Regulation of Growth of Human Epidermoid Cancer Cell Line (A431): Role of 12-Lipoxygenase and Cyclooxygenase-2

Thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

By

SMITA AGARWAL

Department of Animal Sciences
School of Life Sciences
University of Hyderabad
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Enrolment No. 04LAPH02
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DECLARATION

I hereby declare that the work embodied in this thesis entitled **"Regulation of Growth of Human Epidermoid Cancer Cell Line (A431): Role of 12-Lipoxygenase and 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)

Smita Agarwal
(Research Scholar)

University of Hyderabad
(A Central University established in 1974 by act of parliament)
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CERTIFICATE

This is to certify that **Ms. Smita Agarwal** 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 entitled **"Regulation of Growth of Human Epidermoid Cancer Cell Line (A431): Role of 12-Lipoxygenase and Cyclooxygenase-2"** for submission for the degree of **Doctor of Philosophy** of this University.

Prof. P. Reddanna
Research Supervisor _____

Head
Department of Animal Sciences _____

Dean
School of Life Sciences _____

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Smita….✍

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List of Abbreviations

ºC	:	Degree centigrade/ Degree Celsius
µM	:	Micro molar
AA	:	Arachidonic Acid
Ac-DEVD-AFC	:	N-acetyl-(Asp-Glu-Val-Asp)- trifluoromethyl coumarin
AP-1	:	Activator protein-1
ARCI	:	Autosomal recessive congenital Ichthyosis
BE	:	Baicalein
CHAPS	:	3-[(3-Cholamidopropyl) Dimethyl ammonio]-1-propanesulfonate
CMNS	:	Congenital Melanocytic Nevi Syndrome
COX	:	Cyclooxygenase
DAPI	:	4',6-diamidino-2-phenylindole
DMBA	:	7, 12-dimethylbenz (a) anthracene
DMEM	:	Dulbecco's Modified Eagle Medium
DMSO	:	Dimethyl sulfoxide
DNA	:	Deoxyribonucleic acid
DTT	:	Dithiothreitol
dUTP	:	2'-Deoxyuridine 5'-Triphosphate
ECL	:	Electrochemiluminescence
EDTA	:	Ethylene diamine tetra acetic acid
EDTA	:	Ethylenediaminetetraacetic acid
EET	:	Epoxyeicosatrieoic acids
EGF	:	Epidermal growth factor
ERK	:	Extracellular signal regulated kinase
FACS	:	Fluorescence activated cell sorter
FBS	:	Fetal bovine serum
FDA	:	Food and Drug Administration
FITC	:	Fluorescein isothiocyanate
g	:	Gram
GI	:	Growth inhibitory
h	:	Hour(s)
HE	:	Haematoxylin and eosin
HETE	:	Hydroxyeicosatetraenoic acid
HODE	:	Hydroxyoctadecadienoic acid
HPETE	:	Hydroperoxyeicosatetraenoic acid
I.P.	:	Intra-peritonial
JNK	:	c-Jun N-terminal kinase
kDa	:	Kilodalton

l	:	Litre
LA	:	Linoleic acid
LOX	:	Lipoxygenase
LT	:	Leukotriene
MAPK	:	Mitogen activated protein kinase
mg	:	Milligram
min	:	Minute(s)
ml	:	Milliliter
mM	:	Millimolar
MTT	:	[3-(4, 5-dimethylthiazol-2-yl)-2,5- Diphenyl tetrazolium bromide]
NDGA	:	Nordihydroguaretic acid
NF-κB	:	Nuclear factor-kappa B
nm	:	Nanometers
NSAIDs	:	Non-steroidal anti-inflammatory drugs
PAGE	:	Polyacrylamide gel electrophoresis
PARP	:	Poly (ADP) ribose polymerase
PBS	:	Phosphate buffered saline
PCR	:	Polymerase chain reaction
PG	:	Prostaglandin
PI	:	Propidium iodide
PI3K	:	Phosphatidylinositol-3-kinase
PIPES	:	2-[4-(2-sulfoethyl) piperazin-1- yl]ethanesulfonic acid
PMSF	:	Phenylmethanesulphonylfluoride
ROS	:	Reactive oxygen species
RT-PCR	:	Reverse transcriptase-polymerase chain Reaction
SCC	:	Squamous cell carcinoma
SDS	:	Sodium dodecyl sulfate
TEMED	:	N,N,N',N'-tetramethylene diamine
TPA	:	12-O-tetradecanoylphorbol-13-acetate
Tris	:	Tris-(Hydroxymethyl) aminoethane
TUNEL	:	Terminal deoxynucleotidyl transferase mediated dUTP-biotin nick labeling
UV	:	Ultraviolet

