulforaphane (Figure 4.6h) and DADS (Figure 4.6d) suggesting that these two compounds inhibited the activation of procollagenase to active collagenase whereas clear bands were observed with DAS (Figure 4.6e), AITC (Figure 4.6f) and PITC (Figure 4.6h) which suggest that these compounds have no effect on the activation of procollagenases. When trypsin activated conditioned medium was incubated in substrate buffer (to get enzymatic digestion) containing Sulfur compounds, no inhibition of proteolytic activity was observed. This also suggests that the inhibition of proteolytic activity by Sulforaphane and DADS may be related to the activation of metalloproteinases.

4.3.2.9. Effect of Sulfur compounds on iNOS gene expression

Highly elevated expression of iNOS gene was observed in the metastatic B16F-10 melanoma cells. Four hour treatment of the B16F-10 monolayer with Sulfur compounds at a concentration of 5µg/ml resulted in the suppression of iNOS gene. GAPDH is used as the house keeping gene (Figure 4.7a & b)

4.4. Discussion

Different factors produced by tumour cells and surrounding stromal cells play important roles in regulating tumour angiogenesis by activating or blocking different pathways (Hanahan and Folkman, 1996). Vasodilation, by smooth muscle relaxation, mediated by nitric oxide is a pre-requisite for endothelial cells to enter angiogenic cascade (Griffioen and Molema, 2000). Endogenous NO may mediate angiogenesis directly or as a second messenger of other growth factors. Some synthetic curcuminoids have been shown to inhibit tumour specific angiogenesis through the down regulation of

Figure 4.6. Gelatin zymography

a) Conditioned medium from untreated HUVECs without trypsin activation

b) Conditioned medium from untreated HUVECs with trypsin activation + EDTA

c) Conditioned medium from untreated HUVECs with trypsin activation alone

d) Conditioned medium from untreated HUVECs with trypsin activation + DADS (5μg/ml)

e) Conditioned medium from untreated HUVECs with trypsin activation + DAS (5μg/ml)

f) Conditioned medium from untreated HUVECs with trypsin activation + AITC (5μg/ml)

g) Conditioned medium from untreated HUVECs with trypsin activation + PITC (5μg/ml)

h) Conditioned medium from untreated HUVECs with trypsin activation + Sulforaphane (5μg/ml)

Figure 4.6

Figure 4.7. Effect of Sulfur compounds on iNOS gene expression

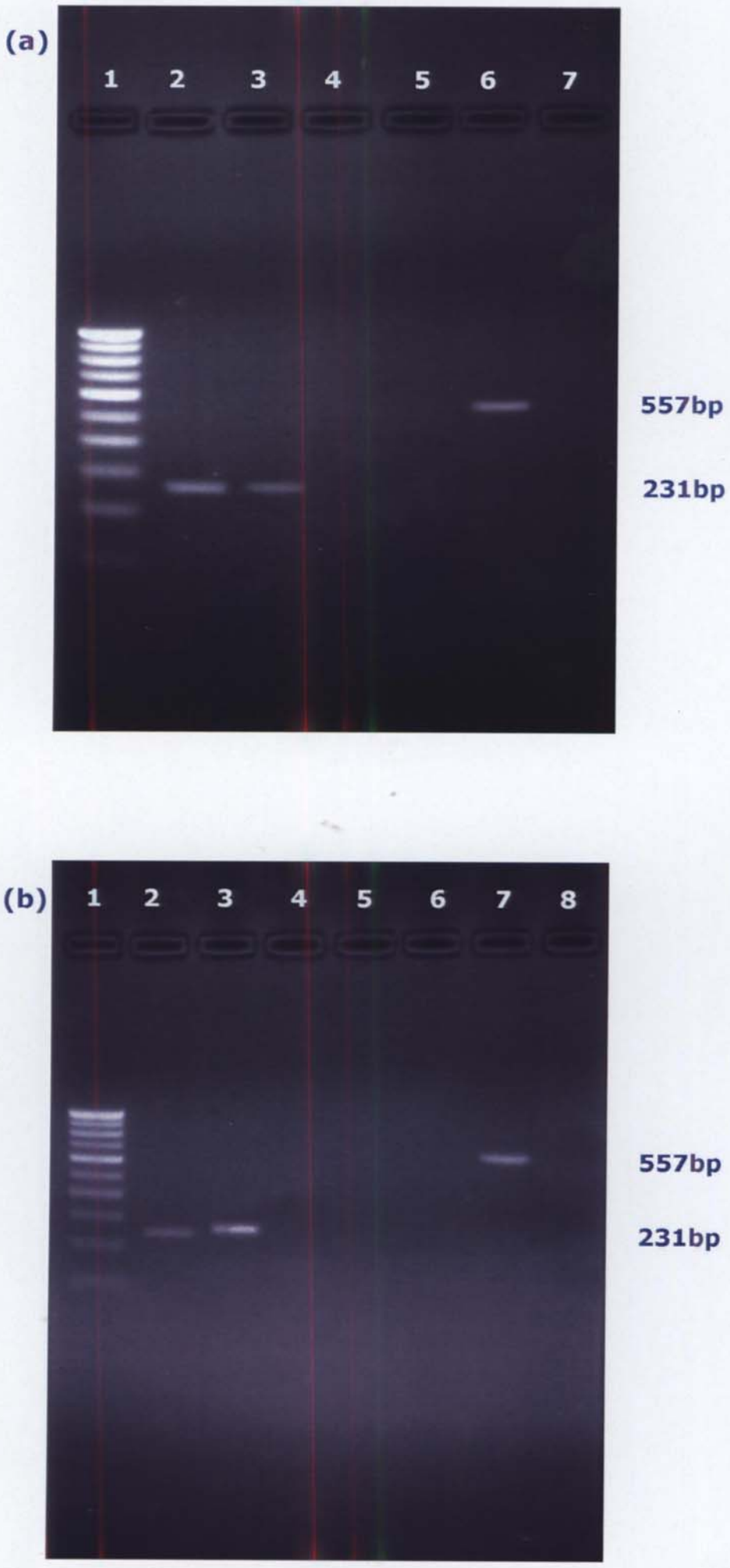
a) Effect of DAS and DADS on iNOS gene expression

1) Lane 1: Molecular weight marker
2) Lane 2: Positive control (231bp)
3) Lane 3: Untreated control
4) Lane 4: Treatment with DAS (5µg/ml)
5) Lane 5: Treatment with DADS (5µg/ml)
6) Lane 6: GAPDH (557bp)

b) Effect of AITC, PITC and Sulforaphane on iNOS gene expression

1) Lane 1: Molecular weight marker
2) Lane 2: Positive control (231bp)
3) Lane 3: Untreated control
4) Lane 4: Treatment with AITC (5µg/ml)
5) Lane 5: Treatment with PITC (5µg/ml)
6) Lane 6: Treatment with Sulforaphane (5µg/ml)
7) Lane 7: GAPDH (557bp)

Figure 4.7



NO production (Leyon and Kuttan, 2003). The results of our present study are in well correlation with these findings. Intradermal introduction of highly metastatic B16F-10 melanoma cells resulted in drastic elevation of serum NO level of untreated angiogenesis induced animals, which may be the reason for increased tumour directed capillary formation in them. But administration of Sulfur compounds significantly reduced the elevated serum NO level after tumour challenge, which in turn resulted in decreased tumour directed capillary formation. Tumour vessel count has been directly correlated with the survival of tumour cells (Weidner *et al.*, 1991) and the administration of Sulfur compounds significantly inhibited tumour specific new blood vessel formation in C57BL/6 mice when they were induced angiogenesis with B16F-10 melanoma cells. Furthermore, NO mediates, VEGF induced vascular permeability increase (Wu *et al.*, 1996). The down regulatory effect of Sulfur compounds was well clear as these compounds suppressed iNOS gene expression in B16F-10 melanoma cells.

VEGF is regarded as one of the earliest and important signals to stimulate multistep cascade of tumour angiogenesis and preferably by promoting endothelial cell proliferation and migration (Coultas *et al.*, 2005). In our study when B16F-10 melanoma cells were introduced intradermally to the mouse, the serum VEGF level was highly elevated as quickly as 24 h of tumour challenge. Treatment with Sulfur compounds significantly inhibited this elevation of serum VEGF level. This may be due to the down regulatory action of Sulfur compounds on the VEGF mRNA expression as there was only low level expression of VEGF mRNA when B16F-10 melanoma cells were treated with Sulfur compounds.

The levels of proinflammatory cytokines, such as IL-1β, IL-6, TNF-α and GM-CSF, evaluated in this study is also important in the process of angiogenesis because of their significant proangiogenic effects. IL-1β and TNF-α were shown to increase HIF-1 activity in the human hepatoma cell line HepG2 (Hellwig-Burgel *et al.*, 1999) which in turn cause the release of VEGF. It has been shown that IL-1β and TNF-α increases HIF-1α levels by activating NF-κB (Jung *et al.*, 2003). In addition, IL-1β (Li *et al.*, 1995) and IL-6 (Cohen *et al.*, 1996) have been shown to induce VEGF expression in several malignant and non-malignant cell lines. GM-CSF is expressed in solid tumours and

potentially enhances tumour cell proliferation and migration as well as tumour angiogenesis. It promotes angiogenic reaction by stimulating endothelial cell proliferation and migration (Bussolino *et al.*, 1991). In our study we found that the serum levels of these proinflammatory / proangiogenic cytokines-IL-1β, IL-6, TNF-α and GM-CSF-in angiogenesis induced untreated control animals were highly elevated after 24 h of tumour challenge and by day 9 these levels were either remained as same (IL-1β) or much more elevated (IL-6 and TNF-α). Among the Sulfur compounds studied, Sulforaphane and DADS were found to be significantly potent in inhibiting the elevation of these proinflammatory cytokines after angiogenesis induction and by day 9, the proinflammatory cytokines levels were more or less normalized. Since most of these cytokines are good inducers of VEGF, their down regulation by test compounds in turn resulted in the reduction of serum VEGF level.

Studies on the effect of Sulfur compounds on the serum levels of IL-2 showed that administration of Sulfur compounds significantly enhanced the serum IL-2 level in angiogenesis induced animals, compared to untreated control animals. IL-2 was the first cytokine used clinically for treating cancer (Neville *et al.*, 2001). It has been reported that IL-2 augment innate immunity by stimulating natural killer cells and also promotes proliferation and differentiation of helper T-cells, cytotoxic T-cells and B-cells (Caligiuri *et al.*, 1993).

Vascular remodeling of ECM is an essential part of angiogenesis (Stamenkovic, 2003). Matrix metalloproteinases and tissue inhibitors of metalloproteinases play a major regulatory role in matrix reorganization (Egeblad and Werb, 2002). MMPs are generally secreted as proforms which is activated by the proteolytic cleavage. Zymographic analysis clearly revealed that Sulforaphane and DADS inhibited the activation of MMP-2 and MMP-9. In MMP-9 deficient mice, reduced angiogenesis has been reported highlighting the role of this protease for angiogenesis (Werb *et al.*, 1999). Tube formation by endothelial cells has been found to be inhibited by mouse monoclonal anti-72 kD gelatinase suggesting the role of MMP-2 in angiogenesis (Schnaper *et al.*, 1993).

Activation of endothelial cells is initiated with binding of the proangiogenic factors such as VEGF, bFGF, PDGF and so on to their receptors expressed on the

endothelial cells followed by transduction of the angiogenic signaling in the cells (Relf *et al.*, 1997). Proteinases facilitate endothelial cell sprouting by liberating matrix bound angiogenic activators such as VEGF, FGF, TGF-β and proteolytically activating angiogenic chemokines (such as IL-1β) (Luttun *et al.*, 2000). Recent studies clearly demonstrated that MMP-9 mediates the release and accumulation of VEGF from the cell matrix (Hiratsuka *et al.*, 2002) and triggers angiogenic switch by rendering VEGF bioavailability to its receptors (Bergers *et al.*, 2000). The observed inhibition of endothelial cell proliferation by Sulforaphane and DADS can be attributed to its inhibitory effect on the activation of MMP-9 and MMP-2 which are essential for endothelial cell proliferation by making the proangiogenic growth factors available to their receptors.

The endothelial cells stimulated by the proangiogenic factors migrate and invade the ECM towards the chemotactic angiogenic stimuli. The proteinase exposes new cryptic epitopes in ECM proteins or changes their structure which induces endothelial cell migration (Hangai *et al.*, 2002). It has been reported that MMPs form minute holes in the sheath like basement membrane surrounding blood vessels to allow endothelial cells to invade (Albini, 1998). Because of their prime role in endothelial cell invasion and migration, the inhibition of the activation of MMPs by Sulforaphane and DADS resulted in significant reduction of endothelial cell motility and invasion. Formation of tubes on matrigel involves endothelial cell attachment, migration and production of enzymes capable of remodeling ECM. It has been shown that a reduction in tube formation by endothelial cells was associated with a decrease in the gelatinolytic activities of both MMP-2 and MMP-9, where as an enhancement of activity increased tube formation (Schnaper *et al.*, 1993). The results are consistent with this finding that inhibition of MMP-2 and MMP-9 activities decreases tube formation by endothelial cells, suggesting that Sulforaphane and DADS induced a decrease in MMP-2 and MMP-9 that led to the inhibition of tube formation. Further more, we have shown that when conditioned medium from untreated cells was incubated in substrate buffers containing Sulforaphane and DADS there is no inhibition of proteolytic activity by these compounds suggesting that the effect of Sulforaphane and DADS on angiogenesis may be related to the regulation of metalloproteinase activation.

Treatment with Sulfur compounds significantly enhanced the production of TIMP in angiogenesis induced animals. TIMP directly suppresses the MMP activity, thereby inhibiting ECM remodeling. It also inhibits angiogenic factor induced endothelial cell proliferation both *in vitro* and *in vivo* independent of MMP inhibition (Jiang *et al.*, 2002). Even though DAS, AITC and PITC had no effect on the activation of MMPs, these compounds effectively inhibited endothelial cell proliferation, migration, invasion and tube formation through the up regulation of TIMP production. This also contributed to the down regulation of VEGF by these Sulfur compounds since MMPs are essential for the release and accumulation of VEGF from the cell matrix (Hiratsuka *et al.*, 2002).

The *in vitro* angiogenesis experiments have again established the antiangiogenic activity of these Sulfur compounds. *In vitro* culture of B16F-10 melanoma cells have been shown to secrete several proangiogenic and proinflammatory cytokines such as IL-1β , IL-6, TNF- α and GM-CSF to the culture supernatant (Leyon and Kuttan, 2004). Administration of these Sulfur compounds reduced the production of proangiogenic factors from B16F-10 melanoma cells as the conditioned medium from treated cells showed reduced potential of microvessel outgrowth from the aortic ring. Simultaneous treatment with these Sulfur compounds was also found to inhibit the microvessel outgrowth.

In short, the antiangiogenic activity of these Sulfur compounds can be attributed to their inhibitory effect on angiogenic differentiation of endothelial cells through the down regulation of NO, VEGF and proinflammatory cytokines as well as up regulation of IL-2 and TIMP. Sulforaphane and DADS were found to be more potent because of their inhibitory effect on the activation of procollagenases to active collagenases.

CHAPTER 5

INDUCTION OF APOPTOSIS AND INHIBITION OF TRANSCRIPTION FACTORS BY NATURALLY OCCURRING SULFUR COMPOUNDS

5.1. Introduction

Apoptosis is a highly regulated process that is essential for the development and tissue homeostasis with in all multicellular organisms (Wang, 2001). Caspases, a family of cystein proteases with aspartate specificity, are the major effectors of apoptosis (Martin and Green, 1995). A wide variety of both pro and antiapoptotic controls exist in a cell that can either support or counteract the apoptotic programme. These include the superfamily of Bcl-2 genes or the family of IAP (inhibitor of apoptosis) genes. Members of these two families act either by direct binding and inhibiting caspases or through multiple interactions with transcription factors such as NF-κB and AP-1. Substantial evidences support the notion that NF-κB and AP-1 can act as powerful suppressor of apoptosis through the activation of antiapoptotic gene expression (Beg and Baltimore, 1996).

Dysregulation of NF-κB can lead to the constitutive overproduction of proinflammatory cytokines, which are released during chronic inflammation and were found to promote cell growth and invasion, to induce mutagenesis, to suppress apoptosis and to increase angiognecity. By virtue of these properties, proinflammatory cytokines support transformation and initiation of malignancy and if sustained they may also promote progression (Ben-Baruch, 2006). Since the suppression of NF-κB can inhibit various steps in the tumourigenic process, the design of NF-κB inhibitors that are pharmacologically safe will be critical for the treatment of cancer.

In this study, we evaluated the effect of Sulfur compounds on the regulation of apoptosis in B16F-10 melanoma cells by analyzing p53, caspase-3 and Bcl-2 gene expressions. We also analyzed their effect on the suppression of proinflammatory cytokine gene expression and transcription factors such as NF-κB and AP-1.

5.2. Materials and Methods

5.2.1. Cells

B16F-10 melanoma cells were used for the *in vitro* study

5.2.2. Chemicals and Kits

EGTA, , DEPC, Aprotinin, Leupeptin, PMSF, DTT, Benzamidine, TritonX-100, Cells to cDNA synthesis kit, Message screen mouse inflammatory cytokine multiplex PCR kit, Mouse Bcl-2, caspase-3, p53 and GAPDH primer sets and Mercury transfactor kit were used for this study.

5.2.3. Morphological analysis

One million B16F-10 melanoma cells in serum free DMEM were plated in clean glass slides and incubated for 24 h at 37^0C in 5% CO_2 atmosphere. Different concentrations of DAS, DADS, AITC, PITC and Sulforaphane (1, 2 & 5µg/ml) were added to the cells and incubated further for 48 h under the same conditions. The cells were then washed with PBS, centrifuged and the cell pellets were separated. To detect apoptotic morphology, a smear was prepared with the cell pellet, stained with haematoxylin and eosin and observed under phase contrast microscopy and photographs were taken.

5.2.4. DNA fragmentation analysis

One million B16F-10 melanoma cells were treated with DAS, DADS, AITC, PITC and Sulforaphane as described in the previous experiment. After incubation, DNA was extracted using phenol-chloroform method as described in Chapter 2. The extracted DNA was resolved in 1.5% agarose gel containing ethidium bromide. The resulting DNA fragmentation was visualized and recorded using the gel-documentation system (Vilber Lourmat, France).

