Therapeutic intervention in inflammatory pathologies and cancer : understanding the anti-inflammatory properties of Viscum album

Pushpa Hegde

Par **Pushpa HEGDE**

Les interventions thérapeutiques dans les pathologies inflammatoires et le cancer : compréhension des propriétés immunomodulatrices de Viscum album

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Sujet de la thèse

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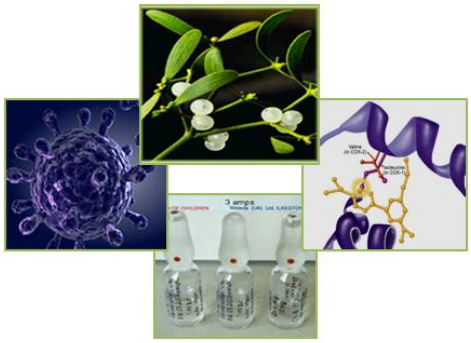

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-Pushpa HEGDE

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ABBREVIATIONS

VA	- *Viscum album*
NSAID	- Non-steroid anti-inflammatory drugs
COX	- Cyclo-oxygenase
PG	- Prostaglandin
APC	- Antigen presenting cells
CTL	- Cytotoxic T- lymphocytes
TLR	- Toll like receptors
NLR	- Nod like receptors
PAMP	- Pathogen associated molecular patterns
DAMP	- Danger associated molecular patterns
TGF-β	- Transforming growth factor- β
CAM	- Complementary and alternative medicine
ML	- Mistletoe lectin
VT	- Viscotoxin
VA Q Spez	- A special preparation obtained from *Viscum album* growing on oak trees
VA P	- Preparation from Viscum album growing on pine trees
VA M Spez	- A special preparation obtained from Viscum album growing on apple trees
RIP	- Ribosome inactivating protein
AA	- Arachidonic acid
NF-κB	- Nuclear factor kappa B
ROS	- Reactive oxygen species
RNS	- Reactive nitrogen species
Mϕ	- Macrophages
DC	- Dendritic cells
NK	- Natural killer cells
TNF	- Tumor necrosis factor
TCR	- T cell receptor
MHC	- Major Histocompatibility Complex
HLA	-Human leukocyte antigen
Ag	- Antigen
Ab	- Antibody

Résumé en Français

Les interventions thérapeutiques dans les pathologies inflammatoires et le cancer: compréhension des propriétés immunomodulatrices de*Viscum album*

par
Pushpa HEGDE

« Biotechnologie et mise en oeuvre des Fonctions Biologiques »

Thèse est présentée à la Faculté de l'Université de Technologie de Compiègne
en vue l'obtention du grades de
Philosophiae Docteur (Ph.D.) de l'Université de Technologie de Compiègne

Les mots clés: Viscum album, médecine complémentaire et alternative, immunomodulation, inflammation, cyclo-oxygenases, PGE2, l'effet anti-inflammatoire, lectin du gui

Les progrès réalisés en immunologie ont orienté les recherches vers des approches et des stratégies de plus en plus prometteuses et innovantes afin de mieux manipuler la réponse immunitaire. Le but de nos recherches est la prévention et le traitement des maladies liées aux dysfonctionnements du système immunitaire, telles que les maladies auto-immunes, inflammatoires et malignes. Bien que l'inflammation constitue un processus physiologique indispensable au maintien de l'homéostasie suite à une infection ou à une lésion, elle est également associée à des pathologies infectieuses, auto-immunes et tumorales. Les stratégies thérapeutiques les plus utilisées pour traiter l'inflammation sont basées sur la neutralisation des médiateurs inflammatoires par des anticorps, des antagonistes moléculaires, des immunoglobulines intraveineuses, des corticostéroïdes, des médicaments anti-inflammatoires non stéroïdiens. En plus des traitements mentionnés, des produits issus de la phytothérapie ont été largement utilisés afin d'atténuer l'inflammation et la douleur dans plusieurs maladies inflammatoires et dans le cancer.

Depuis des décennies, les préparations de *Viscum album*, connu sous le nom de « gui européen », sont largement utilisées dans le traitement du cancer comme thérapie auxiliaire. Bien que les mécanismes d'action soient partiellement connus, plusieurs hypothèses ont été proposées. En effet, les mécanismes anti-tumoraux du *Viscum album* impliquent des propriétés induisant une cytotoxicité, l'apoptose, l'inhibition de l'angiogenèse et plusieurs autres mécanismes immunomodulateurs. Ce travail décrit un nouveau mécanisme anti-inflammatoire de *Viscum album,* qui participe à l'effet thérapeutique de ces préparations. De plus, l'effet bénéfique anti-inflammatoire observé est associé à l'inhibition des voies pro-inflammatoires de COX2 et PGE2 dans les cellules épithéliales issues d'adénocarcinome du poumon.

Ce travail a identifié un des mécanismes moléculaires de *Viscum album* associé à son effet anti-inflammatoire participant à ses bénéfices thérapeutiques. Ainsi, ces préparations pourraient être utilisées en combinaison avec d'autres traitements dans des maladies inflammatoires et dans le cancer.

Abstract in English

Therapeutic intervention in inflammatory pathologies and cancer: understanding the anti-inflammatory properties of *Viscum album*

by

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Thesis is presented at the Faculty of Université de Technologie de Compiègne
for obtaining the degree of
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Key words: Viscum album, complementary and alternative medicine, immunomodulation, inflammation, cyclo-oxygenases, PGE2, anti-inflammatory effect,mistletoe lectin,

Recent advances in immunology research have led us towards more promising approaches and strategies to manipulate the immune response to prevent or treat the diseases related to immune dysfunction such as autoimmune, inflammatory pathologies and malignant diseases. Although, immuno inflammation is a basal physiological phenomenon required to eliminate the causative agent and to initiate the healing process, it is a physiopathological symptom in a diverse conditions of infectious, autoimmune and tumoral origin. Various therapeutic strategies have been developed in order to reduce inflammation and pain, including the treatment with cytokine neutralizing antibodies, molecular antagonists, intravenous immunoglobulins, corticosteroids, non-steroid anti-inflammatory drugs (NSAID) and several others. In addition to these well known anti-inflammatory therapeutic strategies, treatment with various phytotherapeutics has also contributed enormously to control inflammation and pain, associated with various severe inflammatory disorders and cancer.

Viscum album (VA) preparations, commonly known as European mistletoe, are extensively used as complementary therapy in cancer for decades. However the mechanisms of action

have been partially understood. Several mutually non-exclusive mechanisms have been proposed such as anti-tumor properties which involve the cytotoxic properties, induction of apoptosis, inhibition of angiogenesis and several other immunomodulatory mechanisms. This study reveals anti-inflammatory mechanism as another important mechanism of action of these phytotherapeutics, which is responsible for their therapeutic benefit and addresses the molecular mechanisms in the pro-inflammatory axis of COX-2 and PGE2 using *in vitro* experimental model of human lung adenocarcinoma.

The present work contributes for a better understanding of mechanisms of action of *Viscum album* preparations underlying their therapeutic benefit and allows us to revitalize the therapeutic strategies used in treatment of inflammatory disorders and cancer.

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Introduction

Immune system

Immune system is a remarkably versatile defense system that has evolved to protect animals from invading pathogenic micro-organisms and physiological dysfunctions such as autoimmune disorders and cancer. It has evolved over of millions of years to respond to invasion by microbes that regularly attempt to infect our bodies rendering the vertebrate immune system an ability to deal with diverse environmental stimuli. Immune response represents a concerted action of many cells and molecules in a dynamic network and uses various strategies to carry out its functions in protecting and maintaining a steady state defence mechanism. Functionally, immune response can be explained as two inter-related processes namely, immune recognition and response. With a unique ability to discriminate between self and non self (foreign), immune system recognises any invading micro organism or related molecular patterns, with an extreme specificity (Janeway, 1992). Once the immune system recognises a foreign substance it recruits a variety of cells and molecules in order to mount an appropriate, specific effector response to eliminate the causative agent from the system. Subsequent exposure to the same micro-organism will result in a more pronounced and rapid immune reaction because of the exceptional property of immune system called immunological memory (Clark and Kupper, 2005). However under certain circumstances failure in distinguishing between self and non-self or any kind of misdirected immune action can result in various immune disorders such as autoimmune diseases and cancer.

Immune system protects organisms from infection with layered defenses of increasing specificity. At a very basic level, physical and mechanical barriers such as skin and epithelia will prevent the pathogens from entering the body. If these barriers are breached by pathogens, innate immune system provides an immediate, but non-specific response (Medzhitov and Janeway, 2000). It provides resistance through several physical, chemical, and cellular approaches. Microbes first encounter the epithelial layers (physical barriers that

line our skin and mucous membranes). Subsequent general defenses include secreted chemical signals (cytokines), antimicrobial substances, fever, and phagocytic activity associated with the inflammatory response. The phagocytes express cell surface receptors that can bind and respond to common molecular patterns expressed on the surface of invading microbes. Through these approaches, innate immunity can prevent the entry, colonization, and spread of microbes. If pathogens successfully evade the innate response, vertebrates possess a third layer of protection, the adaptive or acquired immune system, which is more specific and activated by the innate responses and is more complexly regulated (Medzhitov and Janeway, 1997),(Janeway and Medzhitov, 1998), (Banchereau and Steinman, 1998) . The immune system adapts its response during an infection in order to improve its recognition of the pathogen. This improved response is then retained even after the pathogen has been eliminated, in the form of an immunological memory, and allows the adaptive immune system to mount faster and stronger attacks when this pathogen is encountered (Levy, 2001).

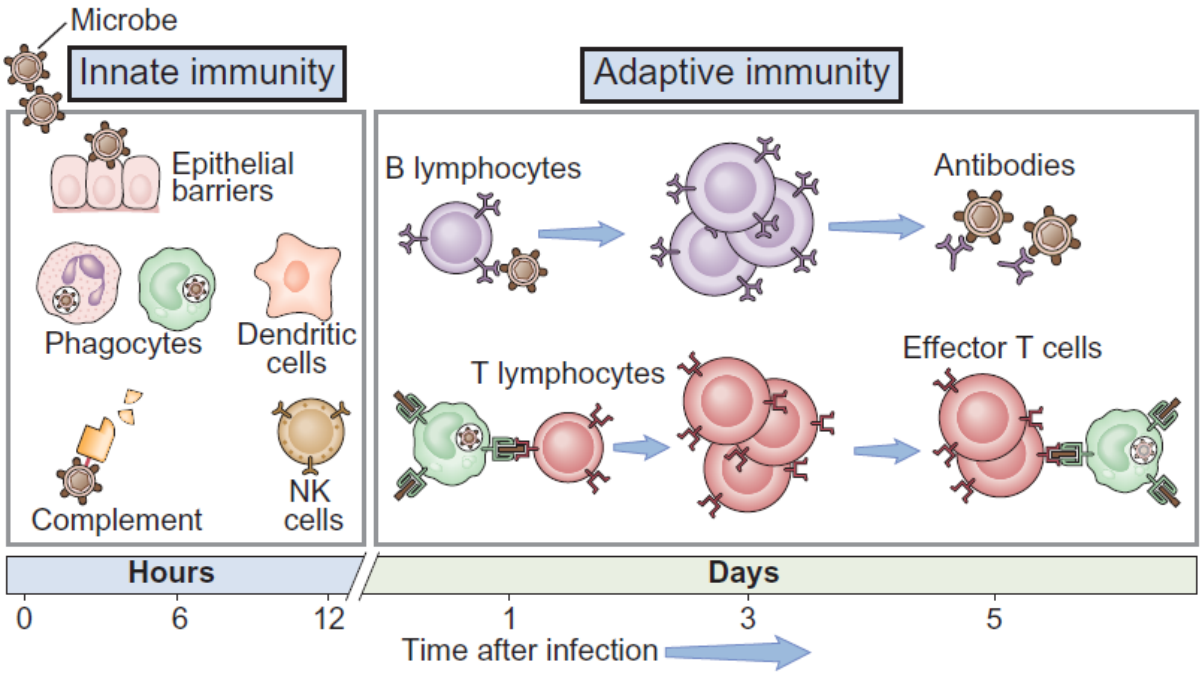

Figure 1: Summary of principal mechanisms of innate and adaptive immunity. Mechanisms of innate immunity provide the initial defense against infections. Some of the mechanisms prevent infections (e.g., epithelial barriers) and others eliminate microbes (e.g., phagocytes, natural killer [NK] cells, the complement system). Adaptive immune responses develop later and are mediated by lymphocytes and their products. Antibodies block infections and eliminate microbes, and T lymphocytes eradicate intracellular microbes.

Both innate and adaptive immunity depend on the ability of the immune system to distinguish between self and non-self molecules, where self molecules are those components of an organism and generally can be distinguished from foreign substances by the immune system. The immune system, if dysregulated, can react to self-antigens, leads to severe complications such as autoimmune diseases as a result of break in tolerance or fail to respond and neutralize infections leading to chronic inflammation and potential mortality, or fail to kill the transformed own cells which will result in various malignant tumors.

Cells of the immune system

The precursor cells of the immune system are derived from pluripotent stem cells in the bone marrow. Cells that are specifically committed to each type of leukocyte are subsequently produced with the assistance of special stimulating factors such as cytokines. Leukocytes derived from pluripotent stem cells in the bone marrow during postnatal life include neutrophils, eosinophils, basophils, monocytes, macrophages (Mφ) and dendritic cells (DC), natural killer (NK) cells, mast cells, and T and B lymphocytes. Cells of the lymphoid system provide highly specific protection against foreign agents and also orchestrate the functions of other parts of the immune system by producing immunoregulatory cytokines. The lymphoid system is divided into 1) central lymphoid organs, the thymus and bone marrow, and 2) peripheral lymphoid organs, lymph nodes (LN), the spleen, and mucosal and submucosal tissues of the intestinal and respiratory tracts. The thymus instructs certain lymphocytes to differentiate into thymus-dependent (T) lymphocytes and selects most of them to die in the thymus (negative selection) and others to exit into the circulation (positive selection) based on their affinity towards self molecules. T lymphocytes circulate through the blood, regulate humoral and cellular immunity and help to defend against infections. Immunologic tolerance (unresponsiveness) prevents the reactions against self-Ags; if immunologic tolerance is broken, autoimmune reactions may occur. Much of the development of tolerance occurs in

the thymus (T cells) or bone marrow (B cells) (central tolerance) by the elimination (clonal deletion) or inactivation (clonal anergy) or receptor editing of self-reactive clones of T & B cells. Other mechanisms of tolerance occur extra-thymically and include activation of antigen-specific T suppressor cells and clonal deletion, which results in the elimination of self-reactive B cells or T cells, and clonal anergy.

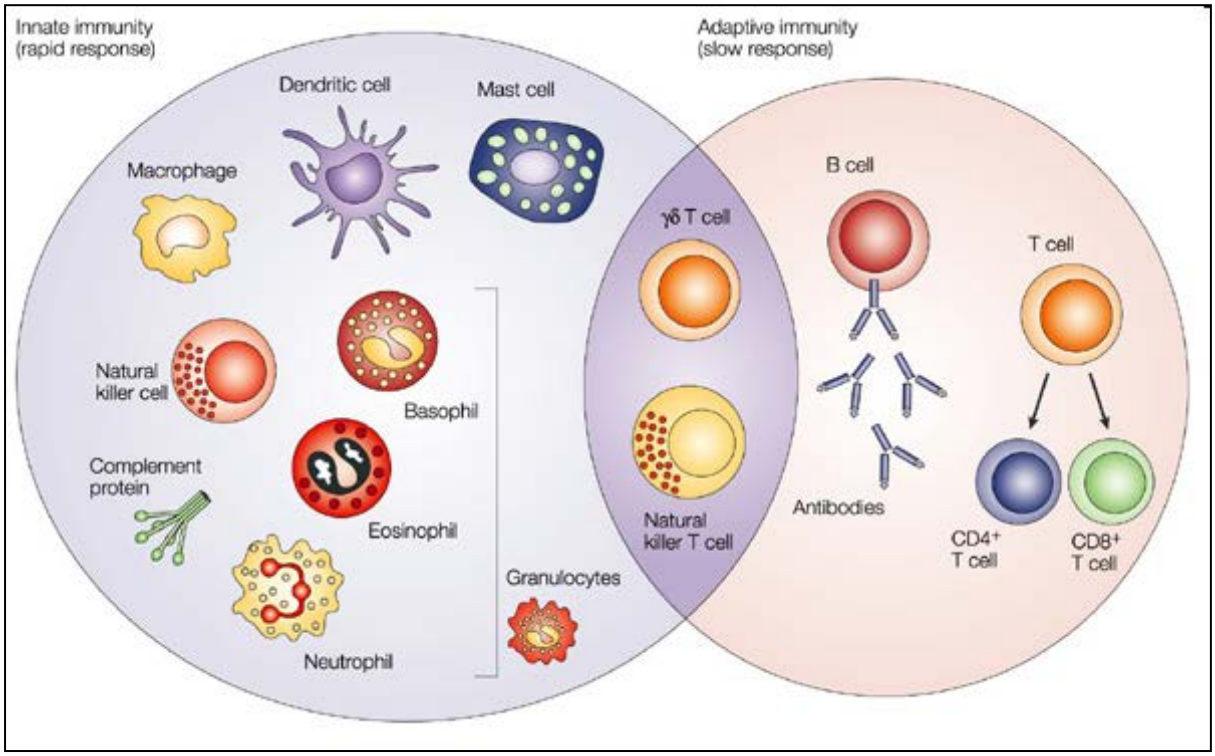

Figure 2: Components of the immune system.

Functions of immune system

The immune system is a complex network of different cellular and molecular components which interact with each other to mount an effective and efficient immune response against pathogens and disease. Immune system works as a co-ordinated crosstalk between innate and adaptive immune compartments (Grakoui et al., 1999). While providing the first line of defense against the evading pathogenic attack, innate immune compartment also primes the adaptive immune system to mount an antigen- specific, long lasting, secondary immune response. T cells play a central role in immune responses by acting as helper cells for B cells

and other cells, as well as by acting as effector cells in cellular responses. T cell precursors mature into T cells in the thymus. During thymic maturation, self-reactive T cells are removed (negative selection), and T cells that recognize antigen in association with MHC molecules are selected (positive selection). T cells express characteristic cell surface molecules, including the CD3 complex and the TCR. The TCR interacts with antigens only when they are processed and displayed in association with MHC molecules by APCs. There are two major subsets of mature T cells that are phenotypically and functionally distinct: T helper (Th) cells, which express the CD4 cell surface marker and provide help to B cells and other immune cells, and CTLs, which are CD8+, and are cytotoxic cells that detect antigens presented on target cells in association with MHC class I molecules. Monocytes, dendritic cells and macrophages are the antigen presenting cells,that play an important role in both innate and adaptive immune responses. APCs (dendritic cells, macrophages and B cells) express MHC class II molecules and are effective antigen presenting cells for CD4 helper T cells. In addition to their role as APCs, macrophages play an important part in innate responses by ingesting and killing microorganisms. B cells produce and secrete antibodies, after exposure to an antigen coupled with appropriate activation signals. NK cells are cytotoxic cells that can kill target cells, including tumor cells, in an antigen-independent fashion. Many viruses subvert cellular immune responses by down-regulating the expression of MHC class I molecules, preventing the killing of such virus-infected cells by CTLs. While the mechanisms involved in the activation and inhibition of NK cells are complex, it is clear that NK cells kill target cells that do not express MHC class I molecules, allowing them to detect and kill virus-infected host cells that have evaded T cell immunity. Immune responses also involve soluble mediator molecules called cytokines. Cytokines are secreted proteins that can have multiple biological effects, are extremely potent, and interact with high-affinity cellular receptors specific for each cytokine. Cytokines binding to the appropriate receptor

results in signal transduction, followed by changes in gene expression, and ultimately, in altered target cell behaviour. Characteristic patterns of cytokine production have been recognized. For example, in innate immune responses, macrophages activated by exposure to microbial molecules such as endotoxin, produce pro-inflammatory cytokines, including IL-6, TNF-γ, and chemokines. These cytokines enhance inflammation, chemotaxis, and promote the development of subsequent adaptive immune responses. Based on the pattern of cytokines produced, CD4 T helper cells can be subdivided mainly into two subsets: "type 1" (Th1) and "type 2" (Th2) cells. Th1 and Th2 subsets control the nature of an immune response by secreting mutually antagonistic sets of cytokines. Th1 responses are characterized by the production of IFNγ and IL-12, and result in enhanced cell-mediated immune and inflammatory responses, including increased macrophage activation. Th2 responses are characterized by the production of IL-10, IL-4 and other B cell-stimulating cytokines, and result in enhanced B cell activation and antibody production, and in the inhibition of cell-mediated immune responses. Nearly all immune responses involve both Th1 and Th2 components. So, the assessment of cytokines that characterize Th1 or Th2 responses may help to elucidate the contribution of immune responses to a particular disease. The most recently described subset, Th17 cells, which preferentially secrete IL-17-family cytokines (IL-17F, IL-22, IL-26 and CCL20), is involved in autoimmune diseases and acute inflammatory responses. Regulatory T cells (Tregs), a functionally defined subset of T cells that can inhibit the activities of other immune cells, have been the focus of great attention. Tregs, which are CD4+, CD25+ and Foxp3+ T cells, produce IL-10 and TGF-ß, cytokines that down-regulate many immune responses. Tregs play a very important role in controlling the self reactive immune system and in regulating the immune homeostasis.

Immune dysfunction and its consequences

A co-ordinated crosstalk between multi-component systems of innate and adaptive immunity confers a specific, controlled, protection against the invading pathogens and cancer. Thus immune system executes dual faceted mechanism to perform this regulatory role. On the one hand, it protects our body by fighting against infection and malignancies while on the other; it can be deceitful, in attacking our own tissues and cells to produce devastating pathologies, and even fatal autoimmune diseases (Matzinger, 1994). Any kind of misdirected or inappropriate immune responses cause a number of human diseases. Whereas a hyperactive or undesirable immune response can lead to various immunological disorders such as allergy, autoimmune disease and graft rejection in transplantation while an insufficient or deprived immune responses can be associated with immunodeficiency, chronic infections or cancer.

Cancer as an example of misdirected immune system

As outlined earlier, cancer is a serious manifestation of misdirected immune system, which results in failure of recognising the transformed cells and their killing by immune attack. According to Hannahan and Weinberg, tumor is characterized by six hallmarks (Hanahan and Weinberg, 2000), which are 1) self-sufficiency in having the growth signals, 2) insensitivity to signals that inhibit growth, 3) ability to resist apoptosis, 4) limitless growth potential, 5) ability to sustain angiogenesis, and 6) an unusual ability to invade surrounding tissues and metastasize to distant organs. These hallmarks define cancer by focusing on the molecular, biochemical, and cellular features of the tumor cells. However, it has recently been described that the **inability of the immune system to eradicate established tumors is the seventh fundamental hallmark of cancer** (Dunn et al., 2004), (Zitvogel et al., 2006).

Immune system can identify and destroy nascent tumor cells in a process termed cancer immunosurveillance, which functions as an important defense against cancer. However,

tumors exert various active mechanisms deceive the host immunity (Zou, 2005). By altering the functions of antigen presenting cells (APC), by promoting dysfunctional T cell co-signaling, and generating an immune-subversive cytokine milieu, they result in the development of a suppressed immunophenotype thereby facilitating the tumor progression (Vesely et al., (2011).

Immunosurveillance against growing cancer

Immunosurveillance is a defence mechanism of the body against the developing tumor which was first proposed that, lymphocytes act as sentinels in recognizing and eliminating continuously arising, nascent transformed cells (Shankaran et al., 2001) ,(Morgan et al., 2006). It is the ability of the immune system to identify the transformed cells of the body and to eventually eliminate it (Pardoll, 2003). Cancer immunosurveillance appears to be an important host protection process that inhibits carcinogenesis and maintains regular cellular homeostasis. Cells of the immune system recognise the evasive mechanisms of the developing tumor cells and help to re-establish the homeostasis though the process of cancer immuno-editing, which occurs in three phases, called elimination, equilibrium and escape (Dunn et al., 2004), (Smyth et al., 2006). These effector immune cells employ extremely diverse mechanisms to control tumor targets including the induction of tumor cell death by mitochondrial and cell death receptor pathways. Tumor microenvironment is composed of immune cells, tumor cells, stromal cells and the extracellular matrix, which acts as battleground during the neoplastic process, fostering proliferation, survival and migration of tumor cells. Not only can tumors survive and disseminate, but also, more importantly, they can mimic some of the signalling pathways of the immune system to propagate conditions that favour tumor immune tolerance thereby escaping the tumor immunity (Dunn et al., 2002).