1. Introduction

1.1 Skin Cancer

Skin cancer is a malignant growth on the skin that generally develops in the epidermis (the outermost layer of skin), and usually clearly visible. This makes most skin cancers detectable in the early stages. Unlike many other cancers, including those originating in the lung, pancreas, and stomach, only a small minority of those afflicted actually die of the disease (www.skincancer.org). Skin cancers are the fastest growing type of cancers in the United States. Skin cancer represents the most commonly diagnosed malignancy, surpassing lung, breast, colorectal and prostate cancer. More than 1 million Americans are diagnosed with skin cancer annually (www.cancer.gov). The annual rates of all forms of skin cancer are increasing each year, representing a growing public concern. It has also been estimated that nearly half of all Americans who live to age 65 will develop skin cancer at least once (www.skincancer.org).

1.2 Types of skin cancer

Skin cancer is of three common types, each of which is named after the type of skin cell from which it arises. The first being basal cell carcinoma which arises from the basal cell. Second is squamous cell carcinoma (also referred as epidermoid carcinoma) which arises from the squamous cells, which are thin, flat cells that look like fish scales under the microscope. These two are the most common forms of skin cancer. Together, these two are also referred to as non melanoma skin cancers. The third type is referred to as melanoma which is the most serious form of skin cancer because it tends to spread (metastasize) throughout the body quickly.

1.3 Basal cell carcinoma

It is the most common form of skin cancer and accounts for more than 90% of all skin cancers in the US. These cancers almost never spread (metastasize) to other parts of the body. They can, however, cause damage by growing and invading surrounding tissue. A basal cell carcinoma usually begins as a small, dome-shaped bump and is often covered by small, superficial blood vessels called telangiectases. The texture of such a spot is often shiny and translucent, sometimes referred to as "pearly." It is often hard to tell a basal cell carcinoma from a benign growth like a flesh-colored mole without performing a biopsy. Some basal cell carcinomas contain melanin pigment, making them look dark rather than shiny. Superficial basal cell carcinomas often appear on the chest or back and look more like patches of raw, dry skin. They grow slowly over the course of months or years to become sizable. Although spread to other parts of the body (metastasis) is very rare, a basal cell carcinoma can damage and disfigure the eye, ear, or nose if it grows nearby.

1.4 Squamous cell carcinoma (Epidermoid Carcinoma)

It begins in the squamous cells, which are thin, flat cells that look like fish scales under the microscope. The word squamous came from the Latin squama, meaning "the scale of a fish or serpent" because of the appearance of the cells. Squamous cells are found in the tissue that forms the surface of the skin, the lining of the hollow organs of the body, and the passages of the respiratory and digestive tracts. Thus, squamous cell carcinomas can actually arise in any of these tissues. Men are affected more often than women. The earliest form of squamous cell carcinoma is called actinic (or solar) keratosis. Actinic keratoses appear as rough, red bumps on the scalp, face, ears, and back of the hands. They often appear against a background of mottled, sun-damaged skin. They can be quite sore and tender, out of proportion to their appearance. In a patient with actinic keratoses,

the rate at which one such keratosis may invade deeper in the skin to become a fully-developed squamous cell carcinoma is estimated to be in the range of 10%-20% over 10 years, though it may take less time. An actinic keratosis that becomes thicker and tenderer raises the concern that it may have transformed into an invasive squamous cell carcinoma.

1.4.1 Melanoma

Melanoma is the most serious form of skin cancers. However, if it is recognized and treated early, it is nearly 100 percent curable. But if it is not, the cancer can advance and spread to other parts of the body, where it becomes hard to treat and can be fatal. While it is not the most common of the skin cancers, it causes the most deaths. Melanoma is a malignant tumor that originates in melanocytes, the cells which produce the pigment melanin that colors our skin, hair, and eyes. The majority of melanomas are black or brown. However, some melanomas are skin-colored, pink, red, purple, blue or white (www.medicine.net).

1.5 Signs and symptoms of skin cancer

There are a variety of different skin cancer symptoms. These include crabs or changes in the skin that do not heal, ulcers in the skin, discoloration, and changes in existing moles.