5.2.5. RT-PCR and Gene expression studies

B16F-10 melanoma cells (2×10^4 cells) in serum free DMEM (250µl) were seeded in 96-well titre plate and incubated for 24 h at 37^0C in 5% CO_2 atmosphere. Sulfur compounds at a concentration of 5µg/ml were added per well and incubation was continued for another 4 h. cDNA was prepared from B16F-10 melanoma cells using cells to cDNA^{TM} II kit (Ambion Inc,USA) as described in Chapter 2. Gene expression analysis was done by PCR. PCR was performed with Biosource message screen^{TM}

Mouse inflammatory cytokine multiplex PCR kit to detect the expression of IL-1β, IL-6, GM-CSF, TNF-α and IL-12p40 against GAPDH. PCR primers with similar Tm's in this kit would enhance amplification of multiple targets. The mouse Bcl-2, caspases-3 and p53 genes were amplified against GAPDH primers obtained from Maxim biotech, USA. The target PCR products generated from a positive control cDNA was also included in this kit. PCR products were analyzed by agarose gel electrophoresis and visualized using gel documentation system as explained in chapter 2.

5.2.6. Preparation of nuclear extracts

Nuclear extracts were prepared by the previously published method (Dignam *et al.*, 1983). B16F-10 cells were grown in 25cm^2 culture flask. The subconfluent cells were treated with DADS, AITC and Sulforaphane (5μg/ml) for 2 h at 37°C in 5% CO_2 atmosphere in serum free medium. The cells were washed with PBS twice and incubated with TNF-α (10ρg/ml) for 30 minutes at 37°C in 5% CO_2 atmosphere. The nuclear extracts were prepared as described in Chapter 2. Protein concentrations of the nuclear extracts were estimated using standard Bradford method and stored at -70^{0}C.

5.2.7. Transcription factor profiling

Each transcription factor profiling kit was provided in a 96-well format with consensus DNA binding sequence. When nuclear extracts added to the well, DNA will bind to their consensus sequences in the well. Bound transcription factors in the DNA were detected by specific primary antibody towards NF-κBp65, NF-κBp50, NF-κB c-Rel, c-Fos, ATF-2 and CREB. A horse radish peroxidase conjugated secondary antibody was then used to detect the bound primary antibody. The enzymatic product was measured with standard microtitre plate reader at 655nm. Percentage inhibition was calculated by the formula, 100-([OD of treated / OD of control] X 100).

5.3. Results

5.3.1. Morphological analysis

Apoptotic cells were characterized with nuclear elongation, margination, fragmentation and sacculation, membrane blebbing and presence of apoptotic bodies. Untreated control normal B16F-10 cells were characterized by less eosinophilic cytoplasm and nucleus with uniform distribution of chromatin material and did not show any morphological modifications or reorganizations (Figure 5.1a). Sulforaphane (Figure 5.1c & d) and DADS (Figure 5.1f & g) at concentrations of 2μg/ml and 5μg/ml produced the membrane blebs, nuclear fragmentation and the budding of membrane bound apoptotic bodies. These two compounds did not produce any characteristic apoptotic features at the lower concentration of 1μg/ml. DAS, AITC and PITC did not give apoptotic morphological features at lower concentrations of 1μg/ml and 2μg/ml but treatment with 5μg/ml displayed the stages of apoptosis as chromatin condensation or nuclear condensation (Figure 5.1i, k & m).

5.3.2. Assessment of DNA fragmentation

In apoptosis there is a two step process of DNA fragmentation: DNA is first cleaved into large fragments of 50-300kb and these are subsequently cleaved into smaller oligonucleosomes in some, but not all cells. DNA fragmentation analysis showed that DNA extract from Sulforaphane and DADS (2μg/ml and 5μg/ml) treated B16F-10 melanoma cells exhibited extensive double strand breaks; thereby yielding a ladder appearance (Figure 5.2a), while the DNA of control B16F-10 melanoma cells did not show any double strand breaks (Lane 2).The other Sulfur compounds such as DAS, AITC and PITC produced DNA fragmentation only at 5μg/ml (Figure 5.2b).

5.3.3. Effect of Sulfur compounds on caspase-3 gene expression in B16F-10 melanoma cells

Effect of Sulfur compounds such as DAS, DADS, AITC, PITC and Sulforaphane on caspase-3 gene expression is shown in Figure 5.3a & b. Lane 1 represents the molecular weight marker. Lane 2 shows the amplified capase-3 positive control cDNA (414 bp) included in the kit. Administration of Sulforaphane (Figure

Figure 5.1. Effect of Sulfur compounds on induction of apoptosis in B16F-10 melanoma cells-Morphological analysis

a) Untreated control B16F-10 melanoma cells

b) B16F-10 melanoma cells treated with Sulforaphane (1μg/ml)

c) B16F-10 melanoma cells treated with Sulforaphane (2μg/ml)

d) B16F-10 melanoma cells treated with Sulforaphane (5μg/ml)

e) B16F-10 melanoma cells treated with DADS (1μg/ml)

f) B16F-10 melanoma cells treated with DADS (2μg/ml)

g) B16F-10 melanoma cells treated with DADS (5μg/ml)

h) B16F-10 melanoma cells treated with DAS (2μg/ml)

i) B16F-10 melanoma cells treated with DAS (5μg/ml)

j) B16F-10 melanoma cells treated with AITC (2μg/ml)

k) B16F-10 melanoma cells treated with AITC (5μg/ml)

l) B16F-10 melanoma cells treated with PITC (2μg/ml)

m) B16F-10 melanoma cells treated with PITC (5μg/ml)

Figure 5.1

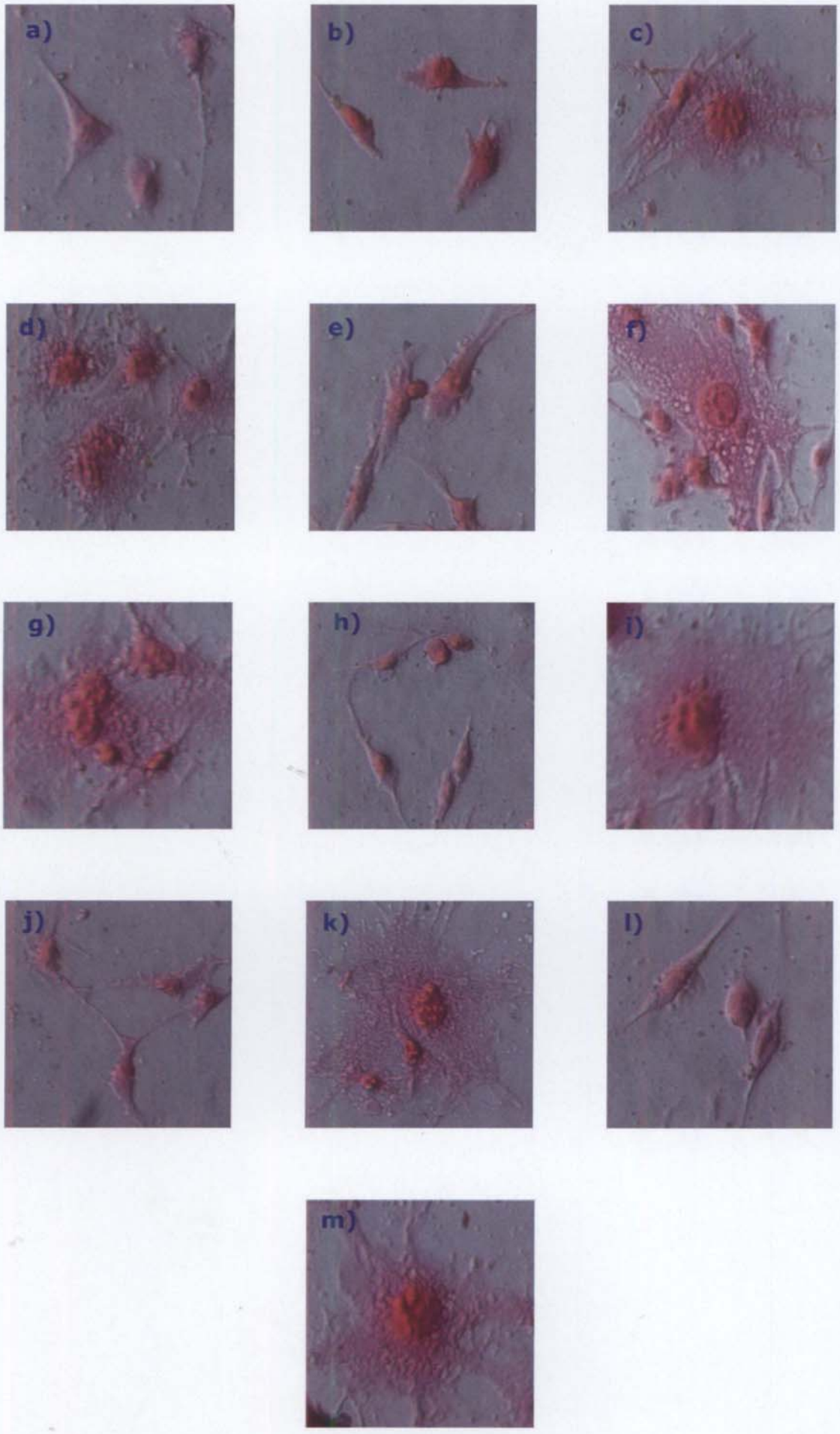


Figure 5.2. Effect of Sulfur compounds on induction of apoptosis in B16F-10 melanoma cells-DNA fragmentation analysis

a) Effect of DADS and Sulforaphane on DNA fragmentation

1) Lane 1: Molecular weight marker
2) Lane 2: Untreated control
3) Lane 3: Treatment with Sulforaphane (1μg/ml)
4) Lane 4: Treatment with Sulforaphane (2μg/ml)
5) Lane 5: Treatment with Sulforaphane (5μg/ml)
6) Lane 6: Treatment with DADS (1μg/ml)
7) Lane 7: Treatment with DADS (2μg/ml)
8) Lane 8: Treatment with DADS (5μg/ml)

b) Effect of DAS, AITC and PITC on DNA fragmentation

1) Lane 1: Molecular weight marker
2) Lane 2: Untreated control
3) Lane 3: Treatment with DAS (2μg/ml)
4) Lane 4: Treatment with DAS (5μg/ml)
5) Lane 5: Treatment with AITC (2μg/ml)
6) Lane 6: Treatment with AITC (5μg/ml)
7) Lane 7: Treatment with PITC (2μg/ml)
8) Lane 8: Treatment with PITC (5μg/ml)

Figure 5.2

(a)

(b)

5.3b; Lane 6), DADS (Figure 5.3a; Lane 5) and AITC (Figure 5.3b; Lane 5) at a concentration of 5μg/ml resulted in the expression of proapoptotic gene caspase-3 while the untreated control B16F-10 melanoma cells did not exhibit the expression of this gene (Lane 3). Treatment with DAS and PITC had no effect on the expression of caspase-3. GAPDH (557bp) is used as the standard house keeping gene which was expressed.

5.3.4. Effect of Sulfur compounds on p53 gene expression in B16F-10 melanoma cells

Effect of Sulfur compounds on p53 gene expression is shown in Figures 5.4a & b. Lane 1 represents the molecular weight marker. Lane 2 shows the amplified p53 positive control cDNA (205 bp) included in the kit. Administration of Sulforaphane (Figure 5.4b; Lane 6) and DADS (Figure 5.4a; Lane 5) at a concentration of 5μg/ml resulted in the expression of proapoptotic gene p53 while the untreated control B16F-10 melanoma cells did not exhibit the expression of this gene (Lane 3). Treatment with DAS, AITC and PITC had no effect on the expression of p53. GAPDH (557bp) is used as the standard house keeping gene.

5.3.5. Effect of Sulfur compounds on Bcl-2 gene expression in B16F-10 cells

Effect of Sulfur compounds on Bcl-2 gene expression is shown in Figure 5.5a & b. Lane 1 represents the molecular weight marker. Lane 2 shows the amplified Bcl-2 positive control cDNA (235 bp) included in the kit. Treatment with Sulforaphane, DADS, AITC, DAS and PITC led to the suppression of Bcl-2 expression which is an antiapoptotic gene. Control B16F-10 melanoma cells showed the expression of Bcl-2 (Lane 3). GAPDH (557bp) is used as the standard house keeping gene.

5.3.6. Effect of Sulfur compounds on the proinflammatory cytokine gene expression.

Highly elevated expression of proinflammatory cytokines such as IL-1β, IL-6, GM-CSF, TNF-α and IL-12 p40 have been observed in the metastatic B16F-10 melanoma cells. Sulforaphane, DADS and AITC (5μg/ml) inhibited the elevated

Figure 5.3. Effect of Sulfur compounds on caspase-3 gene expression in B16F-10 melanoma cells

a) Effect of DAS and DADS on caspase-3 gene expression

Lane 1: Molecular weight marker
Lane 2: Positive control (414bp)
Lane 3: Untreated control
Lane 4: Treatment with DAS (5µg/ml)
Lane5: Treatment with DADS (5µg/ml)
Lane 6: GAPDH (557bp)

b) Effect of AITC, PITC and Sulforaphane on caspase-3 gene expression

Lane 1: Molecular weight marker
Lane 2: Positive control (414bp)
Lane 3: Untreated control
Lane 4: Treatment with PITC (5µg/ml)
Lane 5: Treatment with AITC (5µg/ml)
Lane 6: Treatment with Sulforaphane (5µg/ml)
Lane 7: GAPDH (557bp)

Figure 5.4. Effect of Sulfur compounds on p53 gene expression in B16F-10 melanoma cells

a) Effect of DAS and DADS on p53 gene expression

Lane 1: Molecular weight marker
Lane 2: Positive control (205bp)
Lane 3: Untreated control
Lane 4: Treatment with DAS (5µg/ml)
Lane 5: Treatment with DADS (5µg/ml)
Lane 6: GAPDH (557bp)

b) Effect of AITC, PITC and Sulforaphane on p53 gene expression

Lane 1: Molecular weight marker
Lane 2: Positive control (414bp)
Lane 3: Untreated control
Lane 4: Treatment with AITC (5µg/ml)
Lane 5: Treatment with PITC (5µg/ml)
Lane 6: Treatment with Sulforaphane (5µg/ml)
Lane 7: GAPDH (557bp)

Figure 5.3

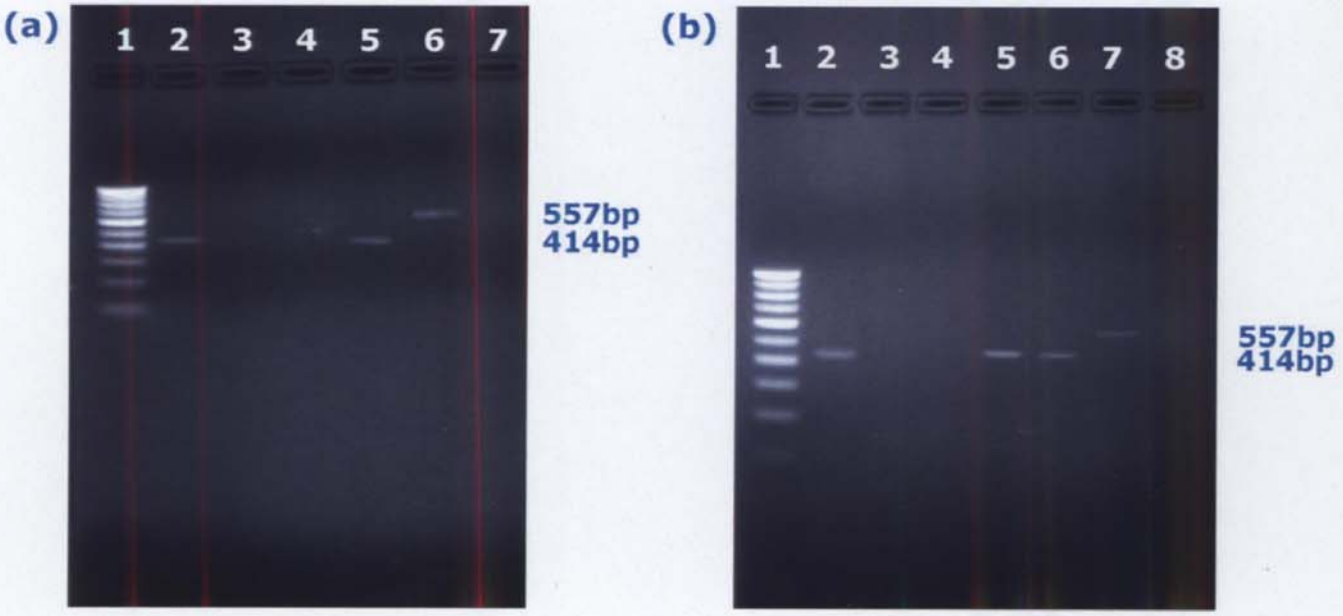


Figure 5.4

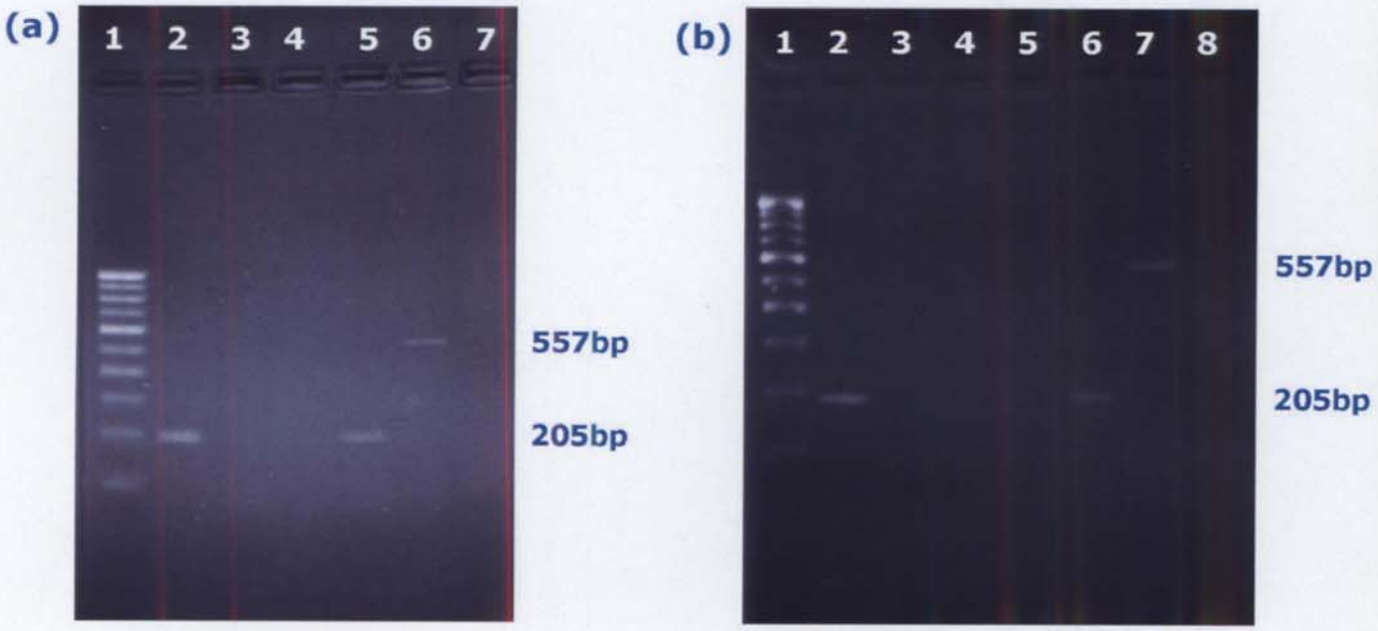


Figure 5.5. Effect of Sulfur compounds on Bcl-2 gene expression in B16F-10 melanoma cells

a) Effect of DAS and DADS on Bcl-2 gene expression

Lane 1: Molecular weight marker
Lane 2: Positive control (235bp)
Lane 3: Untreated control
Lane 4: Treatment with DAS (5µg/ml)
Lane 5: Treatment with DADS (5µg/ml)
Lane 6: GAPDH (557bp)

b) Effect of AITC, PITC and Sulforaphane on p53 gene expression

Lane 1: Molecular weight marker
Lane 2: Positive control (235bp)
Lane 3: Untreated control
Lane 4: Treatment with AITC (5µg/ml)
Lane 5: Treatment with PITC (5µg/ml)
Lane 6: Treatment with Sulforaphane (5µg/ml)
Lane 7: GAPDH (557bp)

Figure 5.6. Effect of Sulfur compounds on the proinflammatory cytokine gene expression.