The immune response to tumors includes CTL-mediated lysis, NK-cell activity, macrophage-mediated tumor destruction, and destruction mediated by ADCC (Smyth et al., 2000), (Girardi et al., 2001). Several cytotoxic factors, including TNF-α and TNF-β, help to mediate tumor-cell killing. Also IFN-γ plays an important role in tumor immunity (Kaplan et al., 1998). Tumors may evade the immune response by modulating their tumor antigens, by reducing their expression of class I MHC molecules, and by antibody mediated or immune complex-mediated inhibition of CTL activity. Both innate and adaptive immune systems play an important role in cancer immunity. Recent evidence has provided several models for the role of innate immunity in recognition and rejection of malignant cells, where innate immune cells can sense transformed cells through expression of molecules up-regulated during the process of malignant transformation and tumor progression (Vesely et al., (2011). With respect to the self-non-self paradigm, two types of receptors on innate immune cells play very important role namely toll-like receptors (TLR) and the NKG2D receptor. Toll-like receptors expressed by APCs recognize non-self molecules, e.g., pathogen-associated molecular patterns (PAMPS) such as bacterial cell wall structures and viral polynucleotides.

On the other hand, activating NKG2D receptor of lymphocytes recognizes self ligands expressed by cancer cells. The discovery of innate immune receptors for self that participate in activation of the innate and adaptive immune system leads to a reconsideration of the framework of "evolution of an immune system to recognize foreign". T cells, NK cells, and NKT cells express NKG2D receptors (Diefenbach et al., 2001). Ligands for NKG2D receptors include major histocompatibility complex (MHC) class I chain related (MIC) A and MIC B on human cells. Innate immune cells do not recognize ligands that are induced only in the context of malignancy, but also recognize ligands that are also up-regulated by non-malignant cells during oxidative stress, heat shock, altered cell cycle regulation, and viral or bacterial infection (Zafirova et al., (2011).

Immune reactions can also potentially promote cancer development and growth. Chronic inflammatory responses, a feature of innate immunity, can contribute to the development of cancer. Additionally, the activation of immune cells places these cells at risk for cancer. For example, the activation B lymphocytes require various DNA modifying activities, errors in which can result in molecular lesions (oncogene mutation, chromosomal translocations) that lead to cancer.

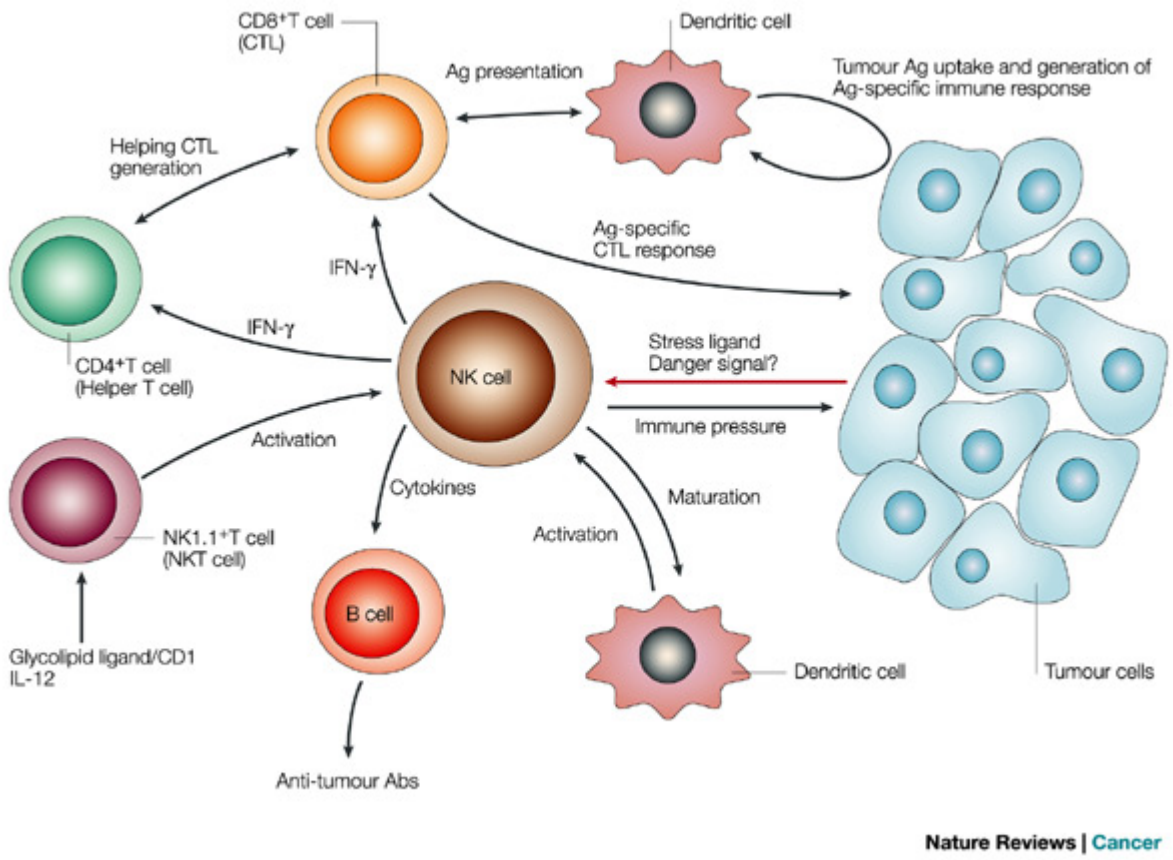

Figure 3: Immunosurveillance against growing tumor.

Inflammation: beyond the physiological wound healing

Inflammation is a physiological immune response that essentially plays a central role in providing the protection against tissue injury and infections, and is extremely important in

maintaining the homeostasis. Inflammation occurs as a set of complex cellular and molecular responses to an infectious agent or to a tissue injury so as to eliminate the causative agent and to initiate the healing process (Medzhitov, 2008). This occurs with five typical symptomatic changes named as “cardinal signs of inflammation” which are dolor (pain), calor (heat), rubor (redness), tumor (swelling) and Functio laesa (loss of function).

Mechanisms of acute and chronic inflammation

A typical inflammatory response consists of four components: inflammatory inducers, the sensors that detect them, the inflammatory mediators induced by the sensors, and the target tissues that are affected by the inflammatory mediators. Pathogens, pathogen associated molecular patterns (PAMP), and danger associated molecular patterns (DAMP) and factors inducing tissue damage act as inducers of inflammation and initiate the inflammatory response and are detected by cellular sensory system. Sensors are the cells of innate immune system which contain the pattern recognition receptors (PRR) such as Toll-like receptors (TLRs), Nod like receptors (NLRs) and act as sentinels of the immune system, such as tissue-resident macrophages, dendritic cells, and mast cells. They induce the production of molecular mediators, including cytokines, chemokines, bioactive amines, eicosanoids, and products of proteolytic cascades, such as bradykinin. These inflammatory mediators further act on various target tissues to elicit changes in their functional states that optimize adaptation to the noxious condition (e.g., infection or tissue injury) associated with the particular inducers that elicited the inflammatory response (Medzhitov, (2010).

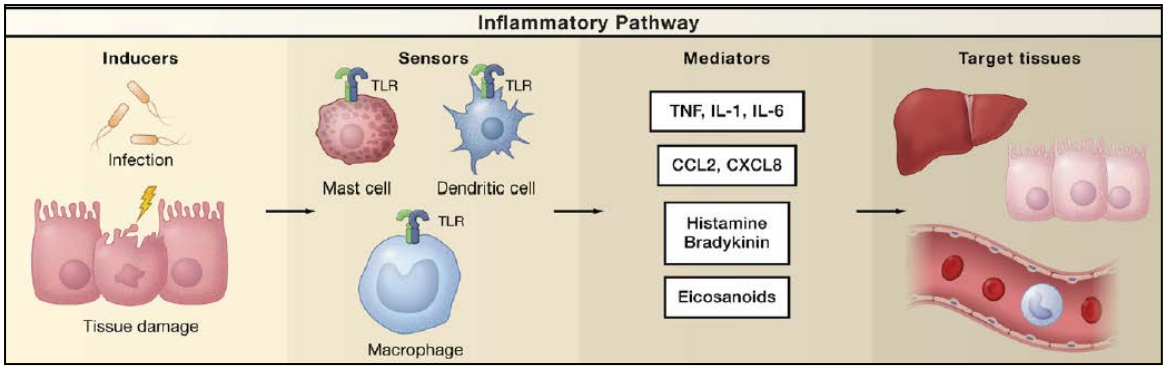

Figure 4: Representation of a typical mode of inflammatory immune response.

The process of inflammation can be explained as acute inflammation or chronic inflammation depending on the type of reaction, intensity of reaction and the participants involved in driving the response. Acute inflammation occurs as an instant and rapid response to bacterial pathogen or tissue injury which normally lasts for a short period of time where as chronic inflammation develops as a complex long term response to a persistent stimulus. Acute inflammation begins within seconds to minutes following the injury of tissues. The damage may be purely physical, or it may involve the activation of an immune response (Butcher and Picker, 1996). Three main processes occur during inflammation are:

Vasodilation: Increased blood flow due to increase in the diameter of the blood vessels (arterioles) supplying the region resulting in an engorgement of the capillary network which is responsible for tissue redness (erythema) and an increase in tissue temperature.

Increased permeability of the capillaries, allowing fluid and blood proteins to move into the interstitial spaces and facilitates an influx of fluid and cells from the engorged capillaries into the tissue. The fluid that accumulates (exudate) has much higher protein content than fluid normally released from the vasculature. contributing to tissue swelling (edema).

Influx of phagocytes from the capillaries into the tissues is facilitated by the increased permeability of the capillaries. The emigration of phagocytes occurs as a multistep process

that includes adherence of the cells to the endothelial wall of the blood vessels **(margination)** due to the action of cell adhesion molecules, followed by their emigration between the capillary endothelial cells into the tissue **(diapedesis** or **extravasation)**, and, finally, their migration through the tissue to the site of the invasion **(chemotaxis)**. As phagocytic cells accumulate at the site of inflammation and begin to phagocytose bacteria, they release lytic enzymes, which can damage nearby healthy cells. The accumulation of dead cells, digested material, and fluid forms a substance called pus (Libby, 2007).

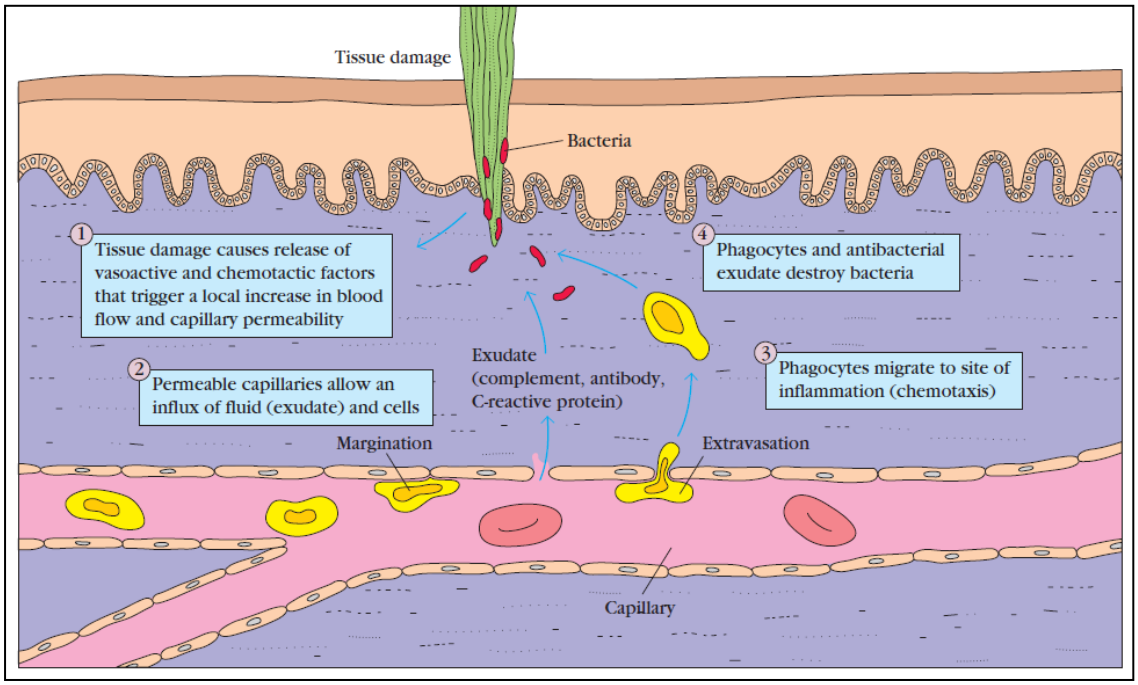

Figure 5: Representation of a typical mode of inflammatory immune response.

Table 1: Characteristic features of acute and chronic inflammation.

Features	Acute inflammation	Chronic inflammation
Causative agent	Bacterial pathogens and tissue injury	Persistent acute inflammation due to non eliminated pathogens, viral infections and autoimmune reactions

Major cells involved	Neutrophils, basophils, eosinophils and mononuclear cells	Mononuclear cells and fibroblasts
Primary mediators	Vasoactive amines and Eicosanoids	Pro-inflammatory cytokines (IFN-γ, TNF-α, IL-6, IL-8,IL-1 etc), reactive oxygen species (ROS), reactive nitrogen species (RNS),growth factors, hydrolytic enzymes,
Onset	Immediate	Delayed
Duration	Short term (few days)	Long term (up to months and years)
Outcomes	Resolution, abscess formation or chronic inflammation	Tissue destruction, fibrosis, necrosis occasionally cancer

The inflammatory response must be actively terminated when it is no longer needed for the tissues. Failure in this process results in chronic inflammation, and cellular destruction. In principle, resolution of inflammation occurs by different mechanisms in different tissues that keep a strong regulation on inflammatory response by the functioning of anti-inflammatory mediators such as cytokines (IL-10), TGF-β, IL-1 receptor antagonist (IL-1RA), TNF receptor and many others, including the apoptosis of pro-inflammatory cells. However, if the regulatory mechanisms of immune system fail to control the inflammatory response by the counteracting mechanisms, various dysfunctional manifestations occur and lead to the emergence of autoimmune, inflammatory pathologies and also cancer. Therefore any degree of inflammation beyond the physiological requirement has to be kept in check which can be achieved by various anti-inflammatory agents including steroids, non-steroidal anti-inflammatory agents, intravenous immunoglobulins, immunosuppressor cells, neutralizing monoclonal antibodies to inflammatory cytokines and also various natural compounds like phytotherapeutics (Balkwill and Mantovani., 2010).

Cancer and inflammation: a critical link

Chronic infections and inflammation are the major risk factors for various types of cancer (Mantovani et al., 2008), (Tan and Coussens, 2007). The construction of an inflammatory network in the tumor microenvironment may be triggered either by conditions that predispose to cancer or by genetic events that cause neoplasm transformation (Bayne et al., 2012). Indeed, alterations in genes representative of all classes of oncogenes drive the production of inflammatory mediators. Immune cells that infiltrate tumors, engage in an extensive and dynamic crosstalk with cancer cells and contribute in constructing an immuno-inflammatory microenvironment and affect malignant cells through production of cytokines, chemokines, growth factors, prostaglandins, and reactive oxygen and nitrogen species, hence play a decisive role at different stages of tumor development including initiation, promotion, malignant conversion, invasion, and metastasis (Balkwill and Mantovani, 2001), (Hagemann et al., 2007), (Lin and Karin, 2007). Inflammation associated with cancer also affects immunosurveillance and response of cancer cells to therapy (Grivennikov et al., 2010). In developing tumors, anti-tumorigenic and pro-tumorigenic immune and inflammatory mechanisms coexist, and act in a dynamic crosstalk, but if the tumor is not rejected, the pro-tumorigenic effect dominates leading to the tumor progression Signaling pathways that mediate the pro-tumorigenic effects of inflammation often lead to a feed-forward loop (for example, activation of NF-kB in immune cells, induces production of cytokines that activate NF-kB in cancer cells to induce chemokines that attract more inflammatory cells into the tumor) (DiDonato et al.), (Yu et al., 2009), (Karin and Greten, 2005). In simple terms tumors distort healthy tissues, setting off tissue repair, which in turn promotes cell proliferation and blood vessel growth, helping cancer cells expand (Karin et al., 2006). Therefore the saying

"inflammation and cancer dance together towards disaster" perfectly suits the way they are intricately associated.

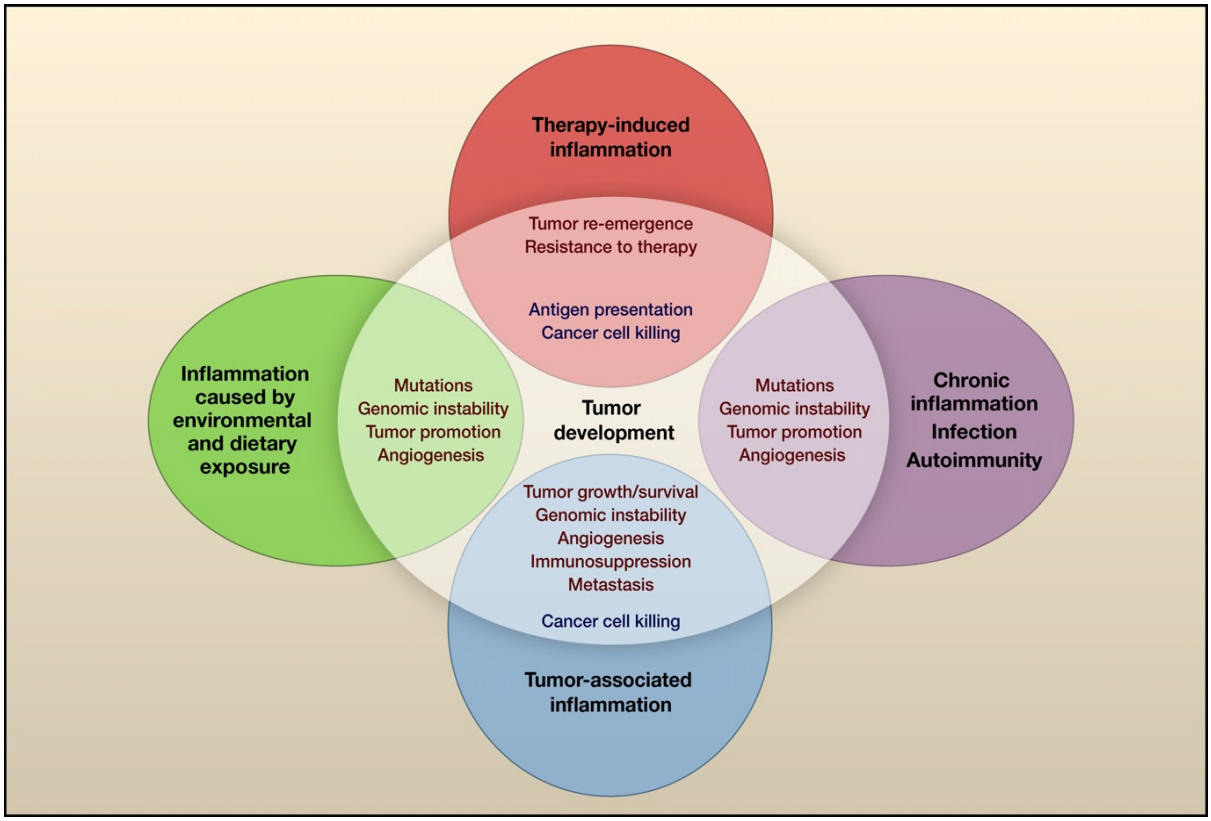

Figure 6: Types of inflammation in Tumorigenesis and Cancer: Chronic inflammation associated with infections or autoimmune disease precedes tumor development and can contribute to it through induction of oncogenic mutations, genomic instability, early tumor promotion, and enhanced angiogenesis. Prolonged exposure to environmental irritants or obesity can also result in low-grade chronic inflammation that precedes tumor development. This inflammatory response can enhance neoangiogenesis, promote tumor progression and metastatic spread, cause local immunosuppression, and further augment genomic instability. Cancer therapy can also trigger an inflammatory response by causing trauma, necrosis, and tissue injury that stimulate tumor re-emergence and resistance to therapy. However, in some cases, therapy-induced inflammation can enhance antigen presentation, leading to immune-mediated tumor eradication. Tumor-promoting mechanisms are in red and antitumorigenic mechanisms are in blue.

Current paradigm of cancer immunotherapy

One of the emerging immunomodulatory strategies used in tumor immunotherapy is, to inhibit the suppressors of the immune system like the suppressor or regulatory T cells (Tregs), dysfunctional APC, suppressive cytokines like TGF-β, and blocking the signaling events promoting the suppressive phenotype (Gajewski et al., 2006), (Nestle et al., 2001). Another promising immunomodulatory approach is to enhance the stimulators of the immune

system, like pro-inflammatory cytokines such as IL-2, IFN-γ, IL-12, stimulating the dendritic cells (Pardoll, 1998), (Liu, 1998) which can further drive the immune response towards a specific cytotoxic T cell functioning and activation of NK cells (Hahn and Weinberg, 2002), (Curiel, 2007), (Rosenberg et al., 1986), (Hurwitz and Watkins., 2012).

Increasing lines of evidence for the critical interplay between inflammation and cancer, which share several signaling pathways and regulatory mechanisms, have clearly shown the involvement of inflammatory processes in malignant disease (Servais and Erez., 2012), (Dunn et al., 2011), (Vendramini-Costa and Carvalho., 2012), (Kundu and Surh., 2012), (Sethi et al., 2011). This has emerged as an attractive target for an important immunotherapeutic strategy for cancer therapy, suggesting that the inflammatory cells and inflammatory mediators in the tumor microenvironment may be targets for treatment or prevention, and therefore anti-inflammatory drugs may be useful in cancer prevention and treatment (Balkwill and Mantovani., 2010), (Mantovani, 2008). Considering crucial role of inflammatory mediators and the regulators of chronic inflammation in tumor development and in generating an inflammatory tumor microenvironment, anti-inflammatory therapeutics play a promising role in designing efficient therapeutic strategies which can be used in the treatment of malignant diseases and vice versa. Therefore the therapeutics with anti-tumor properties can be used in inflammatory conditions and those with anti-inflammatory properties can be used for the treatment of cancer.

Phytotherapy: a promising therapeutic approach in immuno-inflammatory pathologies and cancer

Phytotherapy is one of the promising approaches in the management of inflammatory and tumor pathologies (Shoskes, 2002), particularly in targeting the multiple biological pathways, while leaving the healthy tissues unaffected (Efferth and Koch., 2010), (Giachetti and Monti, 2005), (Bland, 1996). Although there are thousands of phytotherapeutics like curcumin,

which are known to mankind and are used successfully right from the house hold application up to the clinical management; the real scientific dissection of their mechanisms of action is still an active ongoing research (Talero et al., 2012), (Sharma et al., 2006), (Jurenka, 2009), (Cullberg et al., 2013), (Ismail et al., 2012), (Parente et al., 2012). Phytotherapeutics have contributed enormously to the existing list of C*omplementary and Alternative Medicine (CAM).*

C*omplementary and alternative medicine (CAM)* constitutes a collective non-conventional therapeutic approach, using the practices and products that are normally applied either in place of, or in addition to the conventional therapies like surgery, radiotherapy and chemotherapy (Ernst, 2000). Phytotherapeutics such as curcumin, resveratrol, calendula, and a large number plant derived therapeutic molecules have been extensively used as both complementary and alternative medicine (Khan et al., 2007), (Zhang et al., 2003), (Slusarz et al., 2010), (Raina et al., 2007). In view of the increasing lines of evidence on their mechanisms of action they are used worldwide in designing efficient integrative medicine in the effective management of disease and in improving the quality of life (QOL) (Miller et al., 2008).

