Anti-inflammatory properties of $1\alpha25(OH)_2D_3$ and its analogs: Inhibition of Cyclooxygenase-2

Thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

by

R. APARNA

Department of Animal Sciences
School of Life Sciences
University of Hyderabad
Hyderabad-500 046, INDIA

December 2004
Enrolment No. 2KLAPH03

University of Hyderabad

(A Central University established in 1974 by Act of Parliament)

HYDERABAD – 500 046, INDIA

DECLARATION

I hereby declare that the work embodied in this thesis entitled **"Anti-inflammatory properties of 1α25(OH)$_2$D$_3$ and its analogs: Inhibition of Cyclooxygenase-2"** has been carried out by me under the supervision of Prof. P. Reddanna and this has not been submitted for any degree or diploma of any other university earlier.

Prof. P. Reddanna
(Research Supervisor)

R. Aparna
(Research Scholar)

University of Hyderabad

(Central University established in 1974 by Act of Parliament)

HYDERABAD – 500 046, INDIA

CERTIFICATE

This is to certify that **Mrs. R. Aparna** has carried out the research work embodied in the present thesis under my supervision and guidance for a full period prescribed under the Ph.D. ordinance of this University. We recommend her thesis ***"Anti-inflammatory properties of 1α25(OH)$_2$D$_3$ and its analogs: Inhibition of Cyclooxygenase-2"*** for submission for the degree of Doctor of Philosophy of this University.

Prof. P. Reddanna _____

Supervisor

Head _____

Department of Animal Sciences

Dean _____

School of Life Sciences

Acknowledgements

I express my deepest sense of gratitude to my supervisor, ***Prof. P. Reddanna****, for giving me this opportunity to work in his lab and introducing me to the field of inflammatory research. He is persistent source of encouragement and valuable guidance throughout the course of the present investigation. Working under him gave me a true sense of freedom with more confidence in addition to broaden my scientific outlook and positive attitude.*

I am grateful to ***Dr. G. Satya Reddy****, Brown University, USA for providing Vitamin D analogs and for his suggestions time to time.*

I am thankful to ***Prof. Aparna Dutta Gupta****, Head, Dept of Animal Sciences and former heads late* ***Prof. C.H.R.K Murthy*** *and* ***Prof. P. Reddanna*** *for allowing me to use the facilities of the department.*

My thanks to ***Prof. A. S. Raghavendra****, Dean, School of Life Sciences and former Dean* ***Prof. T. Suryanarayana*** *for providing me with the necessary facilities for my research.*

My sincere thanks to my doctoral committee members, Late ***Prof. C.H.R.K. Murthy, Prof. Aparna Dutta Gupta*** *and* ***Dr. Manjula Sritharan*** *for their valuable suggestions. I thank all the other faculty members of School of Life Sciences for their help during the course of my Ph. D. work.*

My heartfelt thanks to ***Prof. P.R.K. Reddy*** *for his timely suggestions and encouragement.*

I acknowledge the financial support from ***AP-Netherlands Biotechnology Program*** *and* ***DBT*** *during my research.*

I express my thankfulness to ***Dr. Nagarajaram****, CDFD, Hyderabad, for his help in modelling studies and* ***Dr. Shashikiran,*** *NIN, Hyderabad for his help in histological studies.*

My special thanks to ***Dr. G.V Reddy*** *for his help and encouragement during my research work. I appreciate the help and constant support provided by* ***Roy*** *during my stay in this University.*

My heartfelt thanks to ***Aruna*** *for her help, support and for the nice times we had at the University.*

I thank ***Subhashini****,* ***Mahipal*** *and* ***Mallikarjun*** *for their help in the lab. Here I also take the opportunity to thank all my lab mates* ***Srikanth, Anil, Sreedevi, Bharat, Ramakrishna,Chandramohan,Vijayabhaskar*** *and* ***Sai.*** *I am very much thankful for constant and enthusiastic help from* ***Mr. Shivakumar.*** *Help from* ***Balaram, Nagesh*** *and* ***Sivaram*** *is appreciated.*

*I consider my-self fortunate to have **Dr. Bindu Madhava Reddy** as my life partner, with out his help and support my thesis wouldn't have been in the final form.*

*A very special thanks to **Venu, Narayana and Pankaj** for their timely help.*

*I thank my seniors **Dr. Kiranmai**, **Dr. Srinivas Reddy**, **Dr. Madhava Reddy**, **Dr Maya and Sunitha.***

*I am gratified by the help provided by **Dr. Kiranmai** and **Dr. Sunitha Rao.***

*I thank **Chenna Reddy, Kalavathi, Roda,** and all other scholars of School of Life Sciences. I cannot forget the great company of **Ramakrishna, Nageswara Rao, Damodar** and **Hari Prasad.***

*This note would be incomplete without mentioning a word to **Aparna** and **Jeyakumar** for "always being there for me."*

*Assistance from the non teaching staff of the School of Life Sciences, especially **Ankineedu garu** and **Jagan** is acknowledged.*

My sincere thanks to all those who have done selfless favors during my stay in this university.

*Last but not the least, my deepest sense of gratitude to **my aunt, my parents, my sisters, brother-in-laws, mother-in law, father-in-law** for their everlasting love, affection and encouragement. Finally an applause to my niece **Sahiti** and nephew **Sunay**.*

Aparna R.

CONTENTS

ABBREVIATIONS

µM	:	Micro molar
^{0}C	:	Degree centigrade/ degree celsius
AA	:	Arachidonic acid
BCIP	:	5-bromo-4-chloro-3-indolyl phosphate
bp	:	Base pair
COX	:	Cyclooxygenase
cpm	:	Counts per minute
DBP	:	Vitamin D binding protein
DNA	:	Deoxy ribonucleic acid
DTT	:	Dithiothreitol
EDTA	:	Ethylene diamine tetra acetic acid
FACS	:	Fluorescence activated cell sorter
FCS	:	Fetal calf serum
g	:	Gram
GPx	:	Glutathione peroxidase
GSH	:	Reduced Glutathione
h	:	Hour(s)
HPETE	:	Hydroperoxyeicosatetraenoic acid
ILs	:	Interleukins
iNOS	:	Inducible Nitric Oxide synthase
kb	:	Kilobase pair
kDa	:	Kilodalton
l	:	Litre
LBD	:	Ligand binding domain
LOX	:	Lipoxygenase
LPS	:	Lipopolysaccharide
LTs	:	Leukotrienes
LXs	:	Lipoxins
MAPK	:	Mitogen activated protein kinase
mg	:	Milligram
min	:	Minutes
ml	:	Milliliter
mM	:	Millimolar
MS	:	Multiple Sclerosis

MTT	:	3-[4,5-dimethylthiazol-2-yl]-2,5-Diphenyltetrazolium bromide
NBT	:	Nitroblue tetrazolium
nm	:	Nanometers
nM	:	Nanomolar
NMMA	:	N^G-monomethyl-L-arginine
NO	:	Nitric Oxide
NSAIDs	:	Non-steroidal anti-inflammatory drugs
N-terminal	:	Amino terminal
OD	:	Optical density
PAGE	:	Polyacrylamide gel electrophoresis
PBS	:	Phosphate buffered saline
PCR	:	Polymerase chain reaction
PGHS	:	Prostaglandin-H-Synthase
PGs	:	Prostaglandins
PMSF	:	Polymethyl sulfonyl fluoride
PTH	:	Parathyroid hormone
RA	:	Rheumatoid Arthritis
ROS	:	Reactive Oxygen Species
rpm	:	Revolutions per minute
RXR	:	Retinoid X receptor
SDS	:	Sodium dodecyl sulfate
SLS	:	Systemic Lupus Erythematosus
TEMED	:	N,N,N',N'-tetramethylene diamine
Tris	:	Tris-(Hydroxymethyl) aminoethane
UV	:	Ultraviolet
VDR	:	Vitamin D receptor
VDRE	:	Vitamin D responsive element

Chapter 1: Introduction

1.1 Vitamin D

The principal physiological function of vitamin D in all vertebrates including humans is to maintain serum calcium and phosphorus concentrations in a range that support cellular processes like neuromuscular function, and bone ossification. Vitamin D accomplishes this goal by enhancing the efficiency of the small intestine to absorb dietary calcium and phosphorous, and by mobilizing calcium and phosphorus from the bone (Holick, 1999). The last couple of decades it has become increasingly apparent that vitamin D also has other important functions in tissues not primarily related to mineral metabolism (Brown, 1999). One example is the haematopoietic system, in which vitamin D affects cell differentiation and proliferation including such effects also in cancer cells. Vitamin D furthermore participates in the process of insulin secretion. The active metabolite of vitamin D, $1\alpha 25(OH)_2D_3$, regulates the transcription of a large number of genes through binding to a transcription factor, the vitamin D receptor (VDR). Blood levels of vitamin D_3 are influenced both by dietary intake and the amount of daylight exposure to the skin. Exposure of the skin to ultraviolet light catalyses the synthesis of vitamin D_3 (cholecalciferol) from 7-dehydrocholesterol. Thus vitamin D is more like a hormone and not strictly a vitamin according to the classical criteria that an essential nutrient is a substance the body can not synthesize in sufficient quantities itself. Deprived of exposure to sunlight vitamin D becomes an essential nutrient. The effectiveness of exposure to sunlight or ultraviolet light in curing or preventing rickets was shown early in the twentieth century (Holick, 1995).

Vitamin D comprises two closely related substances of nutritional importance: vitamin D (cholecalciferol), which is the physiological form, and the synthetic analogue vitamin D_2 (ergocalciferol). The two forms only differ by the side chain to the sterol skeleton (Holick, 1999). It has been assumed, based on studies in the 1930s showing no conclusive difference between vitamin D_3 and D_2 in their preventing effect against infantile rickets, that vitamin D_2 for practical purposes could be regarded as equal to vitamin D_3 from cod liver oil. There is no contemporary evidence showing that vitamin D_3 and D_2 are equally efficient in increasing the circulating metabolite proximate to the active form. Indeed, later studies have shown important biological differences in this respect between these two forms (Trang *et al.*, 1998). Vitamin D_3 and vitamin D_2, together with the provitamins they are made from, are all derivatives of sterols, their chemical structure resembles cholesterol, bile acids and the sex hormones. Vitamin D_2 is formed by UV radiation from its precursor ergosterol. Ergosterol is found in plants, especially in yeast and fungi. The synthesis of ergocalciferol from ergosterol hardly takes place in nature. Plants are thus a poor source of vitamin D_2. Synthetic vitamin D_2, produced by irradiation of ergosterol is the form added to food or given as supplements. During the past two decades, vitamin D_3 has also been used to fortify milk, margarine and other foods worldwide, and although the use of vitamin D_2 in food and supplements still is widely used, its use is less than before. Vitamin D_3 is formed from its precursor 7-dehydrocholesterol, which is found in ample amounts in the skin and fat depots in animals and man (Fig. 1). Vitamin D is relatively stable in fat solutions and is not inactivated by pasteurisation or sterilisation. It oxidises on contact with air and in acid solutions and is inactivated when exposed to sunlight.

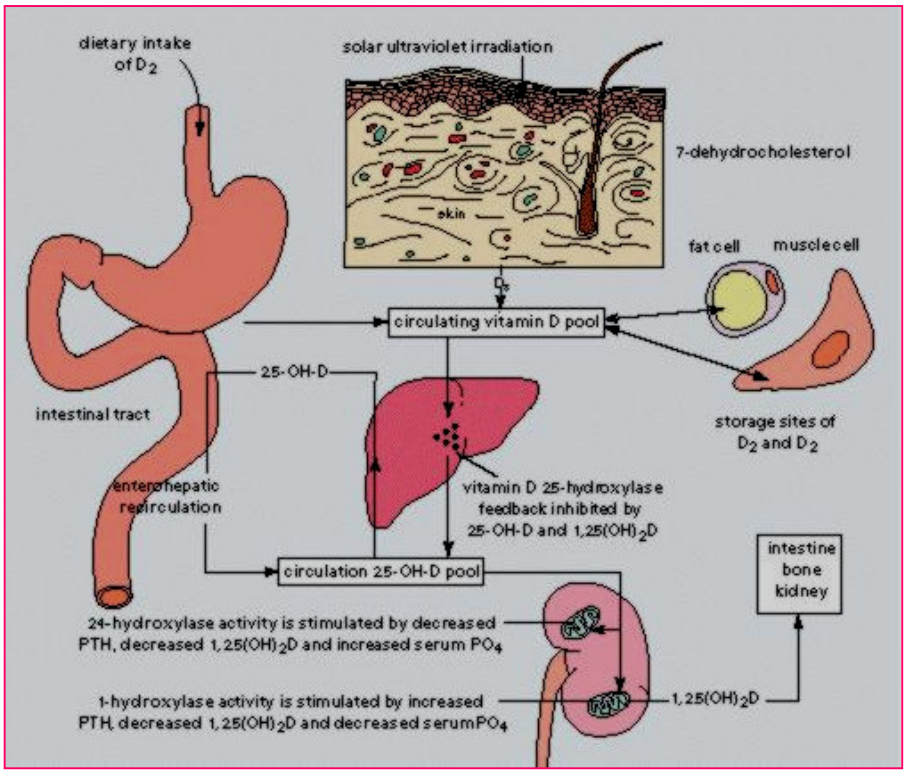


Fig. 1: Synthesis and storage of Vitamin D
(Holick, 2004 *Am J Clin Nutr* 79: 362-371)

1.1.2 Metabolism of Vitamin D

1.1.2.1 Vitamin D activation

Vitamin D (vitamin D_3, cholecalciferol) is biologically inactive. Major metabolic steps involved in the metabolism of Vitamin D_3 requires two obligate hydroxylations to form the active hormone, 1α25-dihydroxyvitamin D_3 ($1\alpha25(OH)_2D_3$) (Fig. 2). The first step of activation takes place by hydroxylation at position C-25, mainly in the liver. The role of other tissues is uncertain. The product, 25-hydroxyvitamin D_3 ($25(OH)D_3$), is transported to the kidneys, where 1α-hydroxylation takes place. The resulting product, 1α25-dihydroxyvitamin D_3 ($1\alpha25(OH)_2D_3$), is the active metabolite (Fig. 3). $1\alpha25(OH)_2D_3$ is transported bound to vitamin D-binding protein (DBP). DBP

is synthesised in the liver and circulates in plasma at concentrations 20 times higher than the total amount of vitamin D metabolites.

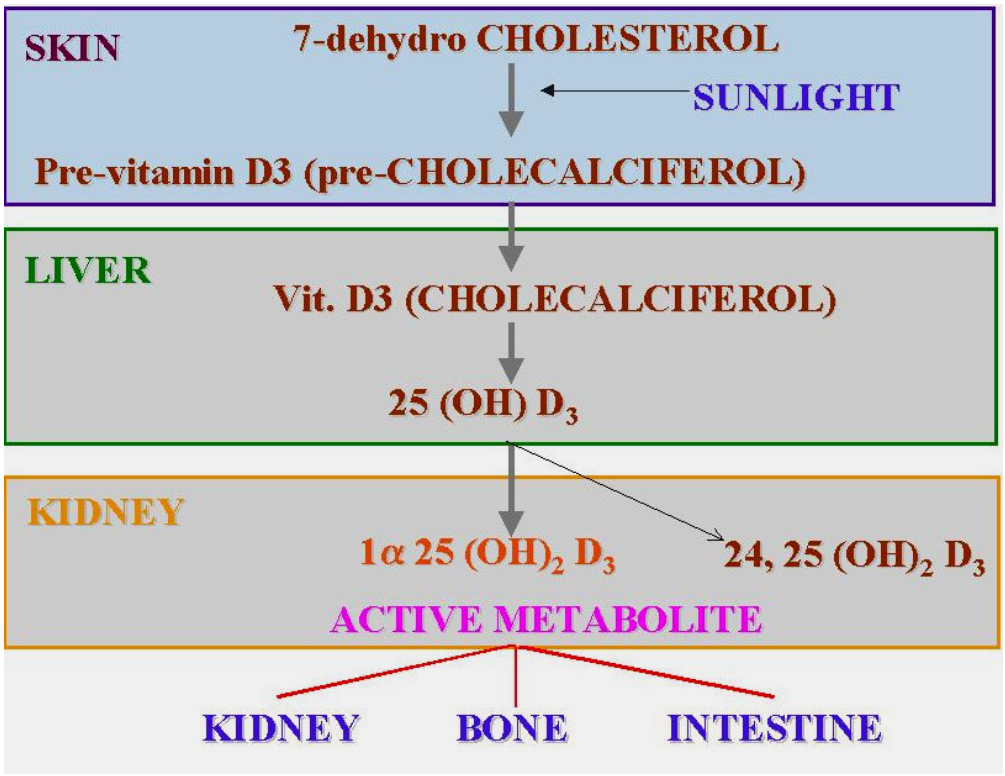


Fig 2: Basic metabolic activation pathway of Vitamin D
(Henry *et al.*, 2001 *Steroids* 66: 391-398)

The role of the large molar excess of DBP is uncertain. Free $1\alpha25(OH)_2D_3$ is in equilibrium with the bound form. It is only free $1\alpha25(OH)_2D_3$, i.e. 0.5% of the total amount of plasma $1\alpha25(OH)_2D_3$, which is hormonally active. The binding to DBP increases the half-life of $1\alpha25(OH)_2D_3$ and makes the hormone available to the cells (Brown, 1999; Gomme & Bertolini, 2004). The concentration of DBP is increases during pregnancy and by oestrogen treatment. It also increases in infants after birth. The 25-hydroxylation of vitamin D is poorly regulated, i.e. the capacity of the 25-hydroxylase in the liver is high. The levels of $25(OH)D_3$ increase is in

proportion to vitamin D intake, and for this reason, plasma $25(OH)D_3$ levels are commonly used as indicator of vitamin D status.

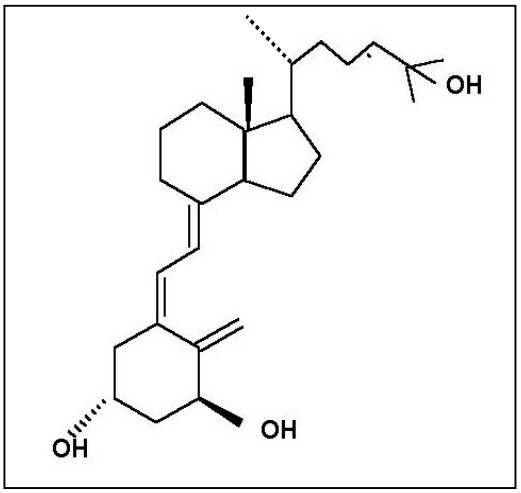


Fig. 3: Structure of $1\alpha 25(OH)_2D_3$

The half-life of $25(OH)D_3$ in circulation is approximately 1-2 months (Vieth, 1999). Steady state in plasma $25(OH)D_3$ concentration would, according to the half-life, not be reached before 4 months after a change in the intake. With concentration-dependent kinetics this could, however, vary. The proportion of $25(OH)D_3$ to vitamin D intake cannot be determined before steady state is reached. In contrast, the production of 1α $25(OH)_2D_3$ is tightly regulated, both by feedback of the $1\alpha 25(OH)_2D_3$, through calcium and phosphate levels in the blood and with the help of parathyroid hormone (PTH). This is illustrated by experiments showing that when large doses of vitamin D are given to animals, the serum concentrations of $25(OH)D_3$ will increase proportionally, while the concentration of $1\alpha 25(OH)_2D_3$ remains normal.

Both the suppression of the kidney 1α-hydroxylase activity and induction of the 24α-hydroxylase activity are VDR mediated. Experiments with rats have shown that tissue specific down-regulation of renal VDR by calcium restriction blocks $1\alpha25(OH)_2D_3$ dependent suppression of renal 1α-hydroxylase or stimulation of renal 24α-hydroxylase (Brown, 1999; Beckman & DeLuca, 2002).

1.1.2.2 Catabolism of vitamin D

The major catabolic enzyme is the 24-hydroxylase, a mitochondrial enzyme, and both $25(OH)D_3$ and $1\alpha25(OH)_2D_3$ are inactivated via hydroxylation pathway. Further oxidation to the ketone, oxidation at C-23 and C-26, and subsequent oxidative cleavage of the side chain is associated with progressive loss of biological activity (Araya *et al*., 2003). In contrast to the limited distribution of the vitamin D-activating enzymes, 24-hydroxylase is ubiquitously present in vitamin D target tissues. This enzyme is highly inducible by $1\alpha25(OH)_2D_3$ providing a regulatory mechanism at the cellular level for attenuating the response of the active compound when abnormally high.

1.1.3 Vitamin D receptor (VDR)

The main mechanism of action of vitamin D is the interaction of $1\alpha25(OH)_2D_3$ with the nuclear vitamin D receptor (Brown, 1999). VDR belongs to the super family of steroid nuclear receptors. Following ligand binding, VDR heterodimerises with retinoid X receptor (RXR) and acts as a ligand-activated transcription factor by binding to genomic vitamin D responsive elements (VDRE) in vitamin D-regulated genes (Fig. 4). These include more than 50 other genes important for mineral homeostasis, vitamin

D metabolism, energy metabolism, cell differentiation and proliferation, extracellular matrix proteins, oncogenes, growth factors, signal transduction

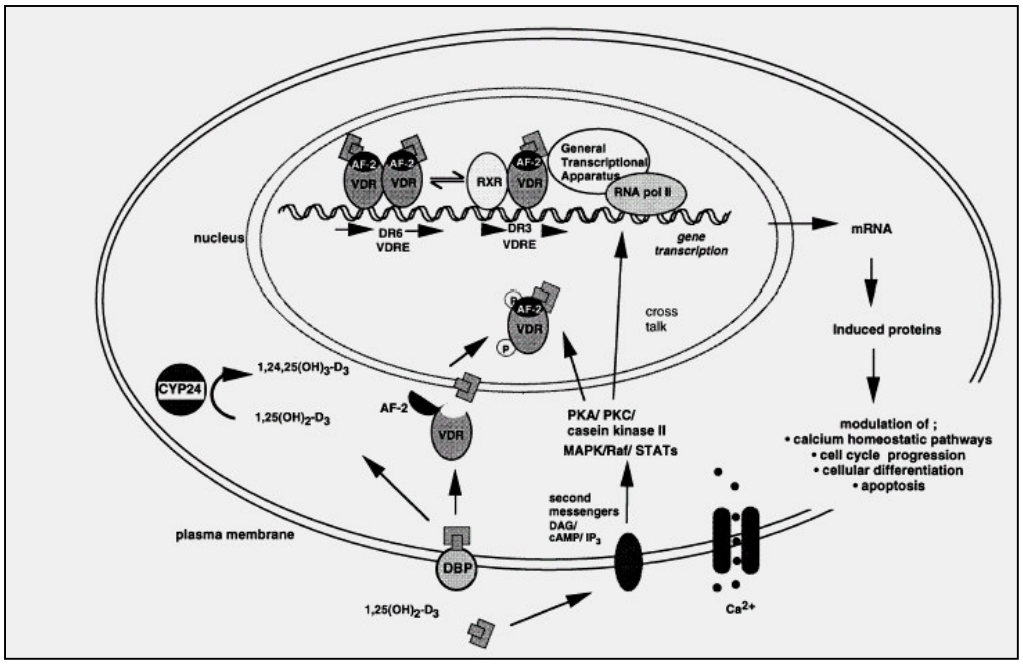


Fig. 4: Mechanism of action of 1α25(OH)$_2$D$_3$
(Jones *et al.*, 2004 *Physiological* R*eviews* 78 : 1193-21)

proteins and peptide hormones. Genes can be both up-regulated or downregulated, but the exact mechanism is unclear. Among genes down-regulated are PTH, osteocalcin, protein-kinase A inhibitors and interleukin-2 genes. Several genetic polymorphisms of VDR have been identified, the exact role of these has not been clarified, but most variants do not affect the protein structure (Brown, 1999; Uitterlinden *et al.*, 2004). In a study on the efficacy of vitamin D supplementation on bone mineral density of the femoral neck in elderly women it was found that those having one or two *VDR* alleles without the Bsm1 restriction site responded better than those with a genotype in which this restriction site was absent (Graafmans *et al.*, 1997). The

cellular response to 1α25$(OH)_2D_3$ is mainly regulated by changing the cellular amount of VDR. Treatment with 1α25$(OH)_2D_3$ increases the receptor level presumably due to stabilisation of the receptor. Some growth factors increase, as IGF-I, while others, such as fibroblast growth factor and mitogens, decrease VDR expression. Activation of protein-kinase C and prednisone treatment inhibit VDR expression whereas oestrogen, retinoic acid and PTH increase VDR expression. VDR expression is also dependent on cell type, and its condition, proliferating or differentiating (Kveiborg *et al.*, 2001; Peleg *et al.*, 2003). VDR can also be regulated at the stage of degradation. VDR interacts directly with SUG1, a component of the proteasome complex important for proteolysis. VDR activity might also be modulated by phosphorylation of serine at different positions (Brown *et al.*, 1999). The homozygous VDR knock out mouse (VDR-/-) shows no sign of defect until end of weaning after which they fail to thrive and die within 15 weeks from birth. They suffer from hypo calcemia, defective fur and females have defects in reproductive organs. Furthermore, bone formation and growth are inhibited and the level of 1α25$(OH)_2D_3$ is increased, indicating a role of VDR in regulation of vitamin D hydroxylation. In some respects the VDR knockout mice show some phenotypic similarities with the disease, vitamin D resistant-rachitis type 2, which is seen in children with inherited mutations in VDR (Kveiborg *et al.*, 2001).

1.1.4 Functions of vitamin D

The principal function of vitamin D (1α25$(OH)_2D_3$) in the body is to maintain intracellular and extracellular calcium concentrations within a physiologically acceptable range. The vitamin accomplishes this goal through

the action of 1α25$(OH)_2D_3$ by regulating calcium and phosphorus metabolism in the intestine and bone.

1.1.4.1 Vitamin D and calcium homeostasis

The most critical role of 1α25$(OH)_2D_3$ in mineral homeostasis is to enhance the efficiency of the small intestine to absorb dietary calcium. This was clearly demonstrated in the VDR null mouse (Yoshizawa *et al.*, 1997; Johnson & DeLuca, 2001). Calcium absorption from the intestine is dependent on the amount of calcium in the diet and on physiological requirements, which is adaptable. When dietary calcium concentrations are low, almost all calcium is absorbed. The same happens in pregnancy and during lactation. 1α25$(OH)_2D_3$ also promotes the intestinal absorption of phosphate. However a significant phosphate absorption also occurs in 1α25$(OH)_2D_3$-deficient states (Brown, 1999). 1α25$(OH)_2D_3$ is essential for development and maintenance of a mineralised skeleton and hence its deficiency results in rickets during growth and osteomalacia in adults. 1α25$(OH)_2D_3$ induces bone formation by regulation of matrix proteins important for bone formation, such as osteocalcin, osteopontine, alkaline phosphatase, matrix-gla- protein and collagen, as well as mineral apposition. The bone forming osteoblasts express VDR and it appears that 1α25$(OH)_2D_3$ inhibits osteoblast proliferation through VDR-dependent signal pathway, and promotes their differentiation (Kveiborg *et al.*, 2001). Vitamin D does not appear to be absolutely essential for the ossification process, but enhances this through increasing serum levels of calcium and phosphate. It has been suggested that not only 1α25$(OH)_2D_3$ is involved in bone mineralisation, but also 24,25$(OH)_2D_3$ may be required (Brown, 1999). 1α25$(OH)_2D_3$ enhances

the mobilisation of calcium and phosphorus stores from bone at times of calcium deprivation. $1\alpha25(OH)_2D_3$ induces stem cell monocytes to become mature osteoclasts. It appears though that this effect is not direct, but is mediated via osteoblasts that secrete a factor promoting osteoclast differentiation (van *et al.*, 2004). $1\alpha25(OH)_2D_3$ regulates calcium homeostasis in close co-operation with PTH, which is the principal hormone regulating extracellular ionised calcium from minute to minute. PTH stimulates $1\alpha25(OH)_2D_3$ synthesis and $1\alpha25(OH)_2D_3$ suppresses the synthesis and secretion of PTH and controls parathyroid growth through negative gene regulation. Studies in the VDR null mouse suggest that VDR is not essential, but works in co-operation with calcium and phosphate (Brown *et al.*, 1999). The most important effects of $1,25(OH)_2D_3$ in the kidney is suppression of 1α-hydroxylase activity and induction of 24-hydroxylase activity. $1\alpha25(OH)_2D_3$ increases renal calcium reabsorption and calcium binding protein expression, and it accelerates PTH dependent calcium transport in the distal tubule, which has the highest level of VDR. The enhancing effect of $1\alpha25(OH)_2D_3$ on renal phosphate absorption might be an indirect action via PTH suppression (Brown,1999; Sulkova *et al.*, 2004).

1.1.4.2 Bone and $1\alpha25(OH)_2D_3$

Bone is one of the classical target organs for $1\alpha25(OH)_2D_3$ action. $1\alpha25(OH)_2D_3$ not only plays a role in bone calcium mobilization but it has an essential role in bone development, mineralization and maintaining the dynamic nature of bone. $1\alpha25(OH)_2D_3$ facilitates those functions by controlling the availability of calcium and phosphate and by regulating the level of hormones such as PTH, parathyroid hormone related peptide and

insulin-like growth factor (Rao *et al*., 2001; Drinka, 2004). It is also involved in the synthesis of bone matrix proteins such as type I collagen, alkaline phosphatases, osteocalcin, osteoponitin and matrix-Gal protein (Gallagher & Riggs, 1990; Glenville *et al*., 1996; Goltzman *et al*., 2001).

1.1.5 Other effects of vitamin D

Synthesis and cellular receptors for $1\alpha25(OH)_2D_3$ have been found not only in the intestine, kidney and bone but also in many other tissues, suggesting that $1\alpha25(OH)_2D_3$ is fundamental in the regulation of gene expression in many cell types in addition to its probable role in intracellular calcium regulation (Brown, 1999; Zehnder *et al*., 2002a). Various functions of Vitamin D in different tissues are listed in table 1. Further local production and action of $1\alpha25(OH)_2D_3$, particularly after inflammatory activation of 1α-hydroxylase activity by, for example, cytokines in endothelial cells, could indicate an important autocrine/paracrine mechanism in peripheral tissues (Zehnder *et al*., 2002). In the skin, $1\alpha25(OH)_2D_3$ plays an important role by inhibiting proliferation and stimulating differentiation of keratinocytes and vitamin D analogues are used in the treatment of psoriasis. In the immune system, $1\alpha25(OH)_2D_3$ modulates synthesis of interleukins and cytokines. Besides stimulating monocytes and macrophages, $1\alpha25(OH)_2D_3$ functions as an immunosuppressive agent by decreasing the rate of proliferation and the activity of both T- and B cells and inducing suppressor T cells (Brown, 1999). In haematopoietic tissue, vitamin D deficiency causes anaemia and decreased cellularity of bone marrow. $1\alpha25(OH)_2D_3$ also inhibits proliferation and promotes differentiation of a number of leukaemia cell lines (Diaz *et al*.,

2000). Also normal myeloid precursor cells mature in the presence of $1\alpha25(OH)_2D_3$.

Table 1: A representation of target tissues and functions of Vitamin D (Rochel *et al.*, 2001 *Eur J Biochem* 268 : 971-79)

Tissue/cell	Action
Hematopoietic tissues	differentiation
myeloid cell precursors	
colony forming units	
Immune system	enhancement of immune function to control viral and
monocyte/macrophages, lymphocyte	bacterial infections and tumor growth
	immunosuppression
Skin	antiproliferative, differentiation
keratinocytes, fibroblasts, hair follicle, Langerhans	
cells and melanocytes	
Muscle	antiproliferative, differentiation
smooth muscle cell, myoblast, heart cardiac muscle	inhibition of antinatriuretic factor synthesis
cell and atrial myocytes	
Pancreas β cells	enhancement of insulin synthesis and secretion
Mammary gland	growth regulation
Cancer cells	antiproliferative, differentiation
Adrenal gland medullary cells	control of catecolamine metabolism
Prostate	antiproliferative, differentiation
Brain hippocampus/selected neurons	neuronal regeneration, enhancement of nerve growth
	factor and neurotrophin synthesis, control of
	sphingomyelin cycle
Cartilage chondrocyte	antiproliferative, differentiation
Female reproductive	antiproliferative, control of foliculogenesis organs
ovarian, myometrial and endometrial cells	
Liver parenchymal cell	enhancement of liver regeneration, control of
	glycogen and transferrin synthesis
Lung	enhancement of maturation, phospholipid synthesis
fetal pneumocytes	and surfactant release
adult pneumocytes	cell growth
Male reproductive organs	enhancement of sertoli cell function and
sertoli/semminiferus tubule	spermatogenesis
Pituitary production	control of T_3-induced growth hormone, prolactin and
	tyrotrophyn
Thyroid	inhibition of calcitonin synthesis
follicular cells	

In addition, VDR is expressed in many other tissues, such as muscle, nervous tissue, liver, intestine, reproductive organs, pancreas, pituitary, thyroid gland and lung, where $1\alpha25(OH)_2D_3$ apparently has important functions in regulation of cell proliferation and differentiation (Brown, 1999; Holick, 1999). In animal experiments and also in epidemiological studies,

vitamin D appears to be a protective factor in colon carcinogenesis (Wali *et al*., 1995).