1) Lane 1: Molecular weight marker
2) Lane 2: Positive control
3) Lane 3: Untreated control
4) Lane 4: Treatment with DAS (5µg/ml)
5) Lane 5: Treatment with DADS (5µg/ml)
6) Lane 6: Treatment with AITC (5µg/ml)
7) Lane 7: Treatment with PITC (5µg/ml)
8) Lane 8: Treatment with Sulforaphane (5µg/ml)

Figure 5.5

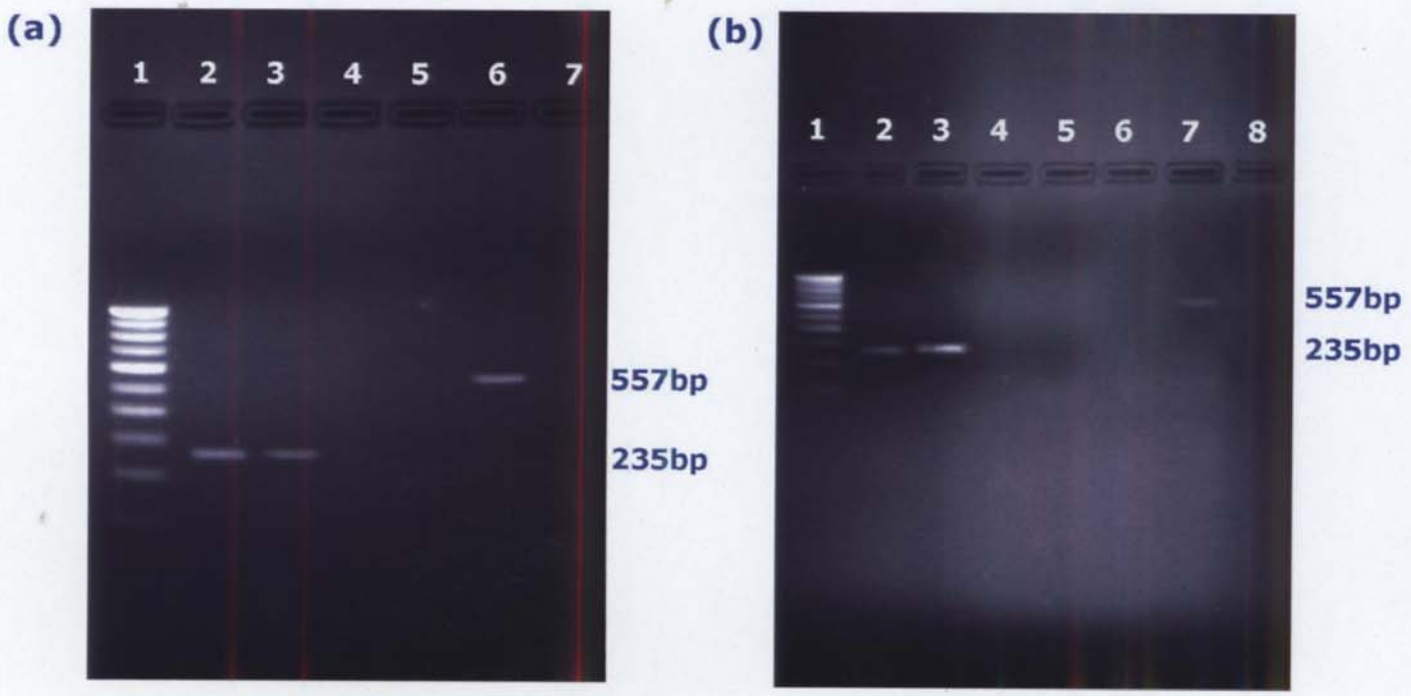
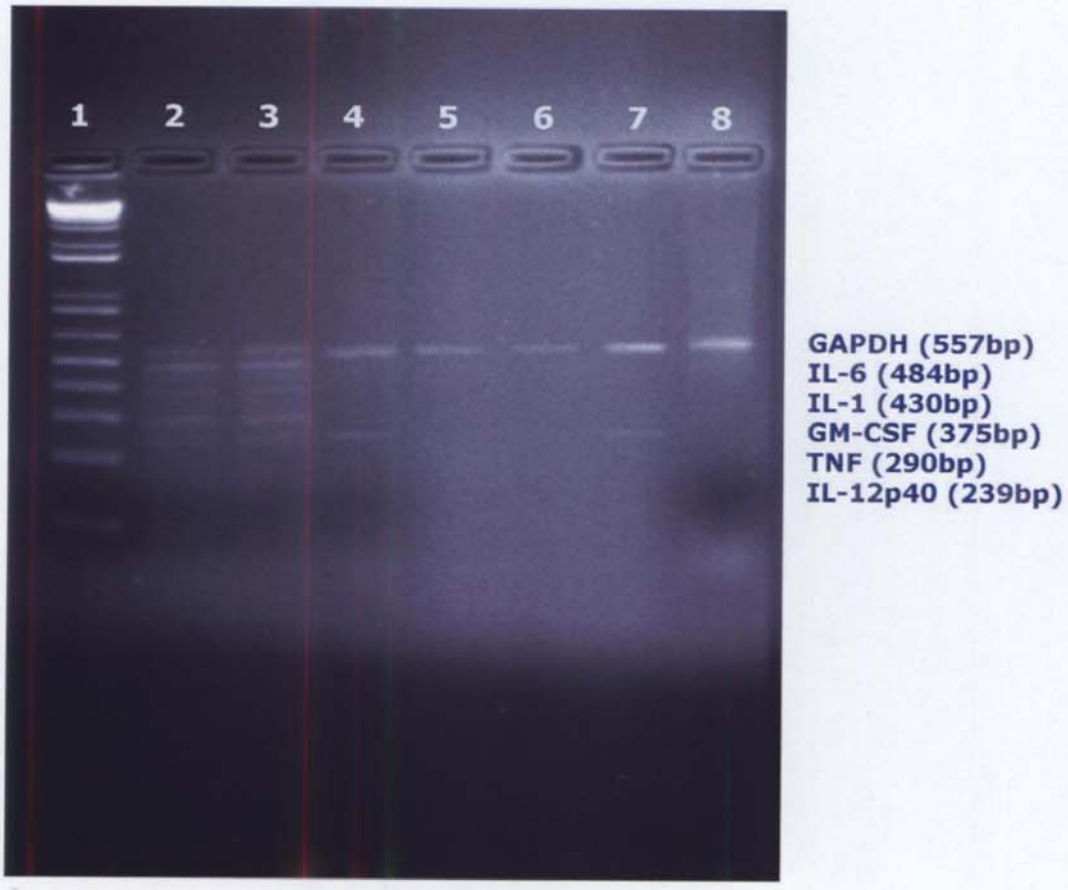


Figure 5.6



expression of proinflammatory cytokines such as IL-1β, IL-6, GM-CSF, TNF-α and IL-12 p40 genes (Figure 5.6). DAS and PITC did not suppress the expression of IL-12 p40; but these two compounds effectively suppressed the expression of other proinflammatory cytokine genes such as IL-1β, IL-6, GM-CSF and TNF-α.

5.3.7. Effect of Sulfur compounds on the translocation of transcription factors

Effect of Sulfur compounds on the nuclear translocation of transcription factors is shown in Table 5.1. The Sulfur compounds such as Sulforaphane, DADS and AITC which inhibited the expression of proinflammatory cytokines were analyzed on the effect of nuclear translocation level of transcription factors. DADS inhibited the translocation of NF-κB subunits such as p65, p50 and c-Rel by 68.61%, 78.59% and 72.8% respectively. We also found that DADS inhibited the nuclear translocation of c-Fos, ATF-2 and CREB by 82.25%, 83.5% and 76.92% respectively. Sulforaphane inhibited translocation of p65 by 74.44%, p50 by 71.21%, c-Rel by 78.4%, c-Fos by 83.5%, ATF-2 by 81.40% and CREB by 67.42%. The inhibition of p65, p50, c-Rel, c-Fos, ATF-2 and CREB by AITC were found to be 51.71%, 58.14%, 51.36%, 37.99%, 42.45% and 47.51% respectively.

5.4. Discussion

Apoptosis is the best defined cell death programme counteracting tumour growth. Since current cancer therapies, such as radiation therapy and chemotherapy, primarily exert their antitumour effect by triggering apoptosis in cancer cells (Herr and Debatin, 2001), defects in apoptosis programmes may result in cancer resistance. Recently, interest has focused on the manipulation of the apoptotic process for the treatment and prevention of cancer. Thus the compounds that can influence apoptosis without producing toxic side effects are of great significance today. Previous studies in our laboratory clearly demonstrated that DAS, DADS, AITC and PITC were potent inhibitors of B16F-10 melanoma induced pulmonary metastasis in C57BL/6 mice (Kuttan and Kuttan, 1999; Manesh and Kuttan, 2003b). The present study indicates that these Sulfur compounds including Sulforaphane are effective inducers of apoptosis in B16F-10 melanoma cells. Apoptosis is characterized by the activation of a specific

Table 5.1. Effect of DADS, AITC and Sulforaphane on the translocation of transcription factors

Transcription factor	% inhibition		
	DADS	AITC	Sulforaphane
NF-κB p65	68.61	51.71	74.44
NF-κB p50	78.59	58.14	71.21
NF-κB c-Rel	72.80	51.36	78.40
c-Fos	82.25	37.99	83.50
ATF-2	83.50	42.45	81.40
CREB-1	76.92	47.51	67.42

B16F-10 melanoma cells were grown in $25cm^2$ culture flask and treated with DADS, AITC and Sulforaphane (5μg/ml) for 2 h at 37^0C in 5% CO_2 atmosphere. Cells were washed with PBS and incubated with TNF-α (10pg/ml) for 30 minutes at 37^0C in 5% CO_2 atmosphere. Experiments were repeated thrice. Nuclear extracts were prepared separately and subjected to transfactor assay.

family of cystein proteases, the caspases, followed by a series of caspase mediated morphological changes such as shrinkage of the cells, the condensation of chromatin and membrane blebbing. When B16F-10 melanoma cells were treated with the Sulfur compounds, these characteristic apoptotic morphological features were observed. Caspase-3 has been shown to play an important role in chemotherapy-, growth factor-, Fas- and retinoic acid-induced apoptosis (Piedrafita and Pfahl, 1997). Once activated, caspase-3 cleaves many substrate proteins including PARP, ICAD and structural proteins such as actin, fodrin or lamin to generate the characteristic apoptotic morphology (Kothakota *et al.*, 1997). Caspase-3 is a most likely candidate to mediate Sulforaphane, DADS and AITC induced apoptosis in B16F-10 melanoma cells as evidenced by increased expression of this gene upon treatment with these Sulfur compounds. In cancerous cells, the expression of caspase-3 is much suppressed and in the present study the untreated control B16F-10 melanoma cells did not exhibit its expression. Cleavage of ICAD by caspase-3 leads to the activation of CAD, which in turn cleaves genomic DNA within internucleosomal regions and generates multimers of nucleosomal domain-sized (ladder like) fragments (Sakahira *et al.*, 1998). DNA extracts from Sulforaphane, DADS and AITC incubated B16F-10 melanoma cells displayed characteristic ladder pattern of discontinuous DNA fragments.

The p53 is a tumour suppressor gene which exerts its antitumour effect through the induction of either cell growth arrest or apoptosis. It is functionally inactivated in 70% of human tumours (Evan *et al.*, 1995). p53 protein stimulates the release of cytochrome-c from mitochondria and pro-caspase-3 activation (Marchenko *et al.*, 2000). The activation of p53 is generally blocked by over expression of antiapoptotic Bcl-2 proteins. Bcl-2 level is highly elevated in a broad range of human cancers including carcinomas of breast, prostate, ovary, colon, and lung (Adams and Cory, 1998). It also blocks release of cytochrome-c from mitochondria and caspase-3 activation (Gross *et al.*, 1999). In B16F-10 melanoma cells, there was no expression of p53 which may be due to over expression of Bcl-2. Treatment of all Sulfur compounds down regulated the expression of Bcl-2 in B16F-10 melanoma cells. Results of this study showed that administration of Sulforaphane and DADS at a low sub lethal dose induced apoptosis in B16F-10 melanoma cells chiefly by stimulating the expression of

caspase-3 and p53 and at the same time by blocking the expression of antiapoptptic Bcl-2. From our study it was clear that AITC induced apoptosis in B16F-10 melanoma cells by up regulation of proapoptotic gene caspase-3 and down regulation of antiapoptotic gene Bcl-2. Even though, DAS and PITC could not up regulate p53 and caspase-3 gene expressions, these two compounds effectively down regulated antiapoptotic Bcl-2 gene expression, thereby promoting apoptosis in B16F-10 melanoma cells.

The activation of transcription factor NF-κB is strongly linked to the inhibition of apoptosis (Barkett and Gilmore, 1999). The most active NF-κB is a heterodimer composed of p50 and RelA subunits. Transcription factor AP-1 consists of 18 different dimeric combinations of proteins from Jun (v-Jun, c-Jun, Jun-B and Jun-D) and Fos (v-Fos, c-Fos, FosB, Fra-1 and Fra-2) families, including Jun homodimers and Jun-Fos heterodimers (Karin *et al.*, 1997). Ap-1 factors can also interact with other proteins including the p65 subunit of NF-κB and CBP (Cyclic AMP response element binding protein-binding protein) and regulates cell proliferation, differentiation, apoptosis and tumour progression (Karin and Delhase, 2000). Activation of AP-1 protein is required for the preneoplastic to neoplastic progression of different cancers such as prostate and epithelial cancer (Huang *et al.*, 1996) and nuclear translocation of c-Fos and activating transcription factor (ATF)-2 has been reported in different cancer cells (Huang *et al.*, 1998; Angel and Karin, 1991). Evidences have shown that c-Fos is required for tumour progression as the c-Fos -/- mice were protected against the progression of benign tumour to carcinoma (Saez *et al.*, 1995). CREB (Cyclic AMP response element binding protein) is activated through protein kinase A (PKA) and mediates phosphorylation in signaling cascade and promotes specific gene transcription (Zhong *et al.*, 1998). Hence the inhibition of subset AP-1 and NF-κB target genes using dietary molecules is a potential strategy for cancer prevention. In the present study, Sulforaphane, DADS and AITC inhibited the activation and nuclear translocation of transcription factors such as NF-κB p65, NF-κB p50, NF-κB c-Rel, c-Fos, ATF-2 and CREB. Since these transcription factors are major negative regulators of apoptosis, their inhibition by these Sulfur compounds also promoted their apoptosis inducing property in B16F-10 melanoma cells.

NF-κB also act as a connecting link between inflammation and cancer as it promotes the expression of major proinflammatory cytokine genes such as IL-1β, IL-6, TNF-α, IL-12p40 and GM-CSF. Tumour promotion and progression largely depend on the production of IL-1β, IL-6 and TNF-α, which serve as growth factors for premalignant cells and already formed tumours. TNF-α was shown to be a growth factor for glioblastoma cells (Aggarwal *et al.*, 1996), IL-1β for acute myelogenous leukemia (Estrov *et al.*, 1998) and IL-6 for multiple myeloma (Bharti *et al.*, 2003). Suppression of NF-κB in these tumours down regulates the cytokine expression and inhibits tumour cell proliferation. GM-CSF has an important role in tissue repair and tumour progression (Bendtzen *et al.*, 2001). IL-12p40 act as a proinflammatory cytokine which can activate the tyrosine kinase 2 and STAT proteins (Oppmann *et al.*, 2002). The IL-12p40 promoter was found to have NF-κB binding sites (Tripathi and Aggarwal, 2006). In our study the expression of proinflammatory cytokines such as IL-1β, IL-6, TNF-α, IL-12p40 and GM-CSF was inhibited by treatment with Sulforaphane, DADS and AITC and this may be due to the inhibition of activation of NF-κB. Even though DAS and PITC had no effect on the expression of IL-12p40, these two compounds effectively suppressed the expression of IL-1β, IL-6, GM-CSF and TNF-α which in turn promoted their apoptosis inducing potential.

In conclusion, Sulforaphane and DADS efficiently induced apoptosis in B16F-10 melanoma cells through up regulation of caspase-3 and p53 and at the same time by the down regulation of Bcl-2 and proinflammatory cytokine gene expression and also by the inhibition of activation and nuclear translocation of antiapoptotic transcription factors such as NF-κB, AP-1, CREB and ATF-2. AITC induced apoptosis in B16F-10 melanoma cells by up regulating caspase-3 expression and down regulating Bcl-2 and proinflammatory cytokine gene expression and activation and nuclear translocation of transcription factors. The apoptosis inducing property of DAS and PITC can be attributed to their down regulatory activity of Bcl-2 and proinflammatory cytokine gene expressions. Thus these Sulfur compounds are promising anticancer agents which can be used along with other cancer therapies as they can affect different targets of tumour progression including apoptotic pathway and transcription factors.