Table 2: List of some selected phytotherapeutics with their therapeutic benefit

Phytotherapeutics	Family	Therapeutic benefit
Curcumin *(Curcuma longa)*	Zingiberaceae	Antiseptic, antioxidant, anti arthritic, anti-ischemic,anti-inflammatory and anti-tumor properties.
Marigold *(Calendula officinalis)*	Asteraceae	anti-viral, anti-genotoxic and anti-inflammatory properties antibacterial and antifungal properties
Ashwagandha	Solanaceae	Anti-tumor and anti-inflammatory

(Withania somnifera)		properties
Resveratrol (Vitis vinifera)	vitaceae	Anti-cancer properties, neuroprotective, cardioprotective, antidiabetic, antiviral, anti-inflammatory
Vinca *(*Catharanthus roseus*)*	Apocyanaceae	Anti-tumor properties
Neem (Azadirachta indica)	Meliaceae	Antiseptic,theramoregulatory, antioxidant, anti-microbial and anti-cancer properties

***Viscum album* in Complementary and Alternative Medicine (CAM)**

Viscum album (*Viscum album L.*) is a traditional phytomedicine of Europe, commonly known as European mistletoe, also found in western and southern Asia. It is a semi-parasitic plant that lives symbiotically with several host plants such as oak, pine, elm and apple. Standardized whole plant extracts of this plant, which mainly contain mistletoe lectins and viscotoxins and also several enzymes, peptides (e.g., viscumamide), amino acids, thiols, amines, polysaccharides, cyclitoles, lipids, phytosterols, triterpines, flavonoids, phenylpropanes, and minerals and many others, are available as commercial preparations called Iscador (Urech, 2006 and Romagnoli, 2003). Considered sacred in ancient times, it has been used for centuries in Europe to treat various diseases such as hypertension, epilepsy, exhaustion, anxiety, arthritis, and degenerative inflammation of the joints. In 1920, Rudolf Steiner, the father of anthroposophic medicine, first proposed the use of mistletoe extract in cancer therapy (Kaegi, 1998). Today, physicians who practice in special anthroposophic clinics in Germany and Switzerland prescribe VA preparations. VA preparations have been demonstrated in numerous clinical studies for the improvements in survival when used along with surgery, chemotherapy or radiotherapy, bringing out an overall improvement in the

quality of life in cancer patients. In addition to an extensive use in the treatment of human malignancies (Bock et al., 2004), (Kienle and Kiene., 2010).

Introduction to *Viscum album*

Taxonomy and morphology

Viscum album is an evergreen, perennial, epiphytic, hemiparasitic shrub that lives on a wide range of woody plant species. It is known in several common names such as, all heal, bird lime, birdlime mistletoe, devil's fuge, European mistletoe, golden bough, herb de la croix (Fr), lignum crucis, mystyldene, visci albi fructus (berries), visci albi herba (leaves), visci albi stipites (stem), and viscum. *Viscum album* belongs to the family Loranthaceae (Viscaceae) of order Santalales.

Viscum album is a mostly globose perennial evergreen shrub with persistent haustoria in the host. It has a globe diameter reaching up to 150 cm (Wangerin 1937) with dichasial branching pattern first forming a fan and with increasing growth forming a globe. Foliage leaves are opposite (equifacial), rarely 3 (–4–5) whorled, sessile, obovate-oblong, obtuse, leathery and (yellowish-) green (Ball 1993). In general leaf length ranges between (1.3–) 2– 8 (–10.7) cm, with a minimum width of 0.3 cm and a maximum of 4.3 cm. Foliage leaf internodes are 1–9 cm long. In adult plants, one dichasial shoot with one short and one long internode and one pair of scale leaves and one of foliage leaves per leaf axil is formed each year. *Viscum album* is dioecious. Staminate and pistillate flowers are usually 3 (–5) in triads with one terminal and two lateral flowers. *Viscum album* contains viscous fruit, berry in a triad at the shoot apex. These berries contain a white epicarp, occasionally yellow with a ring of 4 short dark lines representing the tepalar scars and a central point caused by the stigma. The thick mesocarp consists of viscin, a mucilaginous substance. There are two viscid layers:

an outer one apparently digestible, cellulosic slimy layer, and an inner, indigestible pectin layer. The very thin endocarp adheres to the seed.

Since *Viscum album* plants are semiparasitic, they contain a unique, advanced endophytic system which helps for their integration with the host tree. Endophytic system of Viscum has two parts. Firstly, the haustoria or sinkers which grow radically and reach the host cambium to absorb water and mineral salts. Haustoria do not penetrate the host xylem; they are simply embedded in host xylem tissue. Secondly, the cortical strands run through parenchymatous or phloem tissue and as a result cause lateral spread.

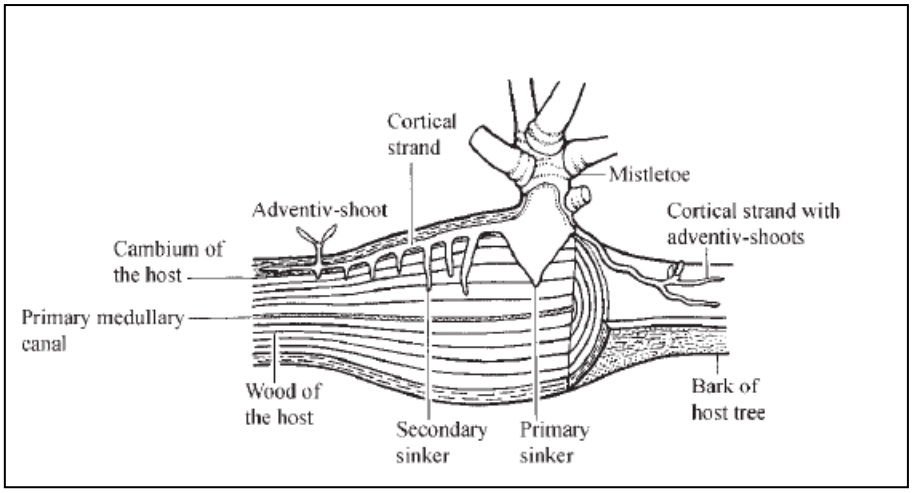

Figure 7: **Perennial mistletoe on a host shoot. Left part shows longitudinal section, right part top view with partly removed Bark.**

Morphological differentiation of the subspecies of *V. album* can only be done through characteristics of the ripe berry. Mistletoe plants from hardwood and from conifers can be distinguished by the the formation of long mucilaginous threads between the inner and the outer layer. These threads are representative for subsp. *album*, whereas no such threads occur in the berries of subsp. *austriacum* and *abietis*. To distinguish between the two subspecies growing on conifers, the shape of the free tip of the hypocotyl is used. In subsp.*abietis* the hypocotyl is cylindrical with a swollen meristematic tip caused by a constriction below. The hypocotyls of subsp. *austriacum* is thin without any constriction.

Life cycle and biology

Viscum album is an evergreen an epiphytic hemiparasitic shoot parasite with the maximum age of about 27–30 years. Birds are the main vectors of mistletoes. The “seeds” survive the passage through the digestive tract unharmed and are released with the faeces.

During the first 4–5 years after germination, *V. Album* produces only internodes with decussate pairs of foliage leaves, one pair each year. After about 4–5 years it develops dichasial shoots and starts flowering. Mature plants flower and fruit once every year.

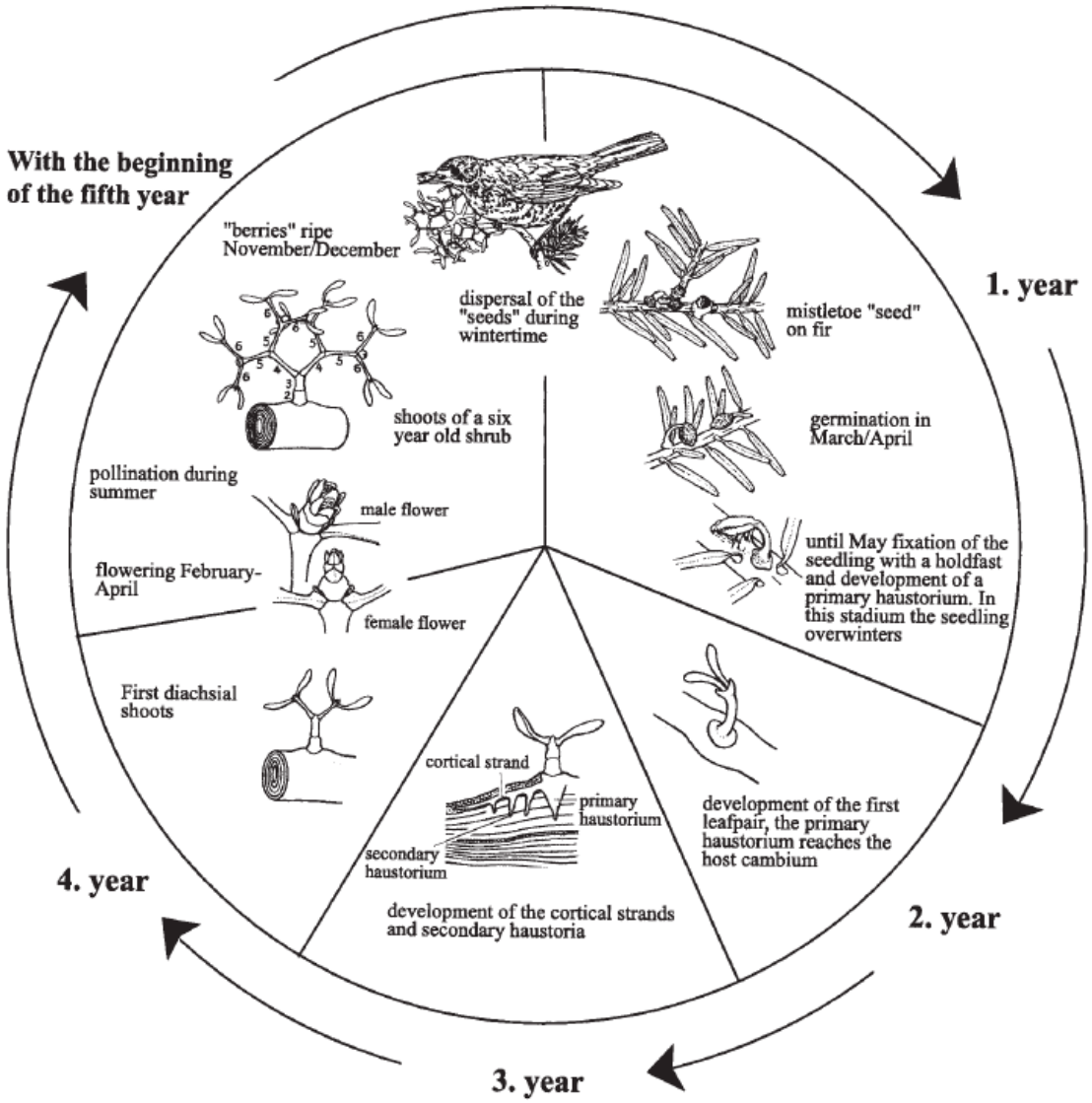

Figure 8: Life cycle of *Viscum album* subsp. *abietis* (Nierhaus-Wunderwald 1997).

Preparation of therapeutic preparation of *Viscum album*

Iscador® is the commercial preparation of VA preparations. It is prepared as an aqueous extract of the whole mistletoe plant with a formulated fermentation with the bacterium *Lactobacillus plantarum*. The product is then mixed and filtered to remove the bacteria before being standardized and packaged in ampules for injection. Fermentation of the mistletoe extract alters its medicinal activity to a significant degree and this change is thought to be related to the degradation of the most toxic lectins. It is believed that the efficacy of mistletoe extracts like Iscador are due to a synergy between both its components that are medicinally active when isolated, and those components like polysaccharides that are medicinally inactive, but can interact with the more active constituents to form complexes.

Clinical use of *Viscum album* preparations

Therapeutic benefit of Viscum preparations has been shown in a variety of pathological conditions of infectious diseases, inflammatory diseases and predominantly in cancer. The VA preparations are normally injected subcutaneously into the abdominal wall, near the tumor site or directly into the tumor if possible. The treatment regimen is designed according to the patients' general condition. The injections are given early in the morning 3-7 times a week with the gradual increase in the dose according to the protocol set by Steiner. A typical course of treatment lasts several weeks. A long-term maintenance dose may be recommended depending on the person's health and tumor status. Experts advise that Mistletoe therapy is compatible with chemotherapy and radiotherapy. It is usually administered before surgery.

Table 3: Clinical uses of Viscum album preparations

- Autoimmune diseases
  - Lupus
  - Arthritis
- Allergic disorders
  - Dermatitis

- Asthma

- Nervous system abnormalities
  - Epilepsy
  - Hypertension
  - Headache

- Sexual abnormalities
  - Infertility
  - Menopausal problems

- Immune disorders
  - HIV

- Veterinary medicine

Composition of therapeutic preparations of *Viscum album:*

Although the proponents of Iscador advise that, optimal beneficial effects require the use of whole-plant products, researchers have ventured to identify the most active constituents. Mistletoe preparations contain a number of biologically active constituents, but they vary widely depending on whether the extract is crude or fermented, on the host species from which the mistletoe has been obtained and on the season during which it was harvested. These variations make it difficult to predict the likely effects of non-standardized mistletoe preparations. However, laboratory research using cell cultures and a variety of animal models has identified the two key components of mistletoe preparations: viscumin (also known as mistletoe lectin I, ML-I or VAL) and viscotoxin. Standardized whole plant extracts of mistletoe are also known to contain in addition, several enzymes, peptides (e.g., viscumamide), amino acids, thiols, amines, polysaccharides, alkaloids, cyclitoles, lipids, phytosterols, triterpines, flavonoids, phenylpropanes, and minerals and many other active biomolecules (Franz, 1985).

Mistletoe lectins:

Lectins constitute the most active component of VA preparations. Mistletoe lectin (ML) I, II, and III belong to the ribosome-inactivating protein (RIP) family of type II, like highly toxic ricin and abrin (Franz et al., 1981). They are composed of one or more protomers consisting

of different disulphide bridge-linked A- and B-chains. RIP of type II contain an N-glycosidase (A chain) and a galactoside-recognizing lectin (B-chain) connected by a disulfide bridge (Ye et al., 2006). The A-chain has N-glycosidase activity and the B-chain has carbohydrate-binding domains. These lectins can catalytically inactivate eukaryotic ribosomes, by the removal of an adenine residue from the 28S ribosomal RNA (rRNA). RIPs can thus be termed polynucleotideadenosine-glycosidases. Studies show that both the chains are important in inducing apoptosis, inhibiting the cellular protein synthesis and resulting in a reduced cell proliferation (Stirpe et al., 1980), (Bantel et al., 1999).

In several in vivo experimental models of tumoral implantation, treatment with VA preparations (Duong Van Huyen et al., 2006) or purified ML has been associated with tumor regression (Yoon et al., 1995), (Lenartz et al., 1998), (Lenartz et al., 2000), (Burger et al., 2003), (Antony et al., 1997). In vitro experimental studies have demonstrated both cytotoxic and immunomodulatory properties of ML that may support its anti-tumoral effect (Hajto et al., 1989), (Kuttan et al., 1990), (Valentiner et al., 2002). For all three MLs, cytotoxic effects by inducing apoptosis in a variety of malignant tumor cells have been shown (Schumacher et al., 1995) , (Lyu and Park, 2007), with ML-I being the predominant cytotoxic agent (Ribereau-Gayon et al., 1986). The growth of a murine non-Hodgkin lymphoma (NHL) tumor has been shown to be reduced by incorporating ML-1 into the diet (Pryme et al., 2002). These results show that ML lectins induce powerful anti-cancer effects.

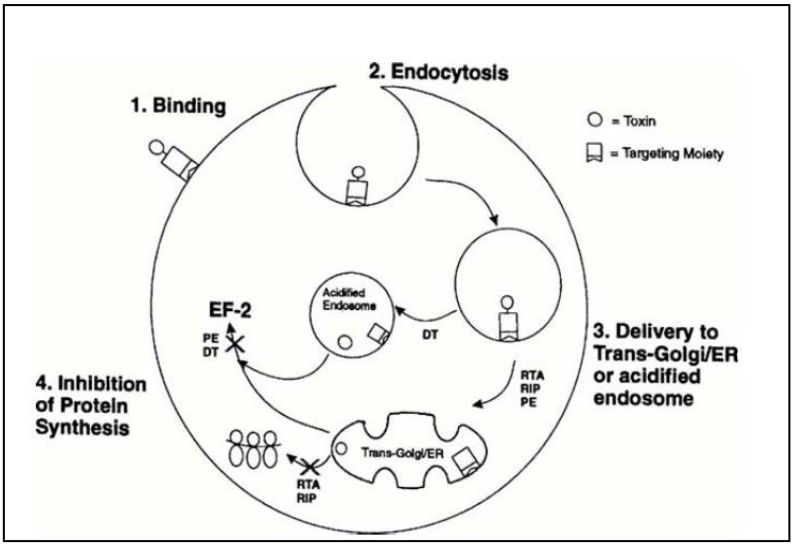

Figure 9: Mechanism of action of type II lectins.

Viscotoxins:

Viscotoxins are the second important family of proteins with the therapeutic properties present in mistletoe preparations (Konopa et al., 1980). Viscotoxins are 46-amino acid peptides that are highly basic and cysteine-rich, related to the family of thionins and resistant to protein denaturing agents (Urech et al., 2006). Thionins are cytotoxic for a large variety of eukaryotic and prokaryotic cells. Toxicity of Thionins is due to their positive charges interacting non pecifically with phospholipids. Thus, by passive insertion into the cell membranes, thionins permeabilize and kill tumoral cells. They have low molecular mass and are thermostable and protease-resistant. Viscotoxins A2, A3 and B (VA2, VA3 and VB respectively) are among the most abundant viscotoxin isoforms that occur in these mistletoe preparations that stimulate NK-cell-mediated lysis (Tabiasco et al., 2002),. Although viscotoxins belong to the thionin family, they are devoid of antimicrobial activity, yet they do display cytotoxic effects towards tumor cells together with immunomodulatory properties (Franz et al., 1983), (Stein et al., 1999b), (Bussing et al., 1999). VA3 shows higher levels of cytotoxicity than other viscotoxins. The different levels of cytotoxicity displayed by VA3,

VA2 and VB on tumor cells depend on their ability to interact with the plasma membrane and modify its supramolecular organization (Coulon et al., 2003).

Polysaccharides:

Colloidal preparation from fresh *Viscum album* L. Berries consists of polymeric carbohydrates. Further fractionation by exchange chromatography revealed a neutral fraction (average molecular weight 30 kDa) and three acidic fractions (average molecular weights 1300 kDa) (Wang and Zhu, 2007), (Jordan and Wagner, 1986). Structural analysis of these fractions and quantitative and qualitative determination of the sugar composition and linkage analysis indicated that all acidic fractions contained an acidic arabinogalactan with a rhamnose-galactoronic acid backbone and highly branched arabinose-galactose side chains attached by the rhamnose residues to the backbone. The neutral fraction consisted of a neutral arabinogalactan beside minor amounts (about 10%) of a low substituted xyloglucan. The main polysaccharide of the green parts of Viscum is a highly esterified galacturonan, whereas in berries, a complex arabinogalactan is predominant (Edlund et al., 2000). These molecules are differentially found in VA preparations with defined changes in molecular weight and structure. Rhamnogalacturonan is known to be involved in the interaction of NK cells and lymphokine activated killer (LAK) cells which target tumor cells. The synergistic enhancement of lysis by mistletoe arabinogalactan is mediated by cytokines secreted from killer cells after its binding to NK cell surface receptors. These cells pulsed with high concentrations of arabinogalactans killed more target cells. IL-12 mediated activation of human MHC-unrestricted cytotoxicity of freshly isolated, highly enriched CD56 +CD3- NK cells, monocytes/macrophages and CD3 +T cells was also increased in the presence of rhamnogalacturonan. Simultaneous enhancement of cytotoxicity indicates the involvement of receptors on effector cells cross-reacting with acetylrhamnose (6-deoxymannose) that might

play an important role in human MHC-unrestricted cytotoxicity against tumor cells (Stein et al., 1999a) (Edlund et al., 2000).

Apart from these, several other bioactive components of *Viscum album* are shown to be important in exerting various biological effects. Studies from isolated flavonoids from *Viscum album* have revealed the importance of flavonoid fraction that is responsible for anti-inflammatory and antinociceptive properties in experimental animals and in vitro experimental models (Nhiem et al., 2013), (Orhan et al., 2006). In view of the fact that, the intact therapeutic extract is a complex mixture of several compounds it is extremely important to understand the mechanisms of action of each component in relation to the effect of whole therapeutic extract.

Table 4: Components of Viscum album preparations and their mechanisms

Molecules	components	functions
Mistletoe Lectins	Mistletoe lectin I (MLI) Mistletoe lectin II (MLII) Mistletoe lectin III (MLIII	Cell cytotoxicity Adjuvant properties Immunomodulation Anti-inflammatory
Viscotoxins	Viscotoxin A2 (VA2) Viscotoxin A3 (VA3) Viscotoxin B (VB)	Tumor cytotoxicity Immunomodulation Interaction with plasma membrane NK cell mediated lysis
Polygosaccharides	Galacturonan Arabinogalactan	Increased NK cell mediated activity Interaction with MLI

Flavonoids	syringin, coniferin, kalopanaxin....	Anti inflammatory and antinociceptive
Other biological molecules	Terpenes, lipids, thiols, Polysterols, amines…	Synergistic effects Not clearly understood

Although the composition of VA preparations is empirically formulated, and can vary to some extent, based on the time of harvesting and method of preparation, amount of two major components, mistletoe lectin and viscotoxin are relatively clear.

Table 5: Mistletoe lectin and viscotoxin contents of three commonly available VA preparations

Type of preparation	Mistletoe lectin	Viscotoxin
VA Q Spez	785 ng/ml	5 μg/ml
VA P	28 ng/ml	6 μg/ml
VA M Spez	548 ng/ml	4 μg/ml
VA A	23 ng/ml	19 μg/ml

Viscum album- Mechanisms of action

Since Viscum preparations are whole plant extracts the known therapeutic benefit cannot be attributed to a single component of the preparation, rather they affect in multiple mechanisms. Several mechanisms of action have been proposed to explain the therapeutic benefit of these preparations which can be broadly classified into three different, but interdependent mechanisms as, Anti-tumor mechanisms, immunomodulatory mechanisms and anti inflammatory mechanisms (Elluru et al., 2006).

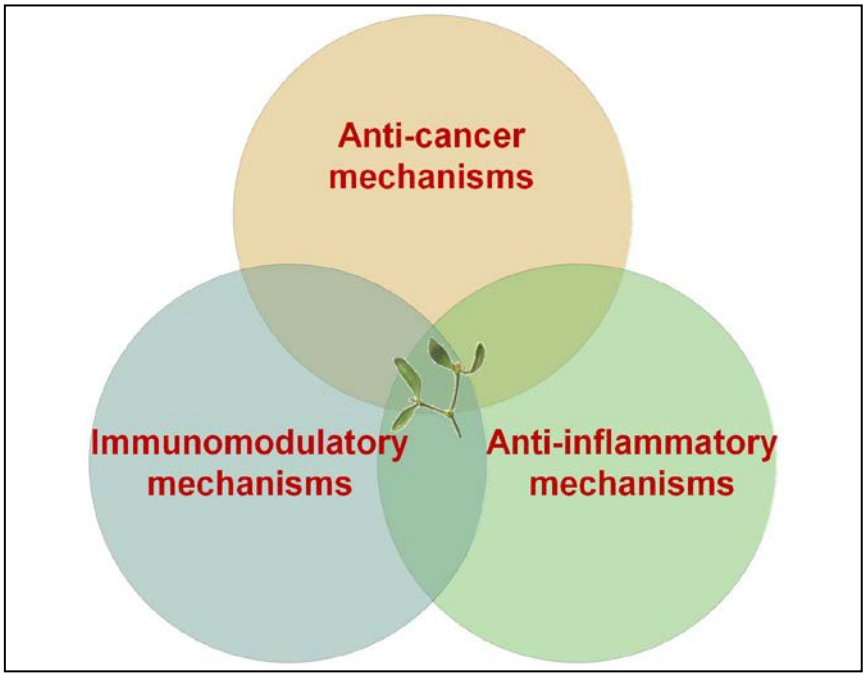

Figure 10: **Proposed mechanisms of action of *Viscum album* preparations**

Anti-tumor mechanisms of *Viscum album*

Viscum preparations are effectively used along with conventional cancer therapies such as, chemotherapy and radiotherapy and have shown to be beneficial in tumor regression and better survival (Bussing et al., 2005). Multiple mechanisms of action have been demonstrated to contribute to this effect. Direct cytotoxic effect of VA preparations on tumor cells is one of their key anti-tumor properties. Various tumor cell types of lymphocytic origin are shown to be affected by different Viscum preparations due to a specifically defined cytotoxic effect depending on variable cellular sensitivity towards the Viscum and also on the host type of the

plant and the method of their preparation (Duong Van Huyen et al., 2002), (Duong Van Huyen et al., 2003), (Duong Van Huyen et al., 2001) , (Duong Van Huyen et al., 2006). Later on it was shown that the tumor cell death caused by mistletoe preparations is often due to the induction of apoptosis, a specifically programmed cell death and involves a dramatic reduction in the expression of anti-apoptotic molecular machinery like caspases, cytochrome c, Bcl-2 family of proteins etc. Several studies have demonstrated the direct cytotoxic effect of isolated mistletoe lectins on primary cells and transformed cells (Mossalayi et al., 2006).