1.1.6 Cancer & Vitamin D

There are several reports indicating that vitamin D plays a role in the prevention of cancer progression. Epidemiological studies have shown that people who live in the northern latitudes have a higher risk to get prostate, colon and breast cancer than individuals living closer to the equator. It has been hypothesised that differences in the amount of vitamin D synthesised in the skin plays a role in this phenomenon (Hanchette & Schwartz, 1992; Mawer *et al*., 1998; Garland *et al*., 1999; Ahonen *et al*., 2000). In addition to that there are lot of *in vivo* and *in vitro* studies indicating that $1\alpha25(OH)_2D_3$ plays a role in the control of proliferation, differentiation and apoptosis of many cell types including cancer cells (Reddy *et al.,* 2001*;* Harris & Go, 2004*;* Guzey *et al.,* 2004 *)*. Many of these effects appear to be dependent on cell type and their growing environment (Hansen *et al*., 2001). Vitamin D has been shown to inhibit cancer cell growth by arresting cell cycle progression. This blocking of cell division is associated with up-regulation of the cyclin dependent kinases p21 and p27 and down regulation of cyclins A and D1 (Liu *et al*., 1996; Verlinden *et al*., 1998; Ryhanen *et al.,* 2003). Other important molecules which are involved in cell growth inhibition by $1\alpha25(OH)_2D_3$ are c-fos and c-myc proto-oncogenes (Jensen *et al*., 2001). Growth factors regulate growth and the differentiation of normal cells. In cancer this regulation is often disturbed, leading to malignant processes. Vitamin D compounds have been shown to block the mitogenic activity of insulin like growth factor I and II in cancer cells (Rozen & Pollak, 1999; Nickerson & Huynh, 1999). Other growth factors such as transforming growth factor-beta (Heberden *et al*., 1998, Wu *et*

al., 1999) epidermal growth factor (Lee *et al*., 2001), keratinocyte growth factor (Lyakhovich *et al*., 2000), vascular endothelial growth factor (Mantell *et al*., 2000) and interleukins are also shown to be targets for the growth inhibitory effects of $1\alpha25(OH)_2D_3$ (Peleg *et al*., 1997; Topiiski *et al*., 2004).Cell death by apoptosis is a natural regulatory process in the body but in cancer, cells are often failed to undergo apoptosis leading to malignant outgrowth (Hansen *et al*., 2001). Vitamin D compounds were shown to induce apoptosis in a number of different cancer cell types by several distinct pathways where MAP kinases, tumor necrosis factor-alpha and apoptosis regulatory protein bcl-2 have been demonstrated to play a critical role (Park *et al*., 2000; Blutt *et al*., 2000; Pirianov & Colston, 2001). In addition, Vitamin D inhibits cancer progression by interfering with specific steps such as angiogenesis and metastasis (Hansen *et al*., 1994; Yudoh *et al*., 1997; Mantell *et al*., 2000; Vanden *&* Chang*,* 2002). $1\alpha25(OH)_2D_3$ inhibits cancer cell invasion by decreasing the activity of certain proteases which degrade extracellular matrix and basement membrane (Koli & Keski-Oja, 2000). Vitamin D has anti proleferative and pro differentiating effects in prostate cancer cells and shows reduced tumor progression in prostate cancer rats (Feldman *et al*., 2000; Jacobe *et al*., 2004).

1.1.7 Vitamin D analogs and their therapeutic applications

The active metabolite of vitamin D, $1\alpha25(OH)_2D_3$, has a wide range of nonclassical actions in the body, such as regulation of cell growth, differentiation and the immune system. This has led to increased interest in using $1,25(OH)_2D_3$ in the treatment of several diseases such as psoriasis, autoimmune diseases, osteoporosis, hyperparathyroidism and cancer. However, the therapeutic applications of $1\alpha25(OH)_2D_3$ are limited due to the

hypercalcemic and phosphataemic activity of this compound (Norman, 1995). The elevated level of calcium and phosphate in serum causes soft tissue calcification especially in the kidney, heart, aorta and intestine that can lead to organ failure and death (Vieth, 1990). In order to avoid these unwanted side effects, a lot of work has been done to synthesize analogs that exhibit weaker effects on calcium metabolism while retaining growth and immune regulating properties (Norman, 1995). It is estimated that over 2,500 analogs have already been synthesized and biologically evaluated. A number of them exhibit potent anti-proliferative activity while still having reduced hypercalcemic toxicity at therapeutic dosages; however, only few analogs are currently in clinical trials or in active use in medicine (Gulliford *et al*., 1998; Jones *et al*., 1998; Diaz *et al.,* 2000; Hansen *et al*., 2001; Wu-wong *et al*., 2004). Most of the analogs are synthesized either by side chain modification or epimerization pathway. Some of the analogs are shown in Fig. 5. The new pathway of $1\alpha25(OH)_2D_3$ metabolism via modification of the A-ring (C-3 epimerization path way) is known to result in the formation of a series of epimers, including $1\alpha25(OH)_2$-3-epi-D_3 (Reddy *et al*., 2000). Epimerization pathway occurs in both normal and cancer tissues, but unlike C-24 oxidation, epimerization is target tissue specific (Reddy *et al*., 2001). Clinically the most promising results were obtained by using analogs, which contain modification in the side chain of $1\alpha25(OH)_2D_3$. It appears that VDR is relatively tolerant of changes in this part of the molecule (Jones *et al*., 1998). The recently published crystal structure of VDR LBD shows that ligand occupies only 56% of the volume of ligand binding cavity and there is an additional space around the aliphatic side chain, which would allow analogs with different chain lengths to fit in (Rochel *et al*., 2000; Tocchini *et al*., 2004).

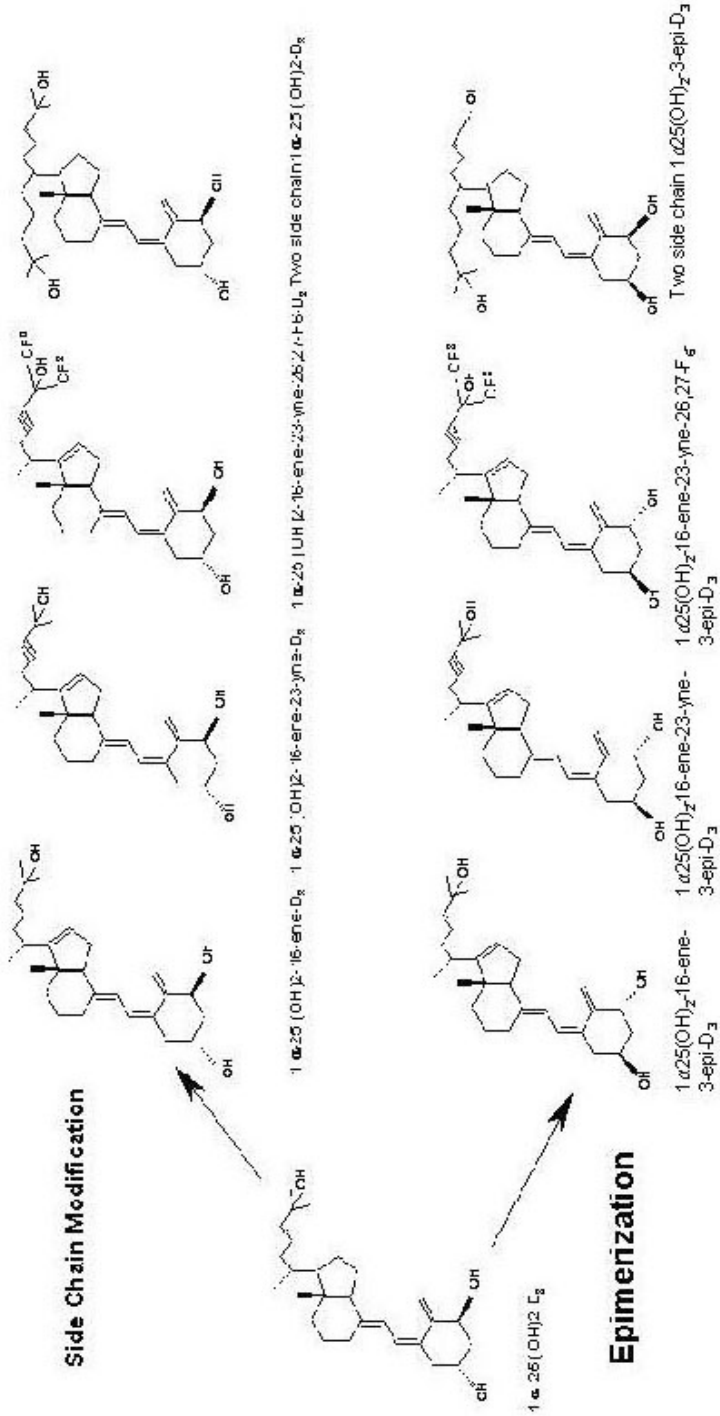


Fig 5: structures of side chain and epi analogs of 1 25(OH)2D3

$1\alpha25(OH)_2D_3$ and its synthetic analogs have also been used to treat osteoporosis (Rao *et al.,* 1996*)* which stimulate bone formation through direct action on bone forming cells and also suppress bone resorption mainly by indirect effect involving PTH suppression. However, the lack of bone specific analogs hampers their wide use in osteoporotic patients (Erben, 2001). In future other possible clinical uses for vitamin D analogs include prevention of graft rejection and autoimmune diseases such as multiple sclerosis, rheumatoid arthritis, scleroderma and insulin-dependent diabetes mellitus. This is based mainly on the immunosuppressive properties of $1\alpha25(OH)_2D_3$, such as its ability to inhibit cytokine secretion, decrease the proliferation rate and activity of the helper T cells, and its property to induce the availability of suppressor T cells (Lemire, 2000; Verstuyf *et al*., 2000; Deluca & Cantorna, 2001; Riachy *et al*., 2001).

Many vitamin D analogs have been shown to have promising anti-cancer effects in cancer animal models and a few analogs (EB1089, OCT) are currently under clinical trials (Diaz *et al*., 2000; Brown, 2001; Hansen *et al*., 2001). However, a real breakthrough in cancer therapy by vitamin D analogs is yet to come. The main reason for these disappointing results is that the use of systemically applied vitamin D analogs causes severe side effects at the supraphysiological doses that are needed in order to reach clinical improvement (Feldman *et al*., 2000; Reichrath *et al*., 2001).

1.1.7.1 Pharmacological and molecular basis for differential actions of vitamin D analogs

Factors that influence the biological profile of the vitamin analogs can be divided into pharmacokinetic and pharmacodynamic factors. There are two main pharmacokinetic factors, which affect the ligand availability for VDR, stability in blood and catabolism of the target cell. Binding of the analogs to DBP or other molecules in blood such as albumin and lipoproteins affects half-life values of the analogs in the blood and the rate of analog uptake by target cell. Those analogs, which have a strong affinity to DBP possess the longest extra cellular half-lifes in the order of days. Analogs with reduced affinity to DBP are metabolized and excreted most rapidly (Bouillon *et al*., 1991; Brown, 2000). Catabolism of $1\alpha25(OH)_2D_3$ and its analogs by target cells is another important factor controlling the concentration of $1\alpha25(OH)_2D_3$ and its analogs inside the target cell. Target cells might inactivate analogs in different ways or create new compounds, which retain significant biological activity (Bouillon *et al*., 1996; Brown, 1999; Peleg & Reddy, 2001; Siu-Caldera *et al*., 1999). It has also been reported that various cell types have different ability for catabolism of vitamin D compounds. This could cause cell specific differences in the action of the analogue and may explain why some analogs have a unique biological profile with favourable dissociation of differentiation versus calcemic potency (Brown, 2000). The pharmacodynamic influences of the vitamin D analogs are based on their ability to modulate VDR functions differently from the natural hormone (Fig. 6). Vitamin D analogs could use different contact amino acid residues for binding at the ligand binding cavity of VDR than $1\alpha25(OH)_2D_3$ does (Gardezi *et al*., 2001). This may result in the formation of additional bonds between the analogue and the ligand binding

cavity that could lead to the prolonged half-lives of activated receptor and long-lasting effects on gene activation (Peleg *et al*., 1998; Tocchini *et al*., 2001). Analogs might induce slightly different structural conformation within the hormone receptor complex (Liu *et al*., 1997), which may modulate the receptor.

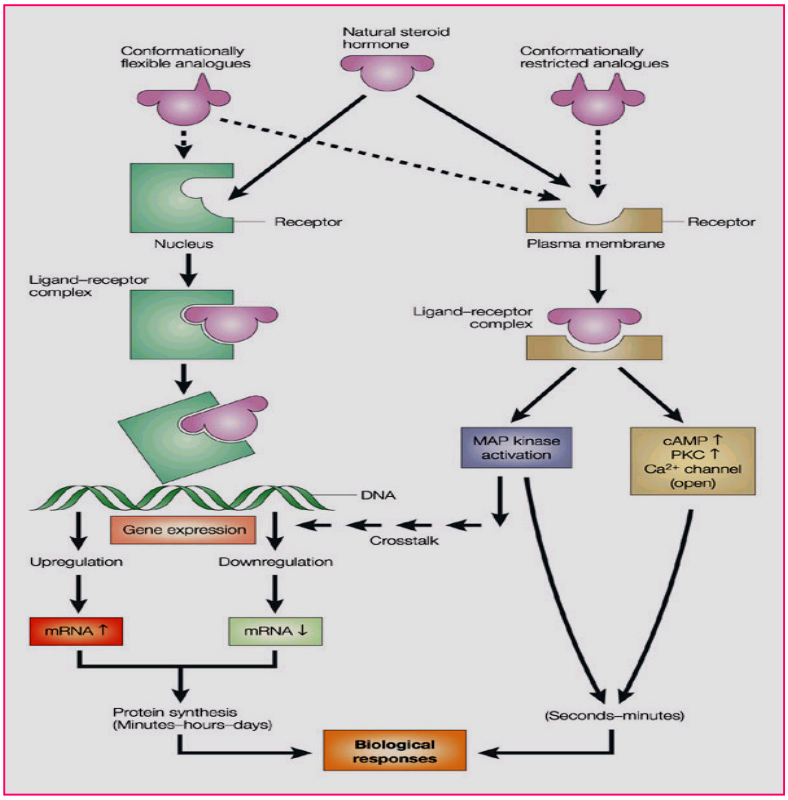


Fig. 6: Mechanism of action of 1α25(OH)$_2$D$_3$ and its analogs
(Norman *et al*., *Steroids* 66 (2001) 147-58**)**

dimerization (Zhao *et al*., 1997; Liu *et al*., 2001), affect the DNA binding properties and even the promoter selectivity of VDR (Quack & Carlberg, 1999). Moreover, analog induced differential conformation might influence receptor interactions with tissue specific cofactors and the stability of RXR/VDR/DNA/coactivator complex (Yang & Freedman, 1999; Herdick *et al*., 2000; Issa *et al*., 2002). It might also increase the intracellular half-life of the receptor-ligand complex by interfering with the SUG1/proteosome mediated

receptor degradation (Masuyama & MacDonald, 1997; Jääskeläinen *et al*., 2000). It has been shown that certain analogs are able to specifically activate nuclear VDR or membrane bound VDR. This could modulate the cross talk between receptors and thus alter genomic activity of the nuclear VDR (Brown *et al*., 1999).

1.2 Inflammation:

Inflammation is a response to infection, antigen challenge or tissue injury that is designed to eradicate microbes or irritants and to potentiate tissue repair. Excessive inflammation may lead to tissue injury and if severe can cause physiological decomposition, organ dysfunction and death. Based on timing and pathological features, inflammation can be divided into two major categories-acute and chronic. Acute inflammation is typically of relatively short duration and is characterized by vasodilation, the exudation of protein-rich fluid and migration of cells into the site of injury (Splettstoesser & Schuff*,* 2002). Hallmarks of inflammation are vasodilation, oedema and leukocyte infiltration. Vasodilatation is characterized by redness and warmth at the site of injury. The purpose of the vasodilatory response is facilitating the local delivery of soluble mediators and inflammatory cells (Fig. 7). Inflammation induced vasodilatation is mediated primarily by nitric oxide (NO) and vasodilatory prostaglandins. NO is produced from L-arginine through the action of nitric oxide synthase (NOS). Three isoforms of NOS have been identified. Endothelial NOS (eNOS) and neuronal NOS (nNOS) are constitutively produced, and their expression is increased by calcium flux. Activated leukocytes produce inducible NOS (iNOS) after exposure to microbial products or pro-inflammatory cytokines (Vallance & Chan, 2001). The NO produced causes subsequent smooth muscle relaxation through

cyclic GMP-dependent mechanisms (Moncada *et al.,* 1991). The primary vasodilatory prostaglandins are prostacyclin (PGI_2), PGD_2, and PGE_2. These lipid mediators are produced from arachadonic acid through the action of

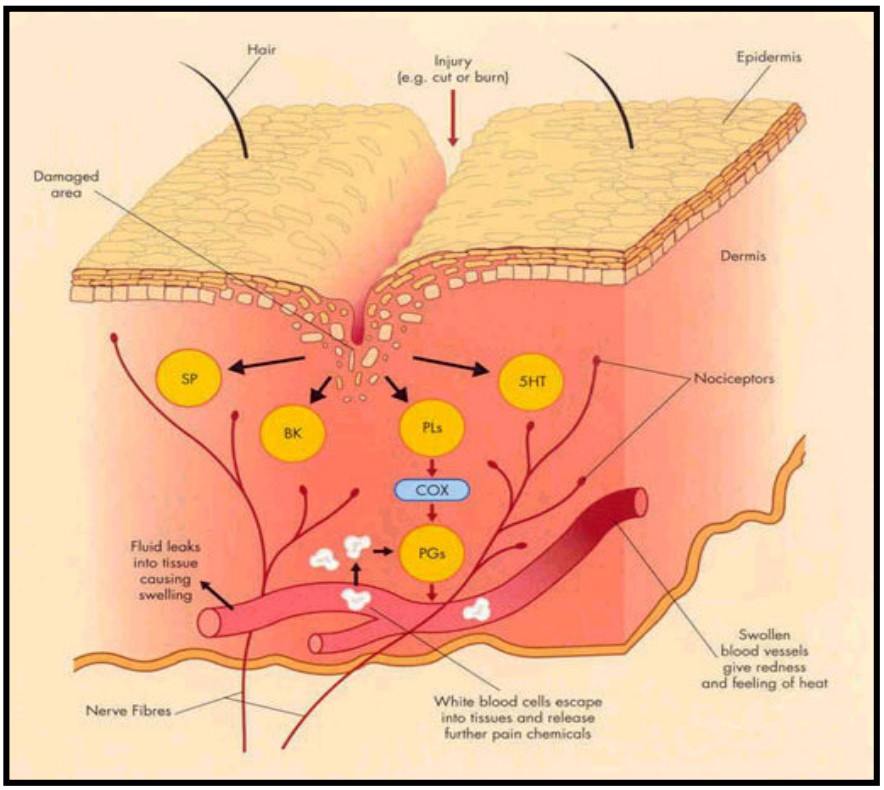


Fig 7: Physiology of inflammation
(Takeuchi *et al*., 2003 *Curr Top Microbiol Immunol* 22: 1767-75)

cyclooxygenase (Vane & Botting, 1998; Tabernero *et al.,* 2003). Oedema is caused by the transvascular flux of protein-rich fluid from the intravascular compartment into the interstitium as a result of the actions of histamine, bradykinin, leukotrienes, complement components, substance P and platelet-activating factor (PAF) (Friedl *et al.,* 1989). Vasodialation and fluid exudation are accompanied by leukocyte margination, adhesion and migration. Neutrophils are the first and most abundant leukocytes to be delivered to a site of infection or inflammation (Downey *et al*., 1993). After migration, a weak

adhesive interaction develops between neutrophils and vascular endothelial cells, causing neutrophils to remain in close proximity to the vascular endothelium. Neutrophils and other leukocytes also require chemoattractants to facilitate their migration to sites of injury or infection. Chemoattractants are soluble molecules such as bacterial byproducts, complement components and chemoattractant cytokines (chemokines) that serve to attract leukocytes to injured tissues (Fig 8). Chemoattractant cytokines or chemokines are leukocyte products that also serve to attract leukocytes into tissues. These

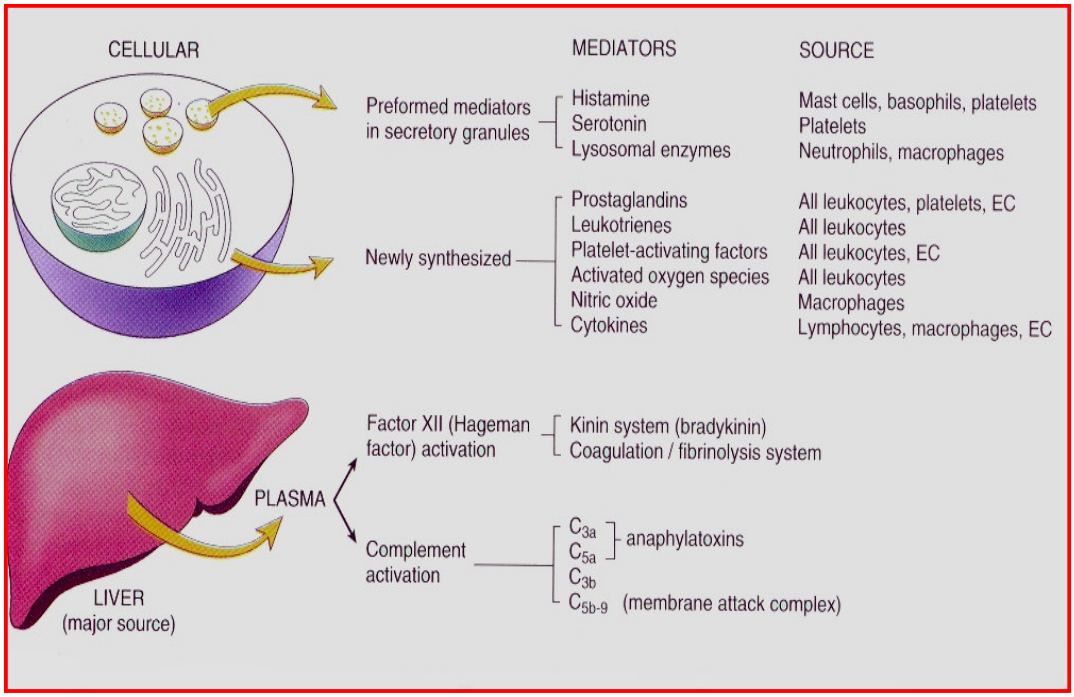


Fig 8: Mediators of Inflammation
(Seely *et al.*, *Critical Care* 7: (2003) 291-307)

are a group of more than 40 peptides with molecular weights of 8–10 kDa that share considerable sequence homology. There are at least four families of chemokines, two of which (the alpha and beta families) have been extensively described (Luster, 1998). The alpha chemokines include interleukins, which

are potent chemoattractant for neutrophils, as well as other members of the family that are lymphocyte chemoattractants. The beta chemokines are chemoattractants for a variety of leukocytes including basophils, monocytes, eosinophils and lymphocytes. The alpha chemokines act in concert with beta chemokines and cytokines to mediate the pro-inflammatory response to injury and infection (Serhan *et al*., 2000; Marcheselli *et al*., 2003; Mukherjee *et al*., 2004).

1.2.1 Mediators of inflammation

1.2.1.1 Lipoxygenase (LOX) pathway

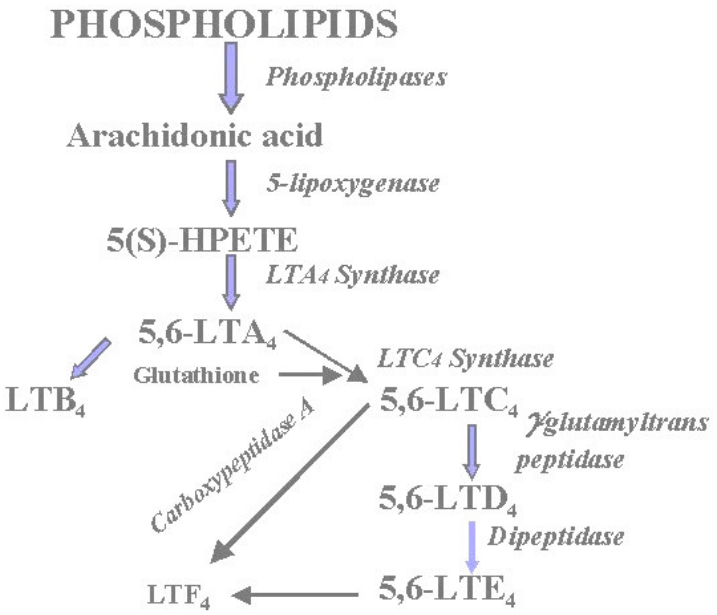
Lipoxygenase pathway leads to the formation of hydroperoxyeicosatetrenoic acid (HPETEs), leukotrienes (LTs) and lipoxins from arachidonic acid. Among these LTs are the most potent biologically active compounds. Lipoxygenases (LOX) comprise a family of non-heme, iron containing dioxygenase enzymes which incorporate molecular oxygen into poly unsaturated fatty acids with 1-cis, 4-cis-pentadiene structures such as arachidonic acid and linolenic acid, which get transformed into 1-hydroperoxy-2,4-trans, cis petadiene product, generating the corresponding hydroperoxy derivative of the fatty acid.

Classification of Lipoxygenases

LOXs have been classified basing on their positional specificity of arachidonate oxygenation (Yamamota, 1992). For example, 15-LOX introduces molecular oxygen at carbon atom 15 of arachidonic acid (formation of 15-HPETE) where as 12-lipooxygenase introduces oxygen at 12-position to generate 12-HPETE. These enzymes are now referred to as 12-,15-,5- and 8-lipooxygenases.

5-Lipoxygenase

5-LOX is involved in the biosynthesis of leukotrienes, pro inflammatory mediators participating in various forms of acute inflammation (Fig. 9). In addition to human allergic asthma, LTs contribute to the pathogenic changes during colitis (Zipser *et al.,* 1987), psoriasis (Chan *et al.,* 1987) glomerulonephritis (Wu *et al*., 1993) and endotoxemia (Keppler *et al*., 1987). 5-LOX in the presence of FLAP catalyzes the oxygenation of arachidonic acid at C-5 into 5-hydroperoxyeicosatetraenoic acid (5-HPETEs) (Samuelsson *et al*., 1987), followed by a second reaction in which 5-HPETE is dehydrated to form the unstable allylic epoxide, LTA_4 by the same enzyme. Once formed LTA4 is further metabolized to LTB_4 (Borgeat & Samuelsson, 1979). Another pathway is the formation of LTC_4 from LTA_4 by an enzymatic conjugation with glutathione at C-6 position, a reaction catalyzed by LTC_4 synthase (Jakschik *et al*., 1982).



F*ig. 9: Lipoxygenase pathway*

Successive elimination of glutamic acid from LTC_4 by γ-glutamyl traspeptidase (Orning *et al*., 1980) and glycine by dipeptidase gives LTD_4 and LTE_4 respectively (Sok *et al.,* 1981). LTE_4 on further action by γ-glutamyl traspeptidase results in the formation of LTF_4 (Anderson *et al*., 1982). LTF_4 can also be formed directly from LTC_4 by the action of carboxypeptidase A (Reddanna *et al.,* 1988). These three sulfidopeptide LTs are commonly referred to as the slow reacting substances of anaphylaxis (Piper, 1985). 5-HPETE is also converted to a dihydroxyeicosatetranoic acid (diHPETE), which may lead to the formation of trihydroxyeicosatetranoic acid, tremed as lipoxins (Rokach & Fitzsimmons, 1988). 5-LOX protein expression and activity is mainly observed in myeloid cells. Granulocytes, monocytes/macrophages, mast cells and B-lymphocytes express 5-LOX, while platelets, endothelial cells, T-cells and erythrocytes are 5-LOX negative (Borgeat & Samuelsson, 1979; Jakobsson *et al.,* 1992).

Biological Role of 5-Lipoxygenase

The Cysteinyl leukotrienes (LTC_4, LTD_4, LTE_4) released from the lung tissue of asthmatic patients on exposure to allergens play a pathophysilogical role in immediate hypersensitivity reactions by increasing the vascular permiability in post capillary venules and stimulating mucus secretion (Bisgaard, 1984; Poff & Balazy, 2004). The dihydroxyl leukotriene, LTB_4 displays chemokinetic activity towards granulocytes and induce granulocyte activation which induces adherence of neutrophils to vessel walls and release of lysosomal enzymes (Ziboh *et al*., 2004). LTC_4 demonstrated to be present in the hypothalamus, is implicated in the release of leutinizing hormone (Hulting *et al.,* 1985). Lipoxins formed by the combined action of 5-LOX, inhibit natural killer cytotoxicity (Samuelsson *et al*., 1987). Lipoxins act as

both immunologic and haemodynamic regulators. Leukitrienes are also implicated in insulin release (Pek & Walsh *et al.,* 1984), steroidogenesis (Dix *et al*., 1984) and ovulation (Reich *et al.,* 1985). Cysteinyl leukotrienes (LTC_4, LTD_4, LTE_4) are identified as important mediators of bronchoconstriction and hypersensitivity reactions.

1.2.1.2 Cyclooxygenase pathway

Cyclooxygenase pathway leads to the formation of PGs, thromboxanes and prostacyclins- a family of autocrine and paracrine mediators that contribute to many physiological and pathophysiological responses (Fig. 10). Cyclooxygenase (COX; Prostaglandin endoperoxide

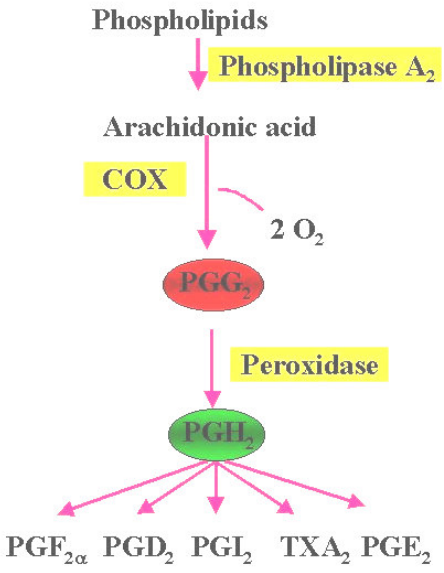


Fig 10: Cyclooxygenase pathway

synthase EC 1.14.99.1) catalyzes two separate enzyme reactions i) the bisoxygenation of arachidonic acid at carbon11 and 15 (cyclooxygenase activity) and ii) the subsequent bi- electron reduction at 15-hydroperoxy group of PGG_2 (Peroxidase activity) to form another endoperoxide derivative, PGH_2

(Smith *et al*., 1996). These two reactions occur at distinct but structurally and functionally interconnected sites. The endoperoxides, PGG_2 and PGH_2 are very unstable with a half life of 4-5min. PGH_2 is transformed to different primary products such as PGD_2, PGE_2, $PGF_{2\alpha}$, prostacyclin and thromboxanes collectively known as prostanoids. The array of PGs produced varies depending on the down stream enzymatic machinery present in a perticular cell type. Prostaglandins are found in animals as primitive as the coelenterates and in a wide variety of human tissues (Mead *et al.,* 1986). PGs not only paly a central role in inflammation, but also regulate other critical physiological responses. In humans, PGs are involved in diverse functions, including blood clotting, ovulation, initiation of labor, bone metabolism, nerve growth and development, wound healing, kidney function, blood vessel tone and immune responces (Smith *et al*., 1996).

Cyclooxygenases

Cyclooxygenases isoforms- cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) are homo-dimeric, glycosylated, heme containing proteins with 2 catalytic sites. Both isoforms have high structural identity but are different in substrate and inhibitor selectivity (Smith *et al*., 1996), and also in their intracellular localization. COX-2 accepts a wider range of fatty acids as substrates when compared to COX-1. COX-2 acetylated by aspirin on serine 530 will still oxidize AA to 15-HETE, whereas similarly acetylated COX-1 will not oxidize AA at all. Unlike COX-1 enzyme, COX-2 has valine at position 523 instead of isoleucine. The difference between valine and isoleucine is of a single methyl group. This substitution allows COX-2 inhibitors to access the secondary internal side pocket of the molecule that is obstructed by isoleucine in the COX-1 isoform (Kurumbail *et al*., 1996; Vane

et al., 1998). The COX-2 active site is about 20 % larger and has a slightly different shape than that of COX-1 (Luong *et al*., 1996). This difference in active site size and shape is due to three amino acid differences between COX-1 and COX-2: isoleucine 523 to valine 523 in the first shell of the active site, and isoleucine 434 to valine 434 and histidine 513 to arginine 513 in the surrounding second shell.