- Basal cell carcinoma usually looks like a raised, smooth, pearly bump on the sun-exposed skin of the head, neck or shoulders. Sometimes small blood vessels can be seen within the tumor. Crusting and bleeding in the center of the tumor frequently develops. It is often mistaken for a sore that does not heal.

- Squamous cell carcinoma (SCC) is commonly a red, scaling, thickened patch on sun-exposed skin. Ulceration and bleeding may occur. When SCC is not treated, it may develop into a large mass.

- Most melanomas are brown to black looking lesions. Signs that might indicate a malignant melanoma include change in size, shape, color or elevation of a mole. The appearance of a new mole during adulthood, or new pain, itching, ulceration or bleeding.

1.6 Risk factors leading to skin cancer

- Exposure to ultraviolet (UV) light is a common cause of skin damage and the major cause of skin cancer in humans (de Gruij *et al*, 1999, Wolf *et al*, 1996, Soter *et al*, 1990). Acute effects of UV irradiation include sunburn, inflammation, erythema, immunosupression, DNA damage, and apoptosis; however, the epidermal damage caused by acute UV exposure will generally disappear within 2 week (Matsumura *et al*, 2004). Chronic or repetitive exposure to UV light on the other hand, while causing similar histological and molecular effects, can lead to photoaging and skin cancer. In the spectrum of UV light, UVB and to a lesser extent UVA are implicated in the development of skin cancer (Akunda *et al*, 2007).
- Chronic non-healing wounds, especially burns are called Marjolin's ulcers based on their appearance, and can develop into squamous cell carcinoma.
- Genetic predisposition, including "Congenital Melanocytic Nevi Syndrome" (CMNS) is characterized by the presence of "nevi" or moles of varying size that either appear at or within 6 months of birth. Nevi larger than 20 mm (3/4") in size are at higher risk for becoming cancerous.

- Skin cancer is one of the potential dangers of ultraviolet germicidal irradiation.

1.7 Treatment of skin cancer

Most skin cancers can be treated by removal of the lesion, making sure that the edges (margins) are free of the tumor cells. These surgical excisions provide the best cure for both early and high-risk disease.

1.7.1 Curettage and desiccation

Dermatologists often prefer this method, which consists of scooping out the basal cell carcinoma by using a spoon like instrument called a curette. Desiccation is the additional application of an electric current to control bleeding and kill the remaining cancer cells. The skin heals without stitching. This technique is best suited for small cancers in non-crucial areas such as the trunk and extremities.

1.7.2 Radiation therapy

Doctors often use radiation treatments for skin cancer occurring in areas that are difficult to treat with surgery. Obtaining a good cosmetic result generally involves 25 to 30 treatment sessions.

1.7.3 Cryosurgery

Some doctors trained in this technique achieve good results by freezing basal cell carcinomas. Typically, liquid nitrogen is applied to the growth to freeze and kill the abnormal cells.

1.7.4 Mohs micrographic surgery

Named for its pioneer, Dr. Frederic Mohs, this technique of removing skin cancer is better termed, "microscopically controlled excision." The surgeon

meticulously removes a small piece of the tumor and examines it under the microscope during surgery. This sequence of cutting and microscopic examination is repeated in a painstaking fashion so that the basal cell carcinoma can be mapped and taken out without having to estimate or guess the width and depth of the lesion. This method removes as little of the healthy normal tissue as possible. Cure rate is very high, exceeding 98%. Mohs micrographic surgery is preferred for large basal cell carcinomas, those that recur after previous treatment, or lesions affecting parts of the body where experience shows that recurrence is common after treatment by other methods. Such body parts include the scalp, forehead, ears, and the corners of the nose. In cases where large amounts of tissue need to be removed, the Mohs surgeon sometimes works with a plastic (reconstructive) surgeon to achieve the best possible post surgical appearance.

1.7.5 Medical therapy

It is using creams that attack cancer cells (5-Fluorouracil--5-FU, Efudex, Fluoroplex) or stimulate the immune system (Aldara). These are applied several times a week for several weeks. They produce brisk inflammation and irritation. The advantages of this method are that it avoids surgery, lets the patient perform treatment at home, and may give a better cosmetic result. Disadvantages include discomfort, which may be severe, and a lower cure rate, which makes medical treatment unsuitable for treating most skin cancers on the face.