Mistletoe lectin (ML) I, II, and III belong to the ribosome-inactivating protein (RIP) family of type II, like highly toxic ricin and abrin. RIP of type II contain an N-glycosidase (A chain) and a galactoside-recognizing lectin (B-chain) connected by a disulfide bridge. Previous studies have shown that both the chains are important in inducing apoptosis, inhibiting the cellular protein synthesis and resulting in a reduced cell proliferation. In several *in vivo* experimental models and *in vitro* experiments, treatment with VA extracts or purified ML has been associated with tumor regression. With these results it seems that the anti-tumor properties of Viscum are due to the presence of mistletoe lectins, however one cannot rule out the possible contribution by several other bioactive compounds which are present in the therapeutically administered plant extract which may act in different pathways in order to exert anti-tumor effect.

Another interesting property of mistletoe preparations is their ability to inhibit the process of angiogenesis. Angiogenesis is the process by which new blood vessels develop from the pre-existing ones. Tumor angiogenesis is a critically important for tumor progression and metastasis (Folkman, 1995). The mechanisms of tumor angiogenesis consists of a variety of processes including an enhanced division of endothelial cells within the tumor, up-regulation of cell adhesion molecules, and production of angiogenic molecules (Carmeliet, 2000). Several anti-tumor therapies are designed based on the process of angiogenesis and its

regulatory mechanisms (Kerbel, 2006),(Yance and Sagar, 2006). Anti-angiogenic effect of mistletoe prep arations has been demonstrated by several *in vivo* and *in vitro* studies using both isolated mistletoe lectins and intact VA preparations (Park et al., 2001), (Duong Van Huyen et al., 2002), (Elluru et al., 2009). VA preparations are shown to induce apoptosis in endothelial cells and to inhibit the cell growth by delaying cell cycle progression thereby contributing to the overall tumor regression. VA preparations are also used successfully in the treatment of several types of cancer in animals (Christen-Clottu et al., 2010).

Immunomodulatory mechanisms of *Viscum album*

Tumors exert various active mechanisms to suppress the host immunity. By altering the functions of antigen presenting cells (APC), by promoting dysfunctional T cell co-signaling, and generating an immune-subversive cytokine milieu, they result in the development of a suppressed immunophenotype (Rabinovich et al., 2007),(Drake et al., 2006), (Finke et al., 1999), (Arens). Viscum preparations exert a strong immunomodulatory effect in order to facilitate an effective anti-tumor immune response (Jurin et al., 1993),(Braedel-Ruoff., 2010), (Hajto et al., 1997). They modulate the cytokine network favouring the anti-tumor immune response, by increasing the secretion of pro-inflammatory cytokines necessary for a moderate immunostimulation (Hajto, 1986), (Kuttan and Kuttan, 1992), (Pelletier et al., 2001). In animal models it has been shown that, they lead to a beneficial tumor regression which is associated with an enhancement of splenocyte proliferation and an up-regulation of IL-12 secretion. In IL-12 deficient mice, VA failed to protect from the developing tumor suggesting a crucial role for IL-12 in mistletoe mediated anti-tumor immune response (Duong Van Huyen et al., 2006).

Mistletoe preparations have a profound stimulatory effect on dendritic cell. They induce the maturation and activation of dendritic cells which will help to mount a tumor specific cytotoxic T cell response. DCs which normally occur within the tumor microenvironment are

found to have a relatively immature phenotype characterized by low level expression of antigen presenting molecule (HLA-DR), and co-stimulatory molecules and are unable to produce pro-inflammatory cytokine. Clinical studies and in vitro experiments have demonstrated that, treatment with *Viscum album* preparations can increase the functioning of DC by increasing the surface expression of HLA-DR and co-stimulatory molecules and enhancing their ability to secrete pro-inflammatory cytokines which will further help to stimulate tumor specific T cells (Elluru et al., 2008).

Although there are several studies which report the influence of mistletoe preparations on various cellular compartment of the immune system, like macrophages and NK cells which will explain at least in part, their mechanisms of action underlying their therapeutic benefit, further investigations will be still helpful in understanding the immunomodulation by *Viscum album* (Yoon et al., 2003), (Antony et al., 2000).

Anti-inflammatory mechanisms of *Viscum album*

Inflammation is a basal physiological phenomenon required for wound healing or to eliminate an infectious agent from the system which occurs as a complex set of cellular and molecular responses. However it is a physiopathological symptom in diverse conditions of infectious, autoimmune and tumoral origin. Inflammatory mediators like pro-inflammatory cytokines, chemokines, bioactive amines, eicosanoids, and products of proteolytic cascades, such as bradykinin, which are produced upon interaction of innate and adaptive immune cells as well as non-immune cells such as endothelial cells and fibroblasts with inflammatory stimulus, act on various target tissues and can exert severe changes in their homeostatic functions and therefore inflammation has to be kept in check (Medzhitov., 2010). This can be achieved by various anti-inflammatory agents including steroids, non-steroidal anti-inflammatory agents (NSAID), intravenous immunoglobulins, immunosuppressor cells, neutralizing monoclonal antibodies to inflammatory cytokines and also by natural products

like various phytotherapeutics (Geng, 2001), (Bayry et al., 2003), (Bayry et al.), (Rao and Knaus, 2008), (Chrubasik et al., 2001).

In view of the increasing evidence that demonstrate the critical interplay between inflammation and cancer which share several regulatory mechanisms, symptomatic associations, signaling events, it is extremely important to dissect and to understand the molecular mechanisms of anti-inflammatory and anti-tumor properties of such interesting therapeutics (DiDonato et al.), (Hagemann et al., 2007). This will allow us to design better immunotherapeutic strategies to inflammatory pathologies and cancer using existing therapeutics. *Viscum album* preparations are shown to exert anti-inflammatory effect in various *in vitro* and *in vivo* experiments by inhibiting the different pro-inflammatory axes. Bacterial *lipopolysaccharide (LPS)*-induced inflammation *can be resolved by VA preparations by inhibiting the neutrophil influx (Lavastre et al., 2004), (Lavastre et al., 2002). However, not many studies have tried to dissect the anti-inflammatory mechanisms of VA preparations.*

Current study identifies one of the key anti-inflammatory properties of Viscum preparations and addresses the molecular mechanisms by investigating the modulation of cyclo-oxygenases (COX) and COX dependant PGE2.

Role of cyclo-oxygenases and COX-derived prostaglandins in inflammation

Cyclooxygenases (COX), also known as prostaglandin-endoperoxide synthase (PTGS) are the enzymes that regulate the biosynthesis of an important family of biological mediators called prostanoids such as prostaglandins, prostacyclin and thrombaxane. They catalyse the first two biochemical reactions in the conversion of arachidonic (AA) acid into prostanoids (Rouzer and Marnett, 2009).

Structure and functions of cyclo-oxygenases

There are three isoforms of COX, COX-1, COX-2 and COX-3 (which is known to be the splice variant of COX-1 and is found to exist in brain and spinal cord) (Chandrasekharan et al., 2002), (Kis et al., 2003). Human COX-1 and COX-2 are homodimers of 576 and 581 amino acids respectively. Both enzymes contain three high mannose oligosaccharides, one of which facilitates protein folding. A fourth oligosaccharide, present only in COX-2, regulates its degradation. Considering the 60% homology in sequence between COX-1 and COX-2, it is not surprising that their three-dimensional structures are nearly super-imposable. Each subunit of the dimer consists of three domains, the epidermal growth factor domain (residues 34–72), the membrane binding domain (residues 73– 116), and the catalytic domain comprising the bulk of the protein, which contains the cyclo-oxygenase and peroxidise active sites on either side of the heme prosthetic group. The two isoforms of COX are almost identical in structure but have important differences in substrate and inhibitor selectivity and in their intracellular locations (Rouzer and Marnett, 2009). These are the oxidation of arachidonic acid (AA) to the hydroperoxy endoperoxide PGG2 and its subsequent reduction to the hydroxyl endoperoxide PGH2. The PGH2 is transformed by a range of enzymes and non enzymatic mechanisms into the primary prostanoids, PGE2, PGF2, PGD2, PGI2, and TXA2. Depending on their availability, either arachidonic acid (AA) or any other polyunsaturated fatty acids of the same family such as Dihomo-γ-linolenic acid (DGLA) and eicosapentaenoic acid (EPA), from the dietary source, cyclo-oxygenases result in the biosynthesis of series 2 prostanids (pro-inflammatory prostanoids) or series-1 and series-3 prostanoids (anti-inflammatory prostanoids). The direct product of the activity of COX enzymes, PGH2, is in turn the precursor of other PGs, including PGE2, PGF2α, PGD2, PGI2(prostacyclin) and thromboxane A2 (TxA2), through the action of cytosolic or microsomal PG synthases. Despite the fact that all the COX isoforms generate the same

PGH2 precursor, the two enzymes control very different biological processes. Whereas COX-2 is predominantly implicated in inflammation, COX-1 is a critical regulator of homeostatic functions (Crofford, 1997), (Kam and See, 2000).

During the tissue injury or a pro-inflammatory stimulus, membrane phospholipids are released into the cytosol and are converted into free arachidonic acid by the action of phospolipases (PLC and PLA_2). This free, cytosolic arachidonic acid acts as substrate for the cyclo-oxygenases for the biosynthesis of prostanoids which are then secreted from the cell and exert their action on the target tissues either by autocrine or by paracrine manner. There are two major isoforms of cyclo-oxygenases. COX-1 is constitutively expressed and in known to mediate the homeostatic functions of gastric mucosa, kidney and vascular endothelium. COX-2 is induced upon a pro-inflammatory stimulus and mediates the over expression of prostaglandins responsible for pain, fever and inflammation. COX-2 derived PGs are also important during ovulation and child birth. The discovery of cyclo-oxygenases has made possible the design of drugs that reduce inflammation without altering homeostatic level of PGs (the protective PGs in the stomach and kidney made by COX-1).

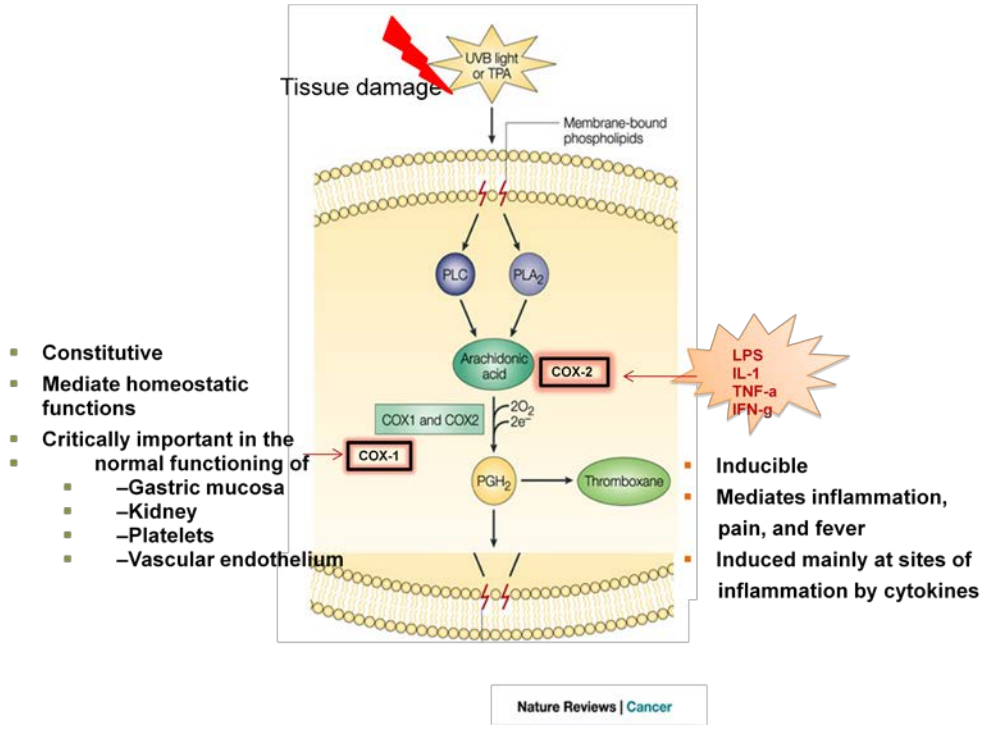

Figure 11: Mechanism of prostanoid synthesis from arachidonic acid by cyclo-oxygenases.

Structural and functional differences of COX-1 and COX-2

COX-1 is widely distributed and constitutively expressed in most tissues where it is found. Its gene, *Ptgs-1*, codes for a 2.8 kb mRNA which is relatively stable. The gene coding for COX-2, *Ptgs-2*, is an immediate early gene that is activated by a wide variety of inflammatory and proliferative stimuli, and the 4 kb COX-2 mRNA turns over rapidly due to the presence of instability sequences in the 3′-untranslated region (3′-UTR). The difference in the pattern of gene expression is responsible for the existence of the two COX isoforms, suggesting that COX-1 provides PGs that are required for homeostatic functions, including gastric cytoprotection and hemostasis, whereas COX-2 plays the predominant role in PG formation during pathophysiologic states, such as inflammation and tumorigenesis. COX-1 is expressed in almost all tissues, with most of the protein localized to the blood vessels, smooth muscle cells, interstitial cells, platelets, and mesothelial cells. Although more highly variable, COX-2 protein was found in nearly all tissues and was most often localized to parenchymal cells.

Constitutive COX-2 expression is well recognized in brain, kidney, and the female reproductive tract, and evidence for induction of COX-1 during the lipopolysaccharide (LPS)-mediated inflammatory response and cellular differentiation has been reported. COX-2 is shown to have wider substrate specificity than that of COX-1. The most significant difference between the isoenzymes, which allows for selective inhibition, is the substitution of isoleucine at position 523 in COX-1 with valine in COX-2. The smaller Val_{523} residue in COX-2 allows access to a hydrophobic side-pocket in the enzyme (which Ile_{523} sterically hinders). Drug molecules, such as DuP-697 and the coxibs derived from it, bind to this alternative site and are considered to be selective inhibitors of COX-2 (Rouzer and Marnett, 2009).

Cyclo-oxygenases as attractive targets of anti-inflammatory therapeutics

Although, PGE2 is a molecular mediator of several homeostatic functions including those of gastric mucosa and vascular endothelium, however, it also exerts potent pro-inflammatory effects including the induction fever and pain. Overproduction of PGE2 occurs in response to a wide variety of pro-inflammatory stimuli like IL-1β, IFN-γ, TNF-α and also pathogen stimuli such as lipopolysaccharides, and correlates with the severity of certain infectious and inflammatory conditions and in many of the tumor conditions (Giulietti et al., 2007), (Martel-Pelletier et al., 2003), (Zhang et al., 2007), (Zhao et al., 2008). Overproduction of PGE2 associated with the over expression of COX-2 is observed in many of the human pathologies related with inflammation and pro-tumoral condition. In these cases COX-2 expression may surpass normal physiological control, resulting in fatally pathological states: namely, autoimmune diseases and cancer. In each of these cases, an aberrant stable expression and activation of COX-2 may be occurring.

In different examples of autoimmune diseases, such as rheumatoid arthritis, systemic lupus erythematosus, or some forms of diabetes, the hyperactivation of immune cells has been shown to be directly dependent on the over-expression of COX-2. The hyperexpression of COX-2 is the result of cross-talk between several mediators of inflammation, including interleukins and cytokines (i.e., IL-1, IL-6 and TNFα), and it occurs via transcriptional activation. Constitutively over expressed COX-2 has been found in a number of cancers which is often associated with a poor prognosis and reduced response to chemotherapy. Therefore, pharmacological strategies to suppress the COX-2 expression and PGE2 secretion are of great interest and are being exploited to develop potent therapeutics to resolve inflammation and several pro-tumor conditions.

One of the well known clinical strategies to suppress pathological COX-2 levels is by inhibiting its activity (Cai et al., 2008). A large family of synthestic inhibitors has been developed on this basis called, Non Steroid Anti-inflammatory Drugs (NSAID) such as aspirin, indomethacin etc. These inhibitors are shown to block the enzymatic activity of cyclo-oxygenases either by modulating their active site or by competing with high affinity for the respective substrate thereby reducing the overall PGE2 biosynthesis. From the viewpoint of an anti-inflammatory therapeutic, inhibition of PGE2 synthesis by acting at the level of cyclo-oxygenases exposes a major concern of reducing the homeostatic levels of PGE2 because of the simultaneous inhibition of COX-1 and COX-2. Owing to the structural and functional homology between the COX-1 and COX-2, most of the NSAID which are synthetically designed to inhibit the enzymatic activity of COX-2 also inhibit COX-1, thereby affecting the overall level of PGE2 and are therefore known to cause several severe side effects. Although these chemical inhibitors *revolutionized the field of drug design to control inflammation with their anti-inflammatory, analgesic and anti-pyretic effects, most of them cause severe side effects such as gastric ulcers, hypertension and risk of cardiovascular*

injury, platelet dysfunctioning, kidney dysfunctions and many others. Therefore specific inhibitors of COX-2 which selectively inhibit the COX-2 without modulating the COX-1 functions, represent attractive therapeutic alternatives for several inflammatory pathologies (Surh and Kundu, 2005). COX-2 selective inhibitors have been developed synthetically such as celecoxib, rofecoxib etc (Marnett, 2009). Although these drugs exert minimal side effects with respect to the gastric dysfunctioning, however there are also reports of side effects associated with cardiovascular dysfunctions and hypertension. Therefore the efficacy of these drugs can vary depending upon the individual's health status and therefore remains unpredictive.

Indeed, several phytotherapeutics and plant-derived molecules like curcumin, some of the flavonoids and polyphenols that have anti-tumor properties also exert anti-inflammatory activity by down-regulating the expression of COX-2 and PGE2 biosynthesis. Several *in vitro* experiments and *in vivo* experiments on animal models and several clinical studies have shown that these compounds interfere either with the expression or regulation of expression of COX or by modulating their biological activity and functioning pathways. Many of the phytotherapeutics are also shown to exert selective COX-2 inhibition with a possible role in reducing the side effects.

Mechanisms of inhibition of COX-2 by phytotherapeutics

Prolonged administration of COX-2 inhibitors has been ineffectual for chemopreventive and chemotherapeutic purposes since the risks prevail over the benefits. Clinical demonstration of severe side effects due to the failure of the classical COX-2 inhibitors to discriminate between an aberrant pathological vs. homeostatic functional activation state raised the concern that direct COX-2 enzymatic inhibition might not represent an eligible clinical strategy to target COX-2 (Cerella et al., 2010).

Since in contrast to COX-1, COX-2 is an early response gene, similar to the genes encoded for cytokines, chemokines and proto-oncogenes, they can be subjected to several mechanisms of expression modulation, ranging from direct transcriptional effects to post-transcriptional and post-translational levels and up to the protein expression and stability of mediating transcription factors. The presence of such multiple levels of modulation of COX-2 expression implies the existence of multiple mechanisms, which may be targeted to finely modulate COX-2 functions. This allows the consideration of COX-2 expression as a more versatile target to modulate the wide array of its enzymatic functions, thus potentially contributing new perspectives in therapeutic and chemopreventive strategies. Therefore the regulatory events which are the determinants of COX-2 expression, in its biological functioning, can be intervened by therapeutic approaches.

Determinants of COX-2 expression

Transcriptional regulation

While COX-1 family member lacks characteristic TATA and GC boxes in its promoter region, and generally acts as a house-keeping gene; in contrast, the COX-2 promoter contains a number of upstream regulatory sequences specific for binding with a variety of transcription factors, such as NF-kB, the SP-1 transcription factor (SP-1), the cAMP responsive element binding protein (CRE), the transcription factor 4 (TCF4), the CCAAT/ enhancer-binding protein beta (c/EPB), and the activator protein 1 (AP-1). This often implicates the participation of several kinase-mediated signal transduction mechanisms including mitogen-activated protein kinases (MAPKs) c-Jun NH2-terminal kinase (JNK), p38 and the extracellular signal regulated protein kinases 1/2 (ERK). Accordingly, a wide range of stimuli including pathogen associated molecular patterns (PAMPS) and pro-inflammatory cytokines may trigger these intracellular signalling pathways, and downstream transcription of COX-2. Therefore understanding of the transcriptional regulation of COX-2 can be

exploited for the designing the therapeutic strategies. Thus COX-2 transcriptional activation can be altered by modulating the three-dimensional conformation of chromatin due to altered methylation status, or by interfering with the binding of transcription factors, or by modulating the expression of regulatory factors required for the transactivation of COX-2.

Post-transcriptional regulation

Post-transcriptional regulation generally refers to the regulation of the stability of an mRNA. Stability of COX-2 is shown to be regulated by two unique mechanisms. Firstly due to the presence of 3′-untranslated region (3′-UTR) which contains a number of copies of the highly conserved cis-acting consensus motif AAUAAA, generally referred as AU-rich elements or AREs and several ARE binding proteins which modulate the physical accessibility and stability of the target mRNAs for translation. Secondly, due to the regulation by several microRNA (miRNA) such as mir-101a, mir-26b, mir-16 and many others which regulate the stability of COX-2 transcripts, and help to maintain the mRNA turn over and mRNA half-life differencially, depending on the cell type and their inflammatory status. Under normal conditions these regulatory mechanisms will maintain the mRNA turnover and silence the COX-2 expression. Thus interfering with the regulatory events at the post-transcriptional level may serve as potential tool to modulate the expression of COX-2 in various pathological situations.

Post-translational regulation

Several lines of evidence have revealed an interesting role of different post-translational modifications such as N-glycosylation, S-nitrosylation, phosphorylation, and acetylation in maintaining stability and integrity of COX-2 protein. Although the role of these regulatory events are not clearly understood with respect to the emergence of inflammatory and pro-tumoral conditions, the fact that they regulate COX-2 protein stability, can provoke their

implication in the potential identification of novel chemopreventive agents acting through the modulation of the post-translational modifications.