COX-1 is constitutively expressed in most cells at physiological conditions, although there is mild increase (2-4 fold) in response to hormonal or growth factor stimulation. Unlike COX-1, COX-2 expression is minimal in most tissues under basal conditions, but is dramatically upregulated up to 80 fold in inflamed tissues (macrophages, fibroblasts, chondrocytes, epithelial, endothelial cells etc) with cytokines, growth factors, human chorionic gonadotropin, phorbol esters, bacterial lipopolysaccharides (LPS), ligands of G-protein-coupled receptors and reactive oxygen intermediates; cyclic adenosine monophosphate and serum. For example COX-2 expression and PGE_2 production are greatly enhanced in rheumatoid synovium compared to the less inflamed osteoarthritic synovium, and in animal models of inflammatory arthritis (Crofford *et al*., 1994; Anderson *et al*., 1996). This provides for a constant level of enzyme in most cell types to synthesize PGs responsible for homeostatic functions. In contrast, the features of COX-2 gene are those of an “immediate early gene” that gets upregulated during inflammation or pathological process. Both the isoforms have a molecular weight of 72 kDa and are almost identical in length, with just over 600 amino acids, of which 60-65 % sequence is identical between COX-1 and COX-2 from the same species and 85 %- 90% identity among individual isoforms from different species (Shimokawa & Smith, 1992; Garavito *et al*., 1996).

However, the human COX-2 gene at 8.3 kb is a small immediate early gene, whereas human COX-1 originates from a much larger 22 kb gene. The gene product also differs, with the mRNA for the inducible COX-2 being approximately 4.5 kb and that of constitutive COX-1 being 2.8 kb. COX-2 mRNA contains long 3' untranslated regions (3' UTR) containing several different polyadenylation signals and multiple 5'-AUUUA-3' instability sequences that act to mediate rapid degradation of the transcript. These features differentiate the gene for COX-1 into a gene consistent with rapid transcription and mRNA processing for a continously transcribed stable message. COX-1 and COX-2 proteins are encoded by separate genes that diverged well before birds and mammals (Reed *et al.*, 1996). The genes for COX-1 and COX-2 are located on separate chromosomes, with COX-1 on chromosome 9 and COX-2 on chromosome 1. The promoter region of the COX-2 gene contains a TATA sequence and transcription factor response elements, which are sensitive to inflammatory mediators accounting for its rapid inducibility. The gene for COX-1 lacks the TATA sequence and immediate early response elements.

1.2.3 NSAIDs

In 1893 German chemist, Felix Hoffman, motivated by concern for his father's severe rheumatoid arthritis set into motion a commercial process for his employer, the Bayer company, to produce a molecule with analgesic activity called salicylic acid or aspirin. What evolved from this historic event was a class of drugs, the non steroidal anti-inflammatory drugs (NSAIDs), that gained immense interest not just because they are the major targets of cyclooxygenases, but also because they are involved in a range of pathologies that for COX-1 include thrombosis and for COX-2 include

inflammation, pain and fever, various cancers, and also Alzheimer's disease (DuBois *et al.*, 1998; Kawai *et al.*, 2002; Marnett, 2002). Specific inhibition of COX-2 might have therapeutic actions similar to those of NSAIDs, but without causing the unwanted side effects, was the rationale for the development of specific inhibitors of the COX-2 enzyme as a new class of anti-inflammatory and analgesic agents with improved gastrointestinal tolerability (Vane, 1998).

1.2.3.1 NSAIDs as chemopreventives

Epidemiological evidences

Epidemiological studies have shown that chronic intake of aspirin is associated with a reduction in the incidence of colorectal cancer (Thun *et al.*, 1991; Giovannucci *et al.*, 1995) and breast cancer (Garcia & Gonzalea, 2004). In a clinical trial, celecoxib (Steinbach *et al.*, 2000) was found to reduce the number and size of polyps in patients with familial adenomatous polyposis (FAP). The effects of several non-steroidal anti infflammatory drugs (NSAIDs) on tumor growth have also been demonstrated in animal models of FAP (Oshima *et al.*, 1996) and chemical colon carcinogenesis (Takahashi *et al.*, 1996; Kawamori *et al.*, 1998; Yoshimi *et al.*, 1999; Kishimoto *et al.*, 2000) and prostate cancer. These observations suggest that NSAIDs have a potent chemopreventive effect.

1.2.3.2 Mechaniam of action of NSAIDs

NSAIDs inhibit the cyclooxygenase (COX) enzymes, COX-1 and COX-2, which catalyse the conversion of arachidonic acid to prostaglandins. COX-1 is expressed constitutively and is required for physiological processes such as maintenance of gastrointestinal mucosa and platelet aggregation, whereas COX- 2 is induced by cytokines, growth factors, and mitogens. NSAIDs vary in their abilities to inhibit COX-1 and COX-2 (Thun *et al.*, 2002).

Classic NSAIDs not only inhibit COX-2, but also inhibit COX-1, resulting in the common side effect of gastric mucosal damage. To reduce the gastrointestinal side effects of NSAIDs, with selective COX-2 inhibitors were developed (Jackson & Hawkey, 2000; Cannon & Breedbeld, 2001; Aisen, 2002). Selective inhibitors of COX-2 have been demonstrated to induce apoptosis in a variety of cancer cells, including those of colon (Hara *et al.*, 1997), stomach (Sawaoka *et al.*, 1998), prostate and breast (Liu *et al.*, 1998). These observations are consistent with the cancer chemopreventive effects of NSAIDs. Tumour inhibition by NSAIDs may be mediated by distinct cellular processes. These processes involve the ability of NSAIDs to restore apoptosis, induce cell-cycle arrest, and inhibit angiogenesis (Chan, 2002; Thun *et al.*, 2002, Madhava *et al.*, 2001;Jagu et al., 2004). One of the main ways by which NSAIDs exert their effects is modulation of apoptosis, although there is considerable debate about how these effects are mediated. Compounds that are structurally similar to NSAIDs, but do not inhibit COX, also have chemopreventive and proapoptotic properties. The precise mechanisms by which, inflammation stimulates cancer development are not fully understood and are likely to be complex and multifactorial (Coussens & Werb, 2002). Indeed, NSAIDs have been shown to inhibit other pathways that contribute to inflammation. For example, NSAIDs inhibit activation of NF-kB, which controls the transcription of a variety of proinflammatory cytokines, independently of COX inhibition (Tegeder *et al.*, 2001; Jagu et al., 2004). It is therefore quite possible that NSAIDs inhibit inflammation stimulated carcinogenesis by affecting the function of these different pathways in addition to those of COX-1 or COX-2. However, because these agents are

potent inhibitors of COX catalytic activity, it might be difficult to judge the relative contribution of these COX and non-COX mechanisms.

1.2.4 Dual COX/LOX inhibition represents a new approach to relief of pain and inflammation

Affecting about 10% of adults in the world' s industrialized countries, Inflammatory diseases like osteo arthritis, a common degenerative disease of the joints characterized by the breakdown of cartilage and the proliferation of new bone and connective tissue. Drugs that can modify disease progression represent the ultimate goal of treatment but are not clinically available. As the search for disease-modifying drugs continues, treatment is currently based on symptomatic relief of pain and inflammation associated with disorders. For years non-steroidal anti-inflammatory drugs (NSAIDs), such as ibuprofen and naproxen, have underpinned symptomatic relief of diseases. NSAIDs work by inhibiting the cyclooxygenase (COX) enzymes, COX-1 and COX-2, so preventing the formation of inflammatory prostaglandins from metabolism of arachidonic acid. However, by inhibiting COX-1 they can also cause serious gastrointestinal (GI) side effects and adversely affect platelet function. Increasingly, conventional NSAIDs are being replaced by COX-2 specific inhibitors, such as Celecoxib, for symptomatic relief of pain and inflammation. At therapeutic concentrations these drugs inhibit COX-2, which is associated with tissue injury, but spare COX-1 and so cause less GI toxicity. Recent studies suggest that NSAIDs induced GI toxicity may involve shunting arachidonic acid metabolism to the 5-LOX pathway, so increasing the production of gastrotoxic leukotrienes (Ziboh *et al*., 2004; Giovanni *et al.,* 2003). 5-lipoxygenase (5-LOX) is an enzyme associated with the production of pro-inflammatory and gastrotoxic leukotrienes (Sala & Folco, 2001).

Inhibition of 5-LOX may therefore offer a new approach to reducing the GI toxicity associated with NSAID use, while retaining the analgesic and anti-inflammatory properties of NSAIDs and COX-2 specific inhibitors (Fiorucci *et al*., 2001). Interestingly, 5-LOX has been implicated in the deterioration of joints in OA (Hinz & Bruna, 2004). Inhibition of 5-LOX may therefore help protect arthrodial cartilage and slow disease progression. Leukotrienes are pro-inflammatory, they recruit pro-inflammatory immune cells, increase vascular permeability, are powerful bronchoconstrictor agents, and are damaging to the gastrointestinal tract (Bias *et al.,* 2004). Due to these limitations research is underway to develop new 5-LOX as well as dual COX/LOX inhibitors as anti-inflammatory agents.

1.3 Vitamin D and inflammatory disorders

Among persons with arthritis of the knee, low levels of vitamin D (fewer than 350 IU daily) were associated with up to a fourfold greater risk of disease progression. Vitamin D deficiency is also associated with numerous illnesses with inflammatory components, such as hypertension, heart disease, diabetes, autoimmune illness (Jahnsen *et al*., 2002). Animal experiments reveal that vitamin D hormone can suppress a variety of animal autoimmune diseases including autoimmune encephalomyelitis (EAE), the animal equivalent of multiple sclerosis (MS) (Van *et al*., 2004). Mice that are VDR deficient develop less severe EAE than their wild-type counterparts (Meehan & DeLuca, 2002). Treatment of mice with $1\alpha25(OH)_2D_3$ completely inhibited EAE induction and progression by synthezing two anti-encephalitogenic cytokines, interleukin-2 and transforming growth factor and influenced inflammatory cell trafficking. So, providing supplemental vitamin D to individuals who are at risk for EAE, MS would be advisable (Hayes, 2000).

Furthermore, immunological studies have shown that vitamin D hormone has a number of immunomodulating functions, all of which contribute to the suppression of inflammatory autoimmune reactions. Many of inflammatory disorders with over expression of COX and 5-LOX were shown to be associated with low vitamin D status. In case of osteoporosis, prostaglandins produced by COX-2 play an important role in the bone resorption (Sylvia *et al.*, 2001) and 5-LOX inhibitors are known to increase bone density (Coffey et al., 1994; Harley et al.,1999*)*. Prostaglandin E_2 antagonist inhibits the osteoclast formation (Inoue *et al.,* 1999). Though an inverse relation ship is reported with regard to the levels of Vitamin D and the incidence of COX/LOX associated disorders, the exact mechanism involved was not elucidated.

1.4 Scope of the present work:

The foregoing studies thus demonstrate the critical role of COX and LOX pathways in the mediation of various immunological disorders and the therapeutic potential of Vitamin D and its analogs towards these disorders. However the precise interaction between these two pathways was not elucidated. The present study was, therefore, undertaken to tese the effects of Vitamin D and its analogs on COX/LOX pathways and their relevance to inflammatory disorders. Specifc objectives of the study are:

- To screen and identify the specific Vitamin D analogs regulating lipoxygenase/cyclooxygenase pathways and thus eicosanoid biosynthesis.
- To evaluate the anti inflammatory effects of specific Vitamin D analogs regulating eicosanoid biosynthesis in:
  - Mouse macrophage cell lines (RAW 264.7)
  - Carrageenan induced rat air pouch model of inflammation
- To understand the molecular mechanisms involved in the anti inflammatory effects of specific Vitamin D analogs.

Chapter 2: Materials & Methods

2.1 Materials

Vitamin D and its analogs were kindly provided by Prof. G. Satya Reddy, Brown University, USA. The mouse macrophage cell line, RAW 264.7, was procured from National Centre for Cell Science (NCCS), Pune, India. Celecoxib and Indomethacin were generous gifts from Unichem Laboratories Ltd., Mumbai, India. RPMI-1640 medium, fetal calf serum (FCS), phenylmethylsulfonyl fluoride (PMSF), leupeptin, aprotinin, pepstatin A, trypsin, tween-20, triton X-100, sodium chloride, lipopolysaccharide (*Escherichia coli* 026: B6), propidium iodide, ethidium bromide, trypan blue, 3-[4,5-Dimethylthiazol-2-yl]- 2,5-diphenyltetrazolium bromide (MTT), hematin, N, N, N', N'-tetramethyl-p-phenylene diamine (TMPD), arachidonic acid, ponceau S, boric Acid, sodium orthovandate, sodium bicarbonate, diethyldithiocarbomate, phosphotidyl choline, EDTA, calcium chloride and acrylamide were purchased from Sigma Chemical Company (St. Louis, USA).

Penicillin, streptomycin, gentamycin, phosphate buffered saline and skim milk powder were purchased from HiMedia Laboratories Limited, India. Ribonuclease A (DNase free), 100 bp DNA ladder, protein A-peroxidase conjugate, goat anti rabbit IgG – alkaline phosphatase conjugate, rabbit anti-goat IgG – peroxidase conjugate, TMB/H_2O_2 and BCIP/NBT were purchased from Bangalore Genei Ltd, India. N, N' –methylene-bis-acrylamide sodium dodecyl sulfate, ammonium persulfate, β-mercaptoethanol and bromophenol blue were purchased from Bio-Rad Laboratories (Hercules, CA, USA).

Culture flasks (25 cm^2 & 75cm^2), 6-well & 96-well multidishes, 65 mm petridishes and serological pipettes were purchased from Nalge Nunc International, USA. Hybond ECL nitrocellulose membrane was purchased from Amersham Life Science, USA. Agarose and Grace's Insect culture

medium were purchased from Gibco BRL, USA. Affinity purified goat polyclonal anti-COX-1, anti-COX-2 and anti-iNOS from Santa Cruz Biotechnology Inc., USA. All other analytical grade chemicals were procured from the local companies.

2.2 Methods

2.2.1 Purification of Cyclooxygenase-1

Ram seminal vesicles were collected from local slaughter house and stored at –80^{0}C until used. Few hours before homogenization, the ram seminal vesicles were removed from the freezer and stored at 4^{0}C for thawing. The tissue was weighed, minced into small pieces and homogenized in the buffer containing 100mM Tris-HCl (pH 8.0), 5 mM EDTA and 5mM DDC. The homogenization was done in a blender initially and later in a Potter-Elvehjem homogenizer. The homogenate was filtered through two layers of cheese cloth to remove fat and waste material. The filtrate was centrifuged at 10,000 rpm for 30 min at 4^{0}C. The supernatant obtained above was again centrifuged at 33,000 rpm for 1 h 10 min at 4^{0}C to obtain microsomal pellet. The microsomal pellet obtained above was suspended in minimum volume of solubilization buffer containing 100 mM Tris-HCl pH 8.0, 5 mM EDTA, 5 mM DDC and 1% Triton X-100, with slow stirring at 4^{0}C for 30 min. The sample was centrifuged again at 42,000 rpm for 1 h 10 min at 4^{0}C and the resulting supernatant was used as the enzyme source for assays.

2.2.2 Expression and extraction of recombinant human COX-2

Spodoptera frugiperda (Sf9) cells were maintained at 28^{0}C in Grace's insect culture medium supplemented with 10% fetal bovine serum, 3.3 g/l yeastolate, 3.3 g/l lactalbumin hydrolysate and 100 IU/ml penicillin and 100

µg/ml streptomycin. Cells at 60% confluency were infected with baculovirus containing human COX-2 (hCOX-2 cDNA sequence cloned into BamH1 site of the baculovirus expression vector pVL941). After 72 h of infection, the cells were collected by centrifugation at 2000 rpm for 5 min at 4^0C. The pellet was suspended in minimum volume of Tris-HCl buffer (50 mM, pH 7.2) containing 5 mM EDTA, 300 mM sucrose, 5 mM diethyldithiocarbomate, 1 µg/ml pepstatin and l mM phenol and sonicated for 3 min. The cell lysate was subjected to centrifugation (1,00,000 g for 1 h) at 4^0C and the microsomal pellet obtained was suspended in Tris-HCl buffer (25 mM, pH 7.2) containing 0.5% glycerol, 0.1% tween 20 and 1 mM phenol. This microsomal fraction was used as enzyme source for further studies.

2.2.3 Purification of potato lipoxygenase

Lipoxygenase from potato tubers was purified as per the method described by Reddanna *et al*., (1990). A 20% crude extract was prepared by homogenizing potato tubers in 40mM potassium phosphate buffer pH 6.3 containing 2mM sodium metabisulfite, 1mM EDTA and 1mM ascorbic acid. The homogenate was passed through four layers of cheesecloth and centrifuged at 10,000 X g for 20 min at 4^oC and used for further purification. Ammonium sulfate fractionation was done initially from 0-20% and later from 20-60% by slow addition of salt to homogenate. The salted out proteins were centrifuged at 10,000Xg for 20min at 4^oC and the 20-60% pellet was dissolved in 40mM potassium phosphate (pH 6.3) buffer and then dialysed against 200 volumes of the same buffer with frequent changes for every 4h. This dialysate was centrifuged and the supernatant was further purified on anion exchange column. Anion exchange column was prepared by suspending 50g of Whatman's DE-52 in 500 ml of 40mM potassium

phosphate pH 6.3 buffer. The gel which settles down was taken and re suspended in 100 ml of 40mM potassium phosphate buffer (pH 6.3). The gel was poured into column and was equilibrated with buffer. Sample obtained after dialysis was loaded on to the column. Column was washed thoroughly and the bound protein was eluted with a linear salt gradient of potassium chloride (0 – 0.4N). The eluted fractions were collected in 2ml fractions at a flow rate of 1ml/min. All the collected fractions were checked for activity and the amount of protein present. All the active fractions were pooled and used as enzyme source.

2.3 Analysis of vitamin D analogs

Vitamin D analogs used in this study were dissolved in absolute ethanol at a concentration of 1mM. Purity of the compounds was checked on straight phase high performance liquid chromatography using C18 column at 264nm using hexane: isoproponol (91:9) as solvent system.

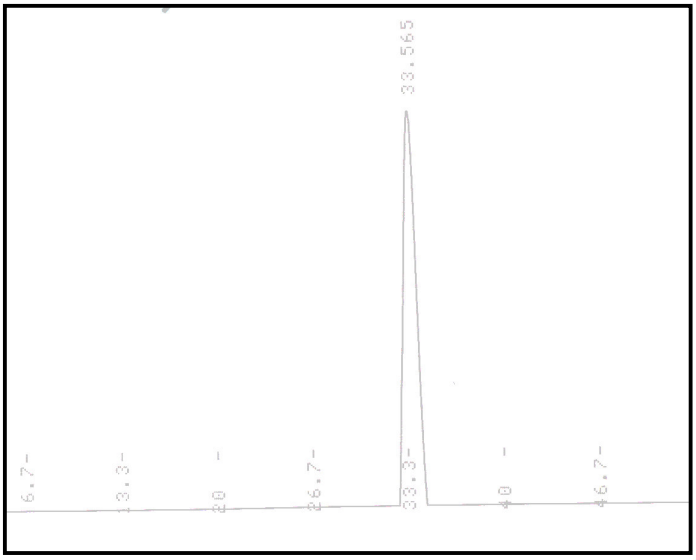


Fig 11: HPLC chromatogram of Vitamin D analogs. Purity of the Vitamin D analogs was checked on straight phase liquid chromatography on c18 column using hexane:isopropanol (91:9) system at 264nm.

2.4 *In vitro* isolated enzyme Assays

2.4.1 Cyclooxygenase Assay

Enzymatic activities of COX-1 and COX-2 were measured according to the method of Copeland *et al.,* (1994) with slight modifications using a chromogenic assay based on the oxidation of N, N, N", N"-tetramethyl-p-phenylene diamine (TMPD) during the reduction of PGG_2 to PGH_2. The assay mixture contained Tris-HCl buffer (100 mM, pH 8.0), hematin (15 µM), EDTA (3 µM), enzyme (100 µg COX-1 or COX-2) and test compound. The mixture was preincubated at 25^0C for 15 min and then the reaction was initiated by the addition of arachidonic acid and TMPD in total volume of 1.0 ml. The enzyme activity was measured by estimating the initial velocity of TMPD oxidation for the first 25 sec of the reaction following the increase in absorbance at 603 nm. A low rate of nonenzymatic oxidation observed in the absence of COX-1 and COX-2 was subtracted from the experimental value while calculating the percent inhibition. The effect of different concentrations of Vitamin D and its analogs, indomethacin and celecoxib on COX-1 and COX-2 activities were examined under the same experimental conditions.

2.4.2 Lipoxygenase Assay

Enzyme activity of lipoxygenase was measured polarographically by using a Clark's oxygen electrode on Gilson model 5/6 oxygraph. The reaction mixture in a volume of 1.6 ml of phosphate buffer pH7.4 and 3mM EDTA enzyme (100µg) and arachidonic acid (133 µM). Decrease in the concentration of oxygen was measured till the slope reduced and maximal slope of O_2 curve was used for the calculation of enzyme activity as per the following equation (Berkely and Galliard, 1976):

$$\text{Enzyme activity} = \frac{\text{Reaction vol. in ml X } O_2 \text{ conc./ ml (0.23 } \mu \text{ moles) X Slope/min}}{\text{Volume of enzyme in ml X Sensitivity}}$$

One unit of enzyme activity is defined as 1 μ mole of oxygen consumed per min. Specific activity is expressed as units/mg protein.

2.5 Generation of ligand and enzyme structures

Docking studies

Docking studies were carried using GOLD (CDDC) docking program. The crystal structures of COX-1 (PDB code: 1Q4G) complexed with α-methyl-4-biphenylacetic acid (BFL) solved at resolution 2 Å and COX-2 (PDB code: 1CVU) complexed with arachidonic acid solved at 2.4 Å resolution were downloaded from PDB database (www.rcsb.org.pdb). Then inhibitor contacts with protein were measured using MOE software. The ligands were removed from the active site and again docked into the active site using GOLD after minimization of protein and inhibitor molecule as standard method for the preparation of protein and ligands molecule before docking. The obtained docked conformation was identical to that in the crystal structure when the scoring function goldscore was used. So, same docking settings were used for further docking studies.

GOLD docking was set for 50 runs. GOLD score (scoring function) was used for scoring the obtained docking solutions. The active site was defined as a sphere of 20 Å radius, with its centre representing the mean of the X, Y, Z coordinates of all the active site residues. The annealing parameters for Vander Waals and hydrogen bonding were set to 4.0 Å and 2.5 Å respectively. The parameters used for genetic algorithm were population size 100, selection pressure 1.1, number of operations 1,00,000,

number of islands 5, niche size 2, migrate 10, mutate 95 and cross-over 95 (all default values).

2.6 Cell culture and treatments

The mouse monocyte/macrophage cell line, RAW 264.7, was maintained in RPMI-1640 medium supplemented with 10% fetal calf serum, sodium bicarbonate (2 g/l), 100 IU/ml penicillin, 100µg/ml gentamycin and 100 µg/ml streptomycin. The cells were maintained at 37°C in a humidified atmosphere of 95% air, 5% CO_2 and were fed every 3-4 days. Cells in the log phase were detached by scraping and were split at a ratio of 1:2 to 1:6, and were routinely fed every 24 h before harvest for experiments. Before each experiment, cells were washed twice with sterile Ca^{2+} and Mg^{2+} free phosphate buffered saline and then were resuspended in sterile PBS at a concentration of 10^6 cells/ml. Cell number and viability were determined by 0.4% trypan blue using hemocytometer with an anticipated accuracy of $\pm$ 10-20%.). A stock solution of 1 mM of RO-23-7553 was prepared in PBS and diluted in standard growth medium to a final concentration of 0.1 nM to 1 µM. Cells were incubated for different periods of time at 37°C in the presence of various concentrations of RO-23-7553, LPS (1µg/ml) and NMMA (1mM). RAW 264.7 cells in the maximal range of 20 passages were used for this study.

2.7 Cell viability assay

The *in vitro* effects of Vitamin D analogs on the growth of RAW 264.7 cells was determined by measuring 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) dye absorbed by living cells (Campling *et al,* 1988). RAW 264.7 cells were cultured in flat-bottomed 96 microtitre plate, at initial concentrations of 1 X 10^4 cells/ well. Following an adherence period,

the medium was changed and cells were treated with LPS (1μg/ml), NMMA (1mM) and with or without Vitamin D analogs dissolved in RPMI medium (0.1 nM, 1 nM, 10 nM, 100 nM and 1 μM) for 4, 8, 12 and 24 h at 37°C. RAW 264.7 cells cultured without Vitamin D analogs were used as control. At the end of each time point, 20 μl of a sterile solution of MTT (5 mg/ml) in PBS was added to each well and the plates were incubated for an additional 4 h at 37°C. A purple-blue formazan precipitate was visualized in wells containing viable cells. MTT solution in the medium was aspirated off. To achieve solubilization of the formazan crystals formed in viable cells, 100 μl of dimethyl sulfoxide was added to each well. The plates were shaken for 30 min at room temperature and absorbance was read immediately at a wavelength of 570 nm on Quant Bio-Tek Instruments, Inc. microtiter plate reader.

2.8 Preparation of cell lysate

For the determination of cyclooxygenases, inducible nitric oxide synthase, and Interleukin-2 in RO-23-7553 treated RAW 264.7 macrophages, the extraction of total proteins was performed as per the method of Mitchell *et al.,* (1994) with slight modifications. RAW 264.7 cells were cultured in 75 cm^2 flask. After reaching the confluence (60%), cells were treated with LPS (1μg/ml), NMMA (1mM) and RO-23-7553 (10nM) were incubated for different time periods (4,8,12 and 24h) in humidified chamber at 37°C. After incubation, cell monolayers were harvested and washed with PBS and incubated with lysis buffer (Tris-HCl (50 mM, pH 7.5) containing 150 mM NaCl, 1 mM EDTA, 1% sodium deoxycholate, 1mM sodium ortho-vandate, 50 mM β–glycero phosphate, 50 mM sodium fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10

μg/ml pepstatin A and 1 mM phenylmethylsulfonyl fluoride. The samples were then placed on ice for 1 h and sonicated for 10 s and then centrifuged for 5 min at 14,000 rpm in Remi cooling microfuge at 4°C to remove the particulate sediment. Cell supernatant was collected and stored at –80°C for further analysis. Protein content in the supernatant was determined by Bradford method (Bradford, 1976).

2.9 SDS-PAGE analysis

SDS-PAGE analysis of proteins was performed by Laemmli method (1970). Whole cell lysates were treated with 4X sample buffer containing 1% SDS, 5% β-mercaptoethanol, 0.01% bromophenol blue and 20% glycerol in 0.063 M Tris-HCl pH 6.8 for 5 min in water bath at 100°C. Samples containing 100 μg of protein from whole-cell extract of both control and treated cells were subjected to SDS-PAGE analysis using 7.5/10/15% separating gel with 5% stacking gel. Electrophoresis was carried out at a constant voltage (100 V) in a buffer containing 25 mM Tris-HCl, 192 mM glycine and 0.1% SDS pH 8.3

2.10 Western blotting

Western blot analysis was carried out according to the procedure of Towbin *et al.,* (1979). The total cell lysate separated on SDS-PAGE were transferred on to the nitrocellulose membrane with a constant current of 50 V in a buffer containing 25 mM Tris-HCl, 192 mM glycine and 20% methanol for 6 h. After electrophoretic transfer of the proteins from the polyacrylamide gel to nitrocellulose membrane, nonspecific binding sites were blocked by incubating the membrane with 5% nonfat dry milk in Tris-buffered saline (TBS) [25 mM Tris-HCl (pH 8.0), 150 mM NaCl] for 1 h at room temperature. After being washed once with TBST (TBS + 0.05% Tween-20) and once with TBS, 15 min each, the membrane was probed with a polyclonal anti- goat

cyclooxygenase-2, anti - goat cyclooxygenase-1, anti mouse nitric oxide synthase-2 and anti rabbit interleukin-2 diluted 1:500 in 1% nonfat dry milk in TBS for overnight at 4°C. The membrane was then washed three times with TBST (1 x 15 min, 2 x 5 min). Each membrane was then incubated with the corresponding secondary antibody: anti-mouse IgG -ALP conjugate and anti-goat IgG-ALP conjugated antibody diluted 1:1000 in 1% nonfat dry milk in TBS. The membrane was washed once with TBST and once with TBS, 15 min each, and the proteins were visualized by incubating with colorigenic substrate, BCIP/NBT. The reaction was stopped by washing the membrane with TBS after which the membrane was dried and stored.

2.11 RT-PCR analysis

Mouse macrophage cells (RAW 264.7) were incubated with LPS(1µg/ml), NMMA(1mM) and RO-23-7553 for different time intervels (4,8,12 and 24h) to determine the levels of Cyclooxygenase mRNA by RT-PCR.Total RNA was isolated by Trizol method. 2µg of total RNA was reverse transcribed according to superscript preamplification system with some modifications. Breifly, the mixture in 1X PCR buffer (20mM Tris-HCl pH 8.4, 50mM KCl, 2.5mM $MgCl_2$,1mg/ml bovine serum albumin) containing 0.5mM dNTP, 0.01 M dithiothreatol, 0.5 µg of oligo (dT) and 200 units of superscript reverse transcriptase in a 50µl reaction volume was incubated for 60 min at 37°C. The reaction was terminated by heating at 95°C for 10min after which the reaction mixture was chilled on ice for 10 min. The reverse transcribed cDNA was diluted 5 times and 10µl of the c DNA was amplified in a final volume of 100µl containing 10µl of 10X buffer, 1µl of 10mM dNTP, 0.5µg of sense primer, the same amount of anti sense primer and 2.5 units of Taq

polymerase. The PCR products obtained were of 450bp and 583bp for COX-1 and COX-2 respectively. The amplification conditions were: COX-1 94°C for 2min for one cycle and 94°C for 1 min (denaturation) and 68°C for 5 min (primer annaeling and extension) for 30 cycles. COX-2: 94°C for 2 min for one cycle and 94°C for 1 min and 55°C for 5min for 30 cycles. Aliquots of PCR products were run on an 1.2% agarose gel.

COX-2: 5' ACTCACTCAGTTTGTTGAGTCATTC3' forward

5' TTTGATTAGTACTGTAGGGTTAATG 3' reverse

COX-1: 5'TGCATGTGGCTGTGGATGTCATCAA 3' forward

5' CACTAAGACAGACCCGTCATCTCCA3' reverse

2.12 DNA extraction and agarose gel electrophoresis

DNA was extracted from both floating and attached cells after 24 h treatment with LPS (1μg/ml) and NMMA (1mM) and RO-23-7553 (10nM) using the SDS/ Proteinase K/ RNase A (Hermann *et al.*, 1994). Five million cells were pelleted, washed in cold PBS and lysed in a buffer containing 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.2 % Triton X-100 for 20 min at 4°C. After centrifugation at 14,000 g for 15 min, the supernatant was treated with Proteinase K (0.5 mg/ml) and 1% SDS for 1h at 50°C. DNA was extracted twice with buffered phenol and precipitated with 140mM NaCl and 2 volumes of ethanol at -20°C overnight. DNA precipitates were washed in 70% ethanol, dissolved in TE, and treated for 1 hour at 37°C with RNase A. Fifteen microliters of DNA was mixed with 3μl of DNA sample buffer (0.25 % bromophenol blue, 0.25 % xylene cyanol and 30 % glycerol), and was resolved on 1 % agarose gel in TBE (44.6 mM Tris, 44.5 mM boric acid and 1 mM EDTA). DNA was visualized upon staining gel with ethidium bromide (0.5

mg/ml) and exposed to UV light. The presence of DNA migration on the gel was recorded.

2.13 Flow cytometric analysis

Flow cytometry was performed according to the procedure of Nicoletti *et al* (1991) with slight modifications. RAW 264.7 cells cultured in 6-well plates were treated with LPS(1μg/ml), NMMA (1mM) and RO-23-7553 (10nM) for 4, 8, 12 and 24 h. The cells were scraped, washed three times with phosphate buffered saline and then fixed by adding 1ml of 70% ethanol gradually for overnight at 4^{o}C. The fixative was removed by centrifugation and washing twice with phosphate buffered saline. These cells were gently resuspended in 1 ml DNA staining reagent (phosphate buffered saline pH 7.4 containing 0.1% Triton X-100, 0.1 mM EDTA, 50 μg/ml of DNase-free RNase A and 50 μg/ml propidium iodide). The cells were then incubated in the dark for over one hour at room temperature and analyzed within 24 h. Flowcytometric assay was performed using FACS Vantage (Becton Dickinson, Biosciences). Ten thousand events were evaluated using the Cell Quest Program.