CHAPTER 6

ANTIMETASTATIC ACTIVITY OF SULFORAPHANE

6.1. Introduction

Metastasis or secondary neoplastic growth defines malignancy and is a major contributor to cancer mortality. The prognosis of cancer is mainly determined by the invasiveness of the tumour and its ability to metastasize. The metastatic process consists of several sequential events including escape of cancer cells from the original tumour, intravasation and dissemination through blood and lymphatic vessels, arrest in the microvasculature of the target organs, extravasation and proliferation at a new site (Fidler, 1978; Nicolson, 1982; Liotta, 1984; Netland and Zetter, 1989). Since basement membrane and extracellular matrix provide the main physical barriers to cancer cell invasion; proteolytic degradation of these structures has been proposed to be important in the metastatic process (Liotta, 1986a; Liotta *et al.*, 1980). Any drug, which can inhibit one of the steps in the cascade, will be useful in the inhibition of tumour metastasis. Although there are several drugs available to control cancer growth in humans, there are no drugs presently available to specifically inhibit the metastasis of cancer cells. This is due to the fact that the cancer cells in different metastases and even in single metastases may respond differently to radiotherapy or chemotherapy. Understanding the molecular basis of metastasis is crucial for the design and effective use of novel therapeutic strategies to combat metastasis.

In this chapter, the antimetastatic activity of Sulforaphane was studied at molecular level using *in vitro* as well as *in vivo* models.

6.2. Materials and Methods

6.2.1. Animals

C57BL/6 mice (4-6 weeks old males) were used for this study

6.2.2. Cell line

B16F-10 melanoma cells were used to induce metastasis

6.2.3. Administration of Sulforaphane

Sulforaphane was suspended in phosphate buffered saline (PBS, pH-7.4) and administered intraperitoneally at a dosage of 500μg/dose/animal.

6.2.4. Determination of the antimetastatic activity in the *in vivo* system

6.2.4.1. Determination of the effect of Sulforaphane on lung tumour nodule formation and rate of survival

C57BL/6 mice were divided into 4 groups (14 mice/group). All the animals were induced metastasis by injecting B16F-10 melanoma cells (10^6 cells/ animal) through lateral tail vein (Liotta, 1986b). To three groups of animals Sulforaphane was administered intraperitoneally at a concentration of 500μg/dose/animal for 10 consecutive days in three different modalities- simultaneously with metastatic tumour cells (Group I); ten days prior to metastatic tumour induction (Group II) and ten days after tumour induction (Group III). Group IV animals were kept as untreated metastatic tumour bearing control treated with vehicle (PBS, pH-7.4) only. Eight animals from each group were sacrificed on the 21st day after tumour challenge, lungs were excised and blood was collected. Lungs were used for morphological examinations of metastatic tumour nodules and for the estimation of collagen hydroxyproline (Bergman and Loxley, 1970), hexosamine (Elson and Morgan, 1933) and uronic acid (Bitter and Muir, 1962) contents (Chapter 2). Serum was separated from the blood and used for the determination of sialic acid (Skoza and Mohos, 1976) and γ-glutamyl transpeptidase (GGT) (Szasz, 1976) levels (Chapter 2). A portion of the lung was used for histopathological analysis. The rest of the six animals in each group were observed for their survival. The mortality of the animals was observed and the percentage increase in life span (%ILS) was calculated.

6.2.4.2. Histopathological analysis

Lung tissues were fixed in 10% formalin, dehydrated in different concentrations of alcohol and embedded in paraffin wax. Sections (4μm) were stained with eosin and haematoxylin.

6.2.4.3. Determination of the effect of Sulforaphane on the TIMP-1 levels in metastatic tumour bearing animals

C57BL/6 animals were divided into two groups (6 mice/group). The animals were induced metastasis by injecting B16F-10 melanoma cells (10^6 cells/animal) via lateral caudal vein. Group I animals were kept as metastatic tumour bearing control treated with vehicle (PBS, pH-7.4) only. Group II animals were treated simultaneously with 5 consecutive doses of Sulforaphane (500µg/dose/animal; i.p). Blood was collected by tail bleeding on 7^{th} and 21^{st} day after tumour inoculation. Serum separated and used for the analysis of TIMP-1.

6.2.5. Determination of antimetastatic activity using *in vitro* models

6.2.5.1. Determination of cell viability by MTT assay

B16F-10 melanoma cells were seeded (5000 cells/well) in 96-well flat bottomed titre plate and incubated for 24 h at 37^0C in 5% CO_2 atmosphere. Different concentrations of Sulforaphane (1-50µg/ml) were added and incubated further for 48 h. The cell viability was assessed by the MTT assay and percentage viability was calculated (Chapter 2).

6.2.5.2. Determination of the effect of Sulforaphane on tumour cell adhesion

Tumour cell adhesion assay was carried out by the method of Inokuchi *et al* (Inokuchi *et al.*, 1991) as described in Chapter 2. Briefly, B16F-10 melanoma cells were seeded on to collagen type I coated wells of flat bottomed titre plates, in the presence and absence of Sulforaphane (1, 2 & 5µg/ml) and incubated at 37^0C in 5% CO_2 atmosphere for 5 h. After the incubation cells were washed, the adhering cells were fixed and stained. Cells were then counted under a microscope. The experiment was done in triplicate.

6.2.5.3. Determination of the effect of Sulforaphane on invasion of B16F-10 melanoma cells

The invasion assay was carried out in Boyden chambers as described by Albini *et al* (Albini *et al.*, 1987) (Chapter 2). Briefly, the lower compartment of the chamber was filled with serum free DMEM and a polycarbonate filter coated with 25μg Type I collagen was placed above this. B16F-10 melanoma cells (10^5 cells/150 μl DMEM) were then seeded on to the upper chamber. To test the effect of Sulforaphane on the invasion of B16F-10 melanoma cells, different concentrations (1, 2 & 5μg/ml) were added along with the cells to the upper chamber (Chapter 2). The experiment was done in triplicate and the results were expressed as percentage inhibition of invasion.

6.2.5.4. Determination of the effect of Sulforaphane on motility of B16F-10 melanoma cells

Tumour cell motility assay was performed in the same manner as the invasion assay except that polycarbonate filters were collagen free. Different concentrations (1, 2 & 5μg/ml) of Sulforaphane were added along with B16F-10 melanoma cells to the upper compartment of the Boyden chamber. After incubation at 37^0C for 24h, the number of cells migrating to the lower chamber was determined using a haemocytometer. The experiment was done in triplicate and the results were expressed as percentage motility.

6.2.5.5. Determination of the effect of Sulforaphane on proliferation of B16F-10 melanoma cells (^{3}H-thymidine incorporation assay)

Tumour cell proliferation assay was carried out as described in Chapter 2. Briefly, B16F-10 melanoma cells (5000 cells/well) were incubated with or without different concentrations of Sulforaphane (1, 2 & 5μg/ml). 18 h prior to the termination of the assay 1μCi of ^{3}H- thymidine was added to each well. After completing incubation, the cells were washed with PBS and then treated with ice cold PCA. The resulting precipitate was dissolved in 0.5N NaOH and transferred to the scintillation fluid and kept overnight in dark. The radioactivity was counted using a Rack Beta liquid scintillation counter.

6.2.5.6. Gelatin Zymography

Gelatin Zymography was followed according to the procedure of Billings *et al* (Billings *et al.,* 1991) with some modification as described in Chapter 2. After determining the protein concentration, supernatant containing the proteases of treated and untreated melanoma cells were subjected to zymographic analysis with or without trypsin activation. Gels were fixed, stained and clear bands were visualized against a dark background.

6.2.6. Determination of the effect of Sulforaphane on the expression of k-ras, ERK1, ERK2, nm-23, prolyl hydroxylase, lysyl oxidase, MMP-2, MMP-9, TIMP-1, TIMP-2 and VEGF

C57BL/6 mice were divided into 2 groups. All the animals were injected with B16F-10 melanoma cells (10^6 cells/ animal) through lateral tail vein. Group I animals were kept as untreated metastatic tumour bearing control and Group II animals were treated with Sulforaphane (500μg/dose/animal/day, i.p) for 5 consecutive days. The animals were sacrificed on 21st day. The lungs were excised and RNA was isolated using guanidium thiocynate by the method of Chomczynski and Sacchi (Chomczynski and Sacchi, 1987; 2006). The cDNA was prepared from RNA by RT-PCR and used for the analysis of expression of k-ras, ERK1, ERK2, nm-23, prolyl hydroxylase, lysyl oxidase, MMP-2, MMP-9, TIMP-1, TIMP-2 and VEGF using specific primers. PCR products were analyzed by agarose gel electrophoresis and visualized using gel documentation system (Chapter 2).

6.3. Results

6.3.1. Determination of antimetastatic activity in the *in vivo* system

6.3.1.1. Effect of Sulforaphane on the lung tumour nodule formation

Metastatic tumour bearing animals treated with Sulforaphane showed significant reduction in tumour nodule formation (Table 6.1). Metastatic control animals had massive tumour growth and were assigned an arbitrary number of 250 (Hill *et al.*, 1994). The three different modalities of compound administration were found to be significantly effective. Of this simultaneous mode of administration produced maximum

Table 6.1. Effect of Sulforaphane on lung colonization of B16F-10 melanoma cells and survival of animals.

Treatment	Number of lung tumour nodules	% inhibition of nodule formation	%ILS
Tumor bearing			
Control	250[a]	----	----
Sulforaphane (500µg/dose/animal)			
Prophylactic	23.71 ± 4.11*	90.51	62.17
Simultaneous	11.25 ± 2.21*	95.50	94.06
Developed	43.87 ± 6.85*	82.45	37.85

The lungs were dissected out and observed for metastases on the 21st day after induction of B16F-10 melanoma cells (10^6 cells) through the lateral tail vein. Prophylactic group of animals received Sulforaphane prior to tumour induction (10 dose, i.p.), simultaneous group received the drug simultaneously with tumour induction where as developed group received drug after 10 days of tumour induction. Values are mean ±S.D

[a] An arbitrary number of 250 is given for massive number of tumour nodules.

*$p<0.001$.

inhibition of 95.5% followed by prophylactic mode of administration (90.51%) and administration after tumour development (82.45%).

Administration of Sulforaphane significantly increased the life span of tumour bearing animals (Table 6.1). The life span was highly enhanced in the case of simultaneous mode of administration (94.06%). Prophylactic administration enhanced the life span by 62.17% whereas administration after tumour development increased the lifespan by 37.85%

6.3.1.2. Effect of Sulforaphane on the biochemical parameters of the metastasis bearing animals

a) Lung collagen hydroxyproline

Effect of Sulforaphane on the lung biochemical parameters is presented in Table 6.2. Control metastatic tumour bearing animals showed an increased level of lung collagen hydroxyproline (21.91 ± 1.16μg/mg protein), which was significantly reduced in animals treated with Sulforaphane by the simultaneous mode of administration (4.99 ± 0.35μg/mg protein). Prophylactic mode of administration was also found to be effective with a reduced level of lung collagen hydroxyproline content (8.95 ± 0.66μg/mg protein). Treatment of Sulforaphane after tumour development also produced considerable reduction of lung collagen hydroxyproline (14.66 ± 0.7μg/mg protein), but not as much as the other two modalities.

b) Lung hexosamine content

Lung hexosamine level was highly increased in control metastatic tumour bearing animals (4.12 ± 0.29mg/100mg tissue dry wt) compared to the normal animals (0.4 ± 0.1mg/100mg tissue dry wt). When Sulforaphane was administered simultaneously with the tumour cells this elevated level was reduced to 0.9 ± 0.12mg/100mg tissue dry wt and when it was administered prophylactically the level was 1.39 ± 0.17mg/100mg tissue dry wt. Administration of Sulforaphane after tumour development also reduced this level to 2.09 ± 0.15mg/100mg tissue dry wt. (Table 6.2).

Table 6.2. Effect of Sulforaphane on the lung biochemical parameters of metastases bearing animals.

Treatment	Hydroxyproline (μg/mg protein)	Uronic acid (μg/100mg tissue wet wt)	Hexosamine (mg/100mg tissue dry wt)
Normal	1.19 ± 0.10	32.20 ± 2.00	0.40 ± 0.10
Tumour bearing			
Control	21.91 ± 1.16	352.62 ± 8.33	4.12 ± 0.29
Sulforaphane (500μg/dose/animal)			
Prophylactic	8.95 ± 0.66*	112.93 ± 5.10*	1.39 ± 0.17*
Simultaneous	4.99 ± 0.35*	85.14 ± 6.09*	0.90 ± 0.12*
Developed	14.66 ± 0.70*	162.81 ± 7.13*	2.09 ± 0.15*

The lungs were dissected out and assayed different biochemical parameters on the 21st day after induction of B16F-10 melanoma cells (10^6 cells) through the lateral tail vein. For Prophylactic group of animals, drug administration started 10 days prior to tumour challenge, simultaneous group received the drug simultaneously with tumour challenge and developed group were given drug, 10 days after tumour challenge. Values are mean ± S.D. *$p<0.001$.

c) Uronic acid levels of the lung

In control metastatic tumour bearing animals, the lung uronic acid level was drastically elevated (352.62 ± 8.33μg/100mg tissue wet wt), as compared to normal level (32.2 ± 2μg/100mg tissue wet wt) which was significantly reduced after the simultaneous administration of Sulforaphane (85.14 ± 6.09μg/100mg tissue wet wt). Considerable reduction in the lung uronic acid level was also obtained after prophylactic (112.93 ± 5.1μg/100mg tissue wet wt) and developed (162.81 ± 7.13μg/100mg tissue wet wt) modalities of administration when compared to control group (Table 6.2).

d) Serum sialic acid level

The effect of Sulforaphane on the serum biochemical parameters is presented in Table 6.3. The serum sialic acid level of control metastatic tumour bearing animals was highly increased (109.68 ± 1.67μg/ml serum) as compared to normal (21.3 ± 1.5μg/ml serum). Here also the simultaneous administration of Sulforaphane significantly reduced the elevated serum sialic acid level to 35.13 ± 0.9μg/ml serum, followed by prophylactic modality (59.51 ± 1.29μg/ml serum) and in developed modality it was reduced only to 92.88 ± 1.23μg/ml serum.

e) Serum γ-glutamyl transpeptidase (GGT) level

Here again, the highly elevated level of GGT in the serum of control metastatic tumour bearing animals (108.26 ± 2.16n mol P-nitroaniline/ml serum) as compared to normal animals (24 ± 0.17n mol P-nitroaniline/ml serum) was significantly reduced by the simultaneous mode of administration of Sulforaphane (39.15 ± 1.13n mol P-nitroaniline/ml serum), followed by prophylactic mode of administration (54.93 ± 1.58n mol P-nitroaniline/ml serum) where as in animals treated after tumour development this level was 76.58 ± 0.9n mol P-nitroaniline/ml serum (Table 6.3).

6.3.1.3. Histopathological analysis of lungs

The haematoxylin and eosin stained sections of lung tissues are shown in Figure 6.1 (X100). The lungs of control metastatic tumour bearing animals (Figure 6.1b)

Table 6.3. Effect of Sulforaphane on serum sialic acid and GGT levels of B16F-10 melanoma bearing animals.

Treatment	Sialic acid (µg/ mL serum)	GGT (nmol p-nitroaniline/ mL serum)
Normal	21.30 ± 1.50	24.00 ± 0.17
Tumour bearing		
Control	109.68 ± 1.67	108.26 ± 2.16
Sulforaphane (500µg/dose/animal)		
Prophylactic	59.51 ± 1.29*	54.93 ± 1.58*
Simultaneous	35.13 ± 0.90*	39.15 ± 1.13*
Developed	92.88 ± 1.23*	76.58 ± 0.90*

The serum was collected on 21st day of tumour challenge by B16F-10 melanoma cells through lateral tail vein and assayed for serum biochemical parameters. Values are mean ± S.D. *$p<0.001$.

Figure 6.1. Histopathological analysis of lungs from metastatic tumour bearing animals

a) Normal lung

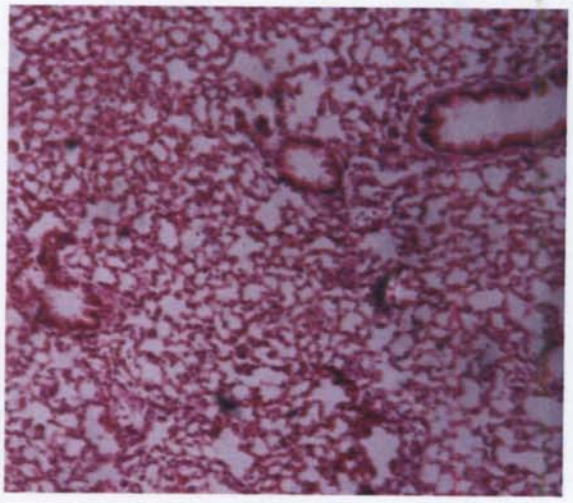
b) Tumour bearing control lung

c) Treated with Sulforaphane by simultaneous treatment modality

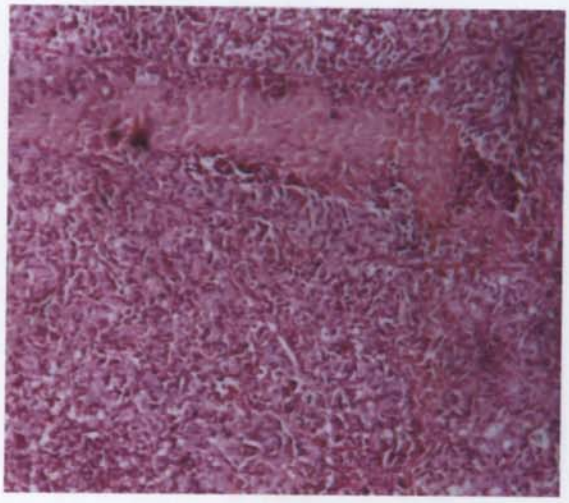
d) Treated with Sulforaphane by prophylactic treatment modality

e) Treated with Sulforaphane by after tumour development

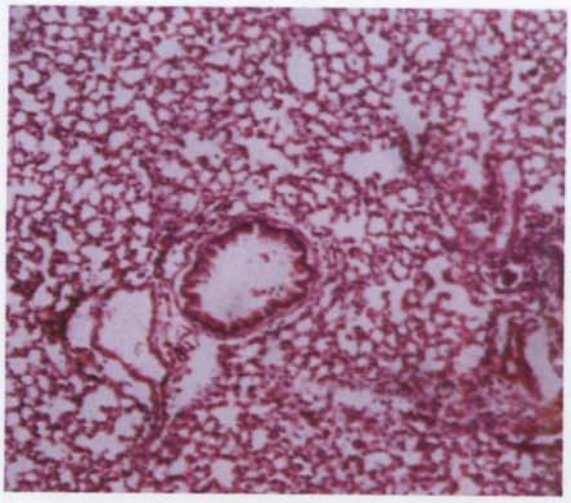
Figure 6.1



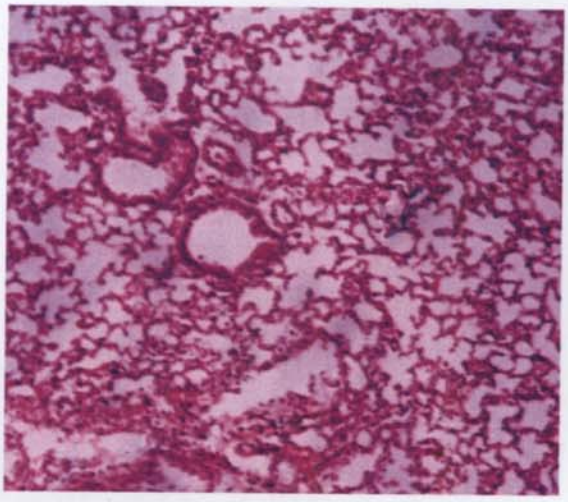
(a)



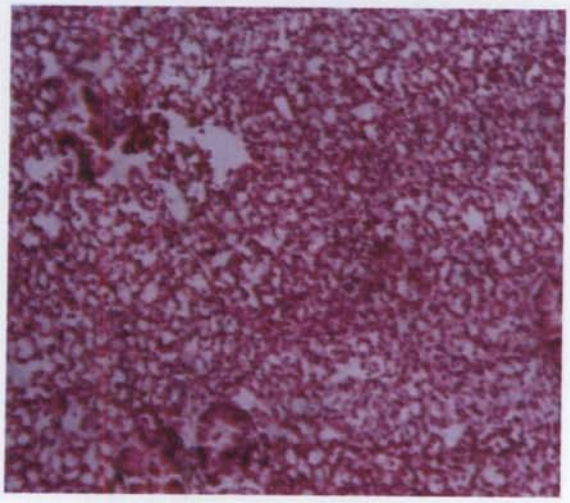
(b)



(c)



(d)



(e)

showed prominent tumour nodules around terminal bronchiole. These tumour nodules are characterized by polygonal tumour cells with prominent nucleolus, intracellular melanin deposition and clear areas of necrosis. This massive infiltration of the neoplastic cells around the main bronchioles, which make alveolar passages indistinguishable, extended to the pleura. This together with fibrosis reduces alveolar space, which in turn leads to reduced vital capacity. Simultaneous administration of Sulforaphane showed significant reduction in tumour mass. Alveoli and pleura were tumour free, alveolar passage lined with healthy ciliated columnar epithelial cells (Figure 6.1c) and almost similar to normal lung (Figure 6.1a). Considerable reduction of tumour mass was also observed in both prophylactic and developed modalities of administration (Figure 6.1d & e).