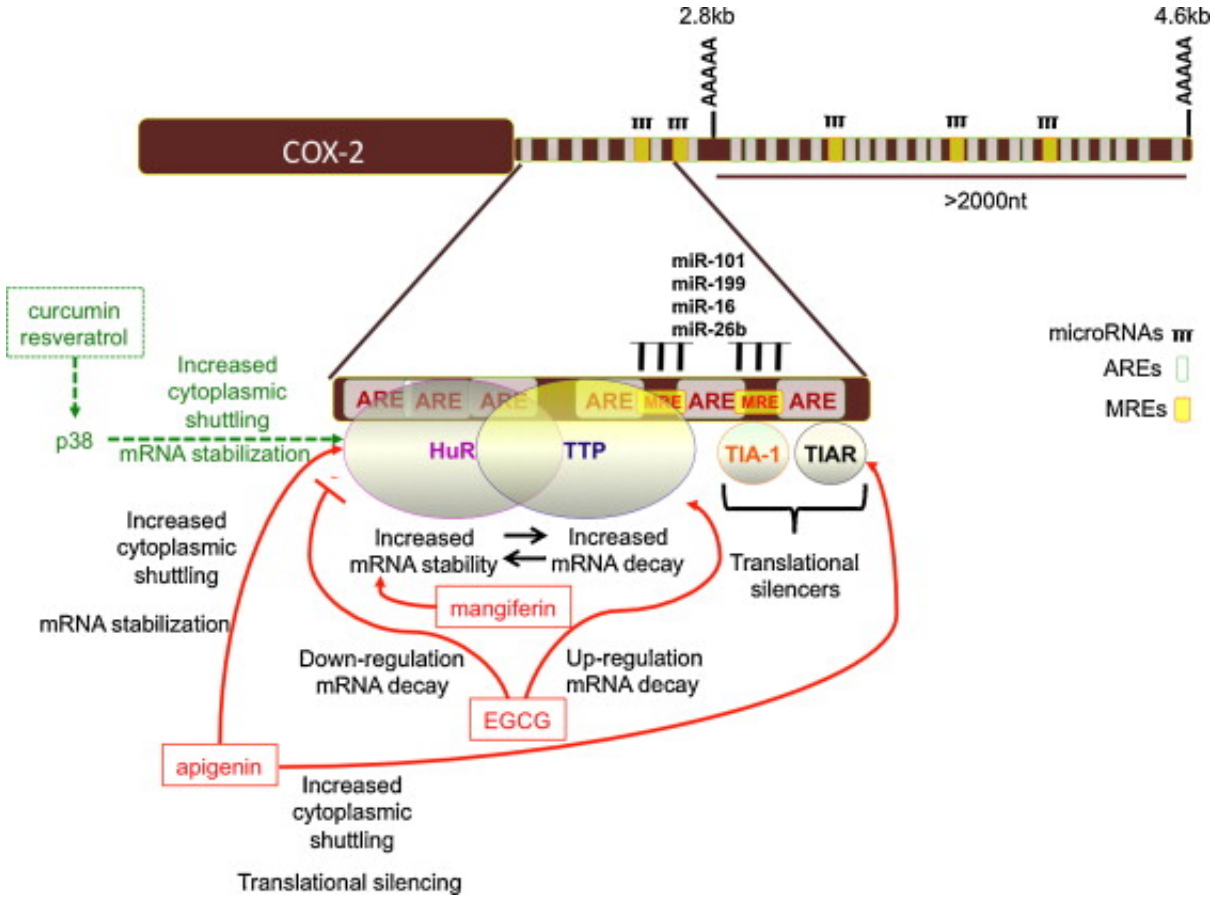

Figure 12: Regulatory mechanisms of COX-2 expression and therapeutic intervention by phytotherapeutics

Long-term side effects of NSAID in various pathological conditions and the increasing body of evidence for anti-inflammatory activity of plant-derived molecules together encourage the conception of phytotherapeutics as potent alternatives to classical anti-inflammatory drugs.

Objectives of the current study

In view of the increasing evidence that demonstrate the critical interplay between inflammation and cancer which share several regulatory mechanisms, symptomatic associations, signaling events, it is extremely essential to dissect and to understand the molecular mechanisms of anti-inflammatory and anti-tumor properties of such interesting

therapeutics. This will allow us to design better immunotherapeutic strategies and integrative medicinal approaches to inflammatory pathologies and cancer using existing therapeutics.Our research team is extremely interested in dissecting and understanding the immunomodulatory strategies in the treatment of autoimmune and inflammatory pathologies. Previous work in the lab has made significant contribution to the understanding of mechanisms of action of Viscum preparation as anti-tumor and immunomodulatory mechanisms.

Owing to the intricate association of inflammation and cancer and the successful utilisation of anticancer therapeutics in the treatment of inflammatory conditions, and in view of the fact that several anti-tumor phytotherapeutics also exert a potent anti-inflammatory effect, this study addresses the anti-inflammatory properties of VA preparations in COX-2 and PGE2 axis to dissect the mechanisms underlying their therapeutic benefit. Following are the objectives of this study.

- **Understanding the anti-inflammatory properties of *Viscum album* in the COX-2-PGE2 pro-inflammatory axis using a cellular model of human lung adenocarcinoma (A549 cell line).**
- **Effect of *Viscum album* on the secretion of PGE2 under the pro-inflammatory conditions.**
- **Exploring the mechanisms of Viscum-mediated PGE2 modulation by analysing the effect at the level of cyclo-oxygenases (COX-1 and COX-2).**
- **Dissecting the molecular mechanisms associated with the Viscum-mediated COX-2 inhibition.**
- **Analysing the anti-inflammatory effects of different Viscum preparations derived from different host plants.**

- **Identifying the possible component of the therapeutic *Viscum album* preparation that can contribute to the anti-inflammatory effect at the level of cyclo-oxygenases.**

Objective 1:

Understanding the anti-inflammatory properties of *Viscum album* in the COX-2-PGE2 pro-inflammatory axis using a cellular model of human lung adenocarcinoma (A549 cell line): analysing the effect of *Viscum album* on the secretion of PGE2 under the pro-inflammatory conditions.

Owing to the intricate association of inflammation and cancer and in view of the fact that several anti-tumor phytotherapeutics also exert a potent anti-inflammatory effect, we hypothesized that VA exerts an anti-inflammatory effect that is responsible for its therapeutic benefit. Since, inflammatory cytokine-induced cyclo-oxygenase-2 (COX-2) and prostaglandin E2 (PGE2) play a critical role in the pathogenesis of inflammatory diseases, we investigated the anti-inflammatory effect of VA on regulation of cyclo-oxygenase expression and PGE2 biosynthesis by using human lung adenocarcinoma cells (A549 cells) as a model. A549 cells were stimulated with IL-1β and treated with VA preparation (VA Qu Spez) for 18 hours. PGE2 was analysed in the culture supernatants by enzyme immunoassay. Expression of COX-2 and COX-1 proteins was analyzed by immunoblotting and the expression of COX-2 mRNA was assessed by semi-quantitative RT-PCR. We found that VA Qu Spez inhibit the secretion of IL-1β-induced PGE2 in a dose-dependent manner. Further, we also show that this inhibitory action was associated with a reduced expression of COX-2 without modulating the COX-1 expression. Together these results demonstrate a novel anti-inflammatory mechanism of action of VA preparations wherein VA exerts an anti-inflammatory effect by inhibiting cytokine-induced PGE2 via selective inhibition of COX-2.

Objective 2:

Dissecting the molecular mechanisms associated with the Viscum-mediated COX-2 inhibition.

Extensive use of *Viscum album* (VA) preparations in the complementary therapy of cancer and in several other human pathologies has led to the increasing number of cellular and molecular approaches to explore the mechanisms of action of *V.* Interestingly in this investigation we observed a significant down-regulation of COX-2 protein expression in VA-treated cells whereas COX-2 mRNA levels were unaltered. In addition to this, VA-mediated COX-2 inhibition was observed only at the early phases of inflammatory process. Therefore we hypothesised that VA induces destabilisation of COX-2 mRNA, thereby depleting the available functional COX-2 mRNA for the protein synthesis and for the subsequent secretion of PGE2.

To address this question, mRNA half-life of COX-2 following transcriptional blockade by actinomycin D in IL-1β-treated A549 cells with or without VA treatment was analysed. COX-2 mRNA levels are analysed at different time points. A marked reduction in the half life of COX-2 mRNA was observed which was due to its rapid degradation in the cells treated with VA compared to that in IL-1β-stimulated cells. These results thus demonstrate that VA-mediated inhibition of PGE2 implicates destabilization of COX-2 mRNA.

Objective 3:

Analysing the anti-inflammatory effects of different Viscum preparations derived from different host plants: identification of possible component of VA which is responsible for COX-2 inhibitory effect.

In spite of a heterogeneous composition and diverse mechanisms of action (due to the host tree and the method of preparation), different preparations are equally beneficial in various pathological condition. But the precise role of various components in these preparations is not yet clear. Given the anti-inflammatory role of *Viscum album* Q Spez (growing on oak trees) in inhibiting the cytokine-induced COX-2 and PGE2, this study was focused to dissect the anti-inflammatory properties of different *Viscum album* preparations derived from different host trees. In this study we demonstrate that Viscum preparations derived from those growing on oak trees and apple trees inhibit the COX-2 expression under pro-inflammatory conditions. However Preparations obtained from those growing on pine trees do not inhibit COX-2 expression. Interestingly it was found that, the COX-2 inhibitory effect in these preparations directly correlates to the presence of mistletoe lectin. Together these results demonstrate one of the important anti-inflammatory properties which can be strongly associated with their mistletoe content.

Results

Article 1- Résumé en Français

Titre: Les extraits de *Viscum album* exercent un effet anti-inflammatoire en inhibant sélectivement l'expression de la cyclo-oxygénase-2(COX-2) induite par les cytokines

Les extraits de *Viscum album* (VA) sont largement utilisés comme thérapie de complément dans les cancers du fait de leurs activités anti-tumorales impliquant des propriétés cytotoxiques, d'induction d'apoptose et d'inhibition de l'angiogenèse ainsi que plusieurs autres mécanismes immuno-modulateurs. En plus de leur intérêt dans le traitement des cancers, les extraits de VA ont également été utilisées avec succès dans le traitement de différentes pathologies inflammatoires. A cause de l'association complexe entre le statut inflammatoire et les cancers, et compte tenu du fait que plusieurs phytothérapeutique anti-tumoraux exercent aussi un effet anti-inflammatoire puissant, nous avons émis l'hypothèse que les

extraits de VA exercent des effets anti-inflammatoires responsables de ses bénéfices thérapeutiques. Étant donné que la cyclo-oxygénase-2 (COX-2) et la prostaglandine E2 (PGE2) sont induites par les cytokines pro-inflammatoires, et jouent un rôle critique dans le développement des pathologies inflammatoires, nous avons étudié les actions anti-inflammatoires des extraits de VA sur l'expression et la régulation de la cyclo-oxygénase-2 et de PGE2, en utilisant un modèle de cellule d'adénocarcinome pulmonaire humaines (A549). Des cellules A549 ont été stimulées avec de l'IL-1β puis traités avec un extrait de VA (VA Qu Spez) pendant 18 heures. La sécrétion de PGE2 a été détectée dans le surnageant de culture par dosage immuno-enzymatique. L'expression des protéines COX-1 et COX-2 ont été analysées par western-blot et l'expression des ARNm de COX-2 a été évaluée par RT-PCR. Nous avons observé que la préparation VA Qu Spez inhibait la sécrétion de PGE2 induite par l'IL-1β, de façon dose-dépendante. De plus, nous avons montré que cette action inhibitrice était associée à une diminution de l'expression de COX-2 sans pour autant affecter l'expression de COX-1. En conclusion, ces résultats révèlent un nouveau mécanisme d'action des préparations de VA, dans laquelle les extraits de VA exercent un effet anti-inflammatoire en réprimant l'expression de la PGE2 induite par les cytokines via l'inhibition sélective de COX-2.

Article 2- Résumé en Français

Titre: L'inhibition de PGE2 et de COX-2 médiée par les extraits de *Viscum album* impliquent la déstabilisation des ARNm de COX-2

L'utilisation extensive des préparations de *Viscum album* (VA) comme traitement complémentaire des cancers ainsi que dans plusieurs autres pathologies humaines a permis la multiplication des approches cellulaires et moléculaires étudiant les mécanismes d'action des préparations de VA. En utilisant un modèle cellulaire d'adénocarcinome de poumon humain, nous avons récemment démontré que les préparations de VA exercent un effet anti-

inflammatoire puissant en diminuant spécifiquement la sécrétion de prostaglandine E2 (PGE2) et l'expression de COX-2, l'une des signatures moléculaires majeure des réactions inflammatoires induite par les cytokines.

De façon surprenante, nous avons observé dans cette étude une diminution significative de l'expression de la protéine COX-2 dans les cellules traitées avec les extraits de VA sans modification des niveaux d'ARNm de COX-2. De plus, l'inhibition de COX-2 médiée par les extraits de VA n'a été observée que dans les premières phases du processus inflammatoire. Nous avons donc émis l'hypothèse que les extraits de VA induisent une déstabilisation de l'ARNm de COX-2, réduisant ainsi la disponibilité des ARNm de COX-2 pour sa synthèse protéique et donc la sécrétion subséquente de PGE2.

Pour évaluer cette hypothèse, nous avons bloqués l'activité transcriptionelle de cellules A549 traitées par l'IL-1β avec de l'actinomycine D et nous avons déterminé la demi-vie des ARNm de COX-2 avec ou sans traitement par des extraits de VA. Les taux d'ARNm de COX-2 ont été analysés à différents temps. Nous avons observé une réduction importante de la demi-vie des ARNm de COX-2 due à sa dégradation rapide dans les cellules traitées avec les extraits de VA par rapport aux cellules non traitées. Ces résultats montrent que l'inhibition de PGE2 médiée par les extraits de VA impliquent la déstabilisation des ARNm de COX-2.

Viscum album-mediated PGE2 and COX-2 inhibition implicates COX-2 mRNA destabilisation

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Abstract

Extensive use of *Viscum album* (VA) preparations in the complementary therapy of cancer and in several other human pathologies has led to the increasing number of cellular and molecular approaches to explore the mechanisms of action of *VA*. By using a cellular model of human lung adenocarcinoma, we have recently demonstrated that, VA preparations exert a potent anti-inflammatory effect by selectively down-regulating the COX-2-mediated cytokine-induced secretion of prostaglandin E2 (PGE2), one of the important molecular signatures of inflammatory reactions.

Interestingly in this investigation we observed a significant down-regulation of COX-2 protein expression in VA-treated cells whereas COX-2 mRNA levels were unaltered. In addition to this, VA-mediated COX-2 inhibition was observed only at the early phases of inflammatory process. Therefore we hypothesised that VA induces destabilisation of COX-2 mRNA, thereby depleting the available functional COX-2 mRNA for the protein synthesis and for the subsequent secretion of PGE2.

To address this question, we analysed the mRNA half-life of COX-2 following transcriptional blockade by actinomycin D in IL-1β-treated A549 cells with or without VA treatment. COX-2 mRNA levels are analysed at different time points. We observed a marked reduction in the half life of COX-2 mRNA due to its rapid degradation in the cells treated with VA compared to that in IL-1β-stimulated cells. These results thus demonstrate that VA-mediated inhibition of PGE2 implicates destabilization of COX-2 mRNA.

Key words: *Viscum album,* anti-inflammatory effect, cyclo-oxygenases, post-transcriptional modification, mRNA stability

Introduction

Cyclo-oxygenase (COX-2) is an early response protein, up-regulated during many pathological conditions and human malignancies. It is over expressed in the most of the cells upon stimulation with diverse pro-inflammatory stimuli such as pro-inflammatory cytokines, chemokines, infectious agents, bacterial lipopolysaccharide etc. COX-2 is a critical enzyme required for the biosynthesis of prostaglandin E2, one of the important molecular mediators of inflammation from cellular arachidonic acid [1]. Two other COX isoenzymes, COX-1 and COX-3, catalyze the same kind of reaction. COX-1 is another important cyclo-oxygenase family member, which is constitutively expressed in cells and tissues. Exact functions need to be still established for COX-3, which will be expressed only in some specific compartments including brain and spinal cord [2],[3]. The mode of expression of COX-1 vs. COX-2 further regulates their differential functions. COX-1 is constitutively and stably expressed at low levels in many tissues. This ensures a constant production of prostaglandins, which are essentially required for the maintenance of important physiological functions, such as platelet aggregation, normal renal functions and gastric mucosal protection. On contrary, COX-2 is mostly silent but the expression can be induced in response to a diverse pro-inflammatory and pathogenic stimuli. When stimulated, its expression is high and transient which leads to a burst of PG production in a regulated time-limited manner [4]. Thus, depending on the COX isoform, the production of the same precursor PGH2 from arachidonic acid differs with respect to the amount and timing of production. This can be differentially decoded by the cells, thereby leading to the activation of various intracellular pathways involving specific classes of prostaglandins and therefore, different responses [5].

In view of the up-regulated COX-2 expression during many pathological conditions and human malignancies, strategies controlling the expression and activity of COX-2 have been developed as potential anti-tumor and anti-inflammatory therapeutics [6-10]. In line with the

therapeutic benefit of Non Steroid Anti-inflammatory Drugs (NSAID) which are synthetically designed mainly to inhibit the enzymatic activity of COX-2, a diverse therapeutics of natural origin such as phytotherapeutics have been characterised to inhibit the COX-2 functioning thereby down-regulating the pathological level of prostaglandins. Considering the severe side effects of NSAID due to the simultaneous inhibition of COX-1 activity which is required to maintain the physiological level of prostaglandins, along with the COX-2 activity due the homology in the two proteins, selective COX-2 inhibitors are of all time interest. Although, a promising class of synthetic COX-2 selective inhibitors called COXIBS have been developed lately, the therapeutic efficacy is still compromised due to various side effects [11],[12]. Several phytotherapeutics have been shown to exert therapeutic benefit via selective inhibition of COX-2 functioning by interfering with the expression and regulatory mechanisms of COX-2 in order to inhibit its functioning [13], [14].

Viscum album (VA) preparations commonly called as mistletoe extracts, are extensively used in the complementary therapy in cancer and also in the treatment of several inflammatory pathologies [15-19],Despite their successful therapeutic application for several years, the underlying mechanisms are not yet clearly understood. Several lines of evidence have revealed that these preparations exert anti-tumor activities which involve the cytotoxic properties, induction of apoptosis, inhibition of angiogenesis and several other immunomodulatory and anti-inflammatory mechanisms [20-30]. These properties collectively define the mechanistic basis for the therapeutic benefit of Viscum preparations. Recently it is shown that, VA preparations exert a potent anti-inflammatory effect by selectively down-regulating the COX-2-mediated cytokine-induced secretion of prostaglandin E2 (PGE2), one of the important molecular signatures of inflammatory reactions [31]. However the molecular mechanisms associated with the Viscum-mediated COX-2 inhibition are not clearly understood yet. *Viscum album* preparations are shown to inhibit the COX-2 protein expression

without modulating its expression at mRNA level suggesting that there may be a possible action of VA on post transcriptional events of COX-2 regulation. In view of the fact that there are several molecules and phytotherapeutics which are known to interfere with the post-transcriptional and post-translation regulation of COX-2 in order to inhibit the COX-2 expression and subsequent reduction in the PGE2 [32-34], in the current study, we investigated the post-transcriptional and post-translational regulation of COX-2 stability by VA preparations.

Materials and methods

Viscum album preparations

VA Qu Spez was a kind gift from Weleda AG (Arlesheim, Switzerland). VA Qu Spez is a therapeutic preparation of *Viscum album* growing on oak trees and is obtained as an isotonic solution of 10mg/ml formulated in 0.9% NaCl. It is free from endotoxin and contains the standardized levels of mistletoe lectins.

Culture of A549 cells

Human lung adenocarcinoma cell line A549 was a kind gift from Dr. Maria Castedo-Delrieu, Institute Gustave Roussy, Villejuif, France. A549 cells were grown in 75 cm^2 culture flasks in Dulbecco's modified Eagle's medium (DMEM) F-12, GIBCO®, BRL Life Technologies, Grand Island, NY, USA)) supplemented with 10% fetal calf serum and 50 U/ml penicillin and 50 µg/ml of streptomycin (GIBCO®, BRL, Cergy Pontoise, France). Cells are incubated at 37^0 C with 5% CO_2 in humidified atmosphere to obtain the cells of about 80-90% confluence and used for all experiments.

Induction of COX-2 and treatment with different VA preparations

Cells grown in complete medium (DMEM with 10%FCS) were harvested by tripsinisation using 0.5% trypsin (Biological Industries, Kibbutz Beit Haemek, Israel) and are seeded in 12 well culture plates (0.5×10^6 cells per well), and incubated at 37° C overnight. Wells containing the adherent A549 were then replenished with the complete medium containing recombinant human IL-1β (10 ng/ml) (Immuno Tools, Friesoythe, Germany) in the presence and absence of different VA preparations and incubated for 18 hours with appropriate duration of VA treatment, at 37° C and 5% CO_2. After 18 hours of incubation cells were harvested by tripsinisation and used for the analysis of COX-2 mRNA by RT-PCR and COX-1/COX-2 protein by flow cytometry

Analysis of COX-2 protein half life by cyclohexamide pulse chase experiment

A549 cells with an appropriate confluency were treated with IL-1β for 18 hours in the presence or absence of VA Q Spez. After 18 hours 10µg/ml of cyclohexamide (Sigma-Aldrich, Lyon), France was added to the cells and cells are harvested by tripsinisation at indicated time points. Expression of remaining COX-2 protein was analysed by intracellular labelling and analysing by flow cytometry and also by western blotting.

Analysis of COX-2 mRNA half life by Actinomycin D pulse chase experiment

A549 cells with an appropriate confluency were treated with IL-1β for 4 hours in the presence or absence of VA Q Spez. After 4 hours 10µg/ml of actinomycin D (Sigma-Aldrich, Lyon), was added to the cells and cells are harvested by tripsinisation at indicated time points. Expression of remaining COX-2 mRNA was analysed by RT-PCR.

Results

Prophylactic treatment of *Viscum album* inhibits the cytokine-induced COX-2 expression but not when the cells are treated with VA at later stages

Since VA inhibited cytokine-induced COX-2 expression, we tried to dissect the window of efficient inhibition by *Viscum album.* Human lung adenocarcinoma (A549) cells were stimulated with IL-1β for 18 hours in the presence or absence of VA Qu Spez, a therapeutic preparation of *Viscum album* that grows on oak trees. *Viscum album* is added to the cells either from the beginning of the experiment (Prophylactic) or after 14 hours of IL-1β induction. Flow cytometric analysis of intracellular COX-2 expression has demonstrated that, *Viscum album* inhibits cytokine induced COX-2 expression only when it is added as a co-treatment with IL-1β but not when it was added after 14 hours (Figure 1). This suggests that, VA-mediated COX-2 inhibition occurs at the early phases of inflammatory process and opens up more exploratory avenues to dissect the regulatory mechanisms of COX-2 mediated by VA at the early phase of inflammation.

Viscum album does not modulate the stability of COX-2 protein

In order to address the effect of *Viscum album* on the molecular stability of COX-2 which could be a potential contributing factor for the observed reduction in COX-2 protein expression, we analysed the stability of COX-2 protein. A549 cells were stimulated with a pro-inflammatory cytokine IL-1β in the presence and absence of VA Qu Spez, for 18 hours. Cells were harvested at different time intervals after blocking the protein synthesis by treating the cells with cyclohexamide. Total cellular protein is extracted for the estimation of COX-2. Western blot analysis and flow cytometric analysis of COX-2 protein have revealed that, there is no considerable difference in the protein degradation profile of COX-2 in VA treated and untreated cells (Figure 2). This suggests that VA has no impact on regulating the protein

stability of COX-2. This may indicate that the regulation COX-2 by VA may occur in an early phase of COX-2 expression but not at the later stages of protein expression and stabilisation.

Viscum album increases the mRNA degradation of cox-2

Due to the indication of interference of *Viscum album* in the early inflammatory responses, we analysed the mRNA stability of cox-2 modulated by VA. Human lung adenocarcinoma (A549) cells were stimulated with a pro-inflammatory cytokine IL-1β in the presence and absence of VA Qu Spez for 4 hours. After 4 hours of IL-1β stimulation cells are blocked with actinomycin D (10μg/ml). Cells were harvested at different time intervals after adding Actinomycin D and total cellular RNA was isolated and used for RT-PCR for the estimation of cox-2 mRNA. Treatment with IL-1β is known to induce the expression of cox-2 mRNA by transcriptional activation and also by increasing the stability of cox-2 mRNA. RT-PCR analysis of cox-2 mRNA expression at different time intervals after Actinomycin D treatment revealed that, at any given time interval the relative expression of cox-2 mRNA is lower in VA treated cells compared to the cells treated with IL-1β (Figure 3A). This suggests that VA increases the rate at which the cox-2 mRNA is degrading when there is no new mRNA synthesis is taking place. Further, results from RT-PCR analysis have also showed cox-2 mRNA half life, time required for 50% of the mRNA degradation in case of VA treated cells was reduced more than two fold compared to that in case of cells stimulated with cytokine alone (Figure 3B). This suggests that VA is reduces the mRNA half life of cox-2 there by leading to its reduced bioavailability for the protein synthesis.