2.14 Animal model and experimental design

2.14.1 Air pouch model of inflammation

Adult Wistar strain male rats (150 to 200 gms) were used for all the experiments in the present study. Air cavities were produced by subcutaneous injections of 20 ml of sterile air into the intracapsular area on the dorsal side of the animal. An additional 10 ml of air was injected into the cavity every three days. Seven days after the initial air injection, 2 ml of 1.5% (w/v) solution of carrageenan dissolved in saline was injected directly into the pouch to produce an inflammatory response. Control animals received 2 ml

of saline only. For the time course studies, animals were sacrified by cervical dislocation at various time points after the injection. Pouch tissue was carefully dissected and cut open to aspirate the inflammatory exudate into graduated tubes. The pouch lining was separated from the muscle and dissected out, and rinsed in saline before processing further.

2.15 Administering of RO-23-7553

All the treatments were given along with carrageenan directly into the pouch cavity. The Vitamin D analog inhibiting specifically COX-2 i.e 1α25 $(OH)_2$-16-ene-23-yne D_3 (RO-23-7553) and celecoxib, a known COX-2 inhibitor were administred into the rat air pouch. The stock solution of RO-23-7553 (1mM) and celecoxib (10mM) was prepared in absolute ethanol and dimethyl sulphoxide respectively and further dilutions were made in carrageenan at the time of experiment.

1. Carrageenan (2ml of 1.5% (w/v) carrageenan in saline)
2. Celecoxib (20mg /Kg body weight)
3. 1α25 $(OH)_2$-16-ene-23-yne D_3(RO-23-7553) (10µg/Kg body weight)

2.16 Air pouch model- microscopic studies

2.16.1 Inflammatory reaction

The degree of inflammation was assessed by measuring the volume of the pouch fluid and the number of cells infiltering into the pouch. The volume of exudate was measured by pipetting the pouch fluid carefully. To measure the total cell population in the fluid the pouch was gavaged using 20 ml of saline repeatedly and the fluid was centrifuged at 3000 rpm for 10 minutes. The pelleted cells were washed twice with RPMI containing 5% FCS, suspended in 1ml of the saline and counted on heamocytometer.

2.16.2 Histology of pouch tissue

Rat air pouch tissue from control and treated animals (Carrageenan and Carrageenan + RO-23-7553) were rinsed in PBS and fixed in Bouin's fixative (70% saturated picric acid, 25 % formaldehyde and 5% glacial acetic acid) overnight followed by thorough washing with distilled water. Tissues were then dehydrated sequentially in 70%, 80%, 90% alcohol and finally absolute alcohol for 10 min each. After dehydration the tissue was processed in alcohol and benzene (3:1 for 10 min, 1:1 for 10 min, benzene and paraffin for 10 min) to embed in paraffin wax. The tissue was placed in molten paraffin for 2-3 hours to allow infiltration of paraffin into the tissue and then allowed to harden. Thin sections (10μM) were taken on Leitz microtome and mounted on ploylysine-coated slides.

2.16.3 Homogenisation of pouch tissue

Pouch lining was carefully dissected out and homogenized in 100 mM Tris-HCl (pH 8.0), 0.3M mannitol, 1mM EGTA, 1mM EDTA, 4mM K_2HPO_4, 1mM DTT, 1mM Sodium ortho vandidate, 0.1% SDS, 2mM PMSF and 40μl/ml of complete protease inhibitor solution, centrifuged for 30 minutes at 10,000 rpm, 4^oC. Protein content in the supernatant was measured by Lowry method (Lowry *et al*., 1951) and this was used for further experiments.

2.17 SDS-PAGE and Western Blotting

SDS PAGE and Western blot analyses for the detection of COX-1, COX-2, iNOS, and IL-2 in the air pouch tissue homogenate were performed by the procedure as mentioned earlier (section 2.9 & 2.10)

2.18 RNA isolation

RNA was isolated using Trizol reagent and the isolation steps were followed as mentioned in the instruction booklet provided by Invitrogen.

100mg of the air pouch tissue was homogenised in 1ml of Trizol reagent, incubated at room temperature for 5 min, followed by centrifugation at 7,500 X g for 10 min and the supernatant was collected. 0.2ml of chloroform was added to the supernatant, and tubes were vigorously mixed for 15 s and incubated at 30º C for 2-3min.This was centrifuged at 12,000 X g for 10 min. at 4º C. The supernatant (aqueous phase) was collected and equal volume of isopropyl alcohol was added. The samples were incubated at 10-30º C for 10min and centrifuged at 12,000 X g for 10 min at 4º C. The RNA pellet was collected and washed in 75% ethanol. The pellet was dried and dissolved in Millipore water/molecular grade formamide. RNA was quantified spectrophotometrically followed by electrophoresis on a formaldehyde/MOPS/EDTA agarose gel (1.2%).

2.19 RT-PCR analysis

Expression of COX-1 and COX-2 was studied from the total RNA isolated by using specific primers. The reaction mixture contained:

10x RT-PCR buffer - 2µl

Forward primer - 5pmoles

Reverse primer - 5pmoles

$MgCl_2$ – 0.6µl

Taq polymerase - 1unit

Reverse transcriptase enzyme - 1 unit

Template RNA - 2µg

The total reaction volume was made to 20µl using double distilled water. The specific primers to COX-2 and COX-1 genes were used to amplify the respective products.

COX-2

5'-CCA TGT CAA AAC CGT GGT -3' – forward

5'-ATG GGA GGG CAG TCA TCA -3' – reverse

These primers would amplify a product of 521bp

COX-1

5' GTG CCG GGT CTG ATG ATG TATG3' forward

5' CAC GGC CCA GAC TAC TACATA C 3 reverse

These primers would amplify a product of 600 bp

2.20 Biochemical parameters

2.20.1 Reduced glutathione (GSH) estimation

250 mg of carrageenan and RO-23-7553 treated rat air pouch tissue was homogenized in 3.5ml of 100mM phosphate buffer pH 7.0 with 1ml of 25% phosphoric acid. Homogenate was centrifuged at 10,000 rpm at 4º C for 30 min. 0.5 ml of supernatant was diluted with 4.5 ml of 100mM phosphate buffer pH 7.0, and this diluted sample was used as the source for GSH estimation. The reaction mixture for each sample consisted of 1800µl of 100mM phosphate buffer pH 7.0, 100µl of tissue homogenate and 100µl of O-phthalaldehyde (OPT) (1% solution). This reaction mixture was incubated at room temperature for 15 min and the emission of fluorescence was monitored at 420nm with excitation at 350nm on Hitachi spectrofluorometer.

2.20.2 Glutathione peroxidase (GPx) activity

Perfused tissues were homogenized (10%w/v) in 50mM phosphate buffer, pH 7.0, containing 1mM EDTA, 1mM PMSF and 250mM sucrose. The cytosolic fraction was used as the enzyme source to estimate the peroxidase activity. The reaction mixture in a final volume of I ml contained 50 mM

sodium phosphate buffer (pH 7.0) containing 1mM EDTA, 0.24 U/ml yeast glutathione reductase, 0.3 mM glutathione (reduced), 0.2 mM NADPH, 1.5 mM cumene hydroperoxide and cytosolic fraction. Reaction was initiated by adding NADPH and its oxidation was monitored at 340 nm by observing the decrease in OD for 1 min. One unit of enzyme activity have been defined as nmoles of NADPH consumed/min/mg protein based on an extinction coefficient of 6.22 mM-1 cm-1. One unit of activity was defined as one nmole of NADPH oxidized per min.

$$ROOH + 2GSH \longrightarrow ROH + H_2O + G\text{-}S\text{-}S\text{-}G$$

Activity of enzyme was calculated according to the following equation:

$$\frac{\text{Difference in absorbance for 1min} \times \text{volume of the reaction mixture in ml}}{\xi\,\text{NADPH}\ (6.2) \times \text{volume of the enzyme in ml}}$$

Specific activity was expressed as unit per mg protein, where one unit was defined as one nmole of NADPH oxidised per minute.

2.21 Statistical analysis

Each experiment was performed in triplicates. Data was expressed as mean ± standard error. Correlations between the various parameters were analyzed using regression analysis. p-value was determined by the Student' s T-test. A p-value of less than 0.05 was considered as a significant difference.

Chapter 3
Effect of 1α,25-(OH)$_2$D$_3$ analogs on 5-lipoxygenase and cyclooxygenases

Arachidonic acid (AA) is the most abundant polyunsaturated fatty acid found in the phospholipid cell membranes. Activation of the phospholipase A_2 in response to various stimuli, releases arachidonic acid from phospholipids which can be further metabolized by two major enzymes, Cyclooxygenase (COX) and Lipoxygenase (LOX) leading to pro inflammatory mediators, prostanoids and leukotrienes respectively. COX has two distinct catalytic activities: cyclooxygenase activity, which oxidizes AA to the hydroperoxy-endoperoxide, PGG_2 and peroxidase activity, which subsequently reduces PGG_2 to the hydroxy endoperoxide PGH_2. PGH_2 is transformed to various prostanoids tissue specifically by specific synthases. COX exists in two isoforms i.e. constitutively expressed COX-1 and inducible COX-2. COX-1 is generally involved in regulation of physiological house keeping functions where as COX-2 isoform is inducible to inflammatory stimuli like cytokines, tumor promoting agents and growth factors etc. Prostaglandins produced by COX-2 play a major role in inflammatory reactions and in various cancers.

Lipoxygenases are the second major metabolizing enzymes of the arachidonic acid pathway and mainly involved in the synthesis of potent inflammatory mediators. LOXs are dioxygenases catalyzing the oxygenation of polyunsaturated fatty acids containing a 1,4-cis-cis-penta diene moiety to produce hydroperoxy derivatives. Until now, three major isozymes have been observed in human beings, classified according to their positional specificity of arachidonic acid oxygenation: the 5-,12- and 15-LOX, which insert molecular oxygen at the C-5, -12 and –15 positions of arachidonic acid respectively. Among these, 5-LOX has been widely studied as it is involved in the synthesis of potent inflammatory mediators, leukotrienes (LTs).

Non steroidal anti inflammatory drugs (NSAIDs) are the most widely used drugs in treatment of various inflammatory diseases such as arthritis, various cancers and in relieve of pain (Garavito, 1996). Their clinical effectiveness is thought to be caused by their ability to inhibit the catalytic activity of COX-2. Because of their non-specific inhibition of both COX isoforms, they are activated with gastric side effects and kidney disorders. These observations provided a rationale for the development of COX-2 selective inhibitors that should retain the potent anti inflammatory and analgesic effects with less side effects. In addition, the 5-LOX pathway, which generates products particularly important in inflammation, is up regulated during COX blockade and is thus potentially responsible for undesirable side effects, such as asthma. Dual inhibition of COX-2 and 5-LOX is therefore an interesting alternative to provide safer NSAIDs. As COX-2 and 5-LOX are up regulated in various cancers and allergic disorders like rheumatoid arthritis, inflammatory bowel disease, asthma and psoriasis, development of drugs targeting both enzymes would be useful direction for chemoprevention.

1α 25$(OH)_2D_3$ is a pluripotent steroid hormone and its physiological roles include calcium and phosphorous homeostasis, regulation of immune system and modulation of the growth and maturity of normal and malignant cells. A number of clinical trails have confirmed that the therapeutic doses of 1α 25$(OH)_2D_3$ induce hypercalcemia. Therefore much effort has been directed to identify analogs with potent cell regulating effects but with less calcemic effects.

The design of new 1α25$(OH)_2D_3$ analogs employed structural modifications of either the side chain or A ring. Promising biological results were obtained from the analogs developed by the introduction of conjugated double bonds, hetero atoms, fluoro atoms and aromatic rings in the side chain of 1α 25$(OH)_2D_3$. After the discovery of epimerization, a new pathway of 1α 25$(OH)_2D_3$ (Reddy *et al*., 2000), a number of analogs have been prepared by the epimerization of A ring of 1α 25$(OH)_2D_3$. A number of studies have demonstrated the potential of 1α 25$(OH)_2D_3$ and its analogs collectively known as deltanoids, in the treatment/prevention of many disorders like inflammation, arthritis, osteoporosis and auto immune disorders. Many of these therapeutic effects were attributed to its immunomodulatory and differentiating properties. Since eicosanoids, the oxygenated metabolites of arachidonic acid via lipoxygenase and cyclooxygenase pathway, are known to mediate many of the above degenerative disorders, an attempt is made in the present study to test the effect of deltanoids on 5-LOX and COX pathway. The various deltanoids employed in the study are given in table 2 and fig 5.

Table 2: Synthetic analogs of 1α 25$(OH)_2D_3$

1α25$(OH)_2D_3$	1α25$(OH)_2$-3-epi-D_3
1α25$(OH)_2$-16-ene-D_3	1α25$(OH)_2$-16-ene-3-epi-D_3
1α25$(OH)_2$-16-ene-23-yne-D_3	1α25$(OH)_2$-16-ene-23-yne-3-epi-D_3
1α25$(OH)_2$-16-ene-23-yne-26,27-F_6-D_3	1α25$(OH)_2$-16-ene-23-yne-26,27-F_6-3-epi-D_3
Two side chain 1α25$(OH)_2D_3$	Two side chain 1α25$(OH)_2$-3-epi-D_3

3.1 Effect of 1α 25(OH)$_2$D$_3$ and its analogs on 5- Lipoxygenase:

Lipoxygenase activity was measured by polarographic method using Clark' s oxygen electrode. In a typical assay, the reaction was initiated by the addition of the substrate, arachidonic acid for 5-LOX. The decrease in oxygen concentration in the reaction mixture was recorded on a graph paper. The rate of decrease in oxygen concentration was used for calculating the enzyme activity.

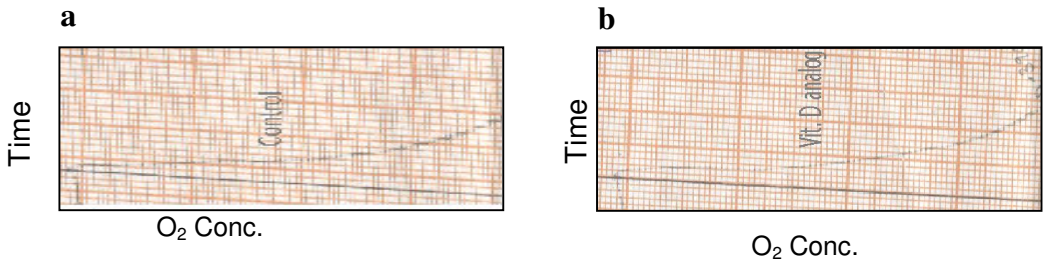


Fig. 12: *Oxygraphic recording of lipoxygenase activity. The typical reaction mixture contained 100mM KH_2PO_4, pH 7.4, 3mM EDTA in a volume of 1.6ml. The reaction was initiated by the addition of arachidonic acid (133μM) and the decrease in O_2 concentration was monitored on an oxygraph. The rate of decrease in O_2 concentration was taken into account for calculating activity a) activity of 5-LOX b) activity of 5-LOX with Vitamin D analog.*

The effect of 1α 25(OH)$_2$D$_3$ and its analogs was studied with potato 5-LOX using polarographic method. Both side chain and epi analogs showed no effect on 5-LOX activity levels up to 5μM concentration. These studies indicate that, the therapeutic effects of 1α 25(OH)$_2$D$_3$ and its analogs may not be mediated through inhibition of 5-lipoxygenase pathway. Hence, no further studies were undertaken on LOX pathway.

3.2 Effect of 1α 25(OH)$_2$D$_3$ and its analogs on COX pathway

The effect of 1α 25(OH)$_2$D$_3$ and its analogs on cyclooxygenase enzyme was studied by employing recombinant human COX-2 and ram seminal vesicles COX-1. The spectrophotometric assay of COX-1 and COX-2 is based on the oxidation of N, N, N' , N' - tetramethyl-p-phenylene diamine (TMPD) during the reduction of PGG_2 to PGH_2. TMPD oxidation for the first 25s of the reaction was monitored by following the increase in absorbance at 603nm. The rate of increase in absorbance was taken for calculating the activity of the enzyme.

Table 3: Effect of 1α25(OH)$_2$D$_3$ and its analogs on COX-1 and COX-2 enzymes

Compound	COX-1 (% +/-)	COX-2(% +/-)
1α25(OH)$_2$D$_3$	No effect	-20
1α25(OH)$_2$-3-epi-D$_3$	No effect	No effect
1α25(OH)$_2$-16-ene-D$_3$	No effect	+25
1α25(OH)$_2$-16-ene-3-epi-D$_3$	+75	No effect
1α25(OH)$_2$-16-ene-23-yne-D$_3$	No effect	-68*
1α25(OH)$_2$-16-ene-23-yne-3-epi-D$_3$	No effect	-38
1α25(OH)$_2$-16-ene-23-yne-26,27-F$_6$-D$_3$	-20	No effect
1α25(OH)$_2$-16-ene-23-yne-26,27-F$_6$-3-epi-D$_3$	+88.4	No effect
Two side chain 1α25(OH)$_2$D$_3$	No effect	No effect
Two side chain 1α25(OH)$_2$-3-epi-D$_3$	No effect	No effect

+ Activation; – Inhibition

*Effect of Vitamin D and its analogs (1μM) on COX-1 and COX-2 activity were tested on a spectrophotometer by employing TMPD assay. * values calculated in the presence of compound at 10nM concentration. The percent increase (+%) or decrease (-%) in the activities were calculated in relation to the activities in the absence of Vitamin D analogs.*

The inhibition of 1α25(OH)$_2$D$_3$ and its analogs on COX enzyme is shown in table 3. From the data It is evident that epi analogs 1α25(OH)$_2$-16-

ene-3-epi-D_3 and 1α25$(OH)_2$-16-ene-23-yne-26,27-F6-3-epi-D_3 showed activation of COX-1 at 1μM concentration by 75% and 88.4% respectively with no effect on COX-2.. Of the compounds tested, only 1α25$(OH)_2$-16-ene-23-yne-26,27-F6-D_3 showed COX-1 inhibition (20%) at 1μM concentration while exhibiting no effect on COX-2. Gemini (two side chain) analogs, Gemini 1α25$(OH)_2D_3$ and Gemini 1α25$(OH)_2$-3-epi-D_3, showed no effect on both enzymes upto 1μM concentration. The effects seen were independent of the period of pre incubation of the enzyme with the compounds.

Control **1α 25$(OH)_2$-16-ene-23-yne-D_3**

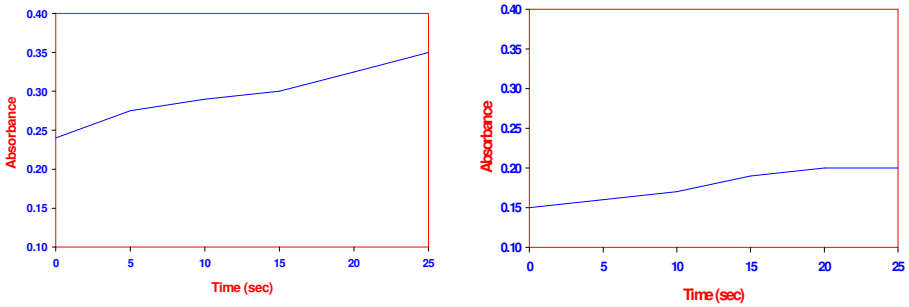


Fig.13 In vitro peroxidase assay of cyclooxygenase as determined on spectrophotometer. The typical assay mixture contained Tris-HCl buffer (100mM,pH 8.0), hematin (15μM), EDTA (3μM) enzyme (100μg of COX-2) and 1α25$(OH)_2$-16-ene-23-yne-D_3 *(RO-23-7553) in a volume of 1ml. The reaction was initiated by the addition of arachidonic acid and TMPD and the increase in absorbance at 603nm was monitored for 25sec.*

3.3 Selective inhibition of COX-2 by 1α 25$(OH)_2$-16-ene-23-yne-D_3 (RO-23-7553)

Fig. 13 shows the spectrophotometric recording of the inhibitory effect of 1α25$(OH)_2$-16-ene-23-yne-D_3 (RO-23-7553) on cyclooxygenase-2 enzyme. Of all the compounds tested only 1α25$(OH)_2D_3$, its side chain analog 1α25$(OH)_2$-16-ene-23-yne-D_3, and 1α25$(OH)_2$-16-ene-23-yne-3-epi-D_3 showed selective COX-2 inhibition. The inhibitory potential of these analogs on COX-2 are in the order of 1α25$(OH)_2$-16-ene-23-yne-D3 (68%) >

1α25(OH)$_2$-16-ene-23-yne-3-epi-D$_3$ (38%)> 1α25(OH)$_2$-D$_3$ (20%). However all these analogs showed no effect on COX-1 enzyme up to 5uM concentration.

Table 4*: The IC_{50} of 1α25(OH)$_2$-16-ene-23-yne-D$_3$, (RO-23-7553) in relation to known inhibitors towards COX-2.*

Compound	COX-2 (IC_{50})	COX-1 (IC_{50})
1α25(OH)$_2$-16-ene-23-yne-D$_3$ (RO-23-7553)	5.8nM	No effect
Celecoxib	0.26µM	16.3µM
Indomethacin	1.74µM	0.22µM

IC_{50} values calculated basing on the activity levels of COX-2 /COX-1 measured in the presence/absence of inhibitors by spectrophotometric assay.

The IC_{50} values of 1α25(OH)$_2$-16-ene-23-yne-D3 and the known inhibitors are presented in table 4. As shown in the table, 1α 25(OH)$_2$-16-ene-23-yne-D$_3$ (RO-23-7553) is a potent inhibitor of human recombinant COX-2 with an IC_{50} value of 5.8nM. This value is much lower than the values

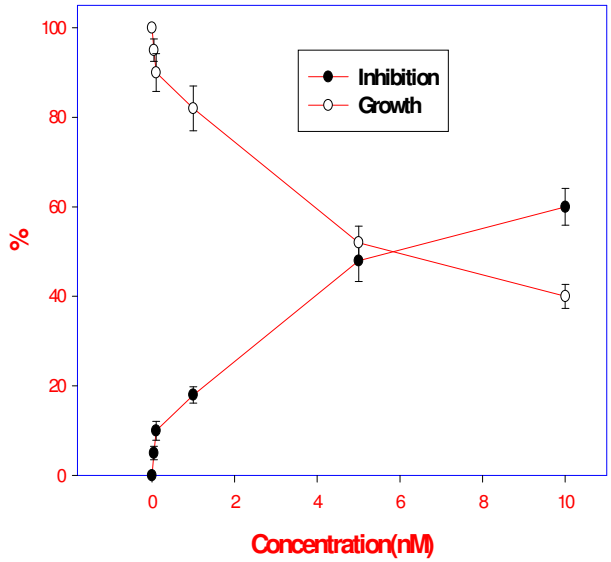


Fig 14: The effect of 1α 25(OH)$_2$-16-ene-23-yne-D$_3$ on COX-2 activity was determined spectrophotometrically basing on the oxidation of TMPD. A graph is plotted between concentration of 1α 25(OH)$_2$-16-ene-23-yne-D$_3$ and % inhibition / activity on COX-2 enzyme. Data represent the mean ± SD values from three separate experiments.

obtained for celecoxib (260nM), the known selective inhibitor of COX-2. The extent of inhibition of COX-2 by RO-23-7553 was independent on the pre incubation period of the enzyme with the inhibitor before the initiation of reaction. In addition, 1α25$(OH)_2D_3$ and epi analog of RO-23-7553, 1α 25$(OH)_2$-16-ene-23-yne-3-epi-D_3 showed maximum COX-2 inhibition of 20% and 38% respectively at 1μM concentration. However all these analogs showed no effect on Cyclooxygenase 1 enzyme.

The *in vitro* potency of an inhibitor is reflected by its IC_{50} value. This is the concentration at which the compound inhibits 50% activity of enzyme. The lower the IC_{50} value, the more potent is the inhibitor. In the present study it has been shown that RO-23 –7553 is a selective inhibitor of COX-2 with an IC_{50} value of 5.8nM with no inhibition of COX-1 upto 5μM concentration. These studies also reveal that 1α 25$(OH)_2$-16-ene-23-yne-D_3 is ~44 times more potent than celecoxib, a selective inhibitor of COX-2 being marketed throughout the world for the treatment of osteoarthritis.

By extensive structure–function studies of analogs of 1α 25$(OH)_2D_3$, it is possible to discriminate various actions of Vitamin D. 1α25$(OH)_2$-16-ene-23-yne-26,27-F_6-D_3 has attracted significant attention because of its less calcemic effects and high activities in growth inhibition of various cancer cell lines and azoxymethane induced colon carcinoma in rats (Wali *et al.,* 1995). However, this analog showed no effect on COX-2. These studies suggest that the anti tumor effects of 1α25$(OH)_2$-16-ene-23-yne-26,27-F_6-D_3 may be mediated through the COX-2 independent mechanism. Development of analogs by introducing blocking groups like fluorine atoms, a methyl group etc

in to the side chain causes the molecule resistant to metabolism. Introduction of fluorine atoms in the side chain makes the compound resistant to 24-hydroxylation and there by increases the half life and potency of the molecule. Fluorination increases the hydrophobic interactions between the ligand and protein there by increasing the affinity to the target protein. In contrast, another analogue, $1\alpha25(OH)_2$-16-ene-D_3 though activating COX-2 enzyme, has potent anti proliferative activities in various cancer and normal cells like keratinocytes (Reddy *et al*., 2001). Multiple lines of metabolic studies suggest that, anti cancerous and immunomodulatory effects of these analogs are attributed to their metabolic stability in the target cells. The combination of structural modifications both in the side chain like introduction of double bond and fluorine atoms and in A-ring prevent the metabolic inactivation and there by increases their potency.

Inhibition of COX-2 is a favorable condition for treating inflammation, arthritis and certain forms of cancer. COX-2 activity modulates critical steps in the initiation, promotion and progression of several epithelial cancers (Masferrer *et al*., 2000). Selective COX-2 inhibitors potentially may combat arteriosclerosis by inhibiting inflammation, in part by blocking Prostaglandin E_2 production. Recent research has clearly established that specific COX-2 inhibitors are associated with less toxicity than the conventional COX inhibitors (Silverstein *et al.,* 2000; Becker *et al*., 2004). Earlier findings on the anti-inflammatory property of RO-23-7553 in various disorders can be explained, in part, by the specific inhibition of COX-2. RO-23-7553 was developed by Hoffmann Roche inc and is very potent in inhibiting proliferation and inducing differentiation of various cancer cell lines and also it increases the survival time of leukemic mice (Zhou *et al.*, 1990; Satchell & Norman,

1996). When compared to toxicities associated with the currently available COX-2 selective anti-inflammatory drugs, RO-23-7553 might provide safer therapeutic alternative. Of all the Vitamin D analogs tested, only RO-23-7553 showed highly potent and selective inhibition of COX-2. In order to understand the mechanism involved in this selectivity further studies were undertaken on molecular modeling of selective COX-2 inhibiting deltanoids.

3.4 Docking studies

Rational drug design deals with the ability to predict new molecules or analogs of known drugs, which will have favorable interactions with a protein of known three-dimensional structure. Based on the information available, one can apply, either structure based or analogue based drug design methods. If the active site is known, design of ligand becomes straightforward that exploits the complementarity of the active surface using ligand docking experiments (Pracard *et al*., 1995). There have been sustained efforts concerning the identification of selective COX-2 inhibitors with an attractive pharmacological profile: NS398, Dup 697, celecoxib and rofecoxib are some highly selective COX-2 inhibitors (Needleman & Isackson, 1998; Li *et al*., 1995; Silverstre *et al*., 1998). A novel series of 1,2-diaryalimidazoles have also been reported as selective COX-2 inhibitors (Bayly *et al*., 1999).

The differential activities of synthetic vitamin D analogs provide a useful method for studying how ligand modification alters the mode of target gene activation. Many of the analogs that have differential activity are modified on the side chain (Yamamoto *et al*., 1996). In the present study, we have analyzed structure based docking studies on analogues of 1α 25$(OH)_2D_3$ (Fig . 15).

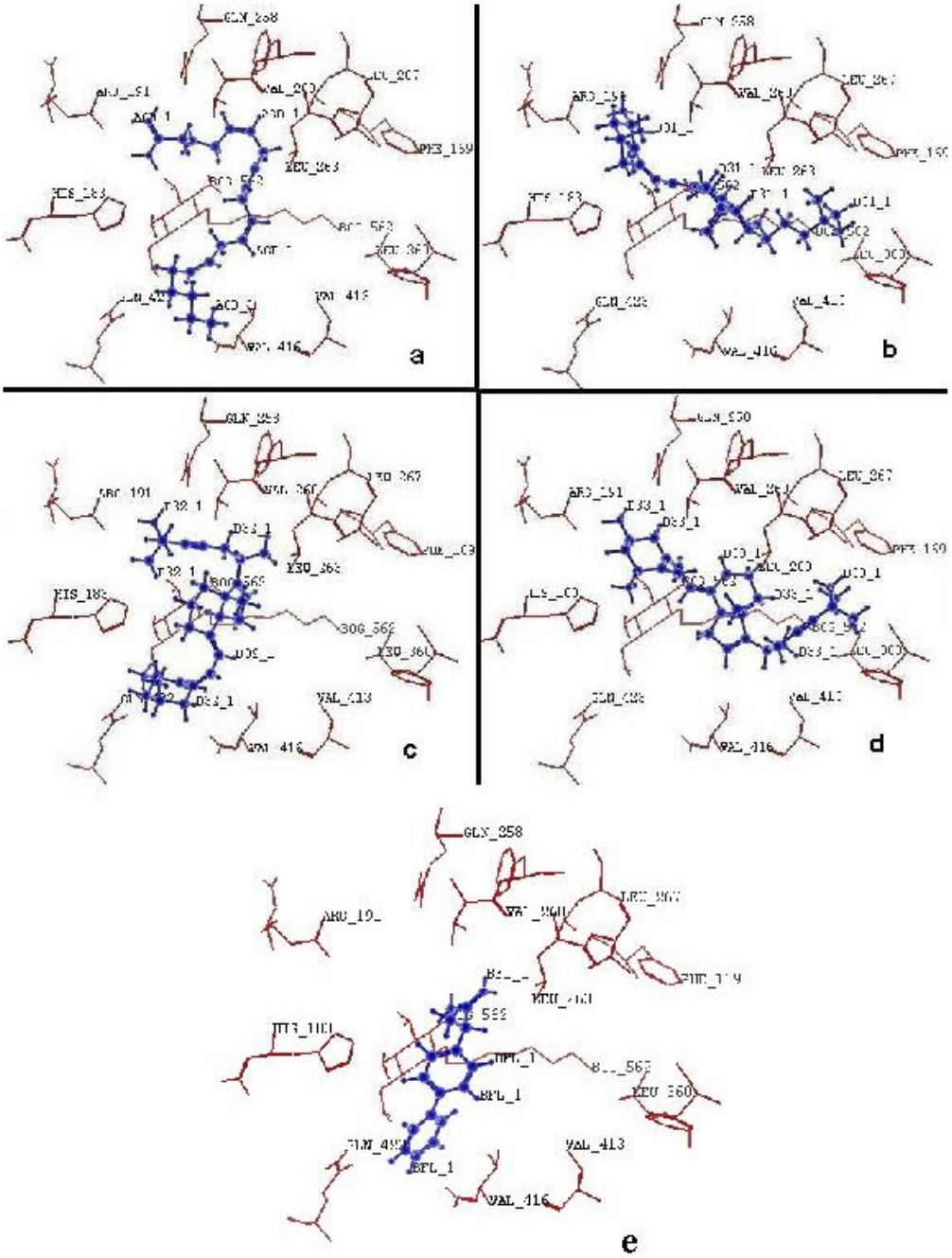


Fig 15 : Binding mode of arachidonic acid, 1α 25(OH)$_2$D$_3$ & its analogs and α-methyl-4-biphenylacetic acid (BFL) *in the active site of COX-2.* Figures illustrating docking of arachidonic acid **(a),** 1α25(OH)$_2$D$_3$ **(b)**, 1α25(OH)$_2$-16-ene-23-yne-D$_3$ (RO-23-7553) **(c),** 1α25(OH)$_2$-16-ene-23-yne-3-epi-D$_3$ **(d),** α-methyl-4-biphenylacetic acid (BFL) **(e)**. These figures were produced using MOE (CCG Inc.).

Table 5 : Docking scores

Compound	GOLD score (COX-1)	GOLD score (COX-2
1α25$(OH)_2D_3$	-77.34	39.92
1α25$(OH)_2$-16-ene-23-yne-D_3	-18.81	51.41
1α25(OH)2-16-ene-23-yne-3-epi-D_3	-16.28	49.78
Arachidonic acid	52.24	56.73
α-methyl-4-biphenyl acetic acid	57.91	43.04

Docking scores of vitamin D analogs, known inhibitor of COX-2 and substrate towards COX-2 enzyme.