6.3.1.4. Effect of Sulforaphane on the TIMP-1 levels in metastatic tumour bearing animals

The level of TIMP in the serum was enhanced by the treatment of Sulforaphane (736.21 ± 9.3pg/ml) compared to normal animals (600 ± 36 pg/ml) on 7th day of tumour inoculation; whereas in metastatic tumour bearing control animals, TIMP level (520.46 ± 19.6pg/ml) was reduced than normal level. On 21st day also, this enhancement in the serum TIMP level was continued in Sulforaphane treated animals (809.36 ± 15.62pg/ml); whereas in metastatic tumour bearing control animals (503.54 ± 16.32pg/ml), the TIMP level still remained reduced than the normal level (Table 6.4).

6.3.2. Determination of antimetastatic activity in the *in vitro* system

6.3.2.1. Cell viability by MTT assay

Fifty eight percent B16F-10 melanoma cells were viable after exposure to 50μg/ml Sulforaphane for 48 h. At concentrations of 1-5μg/ml Sulforaphane, the number of live B16F-10 melanoma cells was more than 95% and were used for further *in vitro* experiments (Table 6.5).

Table 6.4. Effect of Sulforaphane on theTIMP-1 profile of metastasis induced animals.

Treatment	TIMP (pg/ml)	
	Day 7	Day 21
Normal	600 ± 36	
Tumour bearing		
Control	520.46 ± 19.6	503.54 ± 16.32
Sulforaphane	736.21 ± 9.3 *	809.36 ± 15.62 *

Blood was collected from the metastasis induced animals at the indicated time points after tumour challenge. Serum was separated by centrifugation, and level of TIMP-1 was estimated by ELISA method. All the values are mean ± SD (Mean of triplicate). *$p<0.001$ compared to control.

Table 6.5. Cytotoxicity of Sulforaphane towards B16F-10 melanoma cells in Culture

Concentration (μg/ml) of Sulforaphane	% cell viability
1	99.6
2	97.6
5	96.4
10	93.6
20	82.8
25	67.6
50	58.0

B16F-10 melanoma cells were incubated with different concentrations (1-50μg/ml) of Sulforaphane. Percentage of cell viability was determined using MTT assay.

6.3.2.2. Effect of Sulforaphane on adhesion of B16F-10 melanoma cells

The effect of Sulforaphane on the adhesion of B16F-10 melanoma cells to collagen matrix is given in Table 6.6. Maximum inhibition was obtained at a concentration of 5μg/ml of Sulforaphane (24.36%), followed by 2μg/ml (14.21%) and 1μg/ml (5.76%) respectively.

6.3.2.3. Effect of Sulforaphane on invasion of B16F-10 melanoma cells

Metastatic B16F-10 melanoma cells show high invasive property through the collagen matrix. Very high numbers of cells were found in the lower surface of the polycarbonate membrane, but administration of Sulforaphane produced significant inhibition in the invasion of the collagen matrix by the tumour cells. At a concentration of 5μg/ml Sulforaphane significantly inhibited the invasion of B16F-10 melanoma cells by 92.72% whereas at 2μg/ml and 1μg/ml the inhibition of invasion was found to be 61.18% and 36.42% respectively (Figure 6.2).

6.3.2.4. Effect of Sulforaphane on motility of B16F-10 melanoma cells

Inhibition of tumour cell motility by Sulforaphane is given in Table 6.7. At a concentration of 5μg/ml Sulforaphane significantly inhibited the motility of B16F-10 melanoma cells by 72.34% whereas at 2μg/ml and 1μg/ml the inhibition of motility was found to be 48.29% and 22.83% respectively.

6.3.2.5. Effect of Sulforaphane on proliferation of B16F-10 melanoma cells

Proliferation rate was determined by the ^{3}H-thymidine incorporation by the DNA of B16F-10 melanoma cells. Thymidine incorporation is proportional to the potential of the cells to synthesize DNA. Proliferation was expressed as radioactive count per minute. Untreated B16F-10 cells had very high rate of proliferation (4842.33 ± 230.7cpm). Administration of Sulforaphane at a concentration of 5μg/ml significantly reduced the proliferation (1662.66 ± 117.72cpm, 65.66%) of B16F-10 melanoma cells. Considerable inhibition of proliferation was also observed when Sulforaphane was administered at a concentration of 2μg/ml (2840.66 ± 27.57cpm, 41.33%) and 1μg/ml (3316.66 ± 117.55 cpm, 31.5%) (Table 6.8).

Table 6.6. Effect of Sulforaphane on adhesion of B16F-10 melanoma cells

Treatment	% inhibition
Sulforaphane	
1μg/ml	5.76
2μg/ml	14.21
5μg/ml	24.36

B16F-10 melanoma cells ($1x10^5$ cells /ml DMEM) were seeded into collagen Type-I coated wells of flat bottomed titre plates and incubated in presence and absence of different concentrations of Sulforaphane for 4 h at 37^0C. Adhering cells were fixed with 5% formaldehyde, stained with crystal violet and counted.

Figure 6.2. Effect of Suforaphane on collagen matrix invasion

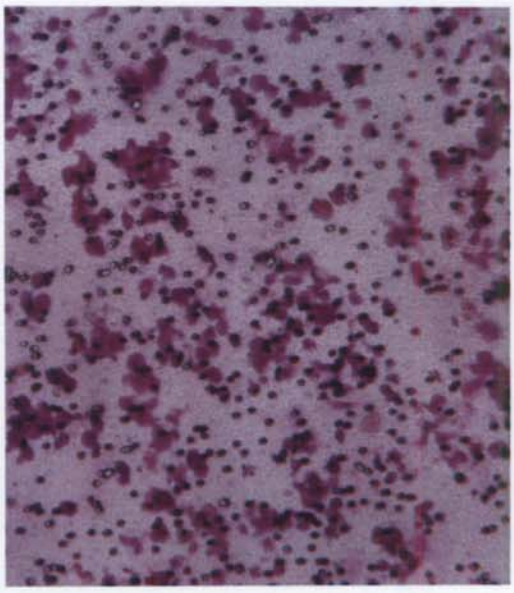
a) Untreated control

b) Treatment with Sulforaphane (1μg/ml)

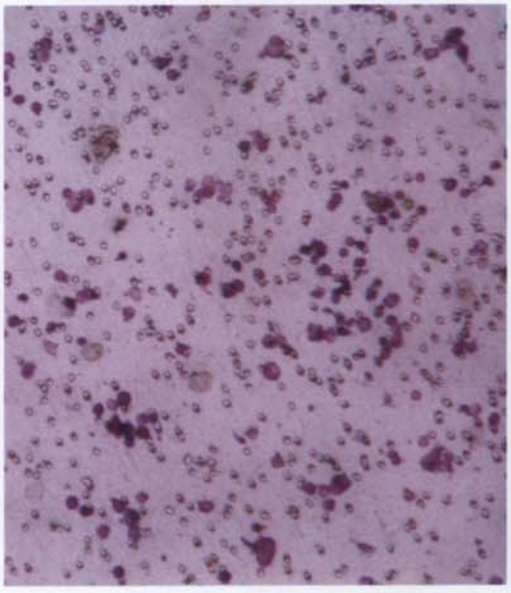
c) Treatment with Sulforaphane (2μg/ml)

d) Treatment with Sulforaphane (5μg/ml)

Figure 6.2



(a)



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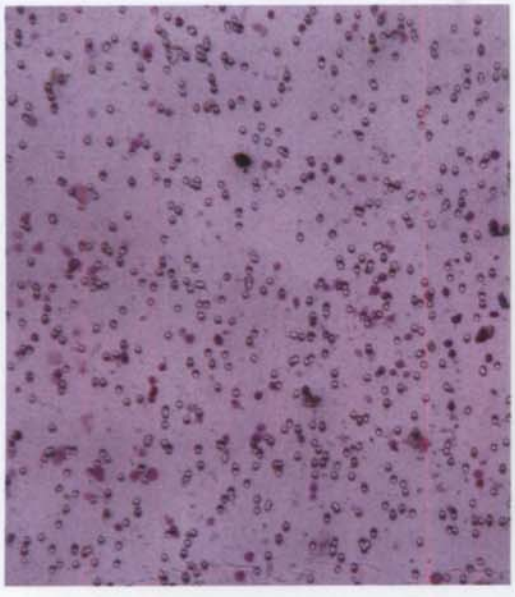

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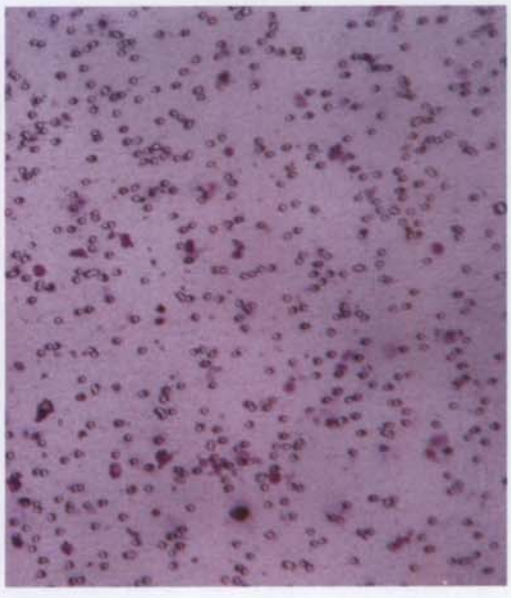


(d)

Table 6.7. Effect of Sulforaphane on migration of B16F-10 melanoma cells through the polycarbonate membrane

Treatment	% inhibition
Sulforaphane	
1μg/ml	22.83
2μg/ml	48.29
5μg/ml	72.34

B16F-10 melanoma cells ($1x10^5$ cells /150μl DMEM) were seeded into collagen free polycarbonate membranes on the upper compartment of the chamber. The lower compartment was filled with DMEM. Cells were incubated in presence and absence of Sulforaphane for 24 h at 37^0C. After incubation the number of cells migrating to the lower chamber was determined using haemocytometer.

Table 6.8. Effect of Sulforaphane on B16F-10 melanoma cell proliferation.

Treatment	Radioactive count/minute (CPM)	% inhibition
Untreated Control	4842.33 ± 230.70	-----
Sulforaphane		
1μg/ml	3316.66 ± 117.55*	31.50
2μg/ml	2840.66 ± 27.57*	41.33
5μg/ml	1662.66 ± 117.72*	65.66

B16F-10 melanoma cells ($5X10^3$ cells/ well) were grown in 96- well flat bottom plate After 24 h various concentrations of Sulforaphane were added and incubation was continued for 48 h. After incubation, ^3H –thymidine was added to each well (1μCi /well) and incubation was continued for 18 h. Cells were lysed and radioactivity was counted by using Rack Beta liquid scintillation counter. Values are mean ± S.D. *$p<0.001$ compared to control.

6.3.2.6. Gelatin zymographic analysis

As shown in Figure 3 Sulforaphane inhibited the activation of matrix metalloproteinases produced by B16F-10 melanoma cells. Conditioned medium after trypsin activation showed digested clear areas at 92 kD and 72 kD which was identical to MMP-9 and MMP-2 activity (Figure 6.3c). Gels loaded with conditioned medium without trypsin activation, did not show any clear degradative areas, indicating the inactive form of the enzyme (Figure 6.3a). Trypsin activated conditioned medium loaded gels, when incubated with 10mM EDTA did not show clear degradative areas which indicates that the enzyme responsible for degradation is metalloproteinase (Figure 6.3b). When conditioned medium was treated with Sulforaphane during trypsin activation, no clear bands were observed (Figure 6.3d & e) indicating that Sulforaphane inhibited the activation of procollagenase to active collagenase at concentrations of 2 and 5μg/ml.

6.3.6. Effect of Sulforaphane on gene expression of k-ras, ERK1, ERK2, nm-23, prolyl hydroxylase, lysyl oxidase, MMP-2 and MMP-9, TIMP-1, TIMP-2 and VEGF

The effect of Sulforaphane on the expression of genes, k-ras, ERK1 and ERK2, is shown in Figure 6.4a. Treatment with Sulforaphane significantly down regulated the expression of k-ras, ERK1 and ERK2, which were highly expressed in the lungs of untreated metastatic tumour bearing control animals.

Figure 6.4b shows the effect of Sulforaphane on the expression of nm-23, prolyl hydroxylase and lysyl oxidase. The tumour suppressor gene nm-23 was highly expressed in the lungs of Sulforaphane treated metastatic tumour bearing animals whereas in the lungs of untreated metastatic tumour bearing animals, the expression of this gene was highly suppressed. The highly expressed Prolyl hydroxylase and lysyl oxidase in the lungs of untreated metastatic tumour bearing animals were significantly suppressed by the administration of Sulforaphane.

The effect of Sulforaphane on the expression of genes, MMP-2, MMP-9 and TIMP-1, is shown in Figure 6.4c. Treatment with Sulforaphane significantly down regulated the expression of MMP-2 and MMP-9, which were highly expressed in the

Figure 6.3. Gelatin zymographic analysis

a) Conditioned medium from untreated B16F-10 melanoma cells without Trypsin activation

b) Conditioned medium from untreated B16F-10 melanoma cells with trypsin activation + EDTA

c) Conditioned medium from untreated B16F-10 melanoma cells with trypsin activation alone

d) Conditioned medium from untreated B16F-10 melanoma cells with Trypsin activation + Sulforaphane (2μg/ml)

e) Conditioned medium from untreated B16F-10 melanoma cells with Trypsin activation + Sulforaphane (5μg/ml)

Figure 6.3

Figure 6.4. Effect of Sulforaphane on gene expression profiles during metastasis

a) Effect of Sulforaphane on the expression of k-ras, ERK1 and ERK2

1) Lane 1: Molecular weight marker
2) Lane 2: Untreated control- k-ras (338bp)
3) Lane 3: Treatment with Sulforaphane
4) Lane 4: Untreated control- ERK 1 (512bp)
5) Lane 5: Treatment with Sulforaphane
6) Lane 6: Untreated control- ERK 2 (216bp)
7) Lane 7: Treatment with Sulforaphane
8) Lane 8: GAPDH (527bp)

b) Effect of Sulforaphane on the expression of nm-23, prolyl hydroxylase and lysyl oxidase

1) Lane 1: Molecular weight marker
2) Lane 2: Untreated control- nm-23
3) Lane 3: Treatment with Sulforaphane
4) Lane 4: Untreated control- Prolyl hydroxylase (317bp)
5) Lane 5: Treatment with Sulforaphane
6) Lane 6: Untreated control- Lysyl oxidase (283bp)
7) Lane 7: Treatment with Sulforaphane
8) Lane 8: GAPDH (527bp)

Figure 6.4. Effect of Sulforaphane on gene expression profiles during metastasis

c) Effect of Sulforaphane on the expression of MMP-2 and MMP-9, TIMP-1

1) Lane 1: Molecular weight marker

2) Lane 2: Untreated control- MMP-2 (354bp)

3) Lane 3: Treatment with Sulforaphane

4) Lane 4: Untreated control- MMP-9 (327bp)

5) Lane 5: Treatment with Sulforaphane

6) Lane 6: Untreated control- TIMP-1 (414bp)

7) Lane 7: Treatment with Sulforaphane

8) Lane 8: GAPDH (527bp)

d) Effect of Sulforaphane on the expression of TIMP-2 and VEGF

I) Lane 1: Molecular weight marker

2) Lane 2: Untreated control- TIMP-2 (525bp)