Discussion

Prolonged administration of COX-2 inhibitors has been ineffectual for chemopreventive and chemotherapeutic purposes since the risks prevail over the benefits. Clinical demonstration of severe side effects due to the failure of the classical COX-2 inhibitors to discriminate

between an aberrant pathological vs. homeostatic functional activation state, raised the concern that direct COX-2 enzymatic inhibition might not sufficiently represent an appropriate clinical strategy to target COX-2. Since in contrast to COX-1, COX-2 is an early response gene, similar to the genes encoded for cytokines, chemokines and proto-oncogenes, they can be regulated under several mechanisms of expression and modulation, ranging from direct transcriptional effects to post-transcriptional and post-translational levels and also indirectly by various transcription factors that mediate the stability [32] ,[35]. Such multiple levels of modulation of COX-2 expression imply the existence of multiple mechanisms, which may be targeted to finely modulate COX-2 functions [36-38]. Several phytotherapeutics have been shown to exert modulatory effect on COX-2 at various levels of its molecular regulation and therefore have been considered as a potent alternative strategy to control the pathogenic expression of COX-2 [39],[33],[40]. Given that *Viscum album* preparations exert a potent anti-inflammatory effect by selective down regulation of COX-2, it is extremely interesting to dissect the COX-2 inhibition mediated by VA at molecular level. The fact that, pre-conditioning the cells with VA before the cytokine stimulation and prophylactic treatment of VA as co-treatment along with cytokine stimulation, inhibits COX-2 expression, however treatment with VA at the later phases of cytokine induction doesn't inhibit suggests that, inhibition of COX-2 by VA occurs in the early stage of pro-inflammatory cytokine stimulation, but not at the later phases (Figure 1). In order to dissect the molecular events of COX-2 regulation we analysed the protein stability of COX-2 in presence of VA by cyclohexamide pulse chase experiments. Results from these experiments clearly showed that there is no considerable difference in the COX-2 protein degradation profile of cytokine stimulated cells with or without *Viscum album.* Therefore it is clear that COX-2 protein stability or protein half life is not affected by VA. Further, reduced level of COX-2 expression at 0 hour in this experiment also suggests that, there may be modulation by

VA to the COX-2 expression before we added the inhibitor of protein synthesis. Significant inhibition of COX-2 protein expression by VA without modulating its stability strongly indicates that, there is a possible modulation by VA at an early stage than the proteins were expressed. However the mRNA of COX-2 was not affected by VA and therefore we analysed the mRNA stability of cox-2 by Actinomycin D pulse chase experiments. We observed that there is a clear reduction in the mRNA half life of cox-2 when cells are treated with VA suggesting that, VA induces destabilisation of COX-2 mRNA, thereby depleting the available functional mRNA for the protein synthesis and for the subsequent secretion of PGE2.

Although this study clearly indicates the post-transcriptional regulation of cox-2 mRNA stability by VA preparations as a possible mechanism for VA-mediated COX-2 inhibition further molecular dissection is required in order to clearly understand the regulatory events of COX-2 regulation, contributing factors and their modulation by *Viscum album* preparations.

Conclusion

Increasing body of evidence for anti-inflammatory activity of plant-derived molecules by modulating the COX-2 functions has evolved as a potent alternative strategy for the conception of novel therapeutic molecules in the treatment of various inflammatory pathologies and in malignancies. In view of the therapeutic benefit of Viscum preparations in diverse pathological situations including inflammatory and cancer conditions, dissecting their molecular mechanisms would contribute enormously to the understanding of role phytotherapy based treatment strategies either in complementary or alternative medicine or in other combinational therapies.

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Figure legends:

Figure1. Inhibition of cytokine-induced COX-2 expression by prophylactic treatment of *Viscum album*: A549 cells were treated with IL-1β (10 ng/ml) and two different increasing concentrations of *Viscum album* Q Spez preparation for 18 hours. Cytosolic COX-2 expression was measured by western blotting and flow cytometric analyses. *Viscum album* is added to the cells either from the beginning of the experiment along with IL-1β (co-treatment) or after 14 hours of IL-1β induction. Normalised percentage COX-2 expression as measured in intracellular staining by flow cytometry (A) and mean fluorescence intensity (MFI) of COX-2 expression (B) are shown.

Figure 2: Effect of *Viscum album* on the stability of COX-2 protein: A549 cells were stimulated with IL-1β in the presence and absence of VA Qu Spez, for 18 hours. 10μg/ml of cyclohexamide was added to inhibit the protein synthesis. Cells were harvested at different time intervals after blocking the protein synthesis. COX-2 expression was measured by intracellular staining in flow cytometry and using the cytosolic extracts by western blot. Normalised percentage COX-2 expression as measured in intracellular staining by flow cytometry (A) and mean fluorescence intensity (MFI) of COX-2 expression (B) are shown. (C), a representative western blot, showing the COX-2 expression after cyclohexamide treatment with or without *Viscum album*.

Figure 3: Increase in the cox-2 mRNA degradation by *Viscum album* treatment: A549 cells were stimulated with a pro-inflammatory cytokine IL-1β in the presence and absence of VA Qu Spez for 4 hours. After 4 hours of IL-1β stimulation cells are blocked with actinomycin D (10μg/ml). Cells were harvested at different time intervals after adding

Actinomycin D and total cellular RNA was isolated and used for RT-PCR for the estimation of cox-2 mRNA. Relative expression of remaining COX-2 mRNA at each time point, in VA treated and untreated cells (A) and the time required for 50% of the mRNA degradation is shown as cox-2 mRNA half life (B).

Figure1.

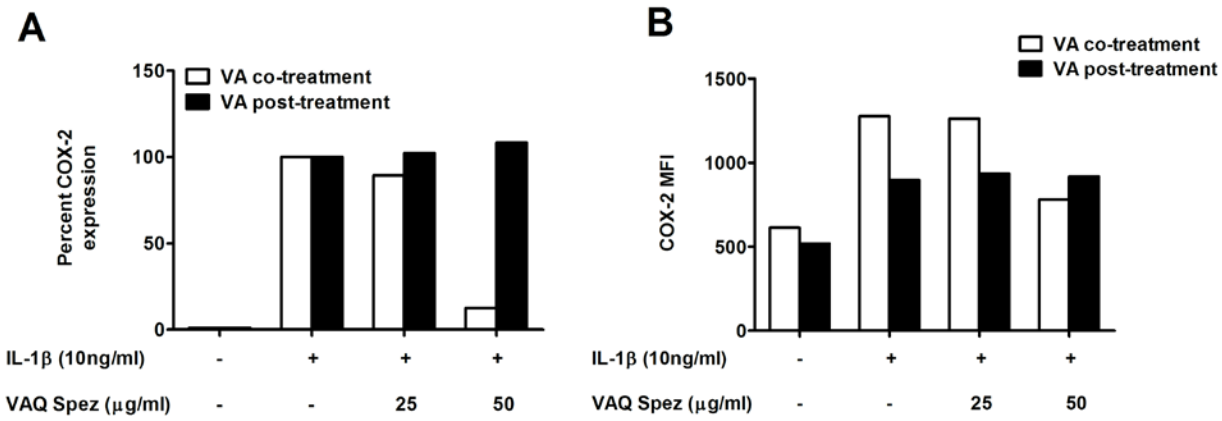
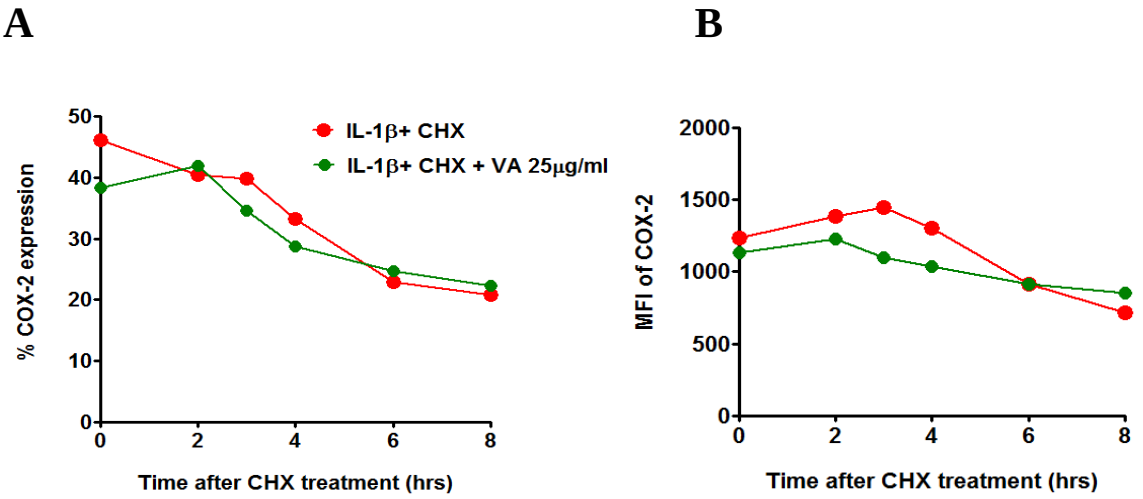


Figure 2.



C

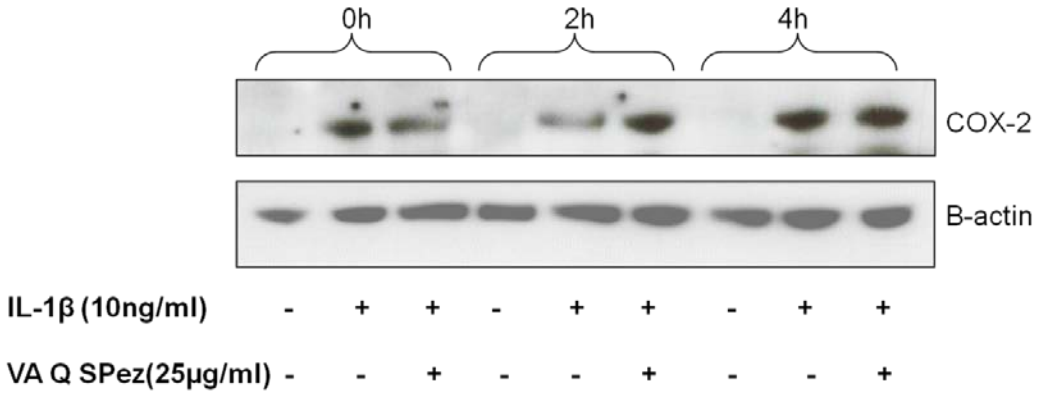
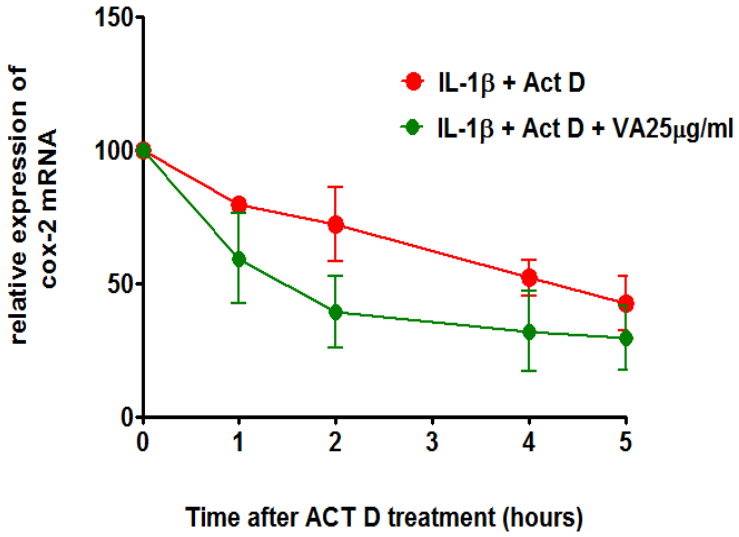
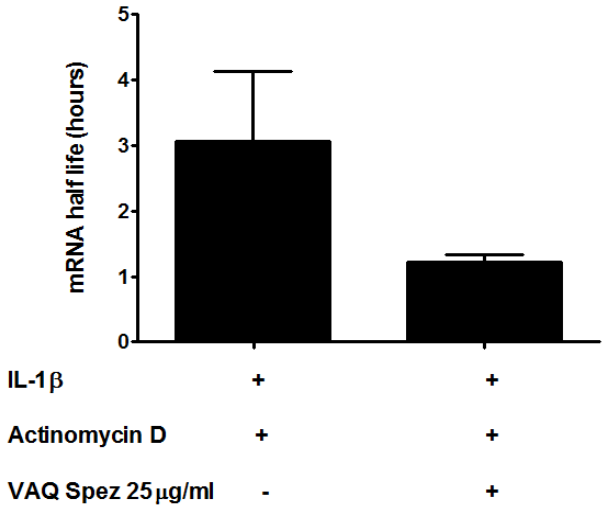

0h
2h
4h
COX-2
B-actin
IL-1β (10ng/ml) - + + - + + - + +
VA Q SPez(25µg/ml) - - + - - + - - +

Figure 3.

A


relative expression of cox-2 mRNA
150
100
50
0
0
1
2
3
4
5
IL-1β + Act D
IL-1β + Act D + VA25µg/ml
Time after ACT D treatment (hours)

B


mRNA half life (hours)
5
4
3
2
1
0
IL-1β + +
Actinomycin D + +
VAQ Spez 25µg/ml - +

Article 3- Résumé en Français

Titre: Etude comparative des propriétés anti-inflammatoires de différentes préparations de *Viscum album*: mistletoe lectin (lectine du gui) comme une molécule clé responsable de l'inhibition de la pro-inflammatoire COX-2

Les différentes préparations de *Viscum album* sont largement utilisées dans la thérapie auxiliaire du cancer impliquant leurs propriétés anti-tumorales, immunmodulatrices et anti inflammatoires. Bien que la composition des différentes préparations soit hétérogène (selon l'arbre sélectionné et la méthode de préparation), ces produits possèdent les mêmes effets bénéfiques thérapeutiques dans plusieurs pathologies. Néanmoins, les molécules impliquées dans ces propriétés ne sont pas identifiées.

Nous avons précédemment montré que la préparation *Viscum album* Q Spez (qui pousse sur les chênes) exerce un effet anti-inflammatoire impliquant l'inhibition de la voie de COX-2 et PGE2 induite par les cytokines. Dans la présente étude, nous avons comparé les propriétés anti inflammatoires de 3 préparations dérivées des chênes, du pin ou du pommier sur l'inhibition des voies de COX2 et PGE2 dans les cellules épithéliales issues d'adénocarcinome du poumon.

Les résultats montrent que les préparations de Viscum dérivées des chênes et des pommiers inhibent l'expression de COX-2 induites par des cytokines pro-inflammatoires. En revanche, les préparations obtenues à partir du pin ne montrent aucune inhibition de l'expression de la voie de COX-2 dans notre modèle cellulaire. De plus, l'inhibition de COX-2 est associée à la présence d'une lectine : la lectine de gui. Considérés dans leur ensemble, ces résultats montrent que l'effet anti-inflammatoire observé est corrélé à la teneur des préparations de *viscum album* en lectine de gui.

Comparative analysis of anti-inflammatory properties of different *Viscum album* preparations: mistletoe lectin as a key molecule responsible for COX-2 inhibition

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Abstract

Viscum album preparations have been successfully used in the complementary therapy of various human malignancies and are shown to be beneficial via several by mutually non exclusive mechanisms such as anti-tumor mechanisms, immunomodulatory mechanisms and anti-inflammatory mechanisms. In spite of a heterogeneous composition and diverse mechanisms of action (due to the host tree and the method of preparation), different preparations are equally beneficial in various pathological condition. But the precise role of various components in these preparations is not yet clear. Given the anti-inflammatory role of *Viscum album* Q Spez (growing on oak trees) in inhibiting the cytokine-induced COX-2 and PGE2 in the experimental model of human lung adenocarcinoma, we are interested to dissect the anti-inflammatory properties of different *Viscum album* preparations derived from different host trees. In this study we demonstrate that Viscum preparations derived from those growing on oak trees and apple trees inhibit the COX-2 expression under pro-inflammatory conditions. However Preparations obtained from those growing on pine trees do not inhibit COX-2 expression. Interestingly we also found that, the COX-2 inhibitory effect in these preparations directly correlates to the presence of mistletoe lectin. Together these results demonstrate one of the important anti-inflammatory properties which can be strongly associated with their mistletoe content.

Key words: *Viscum album*, mistletoe lectin, anti-inflammatory effect, cyclo-oxygenases

Introduction

Inflammation is a co-ordinated cross-talk between innate and adaptive immune system, in order to combat infection and also for the physiological wound healing. However when left unchecked, inflammation can lead to a severe pathological condition including, autoimmune and inflammatory disorders, chronic infections and cancer [1]. Various therapeutic strategies have been developed in order to reduce inflammation and pain, including the treatment with cytokine neutralizing antibodies, molecular antagonists, intravenous immunoglobulin, corticosteroids, non-steroid anti-inflammatory drugs (NSAID) and several others[2-5]. In addition to these well known anti-inflammatory therapeutic strategies, treatment with various phytotherapeutics has also contributed enormously to control inflammation and pain, associated with many of the severe inflammatory disorders and in cancer [6-11].

Therapeutic preparations of *Viscum album* constitute one of the widely used complementary and alternative cancer therapies in Europe. These preparations are standardised whole plant extracts of *Viscum album* L., obtained by various ferment procedures. As a result they are heterogeneous, complex mixtures of various biologically active molecules like mistletoe lectins, viscotoxins, polysaccharides, alkaloids, lipids, phytosterols, triterpines, flavonoids, phenylpropanes, minerals, several peptides and enzymes[12]. Mistletoe lectins constitute considerably good amount of these preparations and are shown to be important for many of their biological mechanisms[13].

Mistletoe lectins (ML) I, II and III are cytotoxic glycoproteins of ribosome-inactivating protein (RIP) family of type II, such as ricin and abrin. They are composed of an N-glycosidase (A chain) and a galactoside-recognizing lectin (B-chain) connected by a disulfide bridge. These proteins are known to inhibit the protein synthesis at the ribosomal level. In a variety of preclinical studies and several *in vivo* experiments of transplantatable tumor

models, treatment with whole plant VA extracts or purified ML has been associated with significant tumor regression [14-16]. In many of the clinical studies, adjuvant therapy with mistletoe extracts has revealed a beneficial impact in reducing the side effects of conventional cancer therapies such as chemotherapy and radiotherapy, thereby improving the quality of life of the patients [17].

Despite the successful therapeutic application of mistletoe preparations, their mechanisms of actions are still partially understood. Several mutually non-exclusive mechanisms have been proposed such as induction of apoptosis and cytotoxicity in cancer cells and inhibition of angiogenesis [18-21]. They are shown to interact with the cells of the immune system and bring about several immunomodulatory effects which are beneficial to potentiate the anti-tumor immunity[22-27]. However the precise mechanisms underlying the therapeutic benefit of these preparations in a diverse pathological conditions and inflammatory pathologies are not yet clearly understood.

In view of the fact that there are several phytotherapeutics which exert both anti-tumor and anti-cancer properties[28-32], recently we have demonstrated that, extracts of *Viscum album* VA Q Spez (that grows on oak trees) inhibits cytokine-induced PGE2 secretion by selectively inhibiting the COX-2 expression[33]. This suggested novel anti-inflammatory mechanisms of action of *Viscum album* preparations where, in addition to being efficient anti-tumor therapeutics they can also act as a potent anti-inflammatory therapeutics by selective COX-2 inhibition. Considering the long term side effects of NSAID in inflammatory conditions phytotherapeutics like *Viscum album* which will not affect the physiological inflammation are of greater interest. Therefore it is extremely encouraging to conceive such phytotherapeutics as potent alternatives to classical anti-inflammatory drugs [34]. In view of the increasing body of evidence for anti-inflammatory properties of phytotherapeutics, in this study we

investigated the anti-inflammatory properties of different Viscum preparations originated from different host plants, in terms of COX-2 inhibition.

Since biological properties of Viscum preparations are variable depending on the host tree and the method of preparations and also to some extent on the time of plant collection, not necessarily all the preparations exert same anti-inflammatory effect. Therefore it is extremely interesting to understand the properties of different preparations and the molecular reasons behind their biological properties.

Materials and methods

***Viscum album* preparations**

VA Qu Spez, VA P and VA M Spez were kindly gifted from Weleda AG (Arlesheim, Switzerland). These are therapeutic preparation of *Viscum album* growing on oak, pine and apple trees respectively and are obtained as an isotonic solution of 10mg/ml formulated in 0.9% NaCl. It is free from endotoxin and contains the standardized levels of mistletoe lectins and viscotoxins. For the comparative analysis of COX-2 expression, preparations are collected from three different batches (named as 1,2 3 for each preparations) of manufacture. Description of mistletoe lectin and viscotoxin content in preparations of different batches is given in Table1.

Table 3: Mistletoe lectin and viscotoxin contents of three commonly available VA preparations

Type of preparation	Mistletoe lectin	Viscotoxin
VA Q Spez	785 ng/ml	5 µg/ml
VA P	28 ng/ml	6 µg/ml
VA M Spez	548 ng/ml	4 µg/ml
VA A	23 ng/ml	19 µg/ml

Culture of A549 cells

Human lung adenocarcinoma cell line A549 was a kind gift from Dr. Maria Castedo-Delrieu, Institute Gustave Roussy, Villejuif, France. A549 cells were grown in 75 cm^2 culture flasks in Dulbecco's modified Eagle's medium (DMEM) F-12, GIBCO®, BRL Life Technologies, Grand Island, NY, USA)) supplemented with 10% fetal calf serum and 50 U/ml penicillin and 50 µg/ml of streptomycin (GIBCO®, BRL, Cergy Pontoise, France). Cells are incubated at 37^0 C with 5% CO_2 in humidified atmosphere to obtain the cells of about 80-90% confluence and used for all experiments.

Induction of COX-2 and treatment with different VA preparations

Cells grown in complete medium (DMEM with 10%FCS) were harvested by tripsinisation using 0.5% trypsin (Biological Industries, Kibbutz Beit Haemek, Israel) and are seeded in 12 well culture plates (0.5×10^6 cells per well), and incubated at 37° C overnight. Wells containing the adherent A549 were then replenished with the complete medium containing recombinant human IL-1β (10 ng/ml) (Immuno Tools, Friesoythe, Germany) in the presence and absence of different VA preparations and incubated for 18 hours at 37° C and 5% CO_2. After 18 hours of incubation cells were harvested by tripsinisation and used for the analysis of COX-2 by western blotting and COX-1/COX-2 by flow cytometry.

Immunoblotting of COX-2

Following the appropriate treatment, cells were harvested by a mild tripsinisation and washed with 1x phosphate buffered saline. Cells were lysed using lysis buffer containing 50 mM Tris-HCl (pH7.4), 0.25% sodium deoxycholate, 150mM NaCl, 1mM EDTA, 1% NP-40, 1 mM PMSF and 1x protease inhibitor cock-tail (Sigma-Aldrich, Lyon, France). Cells were suspended in the lysis buffer (100µl/million cells) and incubated on ice for 30 mins. Supernatants were collected following the centrifugation at 13200 rpm for 20 mins. Total cellular protein is estimated by Bradford method and 20µg of each sample is loaded on SDS

polyacrylamide gel and subjected for electrophoretic migration. Proteins separated on gel were then transferred on to an activated PVDF membrane by wet transfer (45 mins at 75 V). Non-specific binding of antibodies is blocked by treating the membrane with 5% non fat milk in tris buffered saline with tween 20 (TBST-20mM Tris- HCl (pH 7.4), 137mM NaCl and 0.1% Tween 20) for 2 hours at room temperature. Membranes were incubated with primary antibodies diluted according to the manufactures' instructions in 5% BSA overnight at 4° C. Following the three washes with TBST, Blots were then treated with HRP-conjugated secondary antibodies in 5% BSA (1/4000) for 2 hours at room temperature. Primary antibodies against human COX-2, β-Actin, and the HRP labelled secondary antibodies were procured from Cell Signalling Technology (Ozyme, France). Blots were washed well with TBST with minimum three changes for an hour and then revealed using ECL plus western lightening reagent (Perkin Elmer, Waltham, MA, USA) according to manufactures' instructions.