The GOLD score obtained for all the ligands are given in table 4. It can be seen that the molecules showing no inhibitory effect on COX-1/COX-2 gave rise to –ve scores (meaning binding not favorable), where as those showing inhibitory effects gave rise to +ve scores (binding favorable). Docking results of 1α 25$(OH)_2D_3$ and its two analogs are correlated well with the experimental results of isolated enzyme. Docking studies in the cyclooxygenase active site of COX-2 and COX-1 revealed that RO-23-7553 adopts the same orientation and position as the substrate, arachidonic acid (AA). The GOLD score obtained for RO-23-7553 was on par with the arachidonic acid while the epi analog of RO-23-7553 and 1α 25$(OH)_2D_3$ exhibited relatively low GOLD scores when compared with AA. In addition the binding mode i.e. positioning and orientation of epi analog and 1α 25$(OH)_2D_3$ is different from that of AA. This confers that the ability of the RO-23-7553 to accommodate in the active site similar to AA is responsible for its COX-2 inhibition.

Vitamin D side chains are highly flexible as they possess the full carbon atoms structure of cholesterol. Elongation of the side chain increases

the potency of the compound because the ligand and the protein can come closer. Introducing a double and triple bond in the side chain often elevates potency of the analog as this function significantly restricts conformational mobility and the molecule becomes more rigid. Generally rigid molecules are more potent than flexible compounds with a similar structure (Bohm *et al*., 1996). Based on these results RO-23-7553 with high flexible side chain could facilitate the favorable binding to the protein.

Thus the foregoing studies clearly demonstrate that 1α25$(OH)_2$-16-ene-23-yne-D_3 is a potent inhibitor of COX-2 (IC_{50} 5.8 nM) with no effect on COX-1up to 5μM concentration.

Chapter 4

Effect of RO-23-7553 on mouse macrophage cell line, RAW 264.7

From the studies presented in the previous chapter it is evident that 1α 25(OH)$_2$-16-ene-23-yne-D3 (RO-23-7553) selectively inhibits COX-2 with an IC_{50} of 5.8nM, suggesting its possible anti inflammatory properties. Hence, further studies were undertaken to evaluate the anti-inflammatory properties of RO-23-7553 in LPS stimulated mouse macrophage cell line, RAW 264.7. The effect of RO-23-7553 on the growth of LPS stimulated mouse macrophage cell line, RAW 264.7 was analyzed. This cell line was established from a tumor induced by Abelson murine leukemia virus. RAW 264.7 cells are known to over-express COX-2 on stimulation with LPS. Along with COX-2, another immediate early gene, the inducible isoform of nitric oxide synthase also gets induced in LPS stimulated RAW 264.7 macrophages. For these experiments, the nitric oxide synthase inhibitor L-NMMA was necessary to prevent endogenous NO generation that is known to initiate apoptotic cell death in macrophages (Sarith *et al.,* 1993). Hence in the present study L-NMMA was employed along with LPS for all experiments on mouse macrophage cell line, RAW 264.7.

4.1 Effect of RO-23-7553 on growth of Mouse Macrophage Cell Line, RAW 264.7, in the presence of LPS and L-NMMA

To test the effect of RO-23-7553 on growth and multiplication on mouse macrophage cell line, RAW 264.7, cells were incubated with different concentrations of RO-23-7553 (0.1nM to 20nM) and cell viability was examined by MTT assay after 24hrs. A 50% inhibition (IC_{50}) in the growth of RAW 264.7 cells was observed at 10nM concentration of RO-23-7553. A typical growth curve of cells at different concentrations of RO-23-7553 indicating the IC_{50} value is presented in Fig. 16.

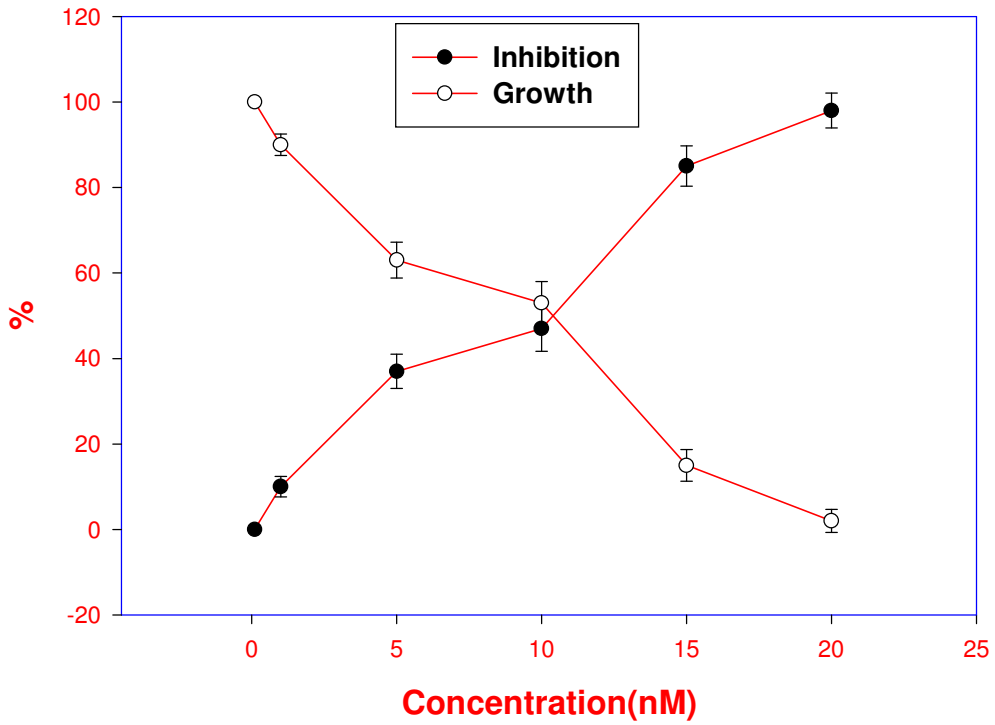


Fig.16: Anti-proliferative effects of RO-23-7553 on RAW 264.7 cells. For determination of IC_{50} Value, RAW cells were incubated with 0.1-20nM concentrations of RO-23-7553 and the cell survival was determined after 24h by MTT assay. The % viable cells were calculated in comparison to untreated cells. The number of cells in the control were taken as 100%. IC_{50} value for RO-23-7553 was around 10nM

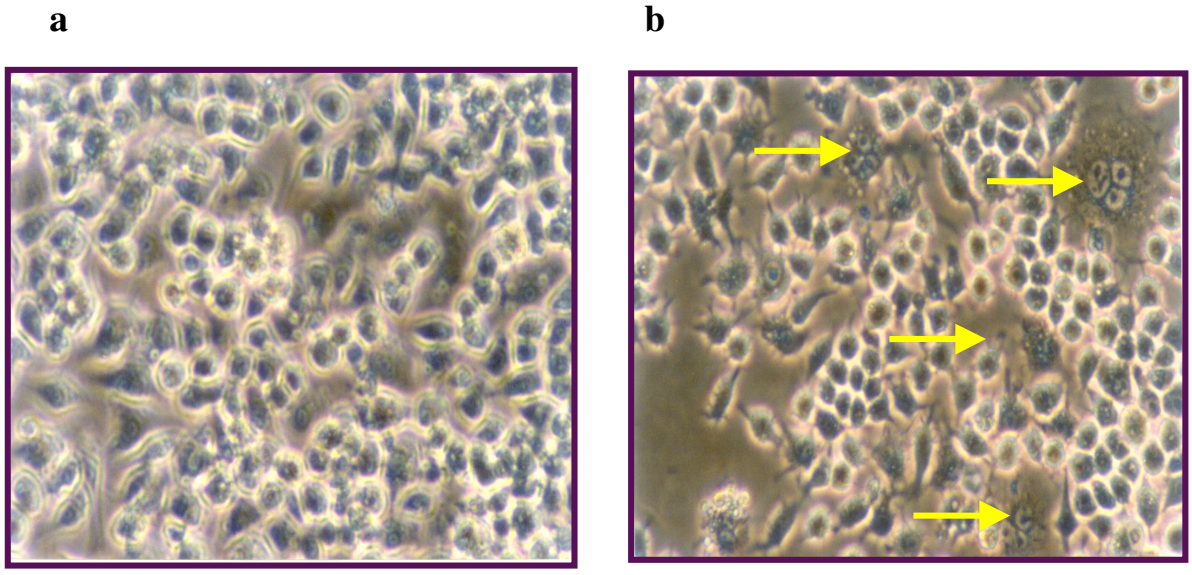


Fig 17: Phase contrast microscopic pictures (800X) of Mouse macrophage cells. a) control b) *Cells treated with RO-23-7553* at 10nM conc for 24h. *Arrows indicate the cells undergoing death.*

Phase contrast microscopic analysis of the RO-23-7553 treated cells revealed pronounced morphological changes. The morphological alterations in RAW 264.7 cells were observed after treatment with 10nM of RO-23-7553 for 24h compared to untreated cells. The cells showed loss of cytoplasm integrity and vacuoles inside the cell (Fig. 17).

4.2 Effect of RO-23-7553 on expression of Cyclooxygenases

To investigate the effect of RO-23-7553 on the expression of COX-1 and COX-2 protein, Western blot analysis was performed with whole cell lysate of the cells treated with or without RO-23-7553 for different time periods after stimulating with LPS. The results presented in Fig. 18 showed no significant changes in COX-1 protein levels in RO-23-7553 treated cells compared to untreated control cells.

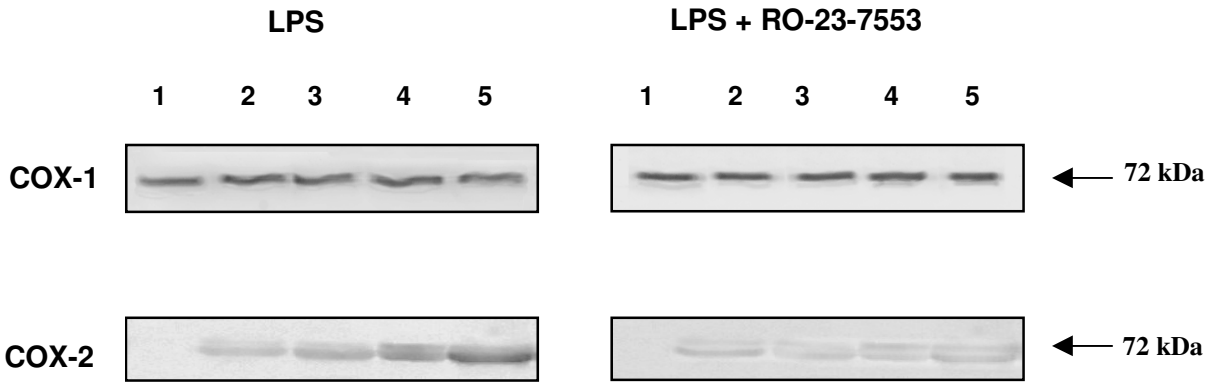


Fig 18: Effect of RO-23-7553 on the expression of COX-1 and COX-2 proteins in LPS treated mouse macrophage cells. Equal amounts of protein (100 µg) from cells treated with either with LPS alone or LPS + RO-23-7553 were loaded and separated on 10 % SDS-PAGE and then probed with goat polyclonal COX-1/COX-2 antibodies. Lane 1: untreated cells; Lanes 2, 3,4 & 5: Samples of the cells at 4h,8h,12h and 24h after treatment with LPS or LPS + RO-23-7553.

As shown in the Fig. 18, no COX-2 protein was detected in untreated cells (lane 1). However, the COX-2 protein was detected within 4h after LPS

treatment. The expression of COX-2 protein progressively increased till 24hours in LPS stimulated RAW 264.7cells. However in RO-23-7553 treated cells, COX-2 expression showed no such progressive increase with increasing time after treatment (Fig. 18). As a result the COX-2 protein expression was relatively less at 24h in RO-23-7553 treated cells compared to untreated cells (Compare lane 5).

4.3 Effect of RO-23-7553 on Cyclooxygenases expression at transcription level

Total RNA was isolated from untreated and RO-23-7553 treated RAW 264.7 cells at different time points by using TRIZOL as described in the methodology. RT-PCR reaction was setup using 2µg total RNA using COX-1 and COX-2 specific primers. The primers yielded the expected fragments of 450 and 583bp for COX-1 and COX-2 respectively, when amplified fragments were run on 1.2% agarose gel (Fig. 19). As shown in the figure, there was no appreciable change in the message levels of COX-1 in both control RO-23-7553 treated cells at different time points studied. In contrast, COX-2 was induced with in 4h after LPS treatment, with progressive increase in the message and reaching maximum at 24h time period. However, no such progressive increase in the message was seen in RO-23-7553 treated cells. The results further confirm that, expression of COX-2 was less in RO-23-7553 treated cells compared to untreated cells even at transcription level.

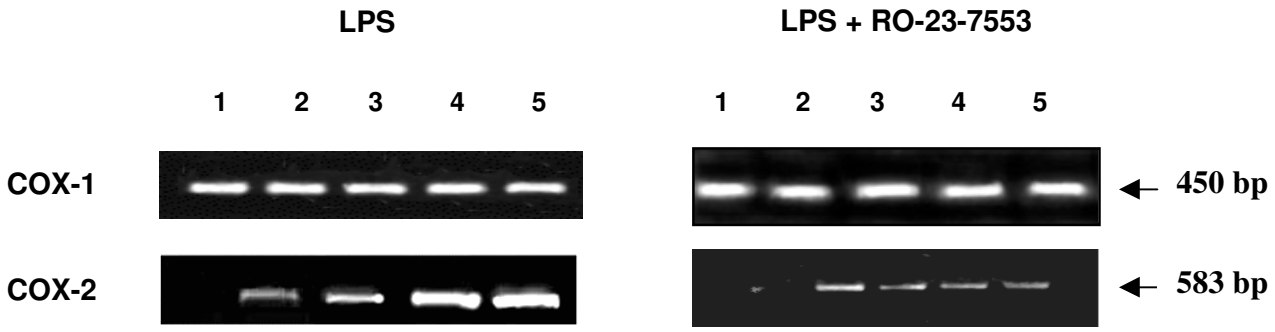


Fig. 19: RT-PCR analysis showing the expression of COX-1 and COX-2 in mouse macrophage cell line, RAW 264.7, treated with or without RO-23-7553. Total RNA was isolated from RAW 264.7 cells at different time points after treatment. COX-1 and COX-2 expression was studied by using specific primers and fragments were resolved on 1.2% agarose gel. Lane 1: Untreated cells; Lanes 2, 3,4 & 5: samples of cells at 4h, 8h, 12h & 24h after treatment with LPS or LPS + RO-23-7553.

4.4 Effect of RO-23-7553 on iNOS protein expression

To investigate the effect of RO-23-7553 on the expression of pro inflammatory enzyme, iNOS, Western blot analysis was performed with whole cell lysate made from the RAW 264.7 cells treated with or without RO-23-7553 after stimulating with LPS. Western blot analysis of the RAW 264.7 cell lysate of control and RO-23-7553 treated cells, probed with iNOS antibodies, showed a single band with molecular weight of 133kDa (Fig. 20).

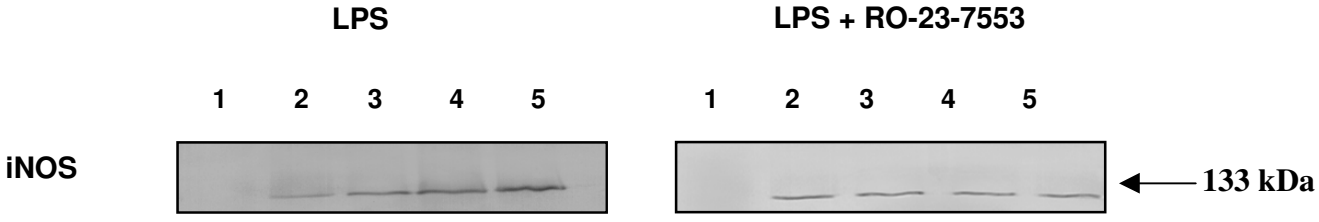


Fig. 20: Effect of RO-23-7553 on iNOS expression in LPS treated RAW 264.7 cells at different time points after treatment. . Equal amounts of protein (100 µg) from cells treated either with LPS alone or LPS + RO-23-7553 were loaded and separated on 7 % SDS-PAGE and then probed with mouse iNOS monoclonal antibodies. Lane 1: Untreated cells, Lanes 2, 3, 4 & 5: Samples of the cells at 4h, 8h, 12h and 24h after treatment with LPS or LPS + RO-23-7553.

As shown in the figure, iNOS was induced with in 4h after LPS treatment in RAW 264.7 cells, with gradual increase and reaching maximum at 24 hours period. However, no such gradual increase in iNOS expression was observed in cells treated with RO-23-7553. As a result the iNOS protein levels in RO-23-7553 treated cells at 24h time interval were much lower compared to the corresponding levels in untreated cells (Compare lane 5).

4.5 Effect of RO-23-7553 on IL-2 protein expression

To investigate the effect of RO-23-7553 on the expression of pro inflammatory cytokine-IL-2 in LPS treated mouse macrophage cell line, RAW 264.7, Western blot analysis was performed with whole cell lysate (Fig 21). As shown in the figure, no IL-2 protein was detected in untreated control cells (lane 1). However IL-2 was induced within 4hours after LPS treatment (lane 2) in RAW 264.7 cells with gradual increase at later periods of time (lanes 3 to 5).

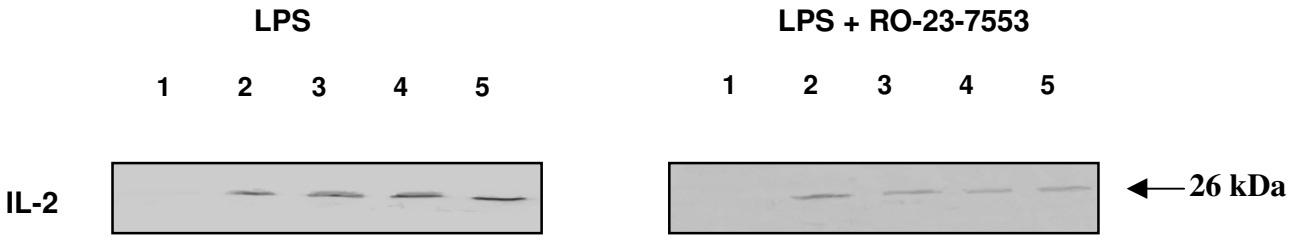


Fig. 21: Effect of RO-23-7553 on IL-2 expression in LPS treated RAW 264.7 cells at various time points after treatment. Equal amounts of protein (100 µg) from control and treated cells at various time points were loaded and separated on 10 % SDS-PAGE and then probed with rabbit polyclonal IL-2 antibodies. Lane 1: Untreated cells, Lanes 2, 3,4 & 5: Samples of the cells at 4h,8h,12h and 24h after treatment with LPS or LPS + RO-23-7553.

The low levels of IL-2 protein expressed at 4h time period in RO-23-7553 treated cells, were further decreased at later periods of time. As a result

the IL-2 protein levels in RO-23-7553 treated cells were much lower to the corresponding levels in untreated cells at all the time points.

4.6 Mechanism of antiproliferative action of RO-23-7553 in RAW 264.7 cells

4.6.1 Flow cytometric Analysis

Loss of DNA is a typical feature of apoptotic cells. Propidium iodide (PI) staining of DNA, which is taken up into the nucleus of apoptotic and necrotic cells, was used to measure the relative numbers of dead cells (Pullen *et al.*, 1981). Furthermore, since apoptosis, but not necrosis, involves degradation of DNA, the staining pattern obtained with PI was used to establish whether cell death was due to apoptosis or necrosis. Two different ways of staining with PI are generally observed: PI in the presence of a permeabilising and fixing agent results in the staining of DNA in living, apoptotic and necrotic cells; and PI in a physiological buffer, stain cells that are dead due to apoptosis or necrosis.

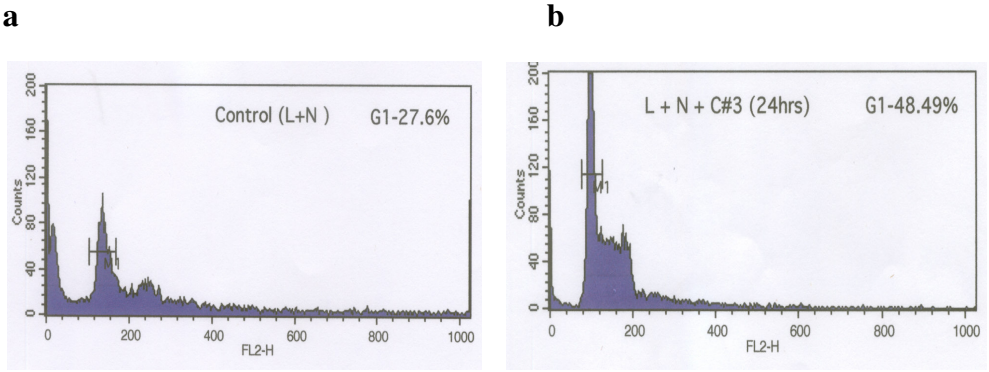


Fig. 22: Flow cytometric analysis of RAW 264.7 cells treated with or without RO-23-7553 at 24h after treatment. (a) RAW 264.7 cells at 24h treatment with LPS (b) RAW 264.7 cells at 24h after treatment with LPS + 10nM RO-23-7553.

To determine the mechanism of antiproliferative action of RO-23-7553, the distribution of DNA was examined in treated cells by flow cytometry.

RAW 264.7 cells cultured with LPS (1µg/mg) were incubated with RO-23-7553 for 24h and then analyzed on FACS. The untreated cells were taken as control. Fig. 22 shows the FACS analysis of control (a) cells treated with LPS and RO-23-7553 treated cells (b). As shown in the figure, 27% cells are in G1 phase at 24h after treatment with LPS. However, in cells treated with RO-23-7553, the percentage of cells in G1 phase was almost double i.e. 48% at 24h time point.

4.6.2 DNA fragmentation by Agarose gel electrophoresis

Detection of DNA fragmentation is widely considered as a biochemical hallmark of apoptosis. During later stages of apoptosis internucleosomal cleavage of cellular DNA by endonucleases to 180 bp or oligimers of 180bp fragments could be detected by separation of nuclear DNA on agarose gel electrophoresis. In the present study RO-23-7553 treated cells, the DNA showed a streaking pattern, typical of cells undergoing necrotic cell death. As shown in Fig. 23, DNA in untreated cells (lane 1), showed no fragmentation. However in RO-23-7553 treated cells, random degradation DNA was observed, which progressively increased at later periods of time and reaching maximum at 24h time period (lane 5).

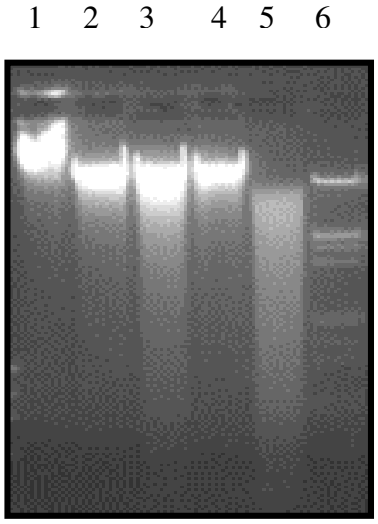


Fig. 23 : Agarose gel electrophoresis of DNA isolated from RAW 264.7 cells treated with RO-23-7553. RAW 264.7 cells were treated with RO-23-7553 at 10nM concentration for various time points. Both floating cells and adherent cells were collected and soluble DNA was extracted and electrophoresed on 1.2% gel containing 0.05mg/ml ethidium bromide. The gels were then photographed under UV illumination. Lane 1: RAW 264.7 cells with LPS; Lane 2: RAW 264.7 cells at 4h after treatment with LPS + 10nM RO-23-7553;Lane 3: RAW 264.7 cells at 8h after treatment with LPS + 10nM RO-23-7553; Lane 4: RAW 264.7 cells at 12h treatment with LPS + 10nM RO-23-7553; Lane 5: : RAW 264.7 cells at 24h treatment with LPS + 10nM RO-23-7553; Lane 6: 100bp DNA ladder

Discussion

1α 25$(OH)_2D_3$ analogs differed from 1α25$(OH)_2D_3$ in their affinity towards Vitamin D receptor and in their ability to mimic the actions of 1α 25$(OH)_2D_3$ on calcium mobilization as well as cell proliferation and differentiation (Khoury *et al*., 1994). Some analogs were 5-10 fold more potent than 1α 25$(OH)_2D_3$ in the promotion of cell differentiation of both benign and malignant cells (Zhou *et al*., 1990; Bouillon *et al*., 1990). RO-23-7553 is one of the most promising synthetic Vitamin D analog and is less calcemic, several fold more potent than 1α 25$(OH)_2D_3$ in suppressing the

growth and inducing differentiation of leukemic cells (Zhou *et al*., 1989; Cross *et al.,* 1991). In the present study we have shown that it is a selective inhibitor of COX-2 with no effects on COX-1, suggesting its possible antiinflammatory and anti cancer properties. In order to test these effects mouse macrophage cells, RAW 264.7 that express COX-2 on stimulation with LPS, were employed in the present study.

Based on cell proliferation (MTT) assay, the concentration of RO-23-7553 required for 50% inhibition of growth of mouse macrophage cells was calculated and it was found to be around 10nM concentration. These studies show that the side chain analog of 1α 25$(OH)_2D_3$, i.e, RO-23-7553 is potent in eliciting anti proliferative activity on LPS stimulated RAW 264.7 cells. Similar inhibition in the growth of colon cancer cells by Vitamin D and its analogs was reported (Marcinkowska *et al*., 1998; Shabahang *et al*., 1994; Thomas *et al.,* 1992). RO-23-7553 was shown to be more potent than 1α 25$(OH)_2D_3$ in inducing differentiation and decreasing the proliferation of various myeloid leukemic cells (Norman *et al.,* 1990; Rao *et al*., 1996) .

To determine the effect of RO-23-7553 on COX enzyme *in vitro*, RAW 264.7 cells treated with RO-23-7553 were analyzed for changes in the levels of COX-1 and COX-2, both at transcriptional and translational levels. These studies have shown no significant changes on COX-1 expression but showed decreased expression of COX-2 in response to RO-23-7553 treatment. These studies suggest that RO-23-7553 down regulates the expression of COX-2 in LPS treated mouse macrophage cell line, RAW 264.7 in addition to its direct effect on COX-2 inhibition. As a result it could form a potential anti inflammatory compound. In rat chondrocytes, 1α 25$(OH)_2D_3$ did not show any significant alterations in COX-1 enzyme (Sylvia *et al*., 2001), but induced the

same in osteoclast supporting stromal cells (Adams *et al.,* 1999). Also the expression of COX-2, in keratinocytes in response to 1α 25$(OH)_2D_3$ treatment is unaffected (Kanekura *et al.,* 1998). These studies suggest that the regulation of COX-1 and COX-2 enzyme expression by Vitamin D analogs may be cell specific.

The effects of 1α 25$(OH)_2D_3$ and its analogs may be either antagonized or enhanced by cytokines depending on the type of the cell, stage of differentiation and duration of exposure to the steroid. The over expression of inducible nitric oxide synthase (iNOS) one of the cytokine, is generally associated with many inflammatory diseases (Amin *et al.,* 1999). Like glucocorticoids and retinoic acid, 1α 25$(OH)_2D_3$ also exerts profound immunosuppressive effects, but little is known about its ability to regulate iNOS. In the present study, RO-23-7553 delayed the expression of iNOS in LPS stimulated mouse macrophage cells. Previous reports suggest that 1α 25$(OH)_2D_3$ down regulates iNOS expression by different cell types, including microglia, astrocytes and macrophages (Sparrow, 1995). Another pro inflammatory cytokine IL-2 was also down regulated by RO-23-7553 in LPS stimulated mouse macrophage cells. From these studies it is clear that RO-23-7553 exhibits potent anti-inflammatory effects on mouse macrophage cells, by down regulating the expression of pro inflammatory mediators like COX-2, iNOS and IL-2, in addition to its selective inhibition of COX-2.

Epidemiological and experimental evidences demonstrate the use of COX-2 inhibitors in reducing the risk of colorectal (Tuynman *et al*., 2004; Zhan *et al*., 2004) and breast cancers (Ranger *et al*., 2004; Saji *et al*., 2004). Clinical trails also have shown the use of NSAIDs in reducing the number and size of colorectal polyps in patients with Familial Adenomatous Polyposis

(FAP). However, the signaling mechanisms responsible for the chemopreventive action of these drugs have not been clearly established. Recent studies have demonstrated the induction of apoptosis in tumor cells by NSAIDs (Subhashini *et al*., 2004; Miedzybrodzki, 2004; Pelzmann *et al.,* 2004). In addition NSAIDs were shown to inhibit COX-2 induced angiogenesis. Similar to other NSAIDs, Vitamin D and its analogs are known to exhibit anticancer properties. In the present study side chain analog of 1α 25$(OH)_2D_3$, RO-23-7553, reduced the growth and multiplication of mouse macrophage cell line RAW 264.7. Although the mechanism behind this effect is not fully understood, based on the present studies, it is suggested that inhibition and down regulation of COX-2 expression by RO-23-7553 may be responsible for this effect.

Tumor cells naturally secrete pro-inflammatory cytokines and chemokines to interact with the microenvironment and regulate neoangiogenesis. Therapies directed against chemokine ligands or receptors may be beneficial in cancer. A substantial amount of evidence has accumulated concerning the interaction of 1α 25$(OH)_2D_3$ and its analogs with cytokines in the regulation of cell growth and differentiation in many cell types including epithelial, mesenchymal, neural, vascular endothelial, immune cells and chondrocytes (Suzuki *et al*., 2000; Staeva & Freedman, 2002; Penna & Adorini, 2000).

Despite an intense activity within the area of Vitamin D field, the exact mechanisms responsible for the anti proliferative effects of 1α 25$(OH)_2D_3$ are still to be identified. Recent investigations have shown that one of the mechanisms by which Vitamin D compounds exert their growth inhibiting effects is by regulating cell cycle progression. Treatment of most cell types

with Vitamin D compounds has been found to cause an arrest of cell cycle progression at G1-phase (Colston *et al*., 1989). Based on the FACS results obtained in the present study, it is very clear that RO-23-7553 arrests the mouse macrophage cells in G1 phase of the cell cycle. Similar results were also reported in colon and breast cancer cells (Zhao *et al.,* 2000). Arresting the cell cycle can hinder cellular proliferation and promote the cell death while death occurs via necrosis or apoptosis (Hershberger *et al*., 1999; Verlinden *et al*., 2000). In the present study RO-23-7553 caused necrosis like death, as evidenced by the DNA streak observed on agarose gel electrophoresis of the DNA extracted from the RO-23-7553 treated mouse macrophage cells.

In summary, motivated by several claims on the therapeutic role of RO-23-7553, particularly its anti inflammatory and anti cancer properties, in the present study, it is planned to address the question as to whether it has any effect on the expression of cyclooxygenase enzyme in LPS treated mouse macrophages cells. The preliminary studies on the mechanism of action revealed that, RO-23-7553 exhibits anti-inflammatory and anti-proliferative effects on LPS treated mouse macrophage cells by delaying the expression of COX-2 and other pro-inflammatory mediators (iNOS and IL-2), induce cell cycle arrest at G1 phase and finally leading to the cell death by necrosis.

In the light of anti-inflammatory effects observed on isolated enzyme, COX-2 and on mouse macrophage cells, it will be interesting to evaluate its effects on animal models of inflammation.

Chapter 5
Effect of RO-23-7553 on rat air-pouch model of inflammation

The studies presented in chapter 3 reveal that RO-23-7553 is a potent and selective inhibitor of COX-2, suggesting its possible anti-inflammatory properties. Further studies on mouse macrophage cells reveal that RO-23-7553 exhibits anti-proliferative effects by the arrest of the cells at G_1 phase leading to necrosis (chapter 4). Based on these results further studies were undertaken to evaluate the effects of RO-23-7553 on animal models of inflammation. Rat air pouch model of inflammation was employed for these studies.

5 Air Pouch Model of inflammation

Adult Wistar strain male rats weighing 150-200g were employed in the study to induce inflammation as described in the methodology. Air pouch was created by injecting 20 ml of sterile air subcutaneously on to the animal's back and this air cavity was maintained for 6 days. Inflammation was induced by injecting 2 ml of 1.5% carrageenan in to the air pouch of rats. Saline treated rats served as controls.

Administration of test Compounds

Vitamin D analog, RO-23-7553, was simultaneously given along with carrageenan directly into the air pouch cavity at 10μg/Kg body weight while celecoxib, a known COX-2 inhibitor, was given at 20mg/Kg body weight along with carrageenan. Animals were sacrificed at 4h, 8h, 12h and 24hrs time points after carrageenan, carrageenan + celecoxib and carrageenan+RO-23-7553 treatment. Saline treated animals served as controls.

5.1 Inflammatory reaction

The classical symptoms of acute inflammation - redness and swelling were clearly observed in the carrageenan treated animal. The inflammatory

reaction gradually progressed with time and reached a peak at 24 hours after carrageenan treatment. In the RO-23-7553 treated animals the inflammatory reaction was relatively less when compared to animals treated with carrageenan alone treated animals (Fig 24).