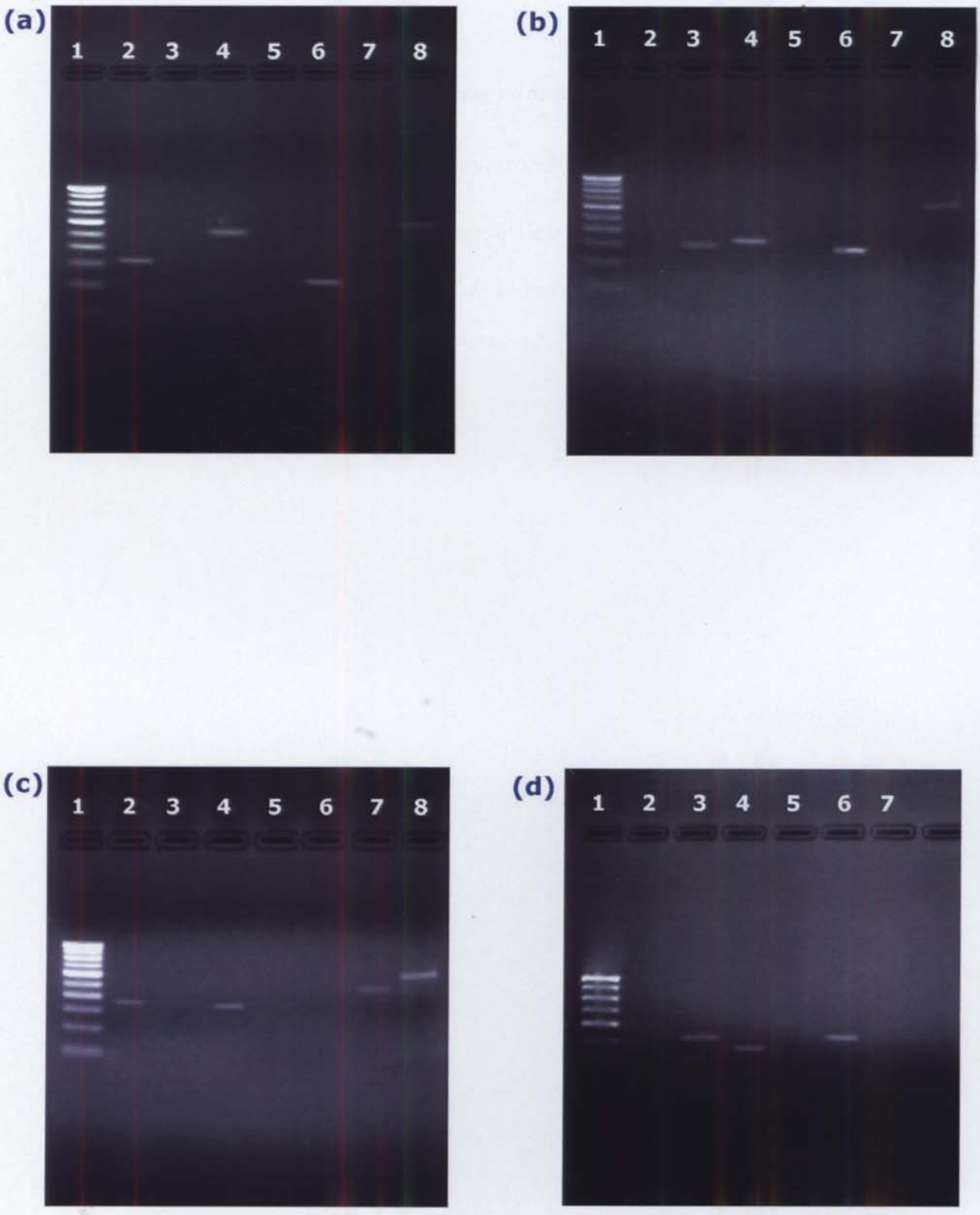
3) Lane 3: Treatment with Sulforaphane

4) Lane 4: Untreated control- VEGF (453bp)

5) Lane 5: Treatment with Sulforaphane

6) Lane 6: GAPDH (527bp)

Figure 6.4



lungs of untreated metastatic tumour bearing control animals. Administration of Sulforaphane significantly up regulated the expression of TIMP-1, which was highly suppressed in the lungs of untreated metastatic tumour bearing control animals.

Figure 6.4d shows the effect of Sulforaphane on the expression of TIMP-2 and VEGF. The inhibitor of MMPs, TIMP-2 was highly expressed in the lungs of Sulforaphane treated metastatic tumour bearing animals whereas in the lungs of untreated metastatic tumour bearing animals, the expression of this gene was highly suppressed. The highly expressed VEGF in the lungs of untreated metastatic tumour bearing animals were significantly suppressed by the administration of Sulforaphane. GAPDH (527bp) is used as the standard house keeping gene in all the experiments.

6.4. Discussion

In the present study, the antimetastatic activity of Sulforaphane and its mechanism of action were analyzed. B16F-10 melanoma cells are highly metastatic and form colonies of tumour nodules in the lungs when administered through tail vain, which in turn promote lung fibrosis and collagen deposition.

Lung collagen hydroxyproline content is a direct marker of lung fibrosis. During lung fibrosis, extra cellular matrix, especially collagen is deposited massively in the alveoli of lungs. 15-30% of collagen is hydroxyproline and it results in defective pulmonary function. Administration of Sulforaphane by 3 different modalities- simultaneous, prophylactic and developed- resulted in significant reduction of hydroxyproline content, which in turn causes marked reduction in lung fibrosis. This was well in correlation with histopathological analysis and significant reduction of tumour nodules in sulforaphane treated animals. This may be the reason for increase in lifespan of Sulforaphane treated animals.

Lysyl oxidase, an ECM protein, is consistently over expressed by hypoxic human tumour cells (Denko *et al.*, 2003). In the ECM, lysyl oxidase initiates the covalent cross linking of collagen and elastin, thereby increasing insoluble matrix deposition and tensile strength (Kagan and Li, 2003). Lysyl oxidase elevation occurs in metastatic and/or invasive breast cancer cell lines and correlates with increased staging in renal cell carcinoma (Kirschmann *et al.*, 2002). The prolyl hydroxylase enzyme plays

a central role in the synthesis of all collagens, as the 4-hydroxyproline residues are essential for the folding of the newly synthesized collagen. The excessive collagen formation was found in patients with various fibrotic diseases (Kivirikko *et al.*, 1992). The expression of lysyl oxidase and prolyl hydroxylase was found to be effectively down regulated by Sulforaphane.

The acidic modification of monosaccharides produced by the oxidation of CH_2OH group to COOH group yield uronic acids where as basic modification yield amino sugars (hexosamines) and these form an integral part of many structural polysaccharides, and glycosaminoglycans found in the ECM. Hyaluronic acid (HA) is a GAG made of repeated disaccharide units of D-glucuronic acid and N-acetyl D-glucosamine (Tammi *et al.*, 2002; Delpech *et al.*, 1997) and is a component of tissue matrix and tissue fluid. Concentration of HA is elevated in several cancers regardless of the tumour grade (Setala *et al.*, 1999) and supports tumour cell migration by interacting with cell surface HA receptors and there by promoting metastasis (Tammi *et al.*, 2002; Delpech *et al.*, 1997; Turley *et al.*, 2002). In the presence of glucuronic acid lactone, which is an esterified form of glucuronic acid, prolyl hydroxylase enzyme converts prohydroxyproline to hydroxyproline (Voet and Voet, 1995), which is a direct marker of lung fibrosis. Hexosamine has an important role in the synthesis of N-acetyl neuraminic acid (sialic acid), which is a component of glycolipids present on the surface of tumour cells (Voet and Voet, 1995). The enhanced level of these monosaccharides in the control metastatic tumour bearing animals indicates the active growth and proliferation of tumour cells. Administration of Sulforaphane significantly reduced the uronic acid and hexosamine content in the tumour bearing animals. The inhibitory effect of Sulforaphane on invasion and proliferation of B16F-10 melanoma cells (*in vitro*) strongly support these results.

Sialic acid, a family of acetylated derivatives of neuraminic acid, occurs as a terminal component at the non-reducing end of carbohydrate chains of glycoproteins and glycolipids. Neoplasm often have an increased concentration of sialic acid on the tumour cell surface and sialoglycoproteins are shed or secreted by some of these cells which increases their concentration in blood (Khadapkar *et al.*, 1975; Kloppel *et al.*, 1977). In the present study, the increased sialic acid level in the control metastatic

tumour bearing animals was significantly reduced in the animals treated with Sulforaphane.

Gamma glutamyl transpeptidase (GGT) is a cellular proliferation marker and high level of GGT was found in the serum of control metastatic tumour bearing animals. GGT catalyses intracellular GSH break down and provide energy to the tumour cells by γ-glutamyl cycle (West *et al.*, 1985). Administration of Sulforaphane significantly reduced the serum GGT level.

Matrix metalloproteinases are a family of more than 20 zinc-dependent endoproteinases that are capable of degrading almost all of the components of the extracellular matrix and thereby up regulates invasion and metastasis (Stetler-Stevenson *et al.*, 1996; Chambers and Matrisian, 1997). Among the MMPs reported earlier, MMP-2 and MMP-9 are key enzymes for degrading type IV collagen, which is a major component of the basement membrane (Zucker *et al.*, 1993; Bernhard *et al.*, 1994). Several experiments also proved that MMPs not only break down the physical barrier of extracellular matrix but also modulates the growth factors and cytokines stored in the extracellular matrix, which may enhance neoplastic progression (Voet and Voet, 1995) by promoting invasion, motility, adhesion and proliferation of tumour cells. In our study, Sulforaphane was found to inhibit these major stages of metastasis, especially invasion and proliferation. The result of zymographic analysis indicates that administration of Sulforaphane inhibited the activation of MMP-2 and MMP-9, which are key promoters of tumour cell invasion and proliferation. More over, the serum level of TIMP, which directly suppresses MMP activity, was significantly enhanced by administration of Sulforaphane. Gene expression profile analysis revealed that Sulforaphane suppressed the expression of MMP-2 and MMP-9 and at the same time up regulated the expression of TIMP-1 and TIMP-2. TIMP-1 is a regulator of MMP-9 while TIMP-2 regulates MMP-2. This also facilitated in the regulation of MMPs by Suforaphane and thereby inhibiting B16F-10 melanoma induced pulmonary metastasis in C57BL/6 mice.

About 25% of all human neoplasms contain mutationally activated forms of the Ras proto-oncogene (Bos, 1989). Constitutive Ras activity has been shown to contribute to increased tumour cell invasiveness through the activation of the matrix

metalloproteinases. Further more, Ras is known to promote endothelial cell dependent tumour angiogenesis, mainly by means of transcriptional up regulation of VEGF and through the repression of antiangiogenic protein thrombospondin-1 (TSP-1) (Rak *et al.*, 2000). The Ras/ Raf-1/ MEK/ ERK module is a ubiquitously expressed signaling pathway that conveys mitogenic and differentiation signals from the cell membrane to the nucleus (Ferrell, 1996). The activated Ras protein binds and activates the Raf-1 kinase. Activated Raf-1 then phosphorylates and activates MEK, a kinase that in turn phosphorylates and activates ERK, the prototypic mitogen activated protein kinase (MAPK). Activated ERKs can translocate to the nucleus and regulates gene expression by the phosphorylation of transcription factors (Robinson and Cobb, 1997). Of the various families of MAPKs, the first to be characterized were ERK1 and ERK2. A broad array of solid tumours is known to express constitutive levels of phosphorylated ERK1 and ERK2 (Hill and Tiesman, 1995). The involvement of this pathway in metastasis as well as angiogenesis has been well documented. Activation of the MAPK pathway occurs in response to integrin mediated cellular adhesion to the extra cellular matrix, which plays a critical role in both tumour metastasis and angiogenesis (Chen *et al.,* 1994). MAPK activation is also required for growth factor induced secretion of angiogenic growth factors from tumour cells (Petit *et al.*, 1997). By down regulating Ras, ERK1 and ERK2 genes, Sulforaphane effectively interfere with this pathway which in turn promoted its antimetastatic activity. Sulforaphane also inhibited the expression of VEGF, the unavailability of which hampers angiogenesis, a major rate limiting step in tumour metastasis. It also up regulated the expression of nm 23, the metastasis suppressor gene.

Thus the interference of Ras/ Raf-1/ MEK/ERK pathway, inhibition of MMP-2, MMP-9, lysyl oxidase, prolyl hydroxylase and VEGF genes and promotion of nm 23, TIMP-1 and TIMP-2 genes by Sulforaphane promoted its antimetastatic activity.

CHAPTER 7

MODULATION OF CELL MEDIATED IMMUNE RESPONSE DURING METASTASIS BY SULFORAPHANE

7.1. Introduction

A large proportion of cancer related deaths are due to metastases that are resistant to conventional therapies. The tumour development, out growth and metastasis, which are under the surveillance of the immune system, are frequently accompanied by a concomitant immunosuppression regardless of tumour location and etiology (Nelson and Nelson, 1987). Additionally, the down regulation of cytotoxic cells, such as T lymphocytes and natural killer (NK) cells, by developing neoplasm may further increase the severity of the disease (Lala *et al.*, 1985). The natural cytotoxicity mediated by NK cells may have a role in the prevention of the development of cancer and in the establishment of metastasis in humans as well as mice. Animals with low levels of NK cell activity have been shown to develop an increased number of spontaneous and experimental tumours and their metastases (Gorelik *et al.*, 1982). Conversely, animals with augmented NK cell activity display increased resistance to the development of metastasis (Hanna, 1982). One of the mechanisms employed by tumours for the evasion of host defenses is the production of proinflammatory cytokines that may affect the function of lymphocytes, macrophages and NK cells. These proinflammatory cytokines promote tumour growth by increasing cell adhesiveness and/ or enhancing tumour angiogenesis and by blocking cell mediated mechanisms for identifying and destroying the tumour (Balkwill and Mantovani, 2001). Obviously, if inflammatory conditions prevail at the tumour site, they may further support the progression of the tumour into more advanced stages and also promote metastasis and associated immunosuppression (Ben-Baruch, 2006).

In this Chapter, we evaluated the effect of Sulforaphane on the cell mediated immune (CMI) response of metastatic tumour bearing animals by analyzing the NK cell activity, antibody dependent cell mediated cytotoxicity (ADCC) and antibody dependent complement mediated cytotoxicity (ACC). We also analyzed the effect of Sulforaphane on serum IFN-γ and IL-2 levels during metastasis. Further, the effect of Sulforaphane on the levels of proinflammatory cytokines such as IL-1β, IL-6, TNF-α and GM-CSF in the serum during metastasis were also studied.

7.2. MATERIALS AND METHODS

7.2.1. Animals

Male C57BL/6 mice (4-6 weeks old) were used for this study.

7.2.2. Cell line

B16F-10 melanoma cells and K-562 human leukemic cells were used for this study.

7.2.3. Administration of Sulforaphane

Sulforaphane was suspended in phosphate buffered saline (PBS, pH-7.4) and administered intraperitoneally at a dosage of 500μg/dose/animal.

7.2.4. Determination of the effect of Sulforaphane on cell mediated immune response in metastatic tumour bearing animals

C57BL/6 mice were divided into 3 groups (12 mice/group). Group I animals received B16F-10 melanoma cells (1×10^6 cells/animal) intravenously and kept as untreated control. Group II animals were treated with Sulforaphane (500μg/dose/animal/day, i.p) for 5 consecutive days. Group III animals received Sulforaphane as in Group II animals and B16F-10 melanoma cells (1×10^6 cells/animal) intravenously after the last dose of Sulforaphane. The animals were sacrificed at different time points after 24 h of tumour induction and spleen and blood were collected. Spleen cells were processed to single cell suspension and used as effector cells for NK cell activity and ADCC by ^{51}Cr-release assay (Kim *et al.*, 1980). Serum was separated from the blood and used for the estimation of ACC by trypan blue exclusion method (chapter 2).

7.2.5. Determination of the effect of Sulforaphane on cytokine production by metastatic tumour bearing animals

C57BL/6 mice were divided into 2 groups (12 mice/group). Group I animals received B16F-10 melanoma cells (1×10^6 cells/animal) intravenously and kept as untreated control. Group II animals were treated with Sulforaphane (500μg/dose/animal/day, i.p) for 5 consecutive days and received B16F-10 melanoma

cells (1×10^6 cells/animal) intravenously after the last dose of Sulforaphane. Blood was collected by tail vein bleeding on 7th and 21st days after tumour inoculation; serum was separated and used for assays. Cytokines such as IL-1β, IL-2, IL-6, GM-CSF, TNF-α and IFN-γ were estimated using respective Elisa kits. The manufacturer's protocol was followed to estimate the level of cytokines.

7.3. RESULTS

7.3.1. Effect of Sulforaphane on NK cell activity

The effect of Sulforaphane on NK cell mediated cytotoxicity of normal and tumour bearing animals is shown in Figure 7.1. Administration of Sulforaphane significantly enhanced the NK cell activity in metastatic tumour bearing as well as normal animals and this occurred much earlier when compared to untreated tumour bearing animals. Maximum lysis of target cells (43.17% cell lysis) was seen on the 5th day after tumour induction in Sulforaphane treated metastatic tumour bearing animals whereas in untreated tumour bearing control animals, the peak lysis (9.76% cell lysis) was observed only on day 9. Normal animals treated with Sulforaphane also showed an earlier enhancement of NK cell activity and the peak activity (41.37% cell lysis) was observed on 5th day.

7.3.2. Effect of Sulforaphane on ADCC

The effect of Sulforaphane on ADCC is presented in Figure 7.2. Treatment with Sulforaphane significantly augmented ADCC in tumour bearing animals. In Sulforaphane treated tumour bearing animals maximum lysis of target cells (41.20% cell lysis) was seen on 9th day while in untreated tumour bearing control animals, maximum activity was observed only on day 15 (12.62% cell lysis). Peak lysis of target cells (39.47% cell lysis) in Sulforaphane treated normal animals was also observed on 9th day.