In the case of disease that has spread (metastasized) further chemotherapy may be required (Doherty et al, 2005). Scientists have recently been conducting experiments on what they have termed "immune- priming". This therapy is still in its infancy but has been shown to effectively attack foreign threats like viruses and also latch onto and attack skin cancers. More recently researchers have focused their efforts on strengthening the body's own naturally produced "helper T cells" that identify and lock onto cancer cells and help guide the killer

cells to the cancer. Researchers infused patients with roughly 5 billion of the helper T cells without any harsh drugs or chemotherapy. This type of treatment if shown to be effective has no side effects and could change the way cancer patients are treated (www.cancer.gov). The possibility of metastasis makes it especially important to diagnose squamous cell carcinomas early and treat them adequately.

1.8 Arachidonic acid metabolism

Arachidonic acid (5, 8, 11, 14-eicosatetraenoic acid, 20:4) is a polyunsaturated fatty acid with four isolated cis double bonds. It is predominantly found esterified in membrane phospholipids in all mammalian cells. It is released from the phospholipids in the cell membrane by the action of phospholipases (Brash, 2001). Among the members of the PLA2 family, the cytosolic PLA2 type IV is known to be an important enzyme that selectively releases arachidonic acid from phospholipids (Neumann *et al*, 2007; Bonventre *et al*, 1997). The double bonds of polyenoic fatty acids provide the possibility to react with molecular dioxygen. Such oxygenation reactions may be catalyzed by transition metals (non-enzymatic) or by various types of oxygenases to form eicosanoids (Brash, 2001). Eicosanoids (from the Greek eikosi for “twenty”) are a family of oxygenated metabolites of the 20-carbon fatty acid, arachidonic acid (AA), that are released from the source cell and act at nano molar concentrations on target cells, typically via G-protein coupled receptors (GPCRs).

Although originally recognized for their capacities to elicit biological responses such as smooth muscle contraction, edema, and platelet aggregation, eicosanoids are now appreciated to influence processes ranging from inflammation and immune responses to chronic tissue remodeling and cancer (Brock *et al*, 2007). In fact, for oxygenation of polyenoic fatty acids three major types of oxygenases have been identified: i) cyclooxygenases (COXs) ii)

lipoxygenases (LOXs) and iii) cytochrome P-450 isozymes (Brash, 2001) (Figure 1).