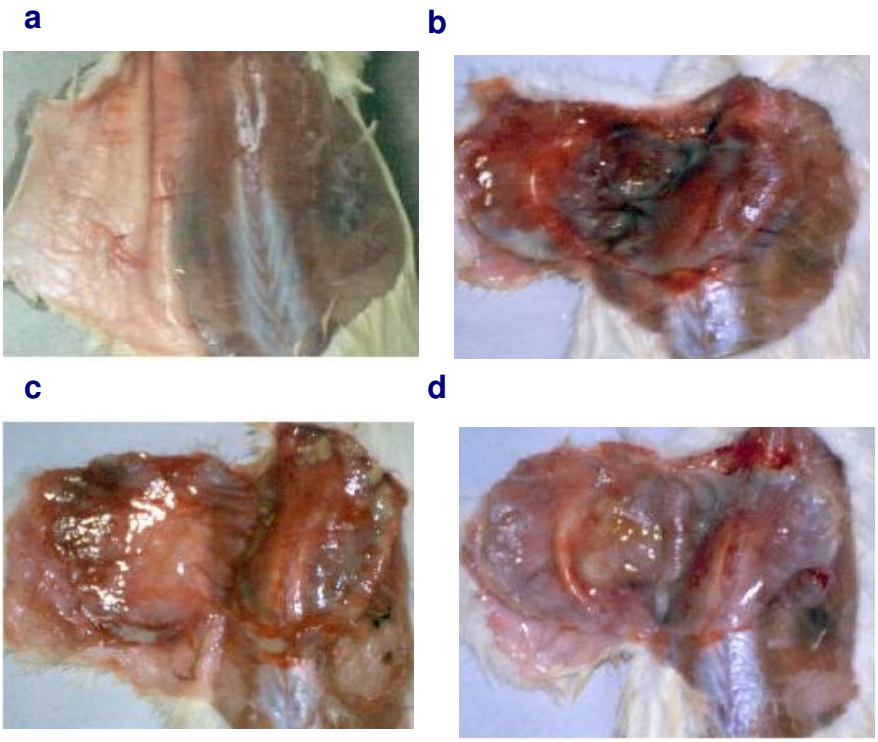


*Fig. 24: Photographs of the exposed air pouch tissue after 24 hours of administration of saline(**a**) carrageenan(**b**) carrageenan + celecoxib (**c**) carrageenan + RO-23-7553 (**d**). The saline treated animal was given 2 ml of saline only while the carrageenan treated animal was given 2ml of 1.5 % carrageenan dissolved in saline. The test compounds celecoxib (20mg/Kg body weight) and RO-23-7553 (10µg/Kg body weight) were given to the animals along with carrageenan.*

These observations were further supported by the histological observations (Fig. 25). The air pouch tissue is a freshly formed granulomatous tissue that is created by the loose association of macrophages, neutrophils,

fibroblasts and plasma cells. The section of the pouch lining showed a large number of air cavities.

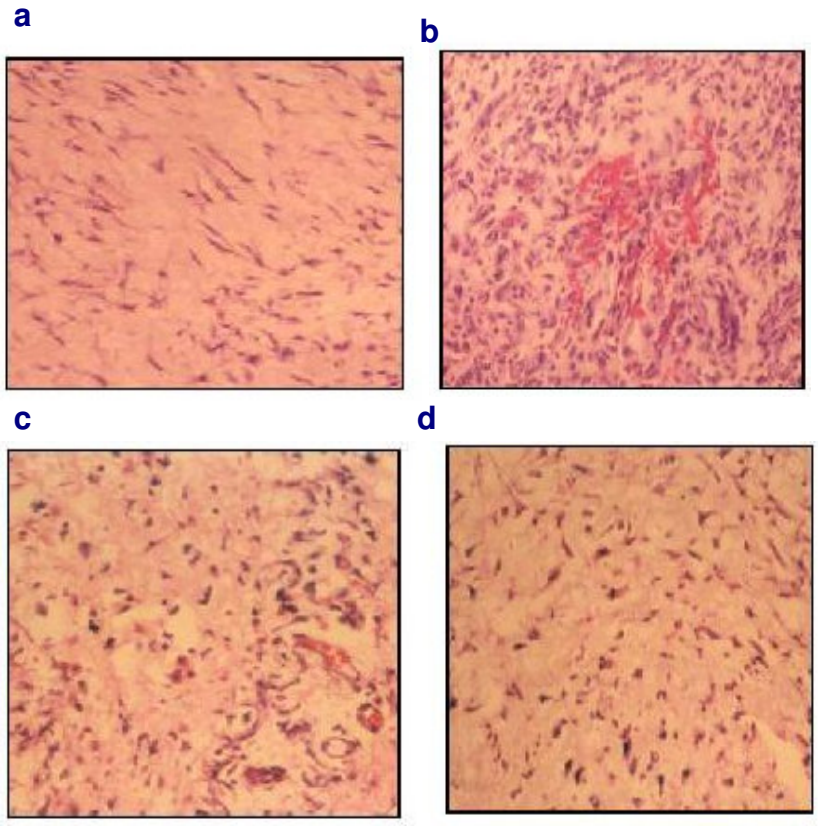


*Fig 25:Photomicrographs (400 X)showing the histological sections of the pouch tissue after 24 hours of saline (**a**)carrageenan (**b**)carrageenan + celecoxib (**c**) carrageenan + RO-23-7553 (**d**) treated rats. The air pouch tissue was fixed in buffered formalin, sectioned and stained with hematoxylin and eosin.*

Carrageenan treated pouch tissue showed heavy infiltration of blood cells at various sites in the tissue, especially near the cavities as it is easy for the migrating cells to move into the open areas (Fig. 25b). These observations clearly demonstrate the induction of inflammation in the air pouch of the carrageenan treated animals. In RO-23-7553 treated animals, however, reduced

inflammatory reaction was seen as indicated by less degree of cellular infiltration (Fig. 25d).

Although the photographs clearly demonstrated inflammation, it is difficult to quantitate the inflammatory reaction based on the appearance of the reaction. For this purpose, the inflammatory exudate was characterized by measuring the volume of the pouch fluid and the population of infiltering cells.

5.2 Exudate volume in the Pouch

The air pouch was carefully dissected by keeping the pouch intact and the fluid was aspirated, collected and measured the volume of fluid.

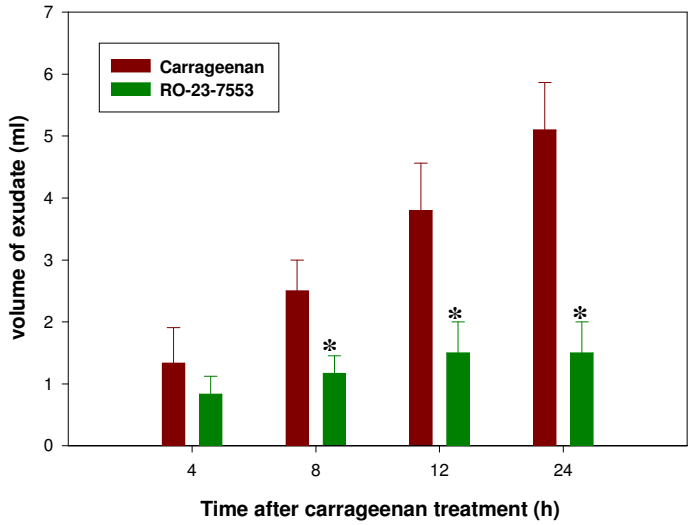


*Fig 26: Effect of RO-23-7553 on accumulated fluid volume in the air pouch of either carrageenan or carrageenan+RO-23-7553 treated rats. Animals were sacrificed at various time points after treatments. The values were the mean ± SE of data obtained from 3 different animals. *p < 0.05 compared to carrageenan treated animals.*

As shown in the Fig. 26, pouch fluid was seen with in 4h after carrageenan treatment with gradual increase at later periods and reaching maximum at 24h. In RO-23-7553 treated animals, fluid volume was found to be

significantly lower i.e. 1.5ml compared to that of 5.5 ml collected in carrageenan treated animals, at 24h time period.

5.3 Infiltration of leukocytes into the pouch fluid

Cell population in the pouch cavity was measured by gavaging about 20 ml of saline into the pouch repeatedly. This procedure ensures the complete recovery of cells from the pouch. For cell counting the collected fluid was centrifuged and the cell pellet was washed in RPMI medium twice to remove the debris and dissolved in saline, then counted on hemocytometer.

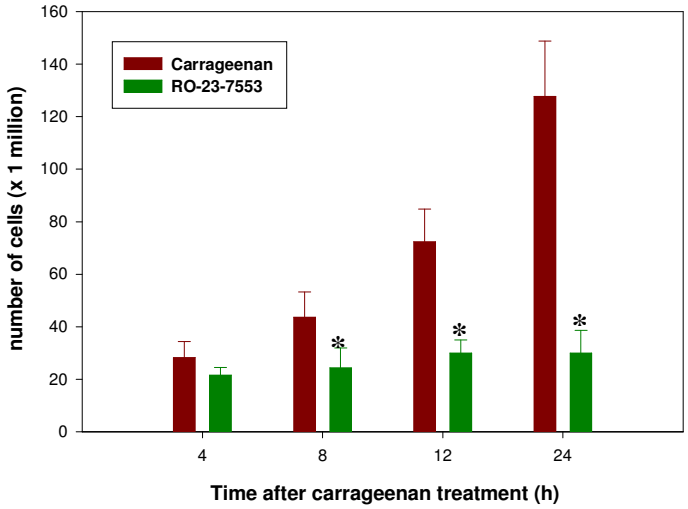


Fig. 27: *Effect of RO-23-7553 on number of cells infiltrated into the air pouch of either carrageenan or carrageenan+RO-23-7553 treated rats. Animals were sacrificed at various time points after treatments. The values were the mean ±SE of data obtained from 3 different animals. *p < 0.05 compared to carrageenan treated animals.*

As shown in the Fig. 27, cells started infiltrating into the pouch as early as 4h and increased continuously to about 130 million cells by 24h in carrageenan treated rats. The cell population mainly consisted of neutrophils, monocytes and macrophages. Infiltration of cells into the air pouch was significantly lower in RO-

23-7553 treated animals (28 million) at 24 hours indicating the arrest of inflammatory reaction.

5.4 Effect of RO-23-7553 on expression of Cyclooxygenases

Western blot analysis was performed to analyze the effect of RO-23-7553 on COX protein levels in rat air pouch tissue. The total protein extracted from the pouch tissue of both carrageenan and RO-23-7553 treated rats was separated on 10% SDS PAGE and then transferred to nitrocellulose membrane. Then it was probed with COX-1/COX-2 specific antibodies. Fig. 28 shows the effect of RO-23-7553 on the expression of COX-1 and COX-2 proteins in rat air pouch tissues.

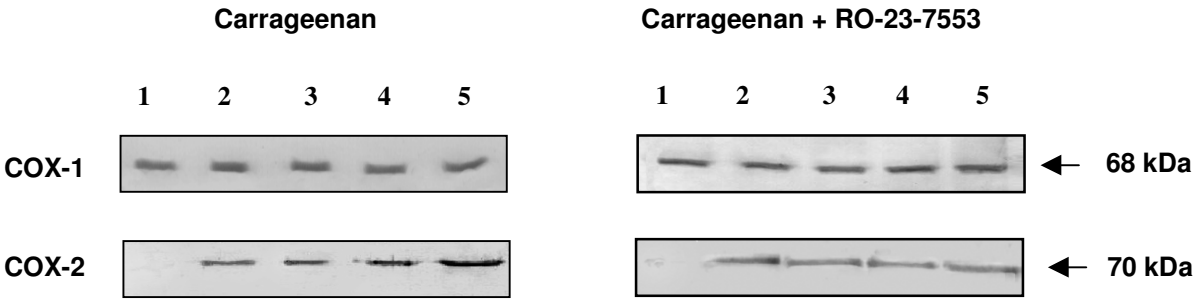


Fig 28: Effect of RO-23-7553 on COX-1 and COX-2 expression in carrageenan and carrageenan + RO-23-7553 treated rat air pouch tissue at various time points after treatment. Equal amounts of protein (100 µg) from both the treated tissues at various time points were loaded and separated on 10 % SDS-PAGE and then probed with goat polyclonal COX-1/COX-2 antibodies. Lanes 1:saline; 2, 3,4 & 5: pouch tissues at 4h, 8h, 12h and 24h after treatment with carrageenan or carrageenan + RO-23-7553.

As shown in the Western blot (Fig. 28), both in carrageenan and carrageenan + RO-23-7553 treated animals, COX-1, a 68kDa band, is constitutively expressed in the pouch tissue in the saline treatment (lane 1) and no change was observed till 24h (lane 5) time period. In contrast, COX-2 protein

was not detected in the saline treatment (lane 1) indicating its lack of expression. However, the COX-2 protein band of 70kDa was detected within 4h (lane 2) after carrageenan treatment. This signal was further amplified at later time periods with maximum levels seen at 24h-time period (45.2 fold) (lane 5). In carrageenan + RO-23-7553 treated pouch tissues, the COX-2 protein levels were more or less stabilized at all time periods. As a result the COX-2 expression at 24h was relatively less in carrageenan + RO-23-7553 tissues when compared to carrageenan alone treated tissues (lane 5 in both).

5.5 Effect of RO-23-7553 on Cyclooxygenases expression at transcriptional level

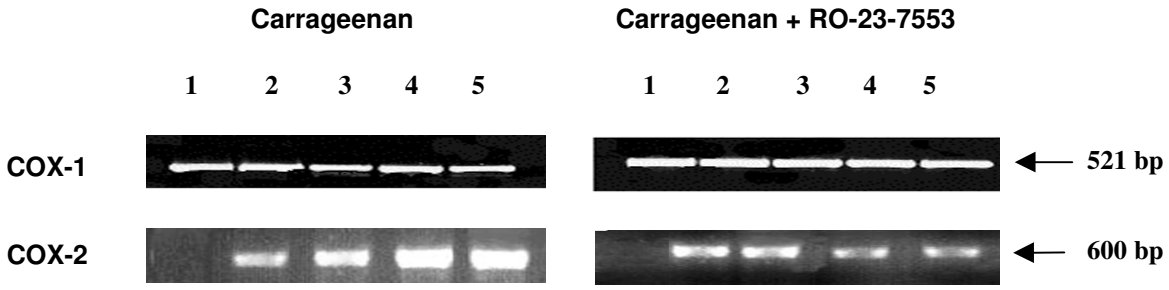


Fig 29: RT-PCR analysis showing the expression of COX-1 and COX-2 in rat air pouch tissue either treated with carrageenan or carrageenan + RO-23-7553. Total RNA was isolated from the pouch tissues at different time points. COX-1 and COX-2 expression was studied by using specific primers and fragments were resolved on 1.2% agarose gel. Lanes 1: saline; 2, 3,4 & 5: pouch tissues at 4h, 8h, 12h and 24h after treatment with carrageenan or carrageenan + RO-23-7553.

The expression of COX genes was also assessed by RT-PCR analysis using COX-1 and COX-2 specific primers. Total RNA was isolated from the pouch tissue using Trizol reagent as described in the methodology. An RT-PCR reaction was setup using 2µg total RNA, AVM reverse transcriptase enzyme and

rat COX specific primers. Fig. 29 shows the levels of COX-1 and COX-2 genes expression in both carrageenan and RO-23-7553 treated animals.

As shown in the figure, a band of 521bp was detected in the saline treatment (lane 1) time interval in carrageenan and RO-23-7553 treated pouch tissue suggesting the constitutive expression of COX-1. There was no change in the levels of COX-1 in response to carrageenan and RO-23-7553 treatment at all the time periods (lanes 2-5 in both). In contrast, a 600bp COX-2 band was not detected in the saline treatment (lane 1) point of carrageenan and RO-23-7553 treated pouch tissue suggesting its lack of constitutive expression. However, COX-2 was detected within 4h (lane 2) after treatment in both the treatments. In carrageenan alone treated pouch tissues, the signal was amplified gradually reaching a maximum at 24h-time point (lane 5). While in RO-23-7553 treated pouch tissues, the COX-2 observed at 4h-time interval (lane 2) gradually decreased thereafter. As a result the expression of COX-2 in RO-23-7553 treated air pouch tissues was lower compared to the corresponding levels in carrageenan alone treated tissues at all time points.

5.6 Effect of RO-23-7553 on iNOS protein expression

To investigate the effect of RO-23-7553 on the expression of pro inflammatory mediator, iNOS, Western blot analysis was performed with tissue homogenates from carrageenan and RO-23-7553 treated rats. Fig 30 shows changes in protein levels of iNOS in carrageenan and RO-23-7553 treated rat air pouch tissues.

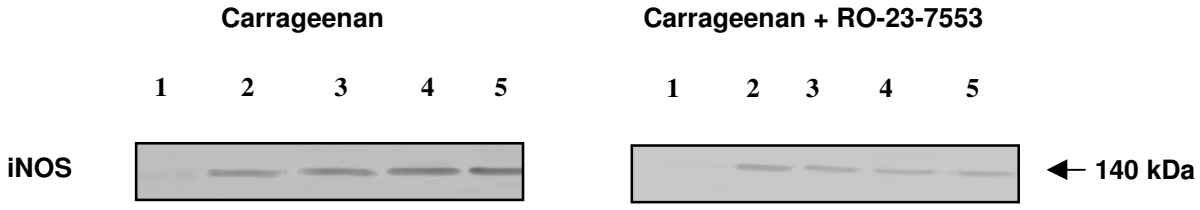


Fig 30: Effect of RO-23-7553 on iNOS expression in carrageenan and carrageenan + RO-23-7553 treated rat air pouch tissue. Equal amounts of protein (100 μg) from either carrageenan or carrageenan + RO-23-7553 treated rat air pouch tissue at various time points was loaded, separated on 7% SDS-PAGE and transferred to nitrocellulose membrane, then probed with mouse monoclonal iNOS antibodies. Lanes 1: saline; 2, 3,4 & 5: pouch tissues at 4h, 8h, 12h and 24h after treatment with carrageenan or carrageenan + RO-23-7553.

As shown in Fig. 30, iNOS protein was not detected in the saline treatment (lane 1), indicating its lack of expression. However, the iNOS protein was detected with in 4hours after carrageenan treatment. This signal was further enhanced at later time periods with maximum levels seen at 24h. In RO-23-7553 treated rats, iNOS protein expression seen at 4h-time interval was gradually decreased at later time periods. As a result the expression of iNOS in RO-23-7553 treated rat air pouch tissues was lower compared to the corresponding levels in carrageenan alone treated rats (compare lane 5).

5.7 Effect of RO-23-7553 on IL-2 protein expression

To investigate the effect of RO-23-7553 on the expression of pro inflammatory cytokine-IL-2, Western blot analysis was performed with tissue homogenates from carrageenan and carrageenan + RO-23-7553 treated rats. Fig. 31 shows changes in protein levels of IL-2 in carrageenan and carrageenan + RO-23-7553 treated rat air pouch tissues.

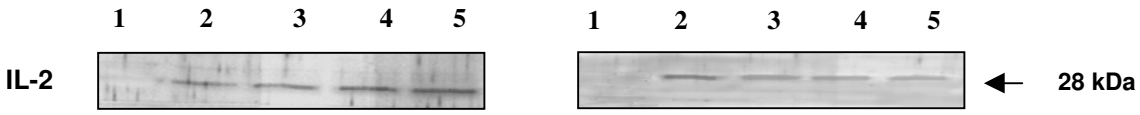


Fig.31: *Effect of RO-23-7553 on IL-2 expression in carrageenan and carrageenan + RO-23-7553 treated rat air pouch tissue. Equal amounts of protein (100 µg) from either carrageenan or carrageenan + RO-23-7553 treated rat air pouch tissue at various time points loaded, separated on 12% SDS-PAGE and transferred to nitrocellulose membrane, then probed with rabbit polyclonal IL-2 antibodies. Lanes 1: saline; 2, 3,4 & 5: pouch tissues at 4h, 8h, 12h and 24h after treatment with carrageenan or carrageenan + RO-23-7553.*

As shown in Fig. 31, IL-2 protein was not detected in the saline treatment (lane 1) is indicating its lack of expression. However, the IL-2 protein was detected with in 4h after carrageenan treatment. This signal was further enhanced at later time periods with maximum levels seen at 24h. In RO-23-7553 treated rats, IL-2 protein levels seen at 4h-time interval were more or less stabilized at later periods of time.

5.8 Antioxidant properties of RO-23-7553

Oxidative stress is another important component of pathophysiology of inflammation. ROS are now recognized to be both mediators and modulators of inflammation. Oxidative stress in any system can be assessed by the levels of reactive oxygen species and the antioxidant potential of the system. ROS are the major cause of tissue damage during inflammation. Hence, reducing oxidative stress should be a part of any anti-inflammatory therapy. Antioxidant enzymes like glutathione peroxidase and reduced glutathione levels were measured in the inflammatory tissue to assess the antioxidant potential of RO-23-7553 in the rat air pouch tissue.

5.8.1 Effect of RO-23-7553 on reduced glutathione levels

Glutathione is a tripeptide and exists either in reduced form (GSH) or in the oxidized form (GSSG) within cells. While GSH is a potent anti-oxidant, GSSG is an indicator of the degree of oxidative stress in the system. Air pouch tissues from the animals were homogenized in phosphate buffer containing phosphoric acid in order to precipitate the proteins as quickly as possible and thus avoid conversion of GSSG to GSH during homogenization. The protein-free supernatant was then used to measure GSH using fluorescent o-pthalaldehyde, which specifically binds to peptide bonds. Protein was correspondingly estimated in an equivalent amount of tissue and the glutathione levels were expressed per mg protein.

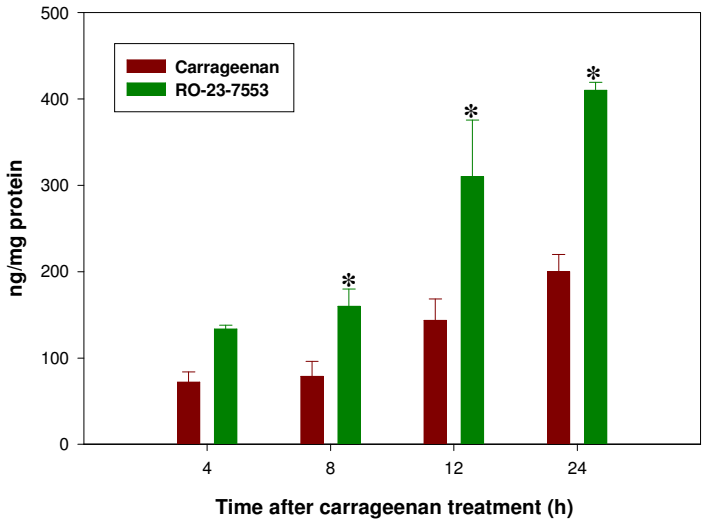


*Fig 32: Effect of RO-23-7553 on levels of GSH in the pouch tissues of carrageenan and carrageenan + RO-23-7553 treated rats at various time intervals. Supernatants from pouch tissues treated with carrageenan alone or carrageenan + RO-23-7553 were assayed for GSH as mentioned in methodology. All the values represent mean ± SE of data obtained from 3 different animals. *p< 0.05 compared to carrageenan treated animals.*

Fig. 32 shows the levels of GSH in the air pouch tissue at various time points after carrageenan and carrageenan + RO-23-7553 treatments. GSH levels increased gradually reaching to a maximum by 24h in both the treatments. However, in RO-23-7553 treated rats GSH levels were significantly higher when compared to carrageenan alone treated rats, implying the protective role of RO-23-7553 against oxidative stress.

5.8.2 Effect of RO-23-7553 on glutathione peroxidase (GPx) activity

The levels of GPx were measured in carrageenan and carrageenan + RO-23-7553 treated rat air pouch tissues and the results were shown in Fig. 33.

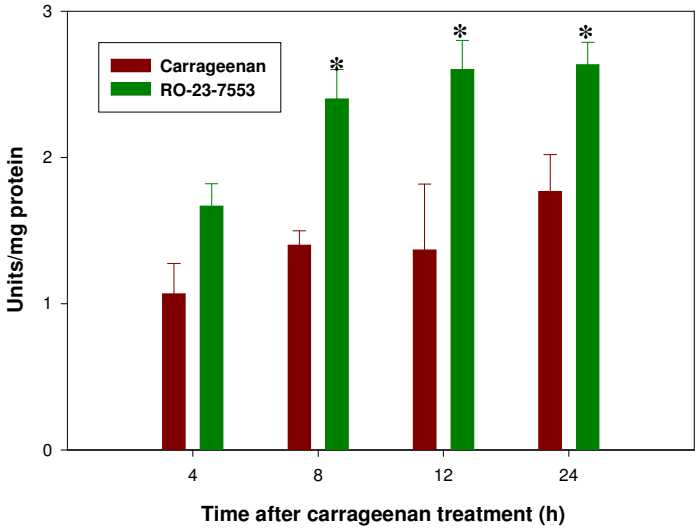


*Fig 33: Effect of RO-23-7553 on levels of GPx in the pouch tissues of carrageenan and carrageenan + RO-23-7553 treated rats at various time intervals. Supernatants from pouch tissues treated with carrageenan alone or carrageenan + RO-23-7553 were assayed for GPx as mentioned in methodology. All the values represent mean ±SE of data obtained from 3 different animals. *p< 0.05 compared to carrageenan treated animals.*

As shown in Fig. 33, the levels of GPx increased gradually with time reaching to a maximum at 24h in both the treatments. There was a significant

increase in the levels of GPx in RO-25-7553 treated rat air pouch tissue when compared to carrageenan treated pouch tissue implying that RO-25-7553 confers a protective sheath to the cell against oxidative stress.

Discussion

Inflammation, which was initially recognized as a simple allergic reaction for centuries is now understood to be an extremely important pathophysiological basis for a large number of very notable diseases. The complex interplay of cellular and particulate mediators during inflammation is unfolding but our understanding of the inflammatory reaction is still incomplete. The inflammatory reaction, which gets ticked off by carrageenan, begins by utilizing the locally released population of mediators. The stimulus and the low level of released mediators specifically LTB_4 lead to chemotactic attraction of leukocytes into the area of stimulus, which in turn leads to induction of COX-2, and other cytokines. This induction of COX-2 leads to enhanced production of PGE_2 in the inflamed tissue. PGE_2 thus released by the induced COX-2, triggers a series of events that initiate resolution of inflammation. One such event is the induction of 15-lipoxygenase that leads to the formation of lipoxins, which are anti-inflammatory and take part in the natural resolution of inflammation (Serhan & Chiang, 2004). Time is of importance here since the stimulus has to be discouraged from growth, which begins by the action of small amounts of ROS released by the local neutrophils and which additionally induce cytokines and growth factors. Monocytes and macrophages arrive within few hours to ingest and phagocytose the foreign substance, undergo apoptosis and remove traces of the inflammatory reaction. If the stimulus persists, apoptosis of the macrophages is discouraged,

continuous infiltration of cells goes on, mediators continue to form and finally cause tissue damage leading to severe inflammatory disorders.

In spite of the relevance of the inflammatory disorders, the clinical studies in this area have been of limited help in dissecting the mechanism of inflammation since whatever samples are available narrate only the final part of the pathway. Animal experiments have been extremely useful in understanding the entire inflammatory reaction since they demonstrate a complete window of events from the time when the stimulus is given till the reaction naturally resolves (Dawson *et al*., 1991; Martin *et al*., 1994; Serhan & Chiang, 2004). These studies can be conducted in both wild type and knockout animals, and also studied with the aid of specific inhibitors at various stages (Langenbach *et al*., 1999). Air pouch model is a popular animal model of inflammation since it is easy, relatively quick and gives reproducible results. Air pouch model is considered very similar to the arthritic condition in humans since the synovial fluid and the air pouch fluid were found to be very similar (Sedgwick & Lees, 1986). In the present study, the air pouch model of inflammation was used to further evaluate the efficacy of RO-23-7553, as anti-inflammatory agent, and to understand the molecular mechanisms involved in its action. This study was carried out by analyzing the expression of COX-1, COX-2, iNOS and IL-2 and different parameters of oxidative stress.

The histological staining of pouch lining shows infiltration of blood cells, in response to carrageenan treatment and thus establishing the role of pouch lining in the initiation and maintenance of inflammatory reaction (Sedgewick *et al*., 1983). The pouch lining cells such as polymorphonuclear leukocytes (PMNLs)

fibroblasts, monocytes and macrophages initiate and elaborate an array of pro-inflammatory mediators, which lead to expression and release of cytokines, specifically LTB_4 (Schumann *et al*., 1994). These molecules act as chemoattractants and bring about changes in the endothelial layer to accommodate movement of infiltrating cells (Yang *et al*., 2003). This process is simultaneously enhanced by induction of different pro-inflammatory proteins such as COX-2 (Kirsching *et al*., 1997). Thus the initiation of an inflammatory reaction is complex and requires the concerted efforts of cells of the pouch lining, endothelial cells and peripheral blood cells. Once initiated, the inflammatory reaction progresses and reaches a maximum at 24hrs after carrageenan treatment and resolves spontaneously by removal of carrageenan either by ingestion by macrophages or by diffusion from the site of injection.

In the present study, the inflammatory response was defined acute since the pouch showed large number of cells mainly PMNLs migrating into the pouch with increasing time of exposure to inflammatory stimulus. PMN leukocytes and macrophages appeared in increasing numbers with increasing time after carrageenan treatment into the pouch. These cells are known to work together with the cells of the pouch lining to bring about the inflammatory reaction. The inflammatory reaction can be quantified by measuring the exudates volume and number of infiltering cells into the pouch - the two parameters that are of significance in assessing the efficacy of anti-inflammatory agents. In the present study, a decrease in these two parameters was observed in response to RO-23-7553 treatment suggesting its anti-inflammatory property. Similar results were also reported with oxo vitamin D analogs (Hirata *et al*., 1994). Angiogenesis, the

formation of new blood vessels from an existing vascular bed, is of fundamental importance in several inflammatory conditions like psoriasis, tumor growth and atherosclerosis. The dependence of many inflammatory diseases on angiogenesis has led to the proposal that such diseases can be controlled by use of antiangiogenic compounds. Studies have revealed that 1α 25$(OH)_2D_3$ inhibits angiogenesis both *in vitro* and *in vivo* there by decreasing the inflammatory reaction (Mantell *et al*., 2000; Hisa *et al*., 1996; Wang *et al*., 1997).

The synthetic analogs of 1α 25$(OH)_2D_3$ require no activation *in vivo* and are resistant to attack by catabolic enzymes because of blocking groups in metabolically sensitive regions. Resistant to metabolism of deltanoids, which increases their half-life in target tissues, depends on type of unsaturation and position of blocking groups. In case of RO-23-7553, introduction of double bond at C-16 position and triple bond at C-23 position reduces 23 hydroxylation and 24-oxidation pathways, which are major inactivating pathways, thus exerting longer period of biological effects. This increased half-life of the compound in the cell may also be responsible for its anti-proliferative property. RO-23-7553 has been shown to be capable of inhibiting prostate tumor growth without affecting serum calcium levels in mice (Schwartz *et al*., 1995) supporting its anti-proliferative property. Epidemiological data also suggest that dietary vitamin D may reduce the risk of bowel cancer, particularly colon cancer (Pritchard *et al*., 1996).

Elevated COX-2 expression has been detected in several human malignancies including colon, esophageal and pancreatic cancers (Van *et al*., 2002). Recent results also suggest a relatively early role for COX-2 in

carcinogenesis of the colorectal and esophagus (Buskens *et al*., 2003). Several studies also indicated that the effects of NSAIDs in the prevention of cancers are partially COX-2 dependent, although it should be noted that some of the anti neoplastic effects of these drugs can not be explained solely by the inhibition of COX-2 (Reddy *et al*., 2001). In the present study, RO-23-7553 down regulated not only COX-2, but also other pro-inflammatory molecules such as iNOS and IL-2, in addition to the selective inhibition of COX-2. This compound, however, did not show any effect on COX-1 expression.

Several *in vivo* results demonstrated that iNOS is involved in inflammatory reaction. The production of iNOS is region specific and expanded with time, thus correlating with the development of inflammatory disorders. It was reported that a majority of female patients suffering from multiple sclerosis have a deficit in Vitamin D (Nieves *et al*., 1994). Some of the acute actions of $1\alpha25(OH)_2D_3$ that influence the immune process are now ascribed to the inhibition of iNOS synthesis (Manolagas *et al*., 1989). In the present study too, similar results of iNOS down regulation was observed with RO-23-7553 in carrageenan induced inflammation.

$1\alpha25(OH)_2D_3$ supplementation in IL-2 knock out mice, that spontaneously develop symptoms of bowel disease, reduced the early mortality of mice (Cartorna *et al*., 1996). Immunoregulatory properties of 1α $25(OH)_2D_3$ may be mediated through the inhibition of IL-2 as 1α $25(OH)_2D_3$ inhibits the IL-2 gene transcript in lymphocytes (Alroy *et al*., 1995; Muller *et al*., 1993). The foregoing studies also support the down regulated expression of IL-2 by RO-23-7553.