7.3.3. Effect of Sulforaphane on ACC

The effect of Sulforaphane on ACC is given in Figure 7.3. Here also treatment of Sulforaphane significantly enhanced ACC in metastatic tumour bearing animals as well

Figure 7.1. Effect of Sulforaphane on NK cell activity

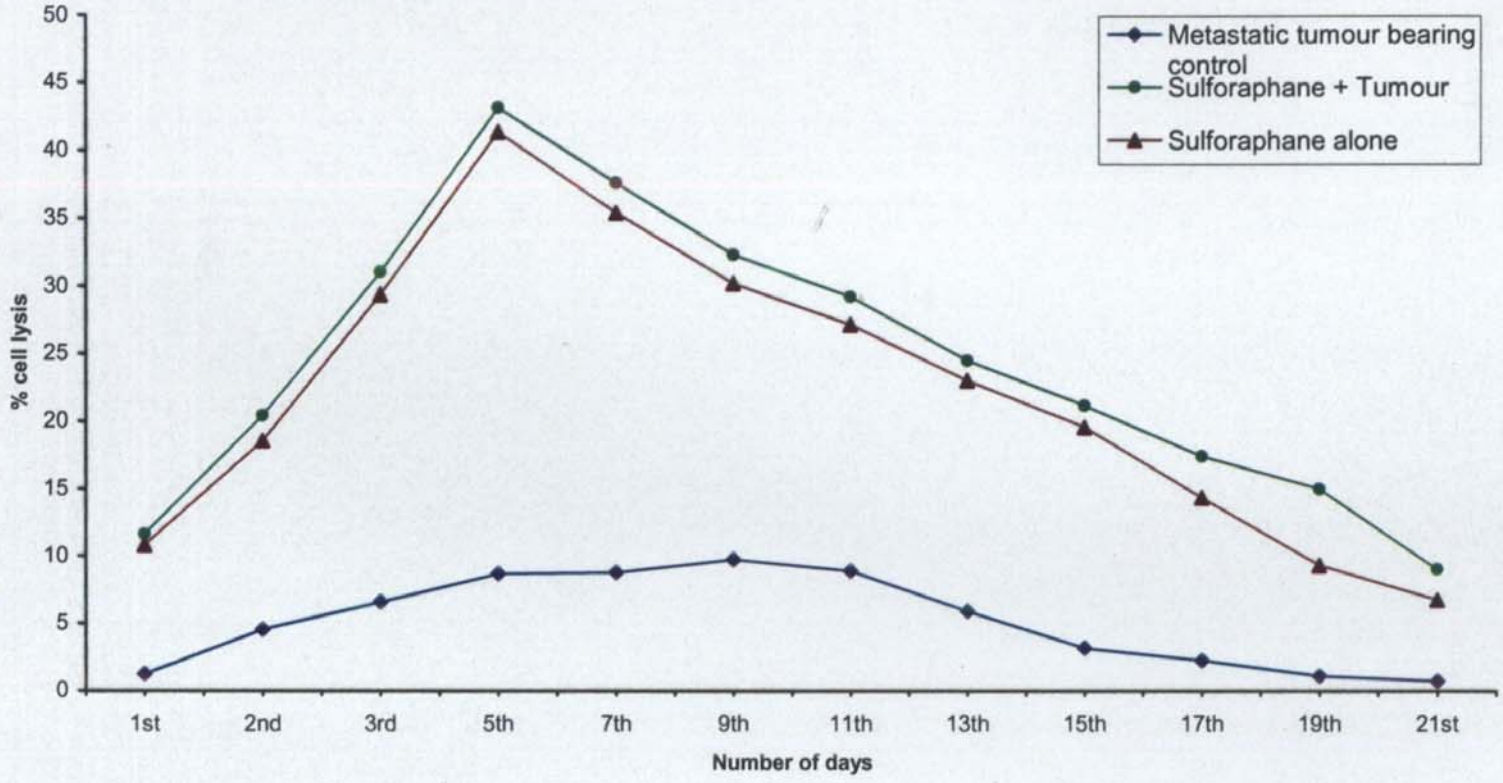


Figure 7.2. Effect of Sulforaphane on ADCC

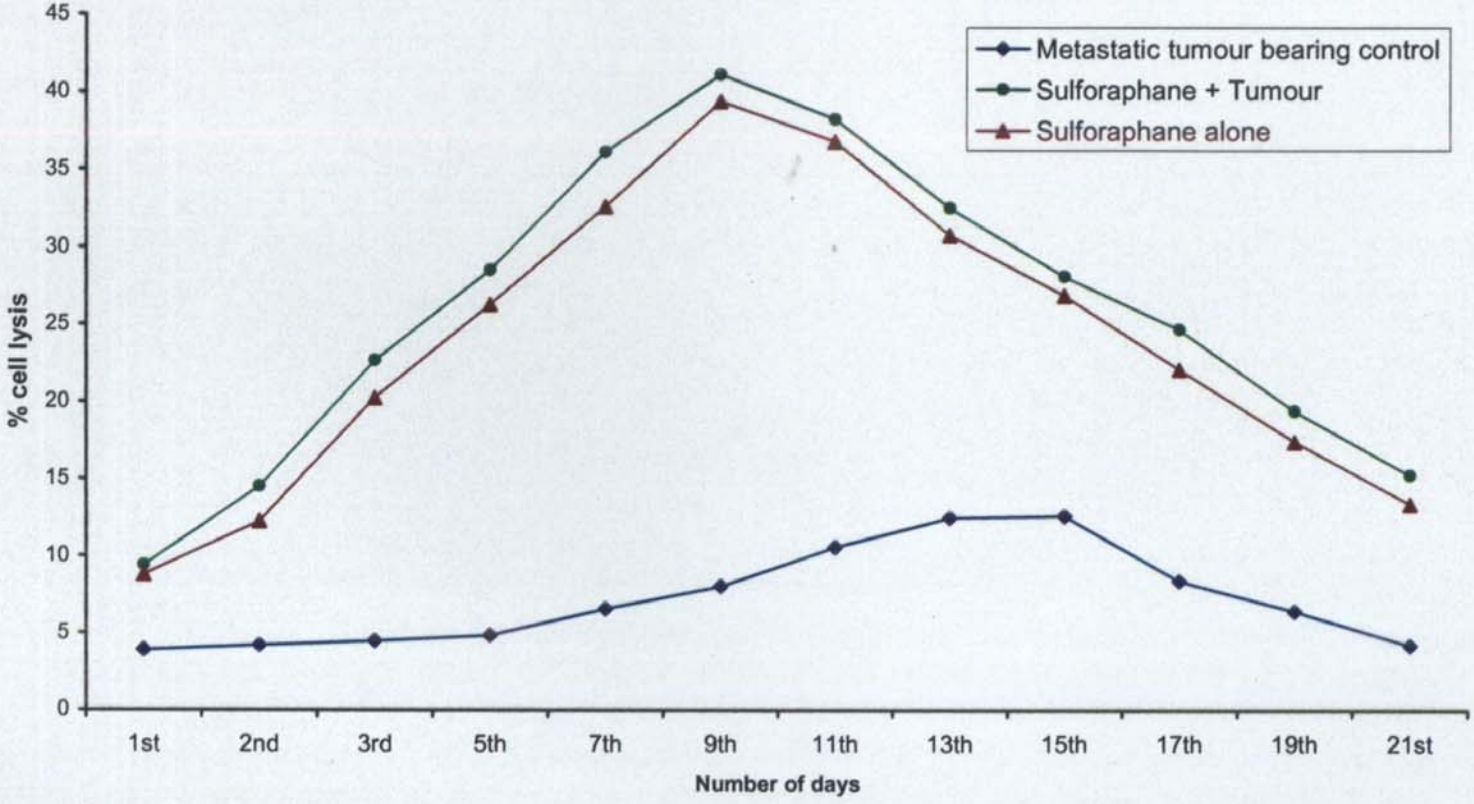
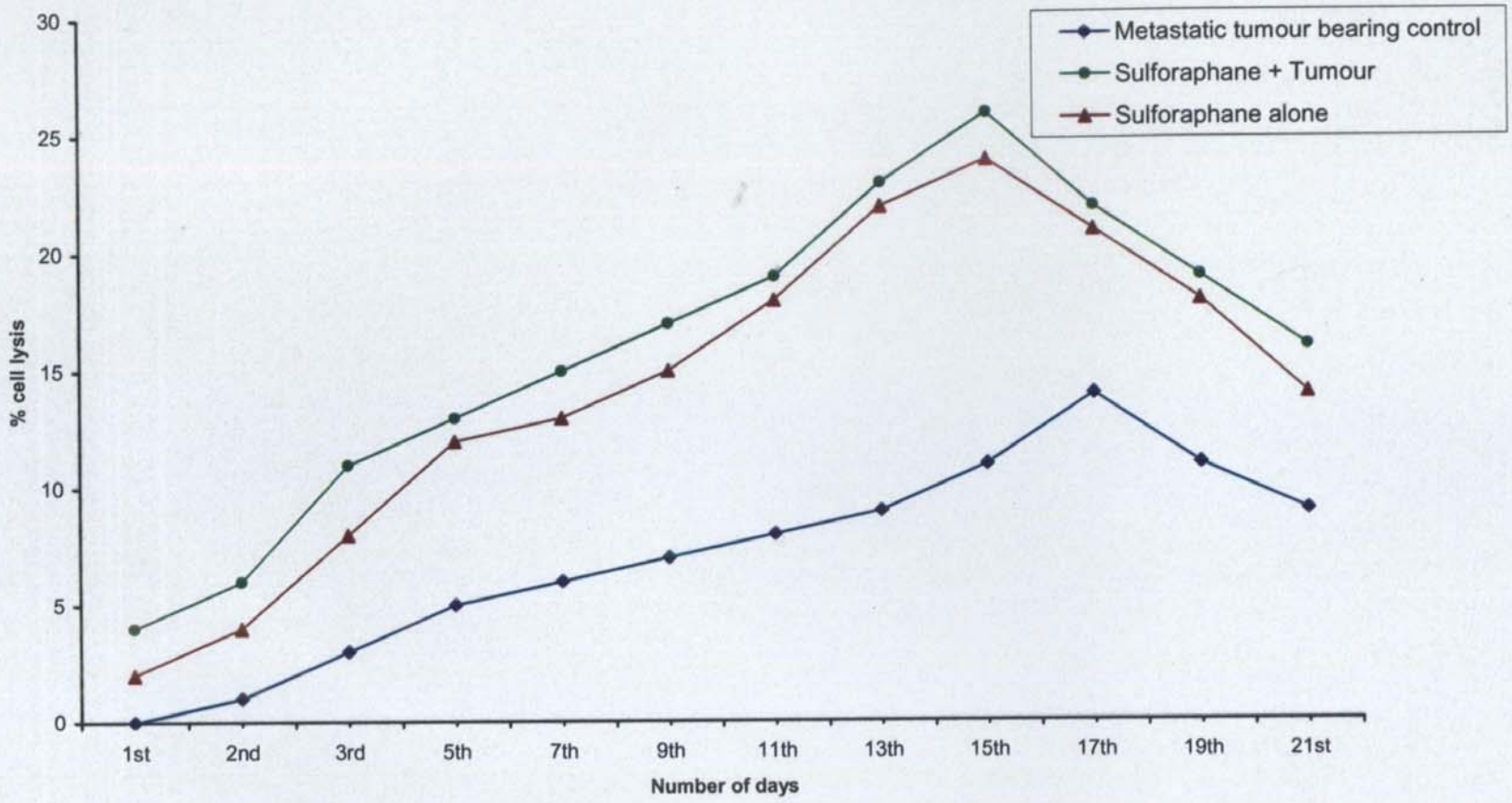


Figure 7.3. Effect of Sulforaphane on ACC



as normal animals and the peak activity was observed on day 15 and the maximum cell lysis was 26% and 24% respectively. In untreated tumour bearing control animals, the peak activity (14% cell lysis) was observed only on day 17.

7.3.4. Effect of Sulforaphane on IL-2 and IFN-γ production during metastasis

The effect of Sulforaphane on serum levels of IL-2 and IFN-γ during metastasis is shown in Table 7.1. The lowered levels of IL-2 in the metastatic tumour bearing control animals (18.67 ± 1.30pg/ml) after 7th day of tumour inoculation was significantly enhanced by the treatment with Sulforaphane (39.11 ± 1.6pg/ml). This enhancement in IL-2 level was still continued and by day 21st it was 52.21 ± 2.11pg/ml upon treatment with Sulforaphane whereas in untreated metastatic tumour bearing control animals, IL-2 level was further lowered to 9.77 ± 1.09 pg/ml which was well below the level of normal value (23 ± 3.2pg/ml).

Introduction of B16F-10 melanoma cells resulted drastic decrease in the level of IFN- γ from the normal (2862.63 ± 132.41pg/ml) and it was 2031.42 ± 116.21 and 2381.43 ± 124.36 pg/ml respectively after 7th and 21st days of tumour induction. Administration of Sulforaphane significantly enhanced the production of IFN-γ in metastatic tumour bearing mice and it was 3231.36 ± 106.81 and 3486.23 ± 118.47pg/ml respectively after 7th and 21st days of tumour induction.

7.3.5. Effect of Sulforaphane on proinflammatory cytokines in metastasis induced animals

Administration of Sulforaphane showed varying pattern of regulation of proinflammatory cytokines such as IL-1β, IL-6, TNF-α and GM-CSF in the serum of metastasis induced animals during the period of study (Table 7.2). After 7th day of tumour challenge, serum level of IL-1β was two fold higher in the metastatic tumour bearing control animals (32.04 ± 1.05pg/ml) than the normal level (16 ± 3.5 pg/ml) and this enhancement still continued (53.64 ± 1.30pg/ml) even after 21 days. Administration of Sulforaphane significantly checked this elevation of IL-1β after7 days (23.82 ± 1.16pg/ml) of melanoma inoculation and was still found to reduce this level to normal after 21 days (18.05 ± 1.49pg/ml).

Table 7.1. Effect of Sulforaphane on the IL-2 and IFN-γ profile of metastasis induced animals.

Treatment	IL-2 (pg/ml)		IFN-γ (pg/ml)	
	Day 7	Day 21	Day 7	Day 21
Normal	23 ± 3.2		2862.63± 132.41	
T.Control	18.67 ± 1.30	9.77 ± 1.09	2031.42 ± 116.21	2381.43 ± 124.36
Sulforaphane	39.11 ± 1.60*	52.21 ± 2.11*	3231.36 ± 106.81*	3486.23 ± 118.47*

Blood was collected from the metastasis induced animals at the indicated time points after tumour challenge. Serum was separated by centrifugation, and levels of IL-2 and IFN-γ were estimated by ELISA method. All the values are mean ± SD (Mean of triplicate). *$p<0.001$ compared to control.

T.Control – Tumour Control

Table 7.2. Effect of Sulforaphane on the proinflammatory cytokine profile of metastasis induced animals.

Cytokines (pg/ml)	Normal	Tumour control		Sulforaphane	
		Day 7	Day 21	Day 7	Day 21
IL-1β	16 ± 3.5	32.04 ± 1.05	53.64 ± 1.30	23.82 ± 1.16*	18.05 ± 1.49*
IL-6	35 ± 6.5	335.08 ± 3.60	580.28 ± 4.80	65.14 ± 3.22*	132.43 ± 4.80*
TNF-α	20 ± 3.2	262.12 ± 8.42	630.80 ± 9.54	137.22 ± 5.75*	62.81 ± 5.13*
GM-CSF	18 ± 3.1	51.01 ± 2.56	24.45 ± 1.75	22.52 ± 1.56*	19.95 ± 1.39*

Blood was collected from the metastasis induced animals at the indicated time points after tumour challenge. Serum was separated by centrifugation and the cytokine level was estimated by ELISA method. All the values are mean ± SD (Mean of triplicate). *$p<0.001$ compared to control.

IL-6 level was found to be drastically elevated in the serum of untreated metastasis induced control animals 7 day after tumour induction (335.08 ± 3.6pg/ml) compared to normal level of 35 ± 6.5pg/ml. Administration of Sulforaphane was found to be very effective in checking the drastic enhancement of IL-6 level on 7th day (65.14 ± 3.22pg/ml) of tumour inoculation and on day 21 the level was 132.43± 4.80pg/ml which was very low when compared to metastatic tumour bearing untreated control animals (580.28 ± 4.8pg/ml).

Drastic enhancement in the level of serum TNF-α (262.12 ± 8.42pg/ml) was observed in untreated metastatic tumour bearing control animals after 7th day of tumour challenge compared to normal serum TNF-α level (20 ± 3.2pg/ml) and was much more elevated on day 21 of tumour challenge (630.80 ± 9.54pg/ml). Even though initial enhancement was observed on 7th day after tumour inoculation (137.22 ± 5.75pg/ml) administration of Sulforaphane significantly reduced the elevated serum TNF-α level to 62.81 ± 5.13pg/ml after 21 days of tumour challenge.

Serum GM-CSF level was highly elevated after 7 days of tumour challenge in the untreated metastatic tumour bearing control animals (51.01 ± 2.56pg/ml) compared to normal level (18 ± 3.1pg/ml) and by day 21 the level was reduced (24.45 ± 1.75pg/ml). Sulforaphane administration reduced the initial elevation to 22.52 ± 1.56pg/ml and by day 21 serum GM-CSF level (19.95 ± 1.39pg/ml) was found to be closer to normal values.

7.4. Discussion

Natural killer (NK) cells play an important role in immunological surveillance in neoplasia and metastasis (North *et al.*, 2007; Brittenden *et al.*, 1996). Human NK cells, comprising approximately 15% of all circulating lymphocytes, can cause early production of cytokines and chemokines and lyse tumour cells without prior sensitization (Cooper *et al.*, 2001). In contrast to cytotoxic T lymphocytes, which are triggered by class I MHC molecules, NK cells mediate lysis of target cells which fail to express MHC class I molecules (Moretta *et al.*, 2002). The absence of MHC class I molecules on tumour cells causes activation of NK cells which recognize the missing self markers, lyse MHC class I^{-} tumour cells and therefore may play an active role in

immunological control of tumour growth. Evidences have shown that experimentally induced and transplanted tumours grow more rapidly in NK-deficient animals (Talmadge *et al.*, 1980).

Animal studies have indicated that immune control over circulating tumour cells and micrometastasis is carried mainly through NK cells which are integral part of CMI (Smyth *et al.*, 2001). Recent studies elucidate the role of NK cells in the destruction of circulating lymphoma and melanoma (Hanna and Burtan, 1981). We analyzed the effect of Sulforaphane on the CMI during metastasis and found that administration of Sulforaphane significantly enhanced the NK cell activity in metastatic tumour bearing animals. Further, Sulforaphane stimulated the activation of NK cells much earlier as there was maximum lysis of tumour cells on day 5 in Sulforaphane treated metastatic tumour bearing animals whereas metastatic tumour bearing control animals had maximum activity on day 9.

The levels of IFN-γ and IL-2 in the serum of metastatic tumour bearing animals were significantly enhanced by the treatment of Sulforaphane. The primary activation of NK cells by the missing self signal may lead to the production of IFN-γ by the activated NK cells and is thought to play key role in NK cell mediated tumour cell rejection. It is well demonstrated that IFNs or IFN-inducers potentiate NK cell reactivity (Djeu *et al.*, 1979). In addition, the lymphokine IL-2, a known T cell growth factor (Misawa *et al.*, 2000), alone or in combination with IFNs, also stimulate the lytic activity of NK cells (Caligiuri *et al.*, 1993) which also produce a variety of immunoregulatory molecules that could synergize with IFNs, IL-2 or IL-2 induced cytokines for induction of antitumour responses (Henney *et al.*, 1981). Thus the observed enhancement in the NK cell mediated tumour cell lysis by Sulforaphane could be attributed to its up regulatory effect on the production of IFN- γ and IL-2.

One of the major cytolytic pathways (ADCC) of the immune system involves NK cells that bind to the Fc effector domain of an antibody resulting in antibody dependent lysis of cells that bind to the targeting domain of the antibody (Henney *et al.*, 1981; Mandelboim *et al.*, 1999). ADCC thus represents a link between the humoral and cell mediated immunity. Activation of NK cells by Sulforaphane also produced a sharp increase of ADCC in metastatic tumour bearing animals whereas untreated metastatic

tumour bearing control animals with low NK cell activity showed decreased ADCC activity.