Flow cytometry analysis of COX-1 and COX-2

Upon treatment with cytokine and VA for 18 hours, A549 cells were harvested by tripsinisation. Cells were fixed with 1% para formaldehyde for 15 minute at room temperature and then *permiabilised and* stained with FITC conjugated monoclonal antibody to COX-1 and PE conjugated mAb to COX-2. Ten thousand cells were acquired for each sample, and the data were processed by using FACS DIVA software (BD Biosciences).

Statistical analysis

Densitometric analysis of the immunoblots was performed using BIO-1D analysis software. Densitometric values were expressed as arbitrary units. All the observations are expressed as Mean±SEM of 3-4 experiments. Graphpad Prism 5.0 is used for all the statistical analysis.

Results

Viscum album preparations differentially modulate cytokine-induced COX-2 expression.

Since we had observed earlier that VA Q Spez selectively inhibits cytokine-induced COX-2 expression, we investigated the effect of VA P (that grows on pine trees), and VA M Spez (that grows on apple trees) on cytokine induced COX-2 expression. A549 cells were treated with IL-1β followed by the treatment with different VA preparations for 18 hours. Cells were harvested by tripsinisation and used for the estimation of COX-2 by immunoblotting.

Figure 1A depicts a representative blot of the expression pattern of COX-2 in different culture conditions with VA Q Spez headed by the histograms of densitometric values from 3 independent experiments, expressed in arbitrary units. While untreated cells did not express detectable COX-2 protein, treatment with IL-1β significantly up-regulated the expression of COX-2. Upon treatment with VA Q Spez, we observed a concentration dependant inhibition in the COX-2 expression induced by IL-1β. Interestingly, cells treated with VA P extract did not inhibit the COX-2 expression induced by IL-1β even at the highest concentration used in the study (Figure 1B). Viscum preparation derived from those that grow on apple trees, inhibited COX-2 expression (Figure 1C) as observed in case of VA Q Spez (Figure 1A).

VA Q Spez and VA M Spez but not VA P selectively inhibits cytokine-induced COX-2

Because of the potential therapeutic efficacy with fewer side effects, COX-2 selective inhibitors gained more importance than the COX-1 inhibitors or the dual inhibitors. Since we had demonstrated earlier that VA Q Spez selectively inhibits cytokine-induced COX-2, we were curious to analyse the effect of other VA preparations on the COX-1/COX-2 expression. COX-1/COX-2 expression was measured by flow cytometry, in A549 cells treated with IL-1β

and different VA preparations for 18 hours. Figure 2A depicts the percentage inhibition of COX-2 expression, with the COX-2 expression in case of IL-1β stimulated cells being normalised to 100, as measured by flow cytometry in terms of percent positive cells. While VA Q Spez and VA M Spez show relatively more COX-2 inhibition, VA P does not significantly inhibit COX-2 compared the other two. When we analysed the expression of COX-1 which is a constitutively expressed and is known to be important homeostatic functions, we observed that none of the three preparations used in the study inhibited COX-1(Figure 2B). Indeed, COX-1 expression is not induced by treatment with IL-1β. Figure 2C is a representative dot plot of a flow cytometric analysis of COX-1 and COX-2. While untreated cells are almost negative to COX-2 expression (0.2%), upon treatment with IL-1β, nearly 90% of the cells express COX-2 with high intensity. This cytokine-induced COX-2 expression is inhibited nearly to half by VA Q Spez (57.8%), and 72.7% by VA M Spez. However VA P did not inhibit the expression of COX-2 (88.6%).

Anti-inflammatory property of *Viscum album* is a specific property of each preparation and not variable from batch to batch

The properties of Viscum preparations are known to be dependent on the host plant, method of preparation and also the time of harvesting. Hence, there may be a minor degree of variation in their composition, which may lead to a drastic change in their biological properties In order to verify whether the differential effect of Viscum preparations on COX-2 inhibition is batch dependant or it is a specific property, we analysed the COX-2 inhibitory effect of Viscum preparations from three different batches of manufacture.A549 cells were treated with IL-1β and VA preparations from different batches for 18 hours. Then we analysed the intracellular expression of COX-2 by flow cytometry. We observed that, VA Q Spez preparations from all the three batches tested, inhibited IL-1β induced COX-2

expression (Figure 3A). However there is a minor degree of variation in the preparations from batch to batch with respect to COX-2 inhibition. Interestingly, none of the VA P preparations from any of the tested batches inhibited COX-2 expression induced by IL-1β (Figure 3B). However, only M Spez preparation inhibited COX-2 expression, while non-special preparations of VA M did not inhibit the COX-2 expression (Figure 3C).

COX-2 inhibitory effect of *Viscum album* correlates with the amount of mistletoe lectins but not that of Viscotoxins

Treatment with isolated mistletoe lectin is shown to be beneficial in a variety of experimental results and clinical studies [14]. Because, mistletoe lectin and viscotoxin are the main quantitatively characterized components of therapeutic preparations of *Viscum album*, and in view of the fact that several immunomodulatory properties of Viscum extracts have been shown to be correlated to the amount of mistletoe lectin present in them, we analysed the correlation of mistletoe lectin content and viscotoxin content to the ability of those preparations to inhibit cytokine induced COX-2 (Figure 4A). Interestingly we observed a strong correlation between COX-2 inhibitory effect and amount of mistletoe lectin. However there was no significant correlation between COX-2 inhibition and viscotoxin (Figure 4B).

Discussion

Cyclo-oxygenases play a very important and regulatory role in the biosynthesis PGE2, one of the molecular signatures of inflammatory immune response. While COX-1 is expressed constitutively and mediates the homeostatic functions, COX-2 is induced upon stimulation with pro-inflammatory stimuli[35]. Increase in the biosynthesis and secretion of PGE2 is known to be associated with the over expression of COX-2. Several pro-inflammatory conditions, chronic inflammations also many of the malignant conditions are known to be

associated with the over expression of COX-2[36-38]. Therefore therapeutic strategies to inhibit COX-2 will be used as anti-inflammatory therapies in many of the pathological conditions [39-41]. However homology between COX-1 and COX-2 leads to the simultaneous inhibition of COX-1 along with COX-2 in many of the cases which will disturb the physiological PGE2 repertoire eventually resulting in long term complications and side effects related to gastrointestinal tract[42-46]. This is the reason why COX-2 selective inhibitors came out as attractive anti-inflammatory therapeutic.

Our results demonstrate that Viscum preparations exert anti-inflammatory effect by selectively inhibiting cytokine induced COX-2 expression. Since therapeutic preparations of *Viscum album* are different with respect to their origin of host plant and the method of preparations, there may be a small degree of heterogeneity in their composition which may lead to their differential mechanisms of action at any given modulatory axis [47]. In spite of the heterogeneity at the level of composition and mechanisms of action, all preparations are equally beneficial when they are used in various therapeutic conditions. Several lines of evidence from experimental and clinical studies have revealed that various preparations of *Viscum album* are beneficial in complementary and alternative medicine. However there may several differences in their mechanisms of action. Hence dissecting the mechanisms of action of various preparations and understanding the relative differences would help us to design better therapeutic strategies and in designing the preferential choice of preparation in the treatment of various pathological conditions. Therefore in this study we aimed at understanding the COX-2 inhibitory effect of different VA preparations.

Analysis of COX-2 expression by western blot, in A549 cells upon treatment with IL-1β and different VA preparations showed that all VA preparations do not inhibit COX-2 expression under inflammatory condition. VA Q Spez and VA M Spez inhibited cytokine induced COX-

2 expression in a dose-dependent manner suggesting that both VA Q Spez and M Spez exert anti-inflammatory effect with similar mechanisms of action. Interestingly VA P did not inhibit cytokine induced COX-2 expression even at the highest concentration used in the study. This suggests that VA P does not modulate the COX-2 pathway; however it is possible that it may act as anti-inflammatory effect by other mechanisms.

Further flow cytometric analyses of COX-1 and COX-2 in A549 cells upon treatment with IL-1β and Viscum preparation reconfirmed the inhibitory effect of VA Q Spez and VA M Spez on cytokine induced COX-2 expression. Also, it is interesting to note that both of these preparations did not inhibit COX-1, which suggests that VA Q Spez and VA M Spez both are selective COX-2 inhibitors. However VA P did not inhibit either cytokine induced COX-2 nor the expression of COX-1. This clearly suggests that anti-inflammatory mechanisms of VA P are independent of COX-2 inhibition.

Since biochemical composition and biological properties of Viscum preparations largely depend on the type of preparation, method of preparation and also the time of harvesting[48-50], it is interesting to investigate whether these preparations behave differently from batch to batch. Accordingly when we analysed the COX-2 inhibitory effect of three different preparations from three different batches of preparation, we observed that, all VA Q preparations inhibited cytokine induced COX-2 expression with certain degree of difference in the extent of inhibition. COX-2 inhibition was also observed in case of VA M Spez preparation, but not in other non-special M preparations. This suggests that COX-2 inhibition is one of the predominant anti-inflammatory mechanisms of VA Q Spez and VA M Spez preparations. On the other hand none of the VA P preparations from any batch inhibited COX-2 expression suggesting that, COX-2 inflammatory axis is refractory to inhibition by VA P preparations. In view of the evidence for the therapeutic benefit of all Viscum

preparations, absence of COX-2 inhibitory effect in VA P preparation, clearly suggests that anti-inflammatory mechanisms of VA P are independent of COX-2 inhibition.

Since mistletoe lectin and viscotoxin are the two major components of *Viscum album* preparation, and in view of the fact that several biological effects of Viscum preparation are shown to be mediated through mistletoe lectins [51], [52] we tried to extract the relation between COX-2 inhibition and these two compounds. We demonstrated that COX-2 inhibitory effect of *Viscum album* preparations directly correlates with their mistletoe lectin content. This explains the most probable reason behind highest COX-2 inhibition by VA Q Spez and VA M Spez which contain more mistletoe lectin compared to VA P preparations. On the other hand content of viscotoxin did not significantly correlate with the COX-2 inhibition. This suggests that mistletoe lectin is an important component of Viscum preparations which is responsible for their anti-inflammatory effect.

Conclusions

In view of the long term side effects of NSAID, phytotherapeutics such as *Viscum album*, which selectively inhibit COX-2, can act as effective alternatives. However understanding the mechanisms of action of such preparations, mainly with such empirically formulated therapeutic preparations is a major challenge. In this study, using the whole plant extracts which are therapeutically administered; we have demonstrated the anti-inflammatory properties of Viscum preparations in relation to their content of mistletoe lectins [53]. This will undoubtedly help in designing better therapeutic strategies and help to formulate proficient synergistic combinations along with the conventional therapies in the treatment of inflammatory pathologies and in cancer.

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Figure legends

Figure 1: Effect of different *Viscum album* preparations on IL-1β-induced COX-2 expression.

A549 cells were treated with IL-1β (10 ng/ml) and increasing concentrations of different *Viscum album* preparations for 18 hours. Cytosolic COX-2 expression was measured by western blotting, and the intensity of expression was measured by densitometric analysis and expressed in arbitrary units. Representative blots of COX-2 expression profile headed by the respective densitometry values of IL-1β-induced cells treated with VA Q Spez (A), VA P (B) and VA M Spez (C). Results are mean ± SEM from 4 independent experiments.

Figure 2: Selective inhibition of COX-2 by different *Viscum album* preparations.

Cells were treated with IL-1β (10 ng/ml) and increasing concentrations of different *Viscum album* preparations for 18 hours. Intracellular COX-1 and COX-2 expressions were measured by flow cytometric analysis and the results are presented as percentage COX expression, using the number of percentage positive cells in each case. Comparative profile of percentage expression of COX-2 (A) and COX-1 (B) are shown. (C). Representative dot plot of COX-1 and COX-2 expression by different VA preparations. Results are mean ± SEM from 4 independent experiments.

Figure 3: Comparative analysis of COX-2 inhibitory effect of different preparations from three different batches of preparation.

VA preparations obtained from three different batches of manufacture were analysed for their COX-2 inhibitory effect. Cells were treated with IL-1β (10 ng/ml) and increasing concentrations of different *Viscum album* preparations for 18 hours. Intracellular COX-2

expressions were measured by flow cytometric analysis and the results are presented as percentage COX-2 inhibition, using the number of percentage positive cells in each case and normalizing the COX-2 expression in IL-1β-induced cells as hundred. Results are mean ± SEM from 4-6 independent experiments.

Figure 4: Correlation of COX-2 inhibitory effect of various VA preparations with their content of mistletoe lectin and viscotoxin.

Cells were treated with IL-1β (10 ng/ml) and increasing concentrations of different *Viscum album* preparations for 18 hours. Intracellular COX-2 expressions were measured by flow cytometric analysis. Percentage COX-2 inhibition was calculated using the number of percentage positive cells in each case and normalizing the COX-2 expression in IL-1β-induced cells as hundred. A significant direct correlation between COX-2 inhibition and mistletoe content was observed however there was no significant correlation of COX-2 inhibitory effect with amount of viscotoxins as analyzed by Spearman rank correlation test.

Figure 1.

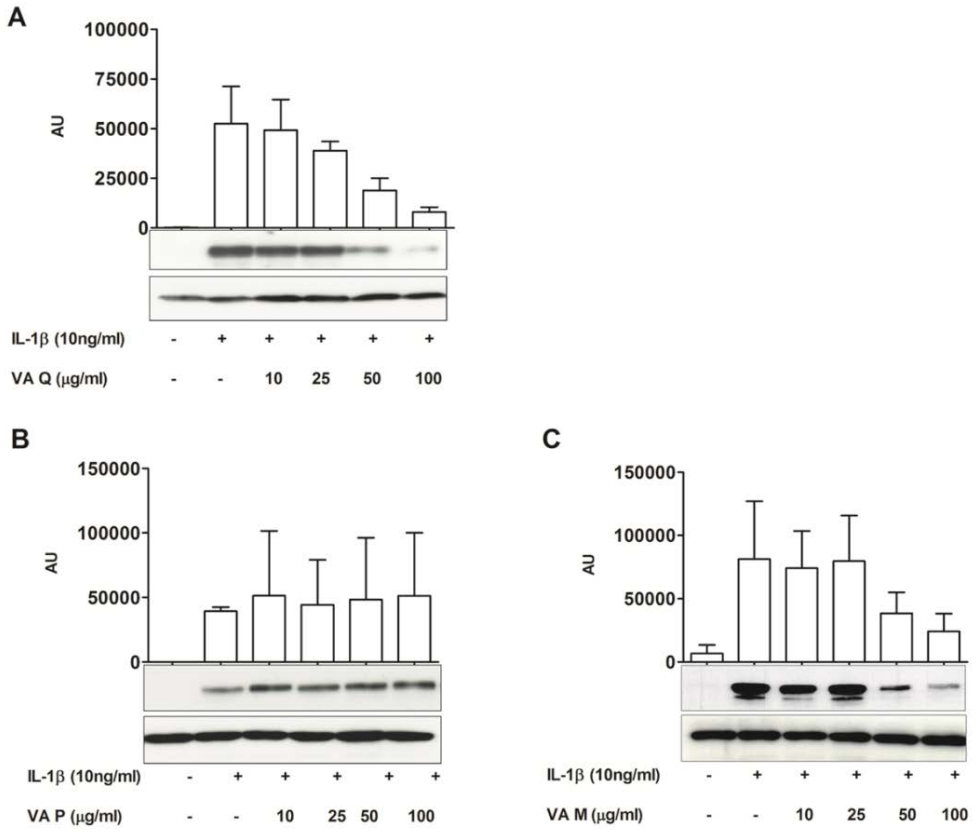
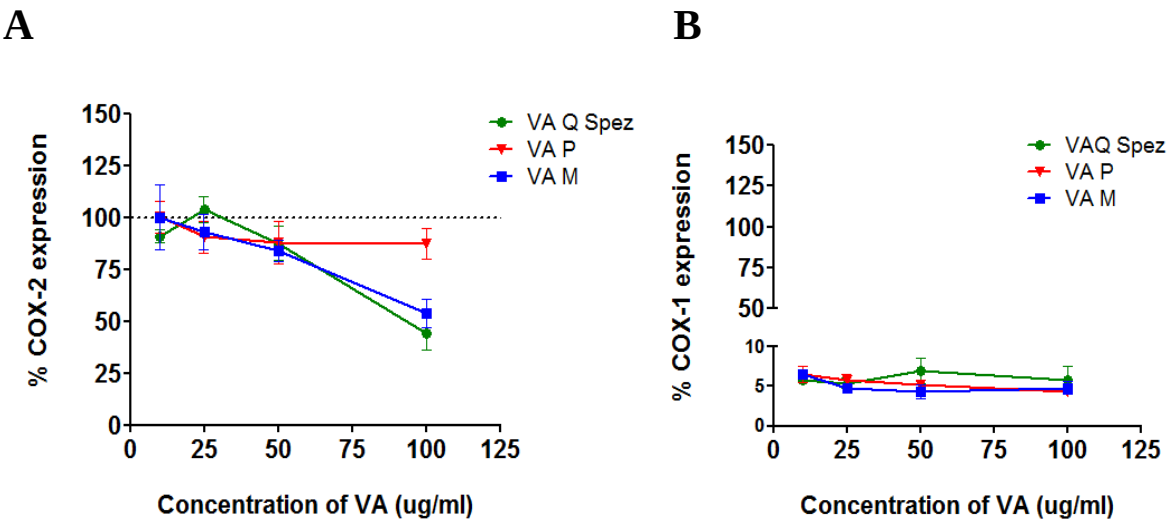


Figure 2.



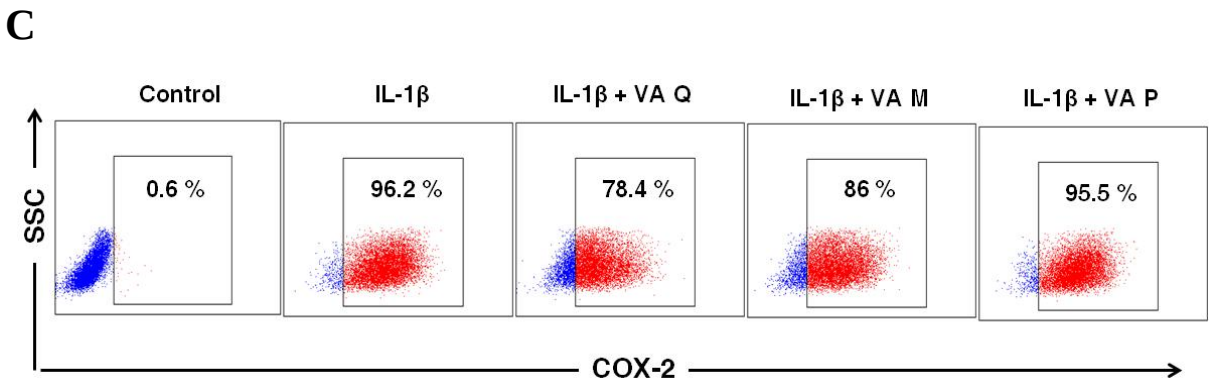

C
Control
IL-1β
IL-1β + VA Q
IL-1β + VA M
IL-1β + VA P
SSC
0.6 %
96.2 %
78.4 %
86 %
95.5 %
COX-2

Figure 3.

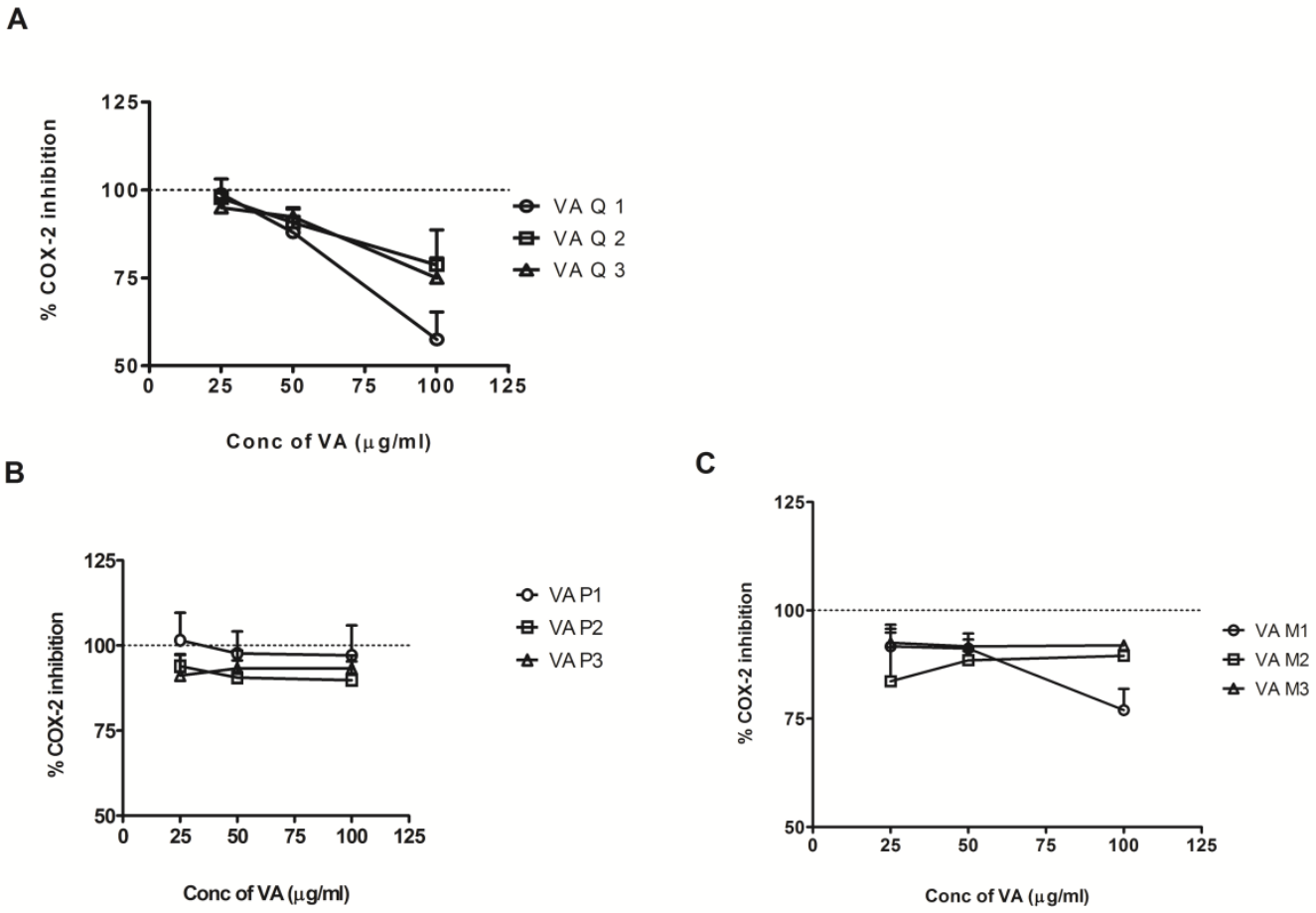
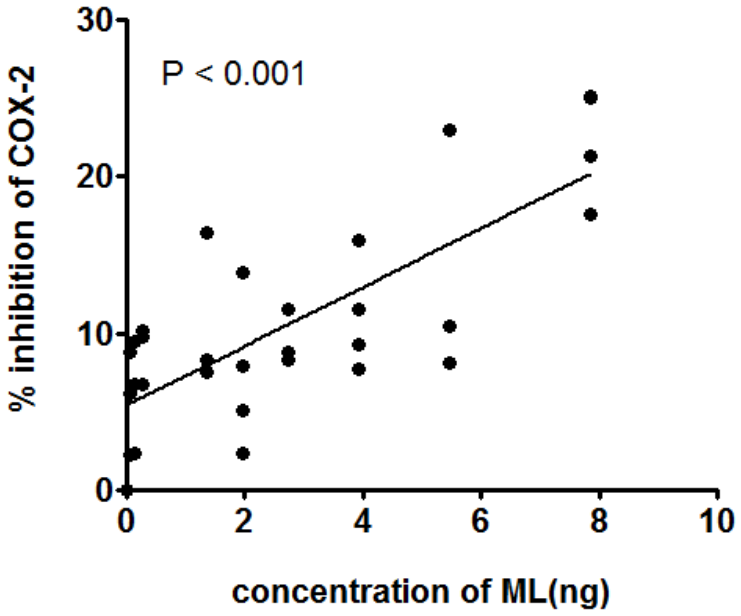
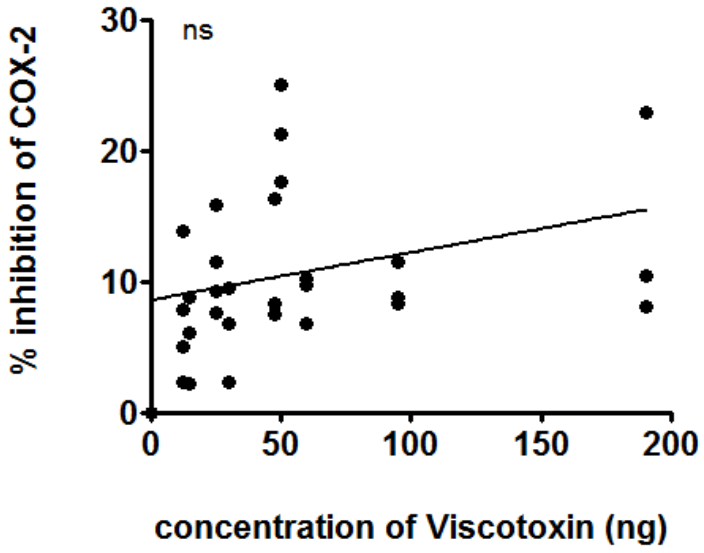

A
% COX-2 inhibition
Conc of VA (µg/ml)
VA Q 1
VA Q 2
VA Q 3
B
% COX-2 inhibition
Conc of VA (µg/ml)
VA P1
VA P2
VA P3
C
% COX-2 inhibition
Conc of VA (µg/ml)
VA M1
VA M2
VA M3

Figure 4.