Free radicals generated from intermediates of metabolism are highly reactive since they contain unpaired electrons (Krinsky, 1989). These reactive species are capable of initiating lipid peroxidation by reaction with polyunsaturated fatty acids inactivating proteins and enzymes by reacting with amino acids and damaging DNA and RNA by reacting with guanine. If the cell is insufficiently protected by enzymatic and non-enzymatic anti oxidants, free radicals can react with bio molecules and thus damage cellular structure. Antioxidants may prevent genetic changes by preventing DNA damage directly induced by free radical attack. Thiol homeostasis is regulated to guarantee basic function and defense mechanism against xenobiotics. Vitamin D was found to be effective antioxidant by elevating glutathione (GSH) levels (Wilson, 1992). In the present study, RO-23-75553 significantly elevated GSH levels suggesting its role in potentiating antioxidant defenses. Glutathione peroxidase (GPx) protects the cell proteins and mechanisms against oxidation by inhibiting the initiation of peroxidative attacks on membrane lipids by reducing the highly reactive organic as well as inorganic peroxides. Enhanced glutathione peroxidase (GPx) levels in RO-23-7553 further strengthens its anti oxidant potential.

In summary, the foregoing study on rat air pouch model of inflammation further demonstrates the anti-inflammatory property of RO-23-7553. The anti-inflammatory action of RO-23-7553 appears to be mediated by the down regulation of the expression of pro inflammatory mediators like COX-2, iNOS and IL-2, in addition to selective inhibition of COX-2. Also this compound appears to improve the anti-oxidant potential of the inflammatory tissues by enhancing the levels of GSH and GPx activity and potentiating tissue antioxidant defenses.

Summary & Conclusions

The principle physiological function of Vitamin D in all vertebrates including humans is to maintain serum calcium and phosphorous concentrations in a range that supports cellular processes, neuromuscular function and bone ossification. During the last couple of decades it has become increasingly apparent that Vitamin D also has other important functions in tissues, not primarily related to mineral metabolism, such as hematopoietic system, insulin secretion etc. 1α $25(OH)_2D_3$ has a wide range of non-classical actions in the body such as regulation of cell growth, differentiation and the immune system. This has led to increased interest in using 1α $25(OH)_2D_3$ in the treatment of several Vitamin D associated diseases such as psoriasis, autoimmune diseases, osteoporosis and hyper parathyroidism. However, the therapeutic application of 1α $25(OH)_2D_3$ is limited due to hypercalcemic activity. In order to avoid the unwanted calcemic effects, efforts were made to synthesize analogs that exhibit less calcemic effects, but with growth and immuno modulating properties. Most of the analogs belong to either side chain or epi modifications, some of them include the natural metabolites of Vitamin D_3.

Inflammation is a response to infection, antigen challenge or tissue injury that is designed to eradicate microbes or irritant and to potentiate tissue repair. Inflammation is characterized by vasodilation, fluid exudation and leukocyte migration to injury site. Arachidonic acid, a derived essential fatty acid, after release from membrane phospholipids forms a precursor for inflammatory mediators like Prostaglandins (PGs) and Leukotrienes (LTs) with a wide range of physiological and pathological effects. PGs and LTs are important mediators of inflammation produced by the action of Cyclooxygenase (COX) and Lipoxygenase (LOX) enzymes respectively.

COX -1 is constitutively active and is responsible for several house keeping physiological functions. On the other hand, COX-2 is the inducible isoform responsible for the up regulation of prostaglandins and other pro-inflammatory agents.

The anti-inflammatory mechanism of NSAIDs is attributed to the inhibition of COX-2 and main undesirable side effects of NSAIDs are due to the inhibition of COX-1. Also the antipyretic and analgesic effects are related to the inhibition of COX-2. Leukotrienes, specifically LTB_4, play an important role in the early phase of inflammatory reaction. The LTB_4 generated at the site of infection attracts leukocytes, which in turn leads to induction of COX-2. The PGE_2 produced by COX-2 in turn initiates a series of events that lead to the resolution of inflammation, which include induction of 15-lipoxygenase and generation of lipoxins.

While COX-2 and 5-LOX play an important role in defense against infection, their uncontrolled stimulation leads to the manifestation of several inflammatory disorders such as arthritis, inflammatory bowel disease, asthma and osteoporosis. Many of these inflammatory disorders with over expression of COX and 5-LOX were shown to be associated with low vitamin D status. In case of osteoporosis, PGs produced by COX-2 play an important role in the bone resorption process. An inverse relationship between vitamin D levels and the occurrence of pro inflammatory and neoplastic disorders was reported. While Vitamin D exhibits anti-inflammatory and anti-proliferative effects, COX-2 and LOX are pro-inflammatory and promote neoplastic transformation. However the precise role of Vitamin D and its analogs in promoting anti-proliferative and anti-inflammatory effects is largely unknown. Hence the present study was undertaken to analyze the role of

prostaglandins and other arachidonic acid metabolites in Vitamin D and its analogs mediated anti-inflammatory and anti-proliferative effects.

In the present study Vitamin D and its analogs were screened for their effects on lipoxygenase and cyclooxygenases *in vitro*. The compounds screened are as follows:

1. $1\alpha25(OH)_2D_3$
2. $1\alpha25(OH)_2$-16-ene-D_3
3. $1\alpha25(OH)_2$-16-ene-23-yne-D_3
4. $1\alpha25(OH)_2$-16-ene-23-yne-26,27-F_6-D_3
5. Gemini (two side chain) $1\alpha25(OH)_2D_3$
6. $1\alpha25(OH)_2$-3-epi-D_3
7. $1\alpha25(OH)_2$-16-ene-3-epi-D_3
8. $1\alpha25(OH)_2$-16-ene-23-yne-3-epi-D_3
9. $1\alpha25(OH)_2$-16-ene-23-yne-26,27-F_6-3-epi-D_3
10. Gemini (two side chain) $1\alpha25(OH)_2$-3-epi-D_3

The specific COX-2 inhibitors were tested for their effects on mouse macrophage cell lines (RAW 264.7) and in rat air pouch model of inflammation.

In vitro studies on isolated 5-lipoxygenase enzyme showed no effect by 1α $25(OH)_2D_3$ and its analogs employed in the present study. However, the studies on cyclooxygenases showed selective inhibition of cycloocygenase-2 enzyme with no inhibition on cyclooxygenase-1 (COX-1) by $1\alpha25(OH)_2D_3$, RO-23-7553 and epi analog of RO-23-7553. Among the three compounds tested, RO-23-7553 showed potent inhibition of COX-2 with an IC_{50} value of 5.8nM.

In order to understand the molecular interaction between COX-2 and RO-23-7553 further studies were undertaken on molecular modeling. These studies showed favorable interactions of RO-23-7553 towards COX-2 as evidenced by GOLD scores. These interactions are more favorable incase of RO-23-7553 than α-methyl-4-biphenylacetic acid, a known selective inhibitor

of COX-2.

To further evaluate the anti-inflammatory effects of RO-23-7553, studies were undertaken on mouse macrophage cell line, RAW 264.7. RO-23-7553 reduced the growth and multiplication of LPS stimulated mouse macrophage cells with IC_{50} of 10 nM. Phase contrast microscopic studies showed the presence of vacuoles and loss of cytoplasm integrity in RO-23-7553 treated mouse macrophage cells. RO-23-7553 down regulated the expression of COX-2 but not COX-1, both at transcriptional and translational level in LPS stimulated mouse macrophage cells. In addition to COX-2, RO-23-7553 down regulated the expression of other pro-inflammatory mediators such as iNOS and IL-2 in mouse macrophage cells. As a result the levels of COX-2, iNOS and IL-2 were markedly lower in RO-23-7553 treated cells when compared to LPS-treated mouse macrophage cells. These studies suggest that RO-23-75553 inhibits growth of mouse macrophages by down regulating the expression of pro inflammatory mediators. In order to understand the mode of cell death induced by RO-23-7553, further studies were undertaken on DNA degradation and flow cytometry. These studies revealed diffused pattern of migration of DNA and arrest of the cells at G_1 phase of cell cycle in response to RO-23-7553 treatment, thus suggesting necrotic pattern of cell death.

Based on the results obtained from isolated enzyme assays and on mouse macrophages, further studies were undertaken to test the anti-inflammatory effects of RO-23-7553 on carrageenan induced rat air pouch model of inflammation. Inflammatory characteristics like exudate volume and no. of infiltrated cells were reduced in RO-23-7553 treated (10μg/Kg body weight) animals compared to carrageenan alone treated animals. Histological

sections of air pouch tissue of RO-23-7553 treated animals also showed reduced inflammatory reaction this was evidenced by the down regulated the expression of COX-2 but COX-1 both at transcriptional and translation levels, in air-pouch tissues of RO-23-7553 treated animals. In addition to COX-2, RO-23-7553 down regulated the expression of other pro-inflammatory mediators such as iNOS and IL-2 in carrageenan induced rat air-pouch tissue. As a result the levels of COX-2, iNOS and IL-2 were markedly lower in RO-23-7553 treated air pouch tissue when compared to carrageenan alone treated animals. In order to understand antioxidant role of RO-23-7553, reduced glutathione (GSH) and glutathione peroxidase (GPx) activity levels were measured in rat air-pouch tissue. These studies revealed that both GSH and GPx levels were increased in RO-23-7553 treated animals compared to carrageenan alone treated animals. These studies suggest that RO-23-7553 potentiates anti-oxidant defenses in inflammatory tissues.

Thus the present study demonstrates that RO-23-7553 is a selective inhibitor of COX-2 and exhibits anti-inflammatory properties by the down-regulation of pro-inflammatory mediators and potentiating the anti-oxidant defenses.

References

- Adams AE, Abu-Amer Y, Chappel J, Stueckle S, Ross FP, Teitelbaum SL & Suva LJ (1999) 1,25 dihydroxyvitamin D3 and dexamethasone induce the cyclooxygenase 1 gene in osteoclast-supporting stromal cells, *J Cell Biochem* 74: 587-95.

- Ahonen MH, Tenkanen L, Teppo L, Hakama M & Tuohimaa P (2000) Prostate cancer risk and prediagnostic serum 25-hydroxyvitamin D levels (Finland), *Cancer Causes Control* 11: 847-52.

- Aisen PS (2002) Evaluation of selective COX-2 inhibitors for the treatment of Alzheimer' s disease,*J Pain Symptom Manage* 23: S35-40. Review.

- Alroy I, Towers TL, Freedman LP (1995) Transcriptional repression of the interleukin-2 gene by vitamin D3: direct inhibition of NFATp/AP-1 complex formation by a nuclear hormone receptor, *Mol Cell Biol* 15: 5789-99.

- Amin AR, Attur M, & Abramson SB (1999) Nitric oxide synthase and cyclooxygenases: distribution, regulation, and intervention in arthritis, *Curr Opin Rheumatol* 11: 202-09.

- Anderson GD, Hauser SD, McGarity KL, Bremer ME, Isakson PC & Gregory SA (1996) Selective inhibition of Cyclooxygenase-2 (COX-2) reverses inflammation and expression of COX-2 and interleukin-6 in rat adjuvant arthritis, *J Clin Invest* 97: 2672-79.

- Anderson ME, Allison DRD & Meister A (1982) Interconversion of leukotrienes catalyzed by purified glutamyl transpeptidase: concomitant formation of leukotriene D4 and glutamyl amino acids, *Proc Natl Acad Sci USA* 79:1088-91.

- Araya Z, Hosseinpour F, Bodin K & Wikvall K (2003) Metabolism of 25-hydroxyvitamin D3 by microsomal and mitochondrial vitamin D3 25-hydroxylases (CYP2D25 and CYP27A1): a novel reaction by CYP27A1, *Biochim Biophys Acta* 1632: 40-47.

- Bayly CI, Black WC, Leger S, Ouimet N, Ouellet M & Percival MD (1999) Structure based design of cox-2 selectivity into flurbiprofen, *Bioorg Med Chem Lett* 9: 307-12.

- Becker JC, Domschke W & Pohle T (2004) Current approaches to prevent NSAID-induced gastropathy - COX selectivity and beyond, *Br J Clin Pharmacol* 58: 587-00.

- Beckman MJ & DeLuca HF (2002) Regulation of renal vitamin D receptor is an important determinant of 1-alpha-25-dihydroxyvitamin D3 levels *in vivo*, *Arch Biochem Biophys* 401:44-52.

- Berkely HO & Galliard T (1976) Measurement of lipoxygenase activity in crude and partially purified potato extracts, *Phytochemistry* 15: 1475-79.

- Bias P, Buchner A, Klesser B & Laufer S (2004) The gastrointestinal tolerability of the LOX/COX inhibitor, licofelone, is similar to placebo and

superior to naproxen therapy in healthy volunteers: results from a randomized, controlled trial, *Am J Gastroenterol* 99: 611-18.

- Bisgaard H (1984) Leukotrienes and prostaglandins in asthma, *Allergy* 39: 413-20.

- Blutt SE, McDonnell TJ, Polek TC & Weigel NL(2000) Calcitriol-induced apoptosis in LNCaP cells is blocked by overexpression of Bcl-2, *Endocrinology* 141: 10-17.

- Böhm HJ & Klebe G (1996) What can we learn from molecular recognition in protein-ligand complexes for the design of new drugs?, *Angew Chem Int Ed Engl* 35: 2588-14.

- Borgeat P & Samuelsson B (1979) Arachidonic acid metabolism in polymorphonuclear leukocytes: effects of ionophore A23187, *Proc Natl.Acad.Sci* 76: 2148-51.

- Bouillon R, Allewaert K, Xiang DZ, Tan BK & van Baelen H (1991) Vitamin D analogs with low affinity for the vitamin D binding protein: enhanced *in vitro* and decreased *in vivo* activity, *J Bone Miner Res* 6: 1051-57.

- Bouillon R, Verstuyf A, Zhao J, Tan BK & Van Baelen H (1996) Nonhypercalcemic vitamin D analogs: interactions with the vitamin D-binding protein, *Horm Res* 45: 117-21.

- Bouillon R.Verhaeghe J, Thomasset M & Van Assche FA (1990) Osteocalcin is vitamin D-dependent during the perinatal period in the rat, *J Dev Physiol* 14: 311-17.

- Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing principle of protein dye binding, *Anal Biochem* 72: 248-54.

- Brown AJ (1999) Regulation of vitamin D action, *Nephrol Dial Transplant* 14: 11-16.

- Brown AJ (2000) Mechanisms for the selective actions of vitamin D analogs, *Curr Pharm Des* 6: 701-16.

- Brown AJ (2001) Therapeutic uses of vitamin D analogs, *Am J Kid Dise* 38: 3-9.

- Brown AJ, Dusso A, Slatopolsky S (1999) Vitamin D. Invited Review, *Am J Physiol* 277 (Renal Physiol 46): F157-75.

- Buskens CJ, Sivula A, van Rees BP, Haglund C offerhaus GJ, van Lanschot JJ & Ristimaki A (2003) Comparison of Cyclooxygenase-2 expression in adenocarcinomas of the gastric cardia and distal oesophagus, *Gut* 52: 1678-83.

- Campling BG, Pym J, Galbraith PR & Cole SP (1988) Use of MTT assay for rapid determination of chemosensitivity of human leukemic blast cells, *Leukemia Research* 12: 823-31.

- Cannon GW & Breedveld FC (2001) Efficacy of cyclooxygenase-2-specific inhibitors, *Am J Med* 110 (3A Suppl): 6S-12S. Review.

- Cantorna MT Hayes CE & DeLuca HF (1996) 1, 25-DihydroxyvitaminD3 reversibly blocks the progression of relapsing encephalomyelitis, a model of multiple sclerosis, *Proc Natl Acad Sci USA* 93: 7861-64.

- Chan CC, Dubois L & Young V (1987) Effects of two novel inhibitors of 15lipoxygenase, L-651, 392 and L-651, 896 in a guinea pig model of epidermal hyperproliferation, *Eur J Pharmacol* 139:11-18.

- Chan TA (2002) Nonsteroidal anti-inflammatory drugs, apoptosis, and colon-cancer chemoprevention, *Lancet Oncol* 3: 166-74.

- Coffey MJ, Wilcoxen SE, Phare SM, Simpson RU, Gyetko MR, Peters-Golden M (1994) Reduced 5-lipoxygenase metabolism of arachidonic acid in macrophages from 1,25-dihydroxyvitamin D3-deficient rats, *Prostaglandins* 313-29.

- Colston KW, Berger U & Coombes RC (1989) Possible role for vitamin D in controlling breast cancer cell proliferation, *Lancet* 1: 188-91

- Copeland RA, Williams JM, Giannaras J, Nurnberg S, Covington M, Pinto D, Pick S & Trzaskos JM (1994) Mechanism of selective inhibition of the inducible isoform or prostaglandin G/H synthase, *Proc Natl Acad Sci USA* 91: 11202-06.

- Coussens LM & Werb Z (2002) Inflammation and cancer, *Nature* 420: 860-67.

- Crofford LJ, Wilder RL, Ristimaki AP, Sano H, Remmers EF, Epps HR & Hla (1994) Cyclooxygenase-1 and –2 expression in rheumatoid synovial tissue, *J Clin Invest* 93: 1095-01.

- Cross HS, Huber C & Peterlik M. 1991 Antiproliferative effect of 1,25-dihydroxyvitamin D3 and its analogs on human colon adenocarcinoma cells (CaCo-2): influence of extracellular calcium, *Biochem Biophys Res Commun* 179: 57-62.

- Dawson J, Sedgwick AD, Edwards JC & Lees P (1991) A comparative study of the cellular, exudative and histological responses to carrageenan, dextran and zymosan in the mouse, *Int J Tissue React* 13: 171-85.

- Deluca HF & Cantorna MT (2001) Vitamin D: its role and uses in immunology, *FASEB* 15: 2579-86.

- Diaz GD, Paraskeva C, Thomas MG, Binderup L & Hague A (2000) Apoptosis is induced by the active metabolite of vitamin D3 and its analogue EB1089 in colorectal adenoma and carcinoma cells: possible implications for prevention and therapy, *Cancer Res* 60: 2304-12.

- Dix CJ, Habberfield AD, Sulllivan MH & Cooke BA (1984) Inhibition of steroid production in leydig cells by non-sterodial anti-inflammatory and released compounds: evidence for the involvement of lipoxygenase products in steroidogenesis, *Biochem J* 219: 529-37.

- Downey GP, Woethen GS, Henson PM & Hyde DM (1993) Neutrophil sequestration and migration in localized pulmonary inflammation. Capillary localization and migration across the interalveolar septum, *American Review of Respiratory disease* 147: 168-76.

- Drinka PJ (2004) The importance of parathyroid hormone and vitamin D status in the treatment of osteoporosis and renal insufficiency, *J Am Med Dir Assoc* 5: 382-86.

- DuBois RN, Abramson SB, Crofford L, Gupta RA, Simon LS, Van De Putte LBA & Lipsky PE (1998) Cyclooxygenase in biology and disease, *FASEB J* 12: 1063-73.

- Erben RG (2001) Vitamin D analogs and bone, *J Musculoskel Neuron Interact* 2: 59-69.

- Feldman D, Zhao X-Y & Krishnan AV (2000) Vitamin D and prostate cancer*, Endocrinology* 141: 5-9.

- Fiorucci S, Meli M, Bucci G & Cirino G. (2001) Dual inhibitors of Cyclooxygenase and 5-lipoxygenase. A new avevue in anti inflammatory therapy?, *Bio Chem Pharmacol*. 63: 1433-38.

- Friedl HP, Till GO, Trentz O & Ward PA (1989) Roles of histamine, complement and xanthine oxidase in thermal injury of skin, *American Journal of Pathology* 135: 203-17.

- Gallagher JC & Riggs BL (1990) Action of 1,25-dihydroxyvitamin D3 on calcium balance and bone turnover and its effect on vertebral fracture rate, *Metabolism* 39 : 30-34.

- Garavito RM (1996) The Cyclooxygenase-2 structure new drugs for an old target?, *Nat Struct Biol* 3: 897-01.

- Garcia Rodriguez LA & Gonzalez-Perez A (2004) Risk of breast cancer among users of aspirin and other anti-inflammatory drugs, *Br J Cancer* 91: 525-29.

- Gardezi SA, Nguyen C, Malloy PJ, Posner GH, Feldman D & Peleg S (2001) A rationale for treatment of hereditary vitamin D-resistant rickets with analogs of 1α,25-dihydroxyvitamin D3, *J Biol Chem* 276: 29148-56.

- Garland CF, Garland FC & Gorham ED (1999) Calcium and vitamin D. Their potential roles in colon and breast cancer prevention, *Ann N Y Acad Sci* 889: 107-19.

- GioVanni de Gaetano, Maria Benedetta Donati & Chiara Ceerletti (2003) Prevention of thrombosis and vascular inflammation: benefits and limitations of selective or combined COX-1,COX-2 and 5-LOX inhibitors, *Trends Pharmacol Sci* 24: 245-52.

- Giovannucci E, Egan KM, HunterDJ, Stampfer MJ, Colditz GA, Whilett WC & Speizer FE (1995) Aspirin and the risk of colorectal cancer in women, *N Engl J Med* 333: 609-14.

- Glenville J, Hogan DB, Yendt E & Hanley DA (1996) Prevention and management of osteoporosis: Consensus statements from the Scientific Advisory Board of the Osteoporosis Society of Canada 8.Vitamin D metabolites and analogs in the treatment of osteoporosis, *Canadian Medical Association J* 155: 955-61.

- Goltzman D, White J & Kremer R (2001) Studies of the effects of 1,25-dihydroxyvitamin D on skeletal and calcium homeostasis and on inhibition of tumor cell growth, *J Steroid Biochem Mol Biol* 76: 43-47.

- Gomme PT & Bertolini J (2004) Therapeutic potential of vitamin D-binding protein, *Trends Biotechnol* 22: 340-45.

- Graafmans WC, Lips P, Ooms ME, van Leeuwn JPTM, Pols HAP & Uitterlinden AG (1997) The effect of vitamin D supplementation on the bone mineral density of the femoral neck is associated with vitamin D receptor genotype, *J Bone Mineral Res* 12: 1241-45.

- Gulliford T, Enlish J, Colston KW, Menday P, Moller S & Coombrs RC (1998) A phase I study of vitamin D analogue EB1089 in patients with advanced breast and colorectal cancer, *Br J Cancer* 78: 6-13.

- Guzey M, Jukic D, Arlotti J, Acquafondata M, Dhir R & Getzenberg RH (2004) Increased apoptosis of periprostatic adipose tissue in VDR null mice, *J Cell Biochem* 93: 133-41.

- Hanchette CL & Schwartz GG (1992) Geographic patterns of prostate cancer mortality: Evidence for a protective effect of ultraviolet radiation, *Cancer* 70: 2861-89.

- Hansen CM, Frandsen TL, Brunner N & Binderup L (1994) 1á,25-Dihydroxyvitamin D3 inhibits the invasive potential of human breast cancer cells *in vitro*, *Clin Exp Metastasis* 12: 195-02.

- Hansen MC, Binderup L, Hamberg KJ & Carlberg C (2001) Vitamin D and cancer: Effects of 1,25(OH)2D3 and its analogs on growth control and tumorigenesis, *Front in Biosci* 6: 820-48.

- Hara A, Yoshimi N, Niwa M, Ino N & Mori H (1997) Apoptosis induced by NS-398, a selective cyclooxygenase-2 inhibitor, in human colorectal cancer cell lines, *Jpn J Cancer Res* 88: 600-04.

- Harle D, Radmark O, Samuelsson B & Steinhilber D (1999) Transcriptional and posttranscriptional regulation of 5-lipoxygenase mRNA expression in the human monocytic cell line Mono Mac 6 by transforming growth factor-beta and 1,25-dihydroxyvitamin D3, *Adv Exp Med Biol* 469: 105-11.

- Harris DM & Go VL (2004) Vitamin d and colon carcinogenesis, *J Nutr* 134: 3463S-71S.

- Hayes CE (2000) Vitamin D: a natural inhibitor of multiple sclerosis, *Proc Nutr Soc* 59: 531-35. Review.

- Heberden C, Denis I, Pointillart A & Mercier T (1998) TGF-beta and calcitriol, *Gen Pharmacol* 30: 145-51.

- Herdick M, Bury Y, Quack M, Uskokovic MR, Polly P & Carlberg C (2000) Response element and coactivator-mediated conformational change of the vitamin D3 receptor permits sensitive interaction with agonists, *Mol Pharmacol* 57: 1206-17.

- Hermann M, Lorenz HM, Voll R, Grunke M, Woith W & Kalden JR (1994) A rapid and simple method for the isolation of apoptotic DNA fragments, *Nucleic Acids Res* 22: 5506-07.

- Hershberger PA, Modzelewski RA, Shurin ZR, Rueger RM, Trump DL & Johnson CS (1999) 1,25-Dihydroxycholecalciferol (1,25-D3) inhibits the growth of squamous cell carcinoma and down-modulates p21(Waf1/Cip1) *in vitro* and *in vivo, Cancer Res* 59: 2644-49.

- Hinz B & Brune K (2004) Pain and osteoarthritis: new drugs and mechanisms, *Curr Opin Rheumatol* 16: 628-33.

- Hirata M, Kato H, Debuchi H, Ikesue A, Mitamura M & Nakagawa H (1994) Anti-inflammatory effect of 22-oxa-1 alpha,25-dihydroxyvitamin D3 on carrageenin-induced inflammation in rats, *Biol Pharm Bull*. 17:1130-31.

- Hisa T, Taniguchi S, Tsuruta D, Hirachi Y, Ishizuka S & Takigawa M (1996) Vitamin D inhibits endothelial cell migration, *Arch Dermatol Res* 288: 262-63.

- Holick MF (1995) Environmental factors that influence the cutaneous production of vitamin D, *Am J Clin Nutr* 61 (Suppl): 638-45.

- Holick MF (1999). Vitamin D. In: Modern nutrition in health and Disease. Shils ME, Olson JA, Shike M, Ross AC (Eds.), 19th edition, Lippincott Willams & Wilkins, Philadelphia, pp 329-45.

- Hulting AL, Lindgren JA, Hokfelt T, Eneroth P, Werner S, Patrono C & Samuelsson B (1985) Leukotriene C_4 as a mediator of lutenizing hormone release from rat anterior pituitary cells, *Proc Natl Acad Sci USA* 82: 3834-38.

- Inoue H, Tsujisawa T, Fukuizumi T, Kawagishi S & Uchiyama C (1999) SC-19220, a prostaglandin E2 antagonist, inhibits osteoclast formation by 1,25-dihydroxyvitamin D3 in cell cultures, *J Endocrinol* 161: 231-36.

- Issa LL, Leong GM, Sutherland PL & Eisman JA (2002) Vitamin D analogs-spesific recruiment of vitamin D receptor coactivators. *J Bone Miner Res* 17: 879-890.

- Jääskeläinen T, Ryhänen S, Mahonen A, DeLuca HF & Mäenpää PH (2000) Mechanism of action of super active vitamin D analogs through regulated receptor degradation, *J Cell Biochem* 76: 548-58.

- Jackson LM & Hawkey CJ (2000) COX-2 selective nonsteroidal anti-Inflammatory drugs: do they really offer any advantages?, *Drugs* 59: 1207-16.

- Jacobs ET, Giuliano AR, Martinez ME, Hollis BW, Reid ME & Marshall JR (2004) Plasma levels of 25-hydroxyvitamin D, 1,25-dihydroxyvitamin D and the risk of prostate cancer, *J Steroid Biochem Mol Biol* 89-90: 533-37.

- Jagu S, Suraneni VKM, Madhava CR, Metukuri MR, Aparna R & Pallu R (2004) Molecular mechanisms in C-Phycocyanin induced apoptosis in human chronic myeloid leukemia cell line –K562, *Biochem Pharmacol* 68: 453-62.

- Jahnsen J, Falch JA, Mowinckel P & Aadland E (2002) Vitamin D status, parathyroid hormone and bone mineral density in patients with inflammatory bowel disease, *Scand J Gastroenterol*. 37: 192-99.

- Jakobsson PJ, Steinhilber D, Odalander B, Radmark O, Claesson HE & Samuelsson B (1992) On the expression and regulation of 5-lipoxygenase in human lymphocytes, *Proc Natl Acad Sci USA* 89: 3521-25.

- Jakschik B, Harper T & Murphy RC (1982) Leukotriene C_4 and D_4 formation by particulate enzymes, *J Biol Chem* 257: 5346-49.

- Jensen SS, Madsen MW, Lukas J, Binderup L & Bartek J (2001) Inhibitory effects of 1á,25- dihydroxyvitamin D3 on the G(1)-S phase-controlling machinery, *Mol Endocrinol* 15: 1370-80.

- Johnson LE & DeLuca HF (2001) Vitamin D receptor null mutant mice fed high levels of calcium are fertile, *J Nutr* 131: 1787-91.

- Jones G, Strungnell SA & DeLuca HF (1998) Current understanding of the molecular actions of vitamin D, *Physiol Rev* 78: 1193-31.

- Kanekura T, Laulederkind SJ, Kirtikara K, Goorha S & Ballou LR (1998) Cholecalciferol induces prostaglandin E2 biosynthesis and transglutaminase activity in human keratinocytes, *J Invest Dermatol* 111: 634-39.

- Kawai S (2002) Recent development of selective cyclooxygenase-2 inhibitors, *Nippon Rinsho* 60: 2370-77. Review.

- Kawamori T, Rao CV, Seibert K & Reddy BS (1998) Chemopreventive activity of celecoxib, a specific cyclooxygenase-2 inhibitor, against colon carcinogenesis, *Cancer Res* 58: 409-12.

- Keppler D, Hagmann W & Rapp S (1987) Role of leukotrienes in endotoxin action *in vivo*, *Rev Infect Dis* 9: S580-84.

- Khoury R, Ridall AL, Norman AW & Farach-Carson MC (1994) Target gene activation by 1,25-dihydroxyvitamin D3 in osteosarcoma cells is independent of calcium influx, *Endocrinology* 135: 2446-53.

- Kirschning C, Unbehaun A, Lamping N, Pfeil D, Herrmann F & Schumann RR (1997) Control of transcriptional activation of the lipopolysaccharide binding protein (LBP) gene by proinflammatory cytokines, *Cytokines Cell Mol Ther* 3: 59-62.

- Kishimoto Y, Takata N, Jinnai T, Morisawa T, Shiota G, Kawasaki H & Hasegawa J (2000) Sulindac and a cyclooxygenase-2 inhibitor, etodolac, increase APC mRNA in the colon of rats treated with azoxymethane, *Gut* 47: 812-19.

- Koli, K & Keski-Oja J (2000) 1á,25-Dihydroxyvitamin D3 and its analogs down-regulate cell invasion-associated proteases in cultured malignant cells, *Cell Growth Differ* 11: 221-29.

- Krinsky NI (1989) Antioxidant functions of carotenoids, *Free Rad Biol Med* 7: 617-35.

- Kurumbail RG, Stevens AM, Gierse JK, McDonald JJ, Stegeman RA, Pak JY,,ildehaus D, Miyashiro JM, Penning TD, Seibert K, Isakson PC & Stallings WC (1996) Structural basis for selective inhibition of cyclooxygenase-2 by anti-inflammatory agents, *Nature* 384: 644-48.

- Kveiborg M, Rattan SI, Clark BF, Eriksen EF & Kassem M (2001) Treatment with 1,25-dihydroxyvitamin D3 reduces impairment of human osteoblast functions during cellular aging in culture, *J Cell Physiol* 186: 298-06.

- Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature* 227: 680-85.

- Langenbach R, Loftin CD, Lee C & Tiano H (1999) Cyclooxygenase-deficient mice. A summary of their characteristics and susceptibilities to inflammation and carcinogenesis, *Ann N Y Acad Sci* 889: 52-61.

- Lee E, Jeon SH, Yi JY, Jin YJ & Son YS (2001) Calcipotriol inhibits autocrine phosphorylation of EGF receptor in a calcium-dependent manner, a possible mechanism for its inhibition of cell proliferation and stimulation of cell differentiation, *Biochem Biophys Res Commun* 284: 419-25.

- Lemire J (2000) 1,25-Dihydroxyvitamin D3 a hormone with immunomodulatory properties, *Rheumatol* 59: 24-27.

- Li SH, Black WC, Chan CC Ford-Hutchinson AW, Gauthier JY, Gordon R; et al (1995) COX-2 inhibitors. Synthesis and pharmacological activities of 5-methane sulfonamido-1-indanone derivatives, *J Med Chem* 38: 4895-97.

- Liu M, Lee M-H, Cohen M, Bommakanti M & Freedman LP (1996) Transcriptional activation of the cdk inhibitor p21 by vitamin D3 leads to the induced differentiation of the myelomonocytic cell line U937, *Genes & Dev* 10: 142-53.