ACC, another cytolytic pathway of the immune system involves the complement cascade, which is activated when the molecule C1q reacts with the Fc region of antibodies bound to a target cell (Fanger and Guyre, 1991). Complement proteins are responsible for cell lysis and mediation of inflammation and enhanced phagocytosis. Both NK cells and complement can trigger powerful cytolytic immune pathway, which should be directed against the targeted melanoma cells and our data clearly demonstrated that Sulforaphane stimulated the complement proteins, which in turn mediated the efficient lysis of melanoma cells.

The proinflammatory cytokines evaluated in this study is also important in the process of metastasis. Altered levels of proinflammatory cytokines such as IL-1β, IL-6, TNF-α and GM-CSF were observed in various forms of cancer including melanoma (Lazar-Moinar *et al.*, 2000). In this study, when the highly metastatic B16F-10 melanoma cells were introduced through lateral tail vein of C57BL/6 mice, the serum levels of these proinflammatory cytokines got highly elevated whereas treatment with Sulforaphane effectively checked the proinflammatory cytokine elevation. The cytokines IL-1β, IL-6 and TNF-α play a key role in the stimulation of myeloma cell growth and also promote the production of VEGF, a key angiogenic growth factor (Balkwill and Mantovani, 2001). The expression of IL-6 in tumour tissues is high and it also contributes to a local immunosuppressive effect that protects the tumour cells from the host immune system (Tilg *et al.*, 1997). Previous reports on different tumour models indicated that GM-CSF may promote tumour progression by enhancing cell migration and thus favouring invasion and metastasis (Young *et al.*, 1992). Thus the elevation of these cytokines from the normal levels are strictly prometastatic and administration of Sulforaphane significantly down regulated this elevated proinflammatory cytokine production during metastasis. From this data, it is clear that Sulforaphane effectively inhibited the spread of metastatic tumour cells through the stimulation of CMI, up regulation of IL-2 and IFN-γ and down regulation of proinflammatory cytokines IL-1β, IL-6, TNF-α and GM-CSF.

SUMMARY AND CONCLUSION

Angiogenesis is essential for successful tumour growth and metastasis (Folkman, 1995). Different factors produced by tumour cells and surrounding stromal cells play important roles in regulating tumour angiogenesis by activating or blocking different pathways (Hanahan and Folkman, 1996). The process of angiogenesis consists of several steps, which include the stimulation of endothelial cells by growth factors such as VEGF, the subsequent degradation of the ECM by proteolytic enzymes such as MMPs, followed by invasion through ECM, migration and proliferation of endothelial cells and finally the formation of new capillary tubes (Carmeliet, 2000). Since each of these steps is rate limiting in the process of angiogenesis, agents that can inhibit or delay these steps are of great therapeutic implications. The onset of angiogenesis appears to be the result of an imbalance between stimulatory and inhibitory factors that leads to the activation of previously quiescent endothelial cells. To use antiangiogenesis approach successfully as an anticancer therapy, it is essential to identify the agents which can stimulate antiangiogenic factors as well as demote proangiogenic factors. In this study we evaluated the inhibitory effect of some naturally occurring organosulfur compounds such as DAS, DADS, AITC, PITC and Sulforaphane on tumour specific angiogenesis using both *in vivo* as well as *in vitro* models.

Endogenous NO may mediate angiogenesis directly through vasodilation by smooth muscle relaxation, which is a pre-requisite for endothelial cells to enter angiogenic cascade (Griffioen and Molema, 2000) or as a second messenger of other growth factors. The Sulfur compounds which we studied effectively down regulated the expression of iNOS gene, which is usually over expressed during angiogenesis, in B16F-10 melanoma cells and also checked the elevation of serum NO level after angiogenesis induction. VEGF, a survival factor for endothelial cells by inhibiting apoptosis, is a key angiogenic factor frequently used by tumours and tissues to switch on their angiogenic phenotypes. This potent and unique angiogenic protein stimulates capillary formation, endothelial cell migration and proliferation as well as increases vascular permeability (Neufeld *et al.*, 1999; Ferrara and Davis-Smith, 1997; Nor *et al*, 1999; Ferrara, 1999). Drastic elevation of serum VEGF levels in C57BL/6 mice after angiogenesis induction using B16F-10 melanoma cells was significantly reduced by the administration of Sulfur compounds. Studies at the molecular level proved that this inhibitory effect on the elevation of serum VEGF levels after angiogenesis induction by these compounds was due to their down

regulatory effect on the VEGF mRNA expression. The observed inhibition of tumour directed capillary formation by the administration of test compounds in angiogenesis induced C57BL/6 mice may be related to their inhibitory effect on the production NO and VEGF.

Remodeling of extracellular matrix (ECM) by matrix metalloproteinases (MMPs) is a crucial step in angiogenic cascade as it promotes endothelial cell migration, proliferation and tube formation. MMPs facilitate endothelial cell sprouting by liberating matrix bound angiogenic activators such as VEGF, FGF, TGF-β and proteolytically activating angiogenic chemokines (such as IL-1β) (Luttun *et al.*, 2000). MMP-2 and MMP-9 have been shown to be important in tumour invasion because of their ability to breakdown basement membrane. In our study, zymographic analysis revealed that among the Sulfur compounds, DADS and Sulforaphane could inhibit the activation of pro-MMPs to their active forms. Recent studies clearly demonstrated that MMP-9 mediates the release and accumulation of VEGF from the cell matrix (Hiratsuka *et al.*, 2002) and triggers angiogenic switch by rendering VEGF bioavailability to its receptors (Bergers *et al.*, 2000). Further, all the Sulfur compounds tested enhanced the production of TIMP, an endogenous inhibitor of MMPs. Thus these Sulfur compounds checked the level of MMPs during angiogenesis either by inhibiting the activation of MMPs or by increasing the level of TIMP. Since proper functioning of MMPs and VEGF are essential for successful endothelial cell migration, proliferation and tube formation, any hindrance to their activity leads to unsuccessful vessel formation. Studies using HUVECs well supported this notion as these Sulfur compounds at non-toxic concentrations significantly inhibited VEGF induced endothelial cell migration, proliferation and tube formation.

The proinflammatory cytokines such as IL-1β, IL-6, TNF-α and GM-CSF evaluated in this study act as autocrine growth factors for tumour angiogenesis. IL-1β (Li *et al.*, 1995) and IL-6 (Cohen *et al.*, 1996) have been shown to induce VEGF expression in several malignant and non-malignant cell lines. TNF-α is a good inducer of HIF-1 activity (Hellwig-Burgel *et al.*, 1999). GM-CSF promotes angiogenic reaction by stimulating endothelial cell proliferation and migration (Bussolino *et al.*, 1991). Elevations of these cytokines are strictly proangiogenic and treatment with these Sulfur compounds regulated the levels and inhibited angiogenesis. In addition, these Sulfur compounds increased the serum levels of

antiangiogenic cytokine IL-2. So these Sulfur compounds were remained successful to shift the equilibrium of pro and antiangiogenic factors in favour of antiangiogenic condition.

Treatment with Sulfur compounds inhibited the microvessel out growth from the rat aorta ring induced by conditioned medium from the B16F-10 melanoma cells. Interestingly, aortic rings incubated with conditioned media from B16F-10 melanoma cells pre-treated with Sulfur compounds also showed a marked reduction in vessel out growth when compared with rings incubated with conditioned medium from untreated B16F-10 melanoma cells. These results indicate that the treatment with these Sulfur compounds effectively inhibited the proangiogenic factor from the melanoma cells without affecting the viability of the cell. This clearly indicates that the action of these Sulfur compounds is at a molecular level rather than the membrane permeability or direct cellular toxicity.

In short, the Sulfur compounds-DAS, DADS, AITC, PITC and Sulforaphane- that inhibited the proangiogenic factor secretion from the tumour cells also decreased their levels in the serum of tumour-angiogenesis induced animals. They also inhibited the biological activities of HUVECs such as invasion, motility, proliferation and tube formation that are essential for neovessel formation. All these factors contributed to the antiangiogenic activity shown by these Sulfur compounds.

A Successful anticancer drug should kill or incapacitate cancer cells without causing excessive damage to normal cells. This ideal situation is achievable by inducing apoptosis in cancer cells. The Sulfur compounds which we studied were found to induce apoptosis in B16F-10 melanoma cells. The tumour suppressor gene p53 is a regulator of apoptosis, the activation of which is generally blocked by over expression of antiapoptotic Bcl-2 proteins. The Bcl-2 proteins also inhibited caspase-3 activation which is essential for execution of apoptosis. DADS and Sulforaphane were found to be very effective inducers of apoptosis as these compounds up regulated the expression of proapoptotic p53 and caspase-3 genes and at the same time down regulated antiapoptotic Bcl-2 gene expression. AITC induced apoptosis by up regulation of caspase-3 gene and down regulation of Bcl-2 gene. DAS and PITC also down regulated the expression of Bcl-2.

Transcription factor NF-κB regulates the expression of various molecules such as MMPs, COX-2, iNOS and proinflammatory cytokines, all of which promote tumour cell invasion and angiogenesis. Besides NFκB, AP-1 is another ubiquitous eukaryotic transcription factor that also is a target of cell signaling cascade. Like NF-κB, AP-1 also has binding site in the promoter region of inflammatory/angiogenesis mediators such as COX-2 and iNOS and down regulation of these proteins is attributed to the suppression of NF-κB or c-Jun/AP-1 activation (Surh *et al.*, 2001). Both of them may act independently or coordinately to regulate target gene expression and hence are prime targets of diverse classes of chemopreventive phytochemicals. Sulforaphane, DADS and AITC inhibited the nuclear translocation of sub units of NF-κB such as p65, p50, c-Rel and subunits of AP-1, such as c-Fos and ATF-2 and CREB in B16F-10 melanoma cell line. NF-κB and AP-1 have selectively regulated the expression of proinflammatory cytokines such as IL-1β, IL-6, GM-CSF, TNF-α and IL-12p40 as well as the expression of degradative enzymes such as MMPs. These Sulfur compounds down regulated the expression of proinflammatory cytokine genes. Thus the inhibition of activation or nuclear translocation of NF-κB, AP-1 and CREB promoted the inhibition of the expression of proinflammatory cytokines and collagenases essential for tumour angiognesis and metastasis. Since NF-κB and AP-1 are negative regulators of apoptosis, their inhibition by these Sulfur compounds also promoted the induction of apoptosis in B16F-10 melanoma cells.

We have also evaluated the immunomodulatory and antimetastatic activity of Sulforaphane. Immunomodulators are substances, which modify the activity of the immune system. Immunomodulatory agents that are free from side effects and which can be administered for long duration to obtain a continuous immune activation are highly desirable for the prevention of diseases. Sulforaphane, unlike other chemically defined compounds, modulated the immune system without affecting other parameters of the body. Administration of Sulforaphane stimulated heamatopoiesis in BALB/c mice. It augmented both humoral as well as cell mediated immune responses. Marked enhancement in the production of circulating antibodies and antibody producing cells in spleen were clear indications of the stimulatory effect of Sulforaphane on humoral immune system. Administration of Sulforaphane resulted in a marked enhancement in the activity of NK cells in normal and metastatic tumour bearing animals. It has been shown that immune control over

circulating tumour cells and micrometastasis is carried mainly through NK cells which are integral part of CMI (Smyth *et al.*, 2001). Further, Sulforaphane stimulated the activation of NK cells much earlier which is of great significance in defense against developing tumour. Sulforaphane also stimulated the production of cytokines IL-2 and IFN-γ which in turn promotes NK cell activity. Treatment with Sulforaphane resulted a significant enhancement in ADCC and ACC of normal and metastatic tumour bearing animals. Administration of Sulforaphane also stimulated the proliferation of spleen cells, thymocytes and bone marrow cells in the presence of mitogens. These results suggest that Sulforaphane is a promising agent that can be used as adjuvant during immunotherapy.

Antimetastatic activity of Sulforaphane has been studied using both *in vitro* and *in vivo* models. Administration of Sulforaphane significantly inhibited the tumour colony formation in lungs after 21 days of tumour challenge and also enhanced the lifespan of metastasis induced animals. The highly elevated levels of markers of metastasis in lungs such as hydroxyproline, uronic acid and hexosamine in metastasis induced animals were significantly reduced by the administration of Sulforaphane. Lung collagen hydroxyproline, a major component of collagen, is a direct marker of lung fibrosis. Uronic acid and hexosamine form integral part of many structural polysaccharides and glycosaminoglycans found in the ECM and promote metastasis by stimulating tumour cell proliferation, migration and adhesion. Sulforaphane also checked the elevation of serum GGT and sialic acid levels in metastatic tumour bearing animals. GGT is essential for tumour cell proliferation and neoplasm often have an increased concentration of sialic acid on the tumour cell surface and sialoglycoproteins are shed or secreted by some of these cells which increase their concentration in blood (Khadapkar *et al.*, 1975). Administration of Sulforaphane at non-toxic concentrations significantly inhibited migration, invasion and proliferation of B16F-10 melanoma cells *in vitro*. This can be attributed to the inhibitory effect of Sulforaphane on the activation of MMPs, especially MMP-2 and MMP-9 as revealed from zymographic analysis. In addition Sulforaphane enhanced the production of TIMP, the endogenous inhibitor of MMP and also checked the elevation of proinflammatory cytokines such as IL-1β, IL-6, TNF-α and GM-CSF, which are prometastatic. Gene expression profile study revealed that administration of Sulforaphane inhibited the Ras/ Raf-1/ MEK/ ERK signaling pathway, the involvement of which is well documented in both angiogenesis and metastasis.

Sulforaphane also down regulated the expression of prolyl hydroxylase and lysyl oxidase genes, which are over expressed during metastasis. The metastatic tumour suppressor gene nm 23 was up regulated by the administration of Sulforaphane. The antimetastatic activity of Sulforaphane was further evidenced by its down regulatory action of MMP-2 and MMP-9 genes as well as up regulatory action of TIMP-1 and TIMP-2 genes. The expression of VEGF gene, which was over expressed during metastasis and angiogenesis, was also down regulated by Sulforaphane.

In conclusion, the results obtained in our study indicate the effectiveness of naturally occurring organoSulfur compounds such as DAS, DADS, AITC, PITC and Sulforaphane in the inhibition of tumour specific angiogenesis and induction of apoptosis. Since angiogenesis is a major rate limiting step in metastasis, which is the prime cause of cancer mortality, the antiangiogenic and apoptosis inducing activities of these Sulfur compounds make them promising agents for anticancer therapy. Sulforaphane with its immunomodulatory and antimetastatic activities forms a new potential candidate for anticancer therapy.

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LIST OF ABBREVIATIONS

1. **Thejass P** and Girija Kuttan (2005). Inhibition of tumour specific angiogenesis by two naturally occurring isothiocyanates - allyl isothiocyanate (AITC) and Phenyl isothiocyanate (PITC). Abstract of the poster presented in 24th Annual convention of Indian Association for Cancer Research and International Symposium on HPV and Cervical Cancer. Abstracted in **The Indian Journal of Medical Research, 121**(Suppl): 99-100.
2. **Thejass.P** and Girija Kuttan (2006). Antimetastatic activity of Sulforaphane. **Life Sciences**, 78: 3043-3050.
3. **Thejass.P** and Girija Kuttan (2006). Augmentation of natural killer cell and antibody dependent cellular cytotoxicity in BALB/c mice by Sulforaphane, a naturally occurring isothiocyanate from broccoli through enhanced production of cytokines IL-2 and IFN-γ. **Immunopharmacology and Immunotoxicology**, 28: 443-457.
4. **Thejass.P** and Girija Kuttan (2007): Inhibition of angiogenic differentiation of human umbilical vein endothelial cells by diallyl disulfide (DADS). **Life Sciences**, 80: 515-521.
5. **Thejass.P** and Girija Kuttan (2007): Allyl isothiocyanate (AITC) and Phenyl isothiocyanate (PITC) inhibit tumour specific angiogenesis by down regulating Nitric oxide (NO) and Tumour necrosis factor-α (TNF-α) production. **Nitric Oxide: Biology and Chemistry**, 16: 247-257.
6. **Thejass.P** and Girija Kuttan (2007). Antiangiogenic activity of Diallyl Sulfide (DAS). **International Immunopharmacology**, 7: 295-305.
7. **Thejass.P** and Girija Kuttan. Immunomodulatory activity of Sulforaphane, a naturally occurring isothiocyanate from broccoli (*Brassica oleracea)*. **Phytomedicine** (In Press).

ACC	: Antibody dependent complement mediated cytotoxicity
ADCC	: Antibody dependent cell mediated cytotoxicity
AIF	: Apoptosis inducing factor
AITC	: Allyl isothiocyanate
Ang	: Angiopoietin
AP-1	: Activator protein-1
Apaf-1	: Apoptotic protease activating factor-1
ATF-2	: Activator of transcription factor
BAD	: Bcl-2 associated death promoter
bFGF	: Basic fibroblast growth factor
BM	: Basement membrane
CDK	: Cyclin dependent kinase
Con A	: Concanavalin A
COX	: Cyclooxygenase
CRB	: CRE binding protein
DAS	: Diallyl sulfide
DADS	: Diallyl disulfide
EC	: Endothelial cell
ECM	: Extra cellular matrix
EGFR	: Epidermal growth factor receptor
EGTA	: Ethylene glycol-bis (2aminoethylether)tetra acetic acid
ERK	: Extra cellular regulated kinase factor
FADD	: Fas associated death domain
GM-CSF	: Granulocyte-macrophage colony-stimulating factor
HIF	: Hypoxia inducible factor
HUVEC	: Human umbilical vein endothelial cell
IAPs	: Inhibitor of apoptosis proteins

ICAM-1	: Intercellular adhesion molocule-1
IFN	: Interferon
IL	: interleukin
JNK	: Janus kinase
LPS	: Lipopolysaccharide
MAPK	: Mitogen activated protein kinase
MEK	: MAPK kinase/ERK kinase
MMP	: Matrix metalloproteinases
MTT	: 3-(4, 5 dimethyl thiazol-2yl)-2,5 diphenyl tetrazolium
NF-κB	: Nuclear Factor kappa B
NK cells	: Natural killer cells
NO	: Nitric oxide
PA	: platelet activating factor
PDGF	: Platelet derived growth factor
PDGFR	: Platelet derived growth factor receptor
PF4	: Platelet factor 4
PHA	: Phytohaemagglutinin
PIGF	: Placental growth factor
PITC	: Phenyl isothiocyanate
PWM	: Pokeweed mitogen
RHD	: Rel homology domain
TAM	: Tumour associated macrophages
TGF	: Transforming growth factor
TIMP	: Tissue inhibitor of metalloprotease
TNF	: Tumour necrosis factor
TNP-470	: Takeda neoplastic product-470
t-PA/u-PA	: tissue type/urokinase plasminogen activator
TSP-1	: Thrombospondin-1
VEGF	: Vascular endothelial growth factor
vWF	: von Willebrand factor/factor VIII-related antigen