A


P < 0.001
% inhibition of COX-2
concentration of ML(ng)
0
10
20
30
0
2
4
6
8
10

B


ns
% inhibition of COX-2
concentration of Viscotoxin (ng)
0
10
20
30
0
50
100
150
200

Discussion

Recent advances in the understanding of immune system and its role in disease, have enormously contributed to the field of therapeutic intervention. An increasing number of therapeutic approaches owing to the major advances in medical science and technology are being used to deal with a wide spectrum of human diseases. Because of the crucial role of immune system in the maintenance of homeostasis, any dysregulation in its normal function can lead to the emergence of a diverse pathology. As described earlier, due to the rupture in the immune tolerance, an exacerbated activity of the immune system towards self tissues leads to autoimmune diseases characterized by inflammation. On the other hand, failure of the immune system to execute an appropriate immunosurveillance may lead to the initiation of certain cancers. As a consequence, immune system serves as an ideal target for correction of these diseases. And hence a large number of immunomodulatory therapeutics has been developed on a strategic basis of immunointervention.

Our research group is extremely interested in understanding the immunomodulatory strategies in the treatment of autoimmune and inflammatory pathologies over the past several decades. It has made a significant contribution towards deciphering the mechanisms of action of an immunomodulatory therapeutic, IVIg (therapeutic preparation obtained from a pool of IgG from plasma of several thousands of healthy donors) in different autoimmune and inflammatory diseases. In addition, our group is equally interested in elucidating the immunomodulatory effects of *Viscum album* preparations (Iscador®) that are used in the complementary and alternative medicine for several human cancers. Despite the use of these agents for several years, the underlying molecular and cellular mechanisms have not been clearly understood. Therefore my study is focused towards understanding the molecular mechanisms of therapeutic preparation of VA, in the context of inflammation, a crucial central event in immunopathology. While an optimized, controlled level of inflammation is an integral part of an appropriate immune response and is necessary for controlling the

infectious attack and also to mount an immune response against transformed tumorigenic cells, an uncontrolled inflammatory activity can be deleterious in autoimmune diseases and several types of cancer. My studies therefore were aimed at understanding whether *Viscum album* (VA) preparations exert anti-inflammatory effect, and to dissect the underlying mechanisms associated, at cellular and molecular level.

Therapeutic intervention using natural compounds such as plant derived molecules and whole plant extracts has enormously contributed to the field of complementary and alternative medicines (CAM) and supportive care and is known as one of the promising and harmless approaches in the treatment of various inflammatory pathologies and malignant tumors. *Viscum album* (VA) preparations are extensively used in the complementary therapy in cancer and also in the treatment of several inflammatory pathologies. Despite their successful therapeutic application for several years, the underlying mechanisms are not yet clearly understood. Several lines of evidence have revealed that these preparations exert anti-tumor activities which involve the cytotoxic properties, induction of apoptosis, inhibition of angiogenesis and several other immunomodulatory and anti-inflammatory mechanisms. These properties collectively define the mechanistic basis for the therapeutic benefit of Viscum preparations. However in view of the complexity of these diseases of inflammatory or tumor origin and the critical association between inflammation and cancer, it becomes extremely important to delineate the exact mechanism of action of these preparations at systemic, cellular and molecular level. My thesis work explores anti-inflammatory mechanisms, as another important mechanism of action, underlying the therapeutic benefit of VA preparation, both in malignant diseases and inflammatory pathologies and also opens up the potential avenues for an intensive future investigation.

The therapeutic benefit of VA in diverse pathologies is attributed to the method of preparation, the proportion of various bioactive compounds present within the extracts and

also to the host trees. Several lines of evidence have revealed that VA preparations exert an anti-tumoral effect because of their ability to induce apoptosis and to inhibit cell proliferation, providing a strong basis for their application as complementary therapy in cancer. However, successful utilization of these preparations in the treatment of certain inflammatory pathologies raises several questions related to their mechanisms of action. With the aim of understanding the role of VA in modulating the immuno-inflammatory response, current study has addressed the effect of VA on the PGE2 axis and its regulation at cyclo-oxygenase level.

Among the isoforms of cyclo-oxygenases, COX-1 is constitutively expressed in the cells whereas COX-2 is induced in response to inflammatory stimuli and contributes majorly to the induction of PGE2. PGE2 is a molecular mediator of several homeostatic functions including those of gastric mucosa and vascular endothelium. However, it also exerts potent pro-inflammatory effects including the induction fever and pain. Overproduction of PGE2 occurs in response to pro-inflammatory stimuli and correlates with the severity of certain infectious and inflammatory conditions. PGE2 exerts autocrine and paracrine actions on the target cells and can induce pro-inflammatory reactions. Cyclo-oxygenases have been thus used as attractive targets for designing the anti-inflammatory molecules such as non-steroid anti-inflammatory drugs (NSAID) and for the conception of novel phytotherapeutics.

Anti-inflammatory effect by *Viscum album* due to selective inhibition of COX-2

My study demonstrates that, VA preparations exert potent anti-inflammatory effect by selectively inhibiting IL-1β-induced PGE2 biosynthesis. In order to understand the mechanisms underlying the VA-mediated inhibition of PGE2 secretion, we analyzed the expression pattern of cytokine-induced COX-2 in the presence of VA. RT-PCR results confirmed that IL-1β induces the expression of COX-2 mRNA while VA does not modulate

this cytokine-induced expression of COX-2 mRNA. On the contrary, VA significantly inhibited COX-2 protein expression induced by IL-1β. These results suggest that VA exerts a post-transcriptional regulatory effect on COX-2 expression. The effect of VA was not restricted to IL-1β-induced COX-2 alone. VA also showed an inhibitory effect on IFN-γ and TNF-α-induced COX-2 expression. Since, inflammation is a consequence of signaling of various inflammatory cytokines released by innate and adaptive immune cells, endothelial cells and epithelial cells, our results thus indicate that suppression of COX-2 by VA is not restricted to an inflammation created by a particular cytokine rather VA inhibits COX-2 expression mediated by a wide range of cytokines. This COX-2 inhibitory effect of VA at the protein expression level suggests its probable action in interfering with the process of translation resulting in the quantitative reduction of the protein. Further, kinetic analysis of COX-2 expression induced by IL-1β showed a typical time-dependant increase in protein expression reaching a maximum at 18 hours followed by a decline at later time points. At each time point, treatment of the cells with VA resulted in the reduced expression of COX-2 induced by IL-1β. This reduction in the presence of VA was more pronounced at 24 and 36 hours as compared to cells treated with IL-1β alone indicating a possible effect of VA on protein degradation. Together, modulation of COX-2 by VA strongly favours the role of VA as an anti-inflammatory therapeutic.

From the viewpoint of an anti-inflammatory therapeutic, inhibition of PGE2 synthesis by acting at the level of cyclo-oxygenases exposes a major concern of reducing the homeostatic levels of PGE2 because of the simultaneous inhibition of COX-1 and COX-2. Owing to the structural and functional homology between the COX-1 and COX-2, most of the NSAID which are synthetically designed to inhibit the enzymatic activity of COX-2 also inhibit COX-1, thereby affecting the overall level of PGE2 and are therefore known to cause several severe side effects. Thus, specific inhibitors of COX-2 represent attractive therapeutic alternatives

for several inflammatory pathologies. Interestingly, in our study, at all the concentrations of VA which inhibited COX-2, we did not observe any change in the expression of COX-1, irrespective of a robust stimulation by IL-1β. These findings suggest a strong selectivity in anti-inflammatory mechanism of VA by inhibiting COX-2 expression.

Long-term side effects of NSAID in various pathological conditions and the increasing body of evidence for anti-inflammatory activity of plant-derived molecules together encourage the conception of phytotherapeutics as potent alternatives to classical anti-inflammatory drugs. Several clinical studies have revealed the selectivity of certain plant-derived molecules in inhibiting COX-2 that are as efficient as synthetic COX-2-specific antagonists (rofecoxib and celecoxib) in rescuing from both acute and chronic inflammatory conditions and some of these plant-derivatives are even superior to synthetic molecules in their anti-inflammatory and analgesic effect. With the growing interest of promising new generation anti-inflammatory therapeutics, exploring and characterizing the novel phytotherapeutics with strong selectivity towards COX-2 are of great value. Together, our results demonstrate that VA exerts an anti-inflammatory effect by interfering with the cytokine-induced PGE2 biosynthesis through a selective inhibition of COX-2 protein suggesting its beneficial role with minimal side effects. These results are relevant in understanding the mechanism of action of VA and may provide useful insights for further exploration of anti-inflammatory mechanisms of VA and other plant-derived molecules in diverse pathologies.

Molecular dissection of VA-mediated COX-2 inhibitory effect

Prolonged administration of COX-2 inhibitors (NSAID) has been ineffectual for chemopreventive and chemotherapeutic purposes since the risks prevail over the benefits. Clinical demonstration of severe side effects due to the failure of the classical COX-2 inhibitors to discriminate between an aberrant pathological vs. homeostatic functional

activation state, raised the concern that direct COX-2 enzymatic inhibition might not sufficiently represent an appropriate clinical strategy to target COX-2. Since in contrast to COX-1, COX-2 is an early response gene, similar to the genes encoded for cytokines, chemokines and proto-oncogenes, they can be regulated under several mechanisms of expression and modulation, ranging from direct transcriptional effects to post-transcriptional and post-translational levels and also indirectly by various transcription factors that mediate the stability. Such multiple levels of modulation of COX-2 expression imply the existence of multiple mechanisms, which may be targeted to finely modulate COX-2 functions. Several phytotherapeutics have been shown to exert modulatory effect on COX-2 at various levels of its molecular regulation and therefore have been considered as a potent alternative strategy to control the pathogenic expression of COX-2. Given that *Viscum album* preparations exert a potent anti-inflammatory effect by selective down regulation of COX-2, it is extremely interesting to dissect the COX-2 inhibition mediated by VA at molecular level.

The fact that, prophylactic treatment of VA inhibits COX-2 expression, however treatment with VA at the later phases of cytokine induction doesn't inhibit suggests that, inhibition of COX-2 by VA occurs in the early stage of pro-inflammatory cytokine stimulation, but not at the later. In order to dissect the molecular events of COX-2 regulation we analysed the protein stability of COX-2 in presence of VA by cyclohexamide pulse chase experiments. Results from these experiments clearly showed that there is no considerable difference in the COX-2 protein degradation profile of cytokine stimulated cells with or without *Viscum album.* Therefore it is clear that COX-2 protein stability or protein half life is not affected by VA. Significant inhibition of COX-2 protein expression by VA without modulating its stability strongly indicates that, there is a possible modulation by VA at an earlier stage than the proteins were expressed. Since the mRNA of COX-2 was not affected by VA, we analysed the mRNA stability of cox-2 by Actinomycin D pulse chase experiments. We observed that

there is a clear reduction in the mRNA half life of cox-2 when cells are treated with VA suggesting that, VA induces destabilisation of COX-2 mRNA, thereby depleting the available functional mRNA for the protein synthesis and for the subsequent secretion of PGE2.

Comparative analysis of COX-2 inhibitory effect of different VA preparations

In view of the essential implication of an up-regulated COX-2 and PGE2 response in several pro-inflammatory conditions, chronic inflammations also in many of the malignant conditions, therapeutic strategies to inhibit COX-2 to be used as anti-inflammatory therapies are of great interest. Since therapeutic preparations of *Viscum album* are different with respect to their origin of host plant and the method of preparations, there may be a small degree of heterogeneity in their composition which may lead to their differential mechanisms of action at any given modulatory axis. In spite of the heterogeneity at the level of composition and mechanisms of action, all preparations are equally beneficial when they are used in various therapeutic conditions. Several lines of evidence from experimental and clinical studies have revealed that various preparations of *Viscum album* are beneficial in complementary and alternative medicine. However there may several differences in their mechanisms of action. Hence dissecting the mechanisms of action of various preparations and understanding the relative differences would help us to design better therapeutic strategies and in designing the preferential choice of preparation in the treatment of various pathological conditions. Therefore in this study we aimed at understanding the COX-2 inhibitory effect of different VA preparations.

Analysis of COX-2 expression by western blot, in A549 cells upon treatment with IL-1β and different VA preparations showed that all VA preparations do not inhibit COX-2 expression under inflammatory condition. VA Q Spez and VA M Spez inhibited cytokine induced COX-2 expression in a dose-dependent manner suggesting that both VA Q Spez and M Spez exert

anti-inflammatory effect with similar mechanisms of action. Interestingly VA P did not inhibit cytokine induced COX-2 expression even at the highest concentration used in the study. This suggests that VA P does not modulate the COX-2 pathway; however considering the therapeutic benefit of VA P, one cannot rule out the anti-inflammatory effects of VA P by other mechanisms apart from COX-2 inhibition.

Further flow cytometric analyses of COX-1 and COX-2 in A549 cells upon treatment with IL-1β and Viscum preparation reconfirmed the inhibitory effect of VA Q Spez and VA M Spez on cytokine induced COX-2 expression. Also, it is interesting to note that both of these preparations did not inhibit COX-1, which suggests that VA Q Spez and VA M Spez both are selective COX-2 inhibitors. However VA P did not inhibit either cytokine induced COX-2 nor the expression of COX-1. This clearly suggests that anti-inflammatory mechanisms of VA P are independent of COX-2 inhibition.

Since biochemical composition and biological properties of Viscum preparations are largely dependent on the type of preparation, method of preparation and also the time of harvesting, it is interesting to investigate whether different preparations behave differently from batch to batch. Accordingly when we analysed the COX-2 inhibitory effect of three different preparations from three different batches of preparation, we observed that, all VA Q preparations inhibited cytokine induced COX-2 expression with certain degree of difference in the extent of inhibition. COX-2 inhibition was also observed in case of VA M Spez preparation, but not in other non-special M preparations. This suggests that COX-2 inhibition is one of the predominant anti-inflammatory mechanisms of VA Q Spez and VA M Spez preparations. On the other hand none of the VA P preparations from any batch inhibited COX-2 expression suggesting that, COX-2 inflammatory axis is refractory to inhibition by VA P preparations. In view of the evidence for the therapeutic benefit of all Viscum

preparations, absence of COX-2 inhibitory effect in VA P preparation, clearly suggests that anti-inflammatory mechanisms of VA P are independent of COX-2 inhibition.

Role of mistletoe lectins and viscotoxins in COX-2 inhibitory effect of VA preparation

Since mistletoe lectin and viscotoxin are the two major components of *Viscum album* preparation, and in view of the fact that several biological effects of Viscum preparation are shown to be mediated through mistletoe lectins, we tried to extract the relation between COX-2 inhibition and these two compounds. We demonstrated that COX-2 inhibitory effect of *Viscum album* preparations directly correlates with their mistletoe lectin content. This explains the most probable reason behind highest COX-2 inhibition by VA Q Spez and VA M Spez which contain more mistletoe lectin compared to VA P preparations. On the other hand, content of viscotoxin did not significantly correlate with the COX-2 inhibition. This suggests that mistletoe lectin is an important component of Viscum preparations which is responsible for their anti-inflammatory effect. With these results it is seemingly true that, the anti-inflammatory properties of *Viscum album* are due to the presence of mistletoe lectins. However considering the empirical composition of these preparations, one cannot rule out the possible contribution by several other bioactive compounds which are present in the therapeutically administered plant extract which may act in different pathways, in order to exert anti-inflammatory effect.

Long-term side effects of NSAID in various pathological conditions and the increasing body of evidence for anti-inflammatory activity of plant-derived molecules together encourage the conception of phytotherapeutics as potent alternatives to classical anti-inflammatory drug. Considering the crucial role of inflammatory mediators and the regulators of chronic inflammation in tumor development and generating an inflammatory tumor microenvironment, anti-inflammatory therapeutics play a promising role in designing efficient

therapeutic strategies which will be used in the treatment of malignant diseases and vice versa.

Perspectives

Although mistletoe has several therapeutic benefits, the present state of research is still infantile and does not necessarily guarantee the safest use in all situations. An extensive future investigation is required with respect to its mechanisms of action at systemic, cellular and molecular level and to extend up to a broad spectrum clinical trials. In view of the heterogeneous composition and diverse biological effects of *Viscum album* preparation, it is interesting to dissect the molecular mechanisms of anti-inflammatory effects in various inflammatory pathways.

Modulation of cell signalling by *Viscum album* preparations

Considering the master regulatory role of NF-κB, in various biological processes such as inflammation, cell cycle, tumor, etc., understanding the modulatory role of VA at the level of NF-κB may provide useful information regarding a wide range of molecular mechanisms. Further unravelling the modulation by VA to various signaling molecular events during a pro-inflammatory response such as MAPK signalling pathway would also give us valuable information regarding the simultaneous regulation by VA, of various biological pathways which are controlled under these signalling events.

Understanding the immunoadjuvent role of *Viscum album*

Viscum preparations exert a potent immunostimulatory effect that facilitates in mounting an efficient anti-tumor immune response. VA preparations induce maturation and activation of human dendritic cells thereby enabling them to stimulate tumor specific CD8 T cell response; however the underlying cellular and molecular mechanisms of immunostimulation are not clear. Our preliminary results show that immunostimulatory effect of VA may implicates the activation of NLRP3 inflammasome in human dendritic cells thereby activating them for the

secretion of pro-inflammatory cytokine IL-1β. Thus exploring the effect of VA preparations in activating the inflammasome would give us interesting information regarding a novel molecular regulatory mechanism by which *Viscum album* preparations exert immunostimulatory effect.

Understanding the immunoregulatory role of *Viscum album* in restoring the immunosurveillance

Cancer immunosurveillance appears to be an important host protection process that inhibits carcinogenesis and maintains regular cellular homeostasis. During cancer, tumors constantly try to suppress the host immunity by various cellular and molecular mechanisms. The different pathways of immune evasion by tumors involve: induction of immune tolerance, resistance to killing by immune effector cells, and imparting functional paralysis of professional antigen presenting cells (APCs) such as dendritic cells (DCs). Since immunosuppressive mechanisms take over the immune homeostasis in cancer conditions, immunotherapeutic strategies are generally targeted either to stimulate the inducers of immune system or to suppress the suppressors of immune system. In view of the fact that Viscum preparations play an important role in immunostimulation by up regulating the secretion of pro-inflammatory cytokines leading to the induction of anti-cancer CD8 T cell response, on the other hand it is excitingly interesting to explore the role of VA on suppressing the suppressors of the immune system.

Since tumor mechanisms constantly try to suppress the host immunity, increase in the immune tolerance, by increasing the functions of (regulatory T cells) Treg, is one of the known immune dysfunctioning mechanism implied by the tumors to subvert the host immunity. And therefore investigating whether VA preparations pay a role in inhibiting the Tregs functions is of utmost interest.

In addition to these, there are still plenty of questions which can be addressed using simple experimental models to dissect the mechanisms of action of VA preparations both as an immunomodulatory agent and anti-inflammatory agent.

Conclusion

Due to multiple mechanisms of action, VA preparations can be appropriately used in cancer therapy where a balance between tumor cytotoxicity, tumor inflammation and beneficial immune responses can be maintained. The fact that VA preparations are administered at the site of tumor facilitates the cytotoxic effects in the tumor microenvironment, while at the non-cytotoxic concentrations *Viscum album* preparations entering the immune system facilitate stimulation of the immune responses against the tumor. These co-ordinated effects mediated by VA preparations can thus provide answer to the improvement in QOL observed in cancer patients undergoing VA-mediated complimentary therapy. In addition, anti-inflammatory properties of VA preparations provide a strong mechanistic basis for their application in inflammatory pathologies. A delicate balance between anti-inflammatory effect and the necessary low degree inflammation exerted by VA preparations makes them an efficient and harmless therapeutics in diverse pathological conditions including cancer and inflammatory pathologies.

The current research has contributed for a better understanding of mechanisms of action of *Viscum album*, which will help in efficient drug designing in inflammatory and malignant diseases using integrative medicinal approaches with conventional therapeutic tools. Given the multiple biological effects and molecular mechanisms of Viscum, it is exceedingly interesting to design the future therapeutic strategies for inflammatory pathologies and malignant diseases with several synergistic combinations of mistletoe with various conventional therapies. This will help as an effective and proficient approach of disease management.

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Annexes

LIST OF PUBLISHED PAPERS

1. Maddur, M.S., Othy, S., **Hegde, P**., Vani, J., Lacroix-Desmazes, S., Bayry, J., and Kaveri, S.V.(2010) Immunomodulation by intravenous immunoglobulin: role of regulatory T cells. J Clin Immunol *30 Suppl 1*, S4-8.
2. **Hegde P**, Maddur MS, Friboulet A, Bayry J, Kaveri SV (2011) Viscum album exerts anti-inflammatory effect by selectively inhibiting cytokine-induced expression of cyclooxygenase-2. PLoS One 6: e26312.
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7. Maddur MS, Sharma M, **Hegde P,** Lacroix-Desmazes S, Kaveri SV, et al.(2013) Inhibitory effect of IVIG on IL-17 production by Th17 cells is independent of anti-IL-17 antibodies in the immunoglobulin preparations. J Clin Immunol 33 Suppl 1: S62-66.
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11. Trinath J, **Hegde P,** Sharma M, Maddur MS, Rabin M, et al. (2013) Intravenous immunoglobulin expands regulatory T cells via induction of cyclooxygenase-2-dependent prostaglandin E2 in human dendritic cells. Blood.
12. Alsteens D, Aimanianda V, **Hegde P**, Pire S, Beau R, et al.(2013) Unraveling the Nanoscale Surface Properties of Chitin Synthase Mutants of Aspergillus fumigatus and Their Biological Implications. Biophys J 105: 320-327.
13. **Hegde, P**., Friboulet, A., Bayry, J., and Kaveri, S.V. *Viscum album*-mediated PGE2 and COX-2 inhibition implicates COX-2 mRNA destabilisation. (under communication).
14. **Hegde, P**., Friboulet, A., Bayry, J., and Kaveri, S.V. Comparative analysis of anti-inflammatory properties of different *Viscum album* preparations: mistletoe lectin as a key molecule responsible for COX-2 inhibition. (under communication).