- Liu X, Li P, Widlak P, Zou H, Luo X, Garrard WT & Wang X (1998) The 40-kDa subunit of DNA fragmentation factor induces DNA fragmentation and chromatin condensation during apoptosis, *Proc Natl Acad Sci USA* 95: 8461-66.

- Liu YY, Collins ED, Norman AW &Peleg S (1997) Differential interaction of 1alpha,25-dihydroxyvitamin D3 analogues and their 20-epi homologues with the vitamin D receptor, *J Biol Chem* 272: 3336-45.

- Liu YY, Nguyen C, Ali Gradezi SA, Schnirer I & Peleg S (2001) Differential regulation of heterodimerization by 1α,25-dihydroxyvitamin D3 and its 20-epi analog, *Steroids* 66: 203-12.

- Lowry OH, Rosenberg NJ, Fany AL & Randall RJ (1951) Protein measurement with folin-phenol reagent, J Biol Chem, 193: 265-75.

- Luong C, Miller A, Barnett J, Chow J, Ramesha C & Browner MF (1996) Flexibility of the NSAID binding site in the structure of human cyclooxygenase-2, *Nat Struct Biol* 3 :927-33.

- Luster AD (1998) Chemokines-chemotactic cytokines that mediate inflammation, *New England Journal of Medicine* 338: 436-45.

- Lyakhovich A, Aksenov N, Miettinen S, Ahonen MH, Syvälä H, Ylikomi T & Tuohimaa P (2000) Vitamin D induced up-regulation of keratinocyte growth factor (FGF-7/KGF) in MCF-7 human breast cancer cells, *Biochem Biophys Res Commun* 273: 675-80.

- Madhava CR, Vadiraja BB, Kirnamai G, Narsa MR, Pallu R & Madhyastha KM (2000) Selective inhibition of cyclooxygenase-2 by C-phycocyanin, a biliprotein from Spirulina platensis, *Biochem Biophys Res Comm* 277: 599-03.

- Manolagas SC, Hustmyer FG, Yu XP (1989) 1,25-Dihydroxyvitamin D3 and the immune system, *Proc Soc Exp Biol Med* 191: 238-45.

- Mantell DJ, Owens PE, Bundred NJ, Mawer EB & Canfield AE (2000) 1 alpha,25-ihydroxyvitamin D(3) inhibits angiogenesis *in vitro* and *in vivo, Circ Res* 87: 214-20.

- Marcheselli VL, Hong S, Lukiw WJ, Hua Tian X, Gronert K, Musto A, Hardy M, Gimemez JM, Chiang N, Serhan CN & Bazan NG (2003) Novel docosanoids inhibit brain ischemia-reperfusion-mediated leukocyte infiltration and pro-inflammatory gene expression, *J Biol Chem* 278: 43807-17.

- Marcinkowska E, Wiedlocha A & Radzikowski C (1998) Evidence that phosphatidylinositol 3-kinase and p70S6K protein are involved in differentiation of HL-60 cells induced by calcitriol, *Anticancer Res* 18: 3507-14.

- Marnett LJ (2002) Recent developments in cyclooxygenase inhibition, *Prostaglandins Other Lipid Mediat* 68-69: 153-64.

- Martin SW, Stevens A J, Brennan BS, Davies D, Rowland M & Houston JB (1994) The six-day-old rat air pouch model of inflammation: characterization of the inflammatory response to carrageenan, *J Pharmacol Toxicol Methods* 32: 139-47.

- Masferrer J. L., Leahy K. M., Koki A. T., Zweifel B. S., Settle S. L., Woerner B. M., Edwards D. A., Flickinger A. G., Moore R. J & Seibert K (2000) Antiangiogenic and antitumor activities of cyclooxygenase-2 inhibitors, *Cancer Res 60:* 1306-11.

- Masuyama H & MacDonald PN (1997) Proteasome-mediated degradation of the vitamin D receptor (VDR) and a putative role for SUG1 interaction with the AF-2 domain of VDR, *J Cell Biochem* 71: 429-40.

- Mawer EB, Jones G, Davies M, Still PE, Byford V, Schroeder NJ, Makin HLJ, Bishop CW & Knutson J (1998) Unique 24-hydroxylated metabolites represent a significant pathway of metabolism of vitamin D2 in humans: 24-hydroxylavitamin D2 and 1,24 dihydroxyvitamin D2 detectable in human serum, *J Clin Endocrinol Metab* 83: 2156-66.

- Mead JF, Alfin-SlaterRB, Howton DR & Popjak G (1986) Prostaglandins, thrombaxanes and prostacyclin. In *Lipids*: Chemistry, Biochemistry and Nutrition. Eds. Mead JF. *Plenum Press p*p. 149-16.

- Meehan TF & DeLuca HF (2002) The vitamin D receptor is necessary for 1alpha,25-dihydroxyvitamin D(3) to suppress experimental autoimmune encephalomyelitis in mice, *Arch Biochem Biophys* 408: 200-04.

- Miedzybrodzki R (2004) Trends in nonsteroidal anti-inflammatory drug development and application, *Postepy Hig Med Dosw* 58: 438-48.

- Mitchell JA, Belvisi MG, Akaresereenont P, Robbins RA, Kwon OJ, Croxtall J, Barnes PJ & Vane JR (1994) Induction of Cyclooxygenase-2 by cytokines in human pulmonary epithelial cells: Regulation of dexamethasone, *Br J Pharmacol* 113: 1008-14.

- Moncada S, Palmer RM & Higgs EA (1991) Nitric Oxide: physiology, pathophysiology, and pharmacology, *Pharmacological Reviews* 43: 109-42.

- Mukherjee PK, Marsccheselli VL, Serhan CN & Bazan NG (2004) Neuroprotectin D1 a docosahexaenoic acid-derived docosatriene protects human retinal pigment epithelial cells from oxidative stress, *Proc Natl Acad Sci USA* 101: 8491-96.

- Muller K, Odum N & Bendtzen K (1993) 1,25-dihydroxyvitamin D3 selectively reduces interleukin-2 levels and proliferation of human T cell lines *in vitro, Immunol Lett* 35:177-82.

- Needleman P & Isackson PC (1998) Selective inhibition of COX-2, *Sci Med* 5: 26-35.

- Nickerson T & Huynh H (1999) Vitamin D analogue EB 1089-induced prostate regression is associated with increased gene expression of insulin-like growth factor binding proteins, *J Endocrinol* 160: 223-29.

- Nieves J, Cosman F, Herbert J, Shen V & Lindsay R (1994) High prevalence of vitamin D deficiency and reduced bone mass in multiple sclerosis, *Neurology* 44: 16872-92.

- Norman AW (1995) The vitamin D Endocrine system: manipulation of structure-function relationships to provide opportunities for development of new cancer chemopreventive and immunosuppressive agents, *J Cell Biochem* 22: 218-25.

- Norman AW, Zhou JY, Henry HL, Uskokovic MR & Koeffler HP (1990) Structure-function studies on analogues of 1 alpha,25-dihydroxyvitamin D3: differential effects on leukemic cell growth, differentiation, and intestinal calcium absorption, *Cancer Res* 50: 6857-64.

- Ornig L, Hammarstrom S & Samuelsson B (1980) Leukotriene D: a slow racting substance from rat leukemia cells, *Proc Natl Acad Sci USA* 77: 2014-17.

- Oshima M, Dinchuk JE, Kargman SL, Oshima H, Hancock B, Kwong E, Trzaskos JM, Evans JF & Taketo MM (1996) Suppression of intestinal polyposis in Apc delta716 knockout mice by inhibition of cyclooxygenase 2 (COX-2), *Cell* 87: 803-09.

- Park WH, Seol JG, Kim ES, Hyun JM, Jung CW, Lee CC, Binderup L, Koeffler HP, Kim BK & Lee YY (2000) Induction of apoptosis by vitamin D3 analogue EB 1089 in NCI-H929 myeloma cells via activation of caspase and MAP kinase, *Br J Haematology* 109: 576-83.

- Pek SB & Walsh MF (1984) Leukotrienes stimulate insulin release from the rat pancreas, *Proc Natl Acad Sci USA* 81: 2199-02.

- Peleg S & Reddy GS (2001) Tissue specific metabolism of 1α,25-dihydroxy-20-epi-vitamin D3 into new metabolites with significant biological activity: studies in rat osteosarcoma cells (UMR 106 and ROS 17/2.8), *J Cell Biochem* 82: 599-09.

- Peleg S, Ismail A, Uskokovic MR & Avnur Z (2003) Evidence for tissue- and cell-type selective activation of the vitamin D receptor by Ro-26-9228, a noncalcemic analog of vitamin D3, *J Cell Biochem* 88: 267-73.

- Peleg S, Nguyen C, Woodard BT, Lee JK & Posner GH (1998) Differential use of transcription activation function 2 domain of the vitamin D receptor by 1,25-dihydroxyvitamin D3 and it's A ring-modified analogs, *Mol Endocrinol* 12: 525-35.

- Peleg S, Qiu H, Reddy S, Harris D, Van Q, Estey EH, Talpaz M & Estrov Z (1997) 1,25-Dihydroxyvitamin D3 and its analogs inhibit acute myelogenous leukemia progenitor proloferation by suppressing interleukin-1â production, *J Cin Invest* 100: 1716-24.

- Pelzmann M, Thurnher D, Gedlicka C, Martinek H & Knerer B (2004) Nimesulide and indomethacin induce apoptosis in head and neck cancer cells, *J Oral Pathol Med* 33: 607-13.

- Penna G & Adorini L (2000) 1 Alpha,25-dihydroxyvitamin D3 inhibits differentiation, maturation, activation, and survival of dendritic cells leading to impaired alloreactive T cell activation, *J Immuno*l 164: 2405-11.

- Piper PJ (1985) Leukotrienes, potent mediators of airway constriction, Int Arch Allergy Appl Immunol 76:43-48.

- Pirianov G & Colston KW (2001) Interactions of vitamin D analogue CB1093, TNFα and ceramide on breast cancer cell apoptosis, *Mol Cell Endocrinol* 172: 69-78.

- Poff CD & Balazy M (2004) Drugs that target lipoxygenases and leukotrienes as emerging therapies for asthma and cancer, *Curr Drug Targets Inflamm Allergy* 3: 19-33.

- Pracard C (1995) Small molecule crystal structures as a structural basis for drug design, *Acta Crystallogr D Biol Crystallogr* 51: 407-17.

- Pritchard RS, Baron JA & Gerhardsson de Verdier M (1996) Dietary calcium, vitamin D, and the risk of colorectal cancer in Stockholm, Sweden, *Cancer Epidemiol Biomarkers Prev* 5: 897-00.

- Pullen GR, Chalmers PJ, Nind AP & Nairn RC (1981) Criteria of cell killing *in vitro, J Immunol Methods* 43: 87-93.

- Quack M & Carlberg C (1999) Selective recognition of vitamin D receptor conformations mediates promoter selectivity of vitamin D analogs, *Mol Pharmacol* 55: 1077-87.

- Ranger GS, Thomas V, Jewell A & Mokbel K (2004) Elevated cyclooxygenase-2 expression correlates with distant metastases in breast cancer, *Anticancer Res* 24: 2349-51.

- Rao LG, Liu LJ, Rawlins MR, McBroom RJ, Murray TM, Reddy GS, Uskokovic MR, Rao DS & Sutherland MK (2001) The biological activities of 1alpha,25-dihydroxyvitamin D3 and its synthetic analog 1alpha,25-dihydroxy-16-ene-vitamin D3 in normal human osteoblastic cells and human osteosarcoma SaOS-2 cells are modulated by 17-beta estradiol and dependent on stage of differentiation, *Biol Pharm Bull* 24: 242-48.

- Rao LG, Sutherland MK, Reddy GS, Siu-Caldera ML, Uskokovic MR & Murray TM (1996) Effects of 1alpha,25-dihydroxy-16ene, 23yne-vitamin D3 on osteoblastic function in human osteosarcoma SaOS-2 cells: differentiation-stage dependence and modulation by 17-beta estradio, *Bone* 19: 621-27.

- Redadnna P, Whelan J & Reddy CC (1988) Anew pathway for the biosynthesis of leukotriene F_4, *Annal N Y Acad Sci* 524: 12339-45.

- Reddy GS, Rao DS, Siu-Caldera ML, Astecker N, Weiskopf A, Vouros P, Sasso GJ, Manchand PS & Uskokovic MR (2000) 1alpha,25-dihydroxy-16-ene-23-yne-vitamin D3 and 1alpha,25-dihydroxy-16-ene-23-yne-20-epi-vitamin D3: analogs of 1alpha,25-dihydroxyvitamin D3 that resist metabolism through the C-24 oxidation pathway are metabolized through the C-3 epimerization pathway, *Arch Biochem Biophys* 383: 197-05.

- Reddy RC, Chen GH, Tateda K, Tsai WC, Phare SM, Mancuso P, Peters-Golden M & Standiford TJ (2001) Selective inhibition of COX-2 improves early survival in murine endotoxemia but not in bacterial peritonitis, *Am J Physiol Lung Cell Mol Physiol* 28: 537-43.

- Reed DW, Bradshaw WS, Xie W & Simmons DL (1996) *In vivo* and *in vitro* expression of a non-mammalian Cyclooxygenase-1, *Prostaglandins* 52: 269-84.

- Reich R, Kohen F, Slager R & Tsafriri A (1985) Ovarian lipoxygenase activity and its regulation by gonadotropin in the rat, *Prostaglandins* 30: 581-90.

- Reichrath J (2001) Will analogs of 1,25-dihydroxyvitamin D3 (calcitriol) open a new era in cancer therapy?, *Onkologie* 24: 128-33.

- Riachy R, Vandewalle B, Belaich S, Kerr-Conte J, Gmyr V, Zerimech F, d' Herbomez M,Lefebvre J & Pattou F (2001) Beneficial effect of 1,25 dihydroxyvitamin D3 on cytokine-treated human pancreatic islets, *J Endocrinol* 169: 161-68.

- Rochel N, Wurtz JM, Mitschler A, Klaholz B & Moras D (2000) The crystal structure of the nuclear receptor for vitamin D bound to its natural ligand, *Mol Cell* 5: 173-79.

- Rokach J & Fitzsimmons B (1988) The lipoxins, *Int J Biochem* 20: 753-58.

- Rozen F & Pollak M (1999) Inhibition of insulin-like growth factor I receptor signaling by the vitamin D analogue EB1089 in MCF-7 breast cancer cells: A role for insulin-like growth factor binding proteins, *Int J Oncol* 15: 589-94.

- Ryhanen S, Jaaskelainen T, Mahonen A & Maenpaa PH (2003) Inhibition of MG-63 cell cycle progression by synthetic vitamin D3 analogs mediated by p27, Cdk2, cyclin E, and the retinoblastoma protein, Biiochem *Pharmacol* 66: 495-04.

- Saji S, Hirose M & Toi M (2004) Novel sensitizing agents: potential contribution of COX-2 inhibitor for endocrine therapy of breast cancer, *Breast Cancer* 11: 129-33.

- Sala A & Folco G (2001) Neutrophils, endothelial cells and cysteinyl leukotrienes: a new approach to neutrophil dependent inflammation?, *Biochem Biophys Res Commun* 238: 1003-06.

- Samuelsson B, Dahlen SE, Lindgren JA, Rouzer CA & Serhan CN (1987) Leukotrienes and lipoxins: Structure, biosynthesis and biological effects. *Science (Washington DC)* 237: 1171-76.

- Sarith M, Souvannavong V & Adam A (1993) Nitric oxide synthase induces macrophages death by apoptosis, *Biochem Biophys Res Commun* 191: 503-08.

- Satchel DP & Norman AW (1996) Metabolism of the cell differentiating agent 1α25$(OH)_2$-16-ene-23-yne-D3 by leukemic cells, *J Steroid Biochem Mol Biol* 57: 117-24.

- Sawaoka H, Kawano S, Tsuji S, Tsujii M, Gunawan ES, Takei Y, Nagano K & Hori M (1998) Cyclooxygenase-2 inhibitors suppress the growth of

gastric cancer xenografts via induction of apoptosis in nude mice, *Am J Physiol* 274: G1061-67.

- Schumann RR, Lamping N, Kirschning C, Knopf HP, Hoess A & Herrmann F (1994) Lipopolysaccharide binding protein: its role and therapeutical potential in inflammation and sepsis, *Biochem Soc Trans* 22: 80-82.

- Schwartz GG, Hill CC, Oeler TA, Bechih MJ & Bahnson RR (1995) 1, 25-Dihydroxy-16-ene-23-yne-vitamin D3 and prostate cancer cell proliferation in vivo, *Urology* 46: 365-69.

- Sedgwick AD & Lees P (1986) Studies of eicosanoid production in the air pouch model of synovial inflammation, *Agents Actions* 18: 429-38.

- Sedgwick AD, Sin YM, Edwards JC & Willoughby DA (1983) Increased inflammatory reactivity in newly formed lining tissue, *J Pathol* 141: 483-95.

- Serhan CN & Chiang N (2004) Novel endogenous small molecules as the checkpoint controllers in inflammation and resolution: entrée for resoleomics, *Rheum Dis Clin North Am* 30: 69-95.

- Serhan CN, Clish CB, Brannon J, Colgan SP, Chiang N & Gronet K (2000) Novel functional sets of lipid-derived mediators with anti-inflammatory actions generated from omega-3 fatty acids via Cyclooxygenase 2-nonsteroidal anti-inflammatory drugs and transcellular processing, *J Exp Med* 192: 1197-04.

- Serhan CN, Hong S, Gronert K, Colgan SP, Devchand PR, Mirick G & Mousignac R-L (2002) Resolvins: a family of bioactive products of omega-3 fatty acid transformation circuits initiated by aspirin treatment that counter pro-inflammation signals, *J Exp Med* 196: 1025-37.

- Shabahang M, Buras RR, Davoodi F, Schumaker LM, Nauta RJ, Uskokovic MR, Brenner RV & Evans SR. 1994 Growth inhibition of HT-29 human colon cancer cells by analogues of 1,25-dihydroxyvitamin D3, *Cancer Res* 54: 4057-64.

- Shimokawa T & Smith WL (1992) Prostaglandin endoperoxide synthase: The aspirin acetylation region, *J Biol. Chem* 267: 12387-92.

- Silverstein FE, Faich G & Goldstein JL (2000) Gastrointestinal toxicity with celecoxib vs. nonsteroidal anti-inflammatory drugs for osteoarthritis and rheumatoid arthritis. *JAMA*284: 1247-55.

- Silvestre J, Lesson PA & Castaner J (1998) Anti-inflammatory COX-2 inhibitors, *Drugs Future* 23: 598-01.

- Siu-Caldera ML, Sekimoto H, Peleg S, Nguyen C, Kissmeyer AM, Binderup L, Weiskopf A, Vouros P, Uskokovic MR & Reddy GS (1999) Enhanced biological activity of 1alpha,25-dihydroxy-20-epi-vitamin D3, the

C-20 epimer of 1alpha,25-dihydroxyvitamin D3, is in part due to its metabolism into stable intermediary metabolites with significant biological activity, *J Steroid Biochem Mol Biol* 71: 111-21.

- Smith WL, Garavito MR & DeWitt D (1996) Prostaglandin Endoperoxide H Synthases (Cyclooxygenases)-1 and –2, *J Biol Chem* 271: 33157-60.

- Sok DE, Pai JK, Atrache V, Kang YC & Sih CJ (1981) Enzymatic inactivation of SRS-Cys-Gly (leukotriene D), *Biochem Biophys Res Commun* 101: 222-29.

- Sparrow JR (1995) Inducible nitric oxide synthase in the central nervous system, *J Mol Neurosci* 5: 219-29.

- Splettstoesser WD & Schuff-Werner P (2002) Oxidative stress in phagocytes – the enemy within, *Microscopy research and Technique* 57: 441-55.

- Staeva-Vieira TP & Freedman LP (2002) 1,25-Dihydroxyvitamin D_3 inhibits IFN-γ and IL-4 levels during *in vitro* polarization of primary murine $CD4^+$ T cells, *J Immunol* 168: 1181-89.

- Steinbach G, Lynch PM, Phillips RK, Wallace MH, Hawk E, Gordon GB, Wakabayashi N, Saunders B, Shen Y, Fujimura T, Su LK & Levin B (2000) The effect of celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous polyposis, *N Engl J Med* 26: 1946-52.

- Subhashini J, Mahipal SVK & Reddanna P (2004) Anti-proliferative and apoptotic effects of celecoxib on human chronic myeloid leukemia *in vitro, Cancer Lett*. In press.

- Sulkova S, Fortova M, Uhrova J & Zima T (2004) An importance of vitamin D metabolites assessment in patients with impaired renal function, *Vnitr Lek* 50: 510-18. Czech.

- Suzuki T, Sano Y, Sotozono C & Kinoshita S (2000) Regulatory effects of 1alpha,25-dihydroxyvitamin D(3) on cytokine production by human corneal epithelial cells, *Curr Eye Res* 20: 127-30.

- Sylvia VL, Del Toro F Jr, Hardin RR, Dean DD, Boyan BD & Schwartz Z (2001) Characterization of PGE(2) receptors (EP) and their role as mediators of 1alpha,25-(OH)(2)D(3) effects on growth zone chondrocytes, *J Steroid Biochem Mol Biol* 78: 261-74.

- Sylvia VL, Del Toro F, Dean DD, Hardin RR, Schwartz Z & Boyan BD (2001) Effects of 1alpha,25-(OH)(2)D(3) on rat growth zone chondrocytes are mediated via cyclooxygenase-1 and phospholipase A(2), *J Cell Biochem* 81: 32-35.

- Tabernero A, Schneider F & Potenza MA (2003) Cyclooxygenase-2 and inducible nitric oxide synthase in omental arteries harvested from patients with severe liver diseases: immuno localization and influence on vascular tone, *Intensive Care Medicine* 29: 262-70.

- Takahashi M, Fukutake M, Yokota S, Ishida K, Wakabayashi K & Sugimura T (1996) Suppression of azoxymethane-induced aberrant crypt foci in rat colon by nimesulide, a selective inhibitor of cyclooxygenase2, *J Cancer Res Clin Oncol* 122: 219-22.

- Tegeder I, Pfeilschifter J & Geisslinger G (2001) Cyclooxygenase-independent actions of cyclooxygenase inhibitors, *FASEB J* 15: 2057-72.

- Thomas MG, Tebbutt S & Williamson RC (1992) Vitamin D and its metabolites inhibit cell proliferation in human rectal mucosa and a colon cancer cell line, *Gut* 33: 1660-03.

- Thun MJ, Henley SJ & Patrono C (2002) Nonsteroidal anti-inflammatory drugs as anticancer agents: mechanistic, pharmacologic, and clinical issues, *J Natl Cancer Inst* 94: 252-66.

- Thun MJ, Namboodiri MM & Heath CW Jr (1991) Aspirin use and reduced risk of fatal colon cancer, *N Engl J Med* 325: 1593-96.

- Tocchini-Valentini G, Rochel N, Wurtz JM & Moras D (2004) Crystal structures of the vitamin D nuclear receptor liganded with the vitamin D side chain analogues calcipotriol and seocalcitol, receptor agonists of clinical importance. Insights into a structural basis for the switching of calcipotriol to a receptor antagonist by further side chain modification, *J Med Chem* 47: 1956-61.

- Tocchini-Valentini G, Rochel N, Wurtz JM, Mitschler A & Moras D (2001) Crystal structures of the vitamin D receptor complexed to superagonist 20-epi ligands. *Proc Natl Acad Sci* USA 98: 5491-96.

- Topilski I, Flaishon L, Naveh Y, Harmelin A, Levo Y & Shachar (2004) The anti-inflammatory effects of 1,25-dihydroxyvitamin D3 on Th2 cells in vivo are due in part to the control of integrin-mediated T lymphocyte homing, *Eur J Immunol* 34: 1068-76.

- Towbin H, Staehelin T & Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications, *Proc Natl Acad Sci USA* 76: 4350-54.

- Trang H, Cole DE, Rubin LA, Pierratos A, Siu S & Vieth R (1998) Evidence that vitamin D3 increases serum 25-hydroxyvitamin D more efficiently than does vitamin D3, *Am J Clin Nutr* 68: 854-58.

- Tuynman JB, Peppelenbosch MP & Richel DJ (2004) COX-2 inhibition as a tool to treat and prevent colorectal cancer, *Crit Rev Oncol Hematol* 52: 81-01.

- Uitterlinden AG, Fang Y, Van Meurs JB, Pols HA & Van Leeuwen JP (2004) Genetics and biology of vitamin D receptor polymorphisms, *Gene* 338: 143-56.

- Vallance P & Chan N (2001) Endothelial function and nitric oxide: clinical relevance, *Heart* (British Cardiac Society) 85: 342-50.

- Van Den Bemd GJ & Chang GT (2002) Vitamin D and vitamin D analogs in cancer treatment, *Curr Drug Targets* 3: 85-94. Review.

- Van Rees Bp, Saukkhoen K, Ristimaki A, Polkowski W, Tytgat GN, Drillenburg P & Offerhaus GJ (2002) Cyclooxygenase-2 expression during carcinogenesis inn the human stomach, *J Pathol* 196: 171-79.

- Van Amerongen BM, Dijkstra CD, Lips P & Polman CH (2004) Multiple sclerosis and vitamin D: an update, *Eur J Clin Nutr* 58: 1095-09. Review.

- Vane JR & Botting RM (1998) in Selective COX-2 inhibitors (Vane JR & Botting JH. Ed.), *Kluwer Academic Publishers and William Harvey Press, UK*, *pp* 1-18.

- Vane JR, Bakhle YS & Botting RM (1998) Cyclooxygenases 1 and 2, *Annu Rev Pahrmacol Toxicol* 38: 97-20.

- Vane L (1998) Differential inhibition of Cyclooxygenase isoforms: An explanation of the action of NSAIDs, *J Clin Rheumatol* 4 (suppl): S3-S10.

- Verlinden L, Verstuyf A, Convents R, Marcelis S, van Camp M & Bouillon R (1998) Action of 1,25(OH)2D3 on the cell cycle genes, cyclin D1, p21 and p27 in MCF-7 cells, *Mol Cell Endocrinol* 142: 57-65.

- Verlinden L, Verstuyf A, Van Camp M, Marcelis S, Sabbe K, Zhao XY, De Clercq P, Vandewalle M & Bouillon R (2000) Two novel 14-Epi-analogues of 1,25-dihydroxyvitamin D3 inhibit the growth of human breast cancer cells *in vitro* and *in vivo, Cancer Res* 60: 2673-79.

- Verstuyf A, Segaert S, Verlinden L, Bouillon R & Mathieu C (2000) Recent developments in the use of vitamin D analogs, *Expert Opin Investig Drugs* 9: 443-55.

- Vieth R (1990) The mechanisms of vitamin D toxicity, *Bone Miner* 11: 267-72.

- Vieth R (1999) Vitamin D supplementation, 25-hydroxyvitamin D concentrations, and safety, *Am J Clin Nutr* 69: 842-56.

- Wali RK, Bissonnette M, Khare S, Hart J, Sitrin MD & Brasitus TA (1995) 1 alpha,25-Dihydroxy-16-ene-23-yne-26,27-hexafluorocholecalciferol, a non calcemic analogue of 1 alpha,25-dihydroxyvitamin D3, inhibits azoxymethane-induced colonic tumorigenesis, *Cancer Res* 55: 3050-54.

- Wang LY, Hung HY, Hsu CH, Shih SL & Lee YJ (1997) Congenital rickets--a patient report, *J Pediatr Endocrinol Metab* 10: 437-41.

- Wilson (1992) Free radical-induced biological damage and the critical roles of Vitamin A, Vitamin C, Vitamin D and Vitamin E and of copper, iron, selenium and zinc, *J Nutr Sci Vitaminol* 541-44.

- Wu SH, Breshahan BA & Lianos EA (1993) Hemodynamic role of arachidonate 12- and 15-lipoxygenases in nephrotoxic serum nephritis, *Kidney Int* 43: 1280-85.

- Wu Y, Craig TA, Lutz WH & Kumar R (1999) Identification of 1α,25-dihydroxyvitamin D3 response elements in the human transforming growth factor beta 2 gene, *Biochemistry* 38: 2654-60.

- Wu-Wong JR, Tian J & Goltzman D (2004) Vitamin D analogs as therapeutic agents: a clinical study update, *Curr Opin Investig Drugs* 320-26.

- Yamamoto K, Sun WY, Ohta M, Hamada K, DeLuca HF & Yamada S (1996) Confromationally restricted analogs of 1α25-Dihydroxyvitamin D3 and its 20-epimer: Compounds for study of the three-dimensional structure of vitamin D responsible for binding to the receptor, *J Med Chem* 39: 2727-37.

- Yamamoto S (1992) Mammalian lipoxygenases: molecular structures and functions, *Biochim Biophys Acta Lipids Lipid Metab* 1128: 117-31.

- Yang KY, Arcaroli JJ & Abraham E (2003) Early alterations in neutrophil activation are associated with outcome in acute lung injury, *Am J Respir Crit Care Med* 167: 1567-74.

- Yang W & Freedman LP (1999) 20-Epi analogs of 1,25-dihydroxyvitamin D3 are highly potent inducers of DRIP coactivator complex binding to the vitamin D3 receptor, *J Biol Chem* 274: 16838-45.

- Yoshimi N, Shimizu M, Matsunaga K, Yamada Y, Fujii K, Hara A & Mori H (1999) Chemopreventive effect of N-(2-cyclohexyloxy-4-nitrophenyl) methane sulfonamide (NS-398), a selective cyclooxygenase-2 inhibitor, in rat colon carcinogenesis induced by azoxymethane, *Jpn J Cancer Res* 90: 406-12.

- Yoshizawa T, Handa Y, Uematsu Y, Takeda S, Sekine K, Yoshihara Y, Kawakami T, Arioka K, Sato H, Uchiyama Y, Masushige S, Fukamizu A, Matsumoto T & Kato S (1997) Mice lacking the vitamin D receptor exhibit impaired bone formation, uterine hypoplasia and growth retardation after weaning, *Nat Genet* 16: 391-96.

- Yudoh K, Matsui H & Tsuji H (1997) Effects of 1,25-Dihydroxyvitamin D3 on tumor cell invasion to the extracellular matrix in human fibrosarcoma HT1080 cells and its correlation with laminin, *Tumour Biol* 18: 69-79.

- Zehnder D, Evans KN, Kilby MD, Bulmer JN, Innes BA, Stewart PM & Hewison M (2002a) The ontogeny of 25-hydroxyvitamin D3 1-alpha-hydroxylase expression in human placenta and deciduas, *Am J Pathol* 161: 105-14.

- Zehnder D, Bland R, Chana RS, Wheeler DC, Howie AJ, Williams MC, Stewart PM & Hewison M (2002) Synthesis of 1,25-dihydroxyvitamin D(3) by human endothelial cells is regulated by inflammatory cytokines: a novel autocrine determinant of vascular cell adhesion, *J Am Soc Nephrol* 13: 621-29.

- Zhan J, Liu JP, Zhu ZH, Yao HR & Chen CY (2004) Relationship between COX-2 expression and clinicopathological features of colorectal cancers, *Chin Med J (Engl)* 117: 1151-54.

- Zhao XY, Eccleshall TR, Krishanna AV, Gross C & Feldman D (1997) Analysis of vitamin D analog-induced heterodimeization of vitamin D receptor with retinoid X receptor using yeast two-hybrid system, *Mol Endocrinol* 11: 366-78.

- Zhao XY, Peehl DM, Navone NM & Feldman D (2000) 1alpha,25-dihydroxyvitamin D3 inhibits prostate cancer cell growth by androgen-dependent and androgen-independent mechanisms, *Endocrinology* 141: 2548-56.

- Zhou JY, Norman AW, Akashi M, Chen DL, Uskokovic MR, Aurrecoechea JM, Dauben WG, Okamura WH & Koeffler HP (1991) Development of a novel 1,25(OH)2-vitamin D3 analog with potent ability to induce HL-60 cell differentiation without modulating calcium metabolism, *Blood* 78: 75-82.

- Zhou JY, Norman AW, Chen DL, Sun GW, Uskokovic M & Koeffler HP (1990) 1α25$(OH)_2$-16-ene-23-yne-D3 prolongs survival time of leukemic mice, *Proc Natl Acad Sci USA* 87: 3929-32.

- Zhou JY, Norman AW, Lubbert M, Collins ED, Uskokovic MR, Koeffler HP (1989) Novel vitamin D analogs that modulate leukemic cell growth and differentiation with little effect

- Ziboh VA, Naguwa S, Vang K, Wineinger J, Morrissey BM, Watnik M, Gershwin ME (2004) Suppression of leukotriene B4 generation by ex-vivo neutrophils isolated from asthma patients on dietary supplementation with gammalinolenic acid-containing borage oil: possible implication in asthma, *Clin Dev Immunol* 11: 13-21.

- Zipser RD, Nast CD, Lee M, Kao HW & Duke R (1987) *In vivo* production of leukotriene B4 and leukotriene C4 in rabbit colitis: relationship to inflammation, Gastroenterology 92: 33-39.