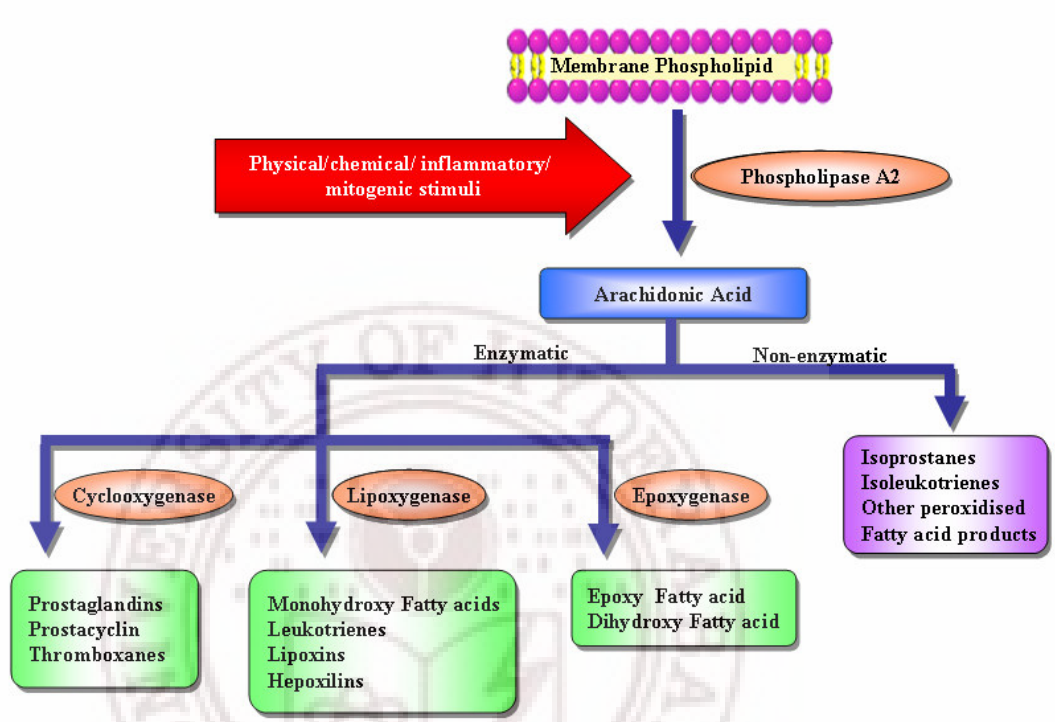


Figure 1. Arachidonic acid metabolism

1.8.1 Cyclooxygenase (COX) pathway

This pathway leads to the formation of cyclisation products of arachidonic acid that include prostaglandins, prostacyclin and thromboxanes. The COX is a bifunctional enzyme and exhibits cyclooxygenase and peroxidase activity. The cyclooxygenase activity introduces two molecules of oxygen into arachidonic acid to form the cyclic hydroperoxy endoperoxide PGG_2, which is subsequently reduced by the peroxidase activity of the enzyme to the hydroxyl endoperoxide, PGH_2 (Smith *et al*, 1991) (Figure 2). PGH_2 constitutes substrate for further enzymatic modifications leading to the formation of the prostaglandins (PGD_2, PGE_2, $PGF_{2\alpha}$,), prostacyclin (PGI_2) or thromboxane A_2 (TXA_2) (Smith *et al*, 1992).

COX-isoforms employ their heme moiety to generate a tyrosyl radical that abstracts hydrogen from C-11 of arachidonic acid. The two isoforms are the target of most prostaglandin synthesis inhibitors, particularly of the nonsteroidal anti-inflammatory drugs (NSAIDs), such as aspirin and indomethacin (Levy, 1997). Prostanoids exert their actions via membrane receptors on the surface of target cells. A family of membrane receptors mediating their actions has been characterized and cloned.

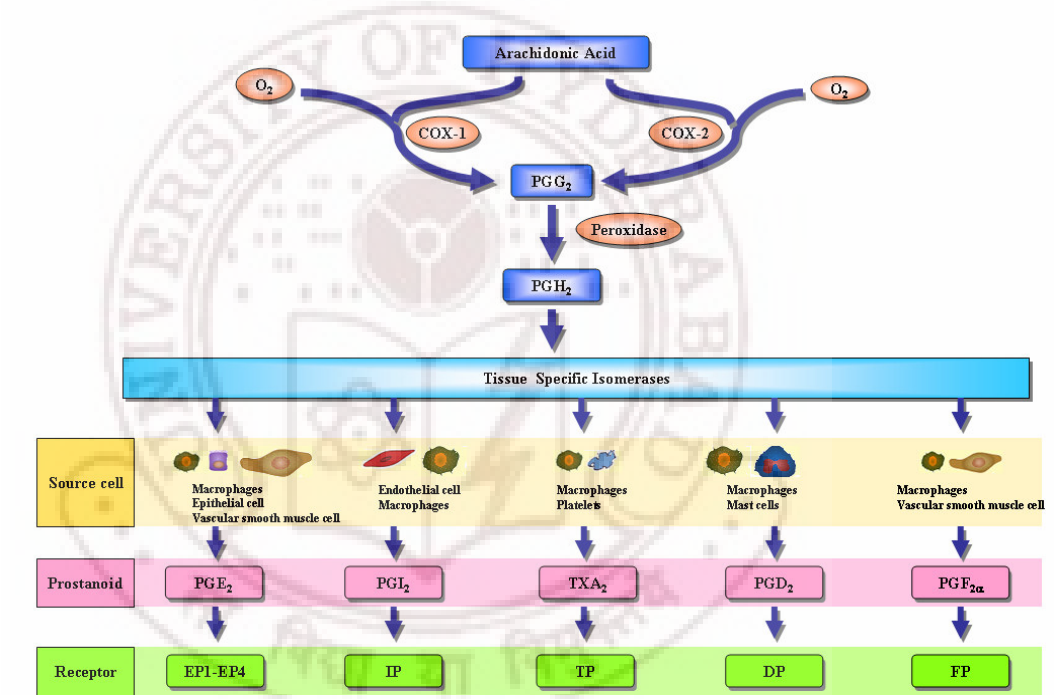


Figure 2: The Cyclooxygenase pathway

There are eight types and subtypes of receptors for prostanoid that are conserved in mammals from mouse to human (Narumiya *et al*, 1999): the PGD receptor (DP), four subtypes of the PGE receptors (EP1, EP2, EP3, and EP4), the PGF receptor (FP), the PGI receptor (IP), and the TXA receptor (TP). All are G-protein coupled rhodopsin-type receptors with seven transmembrane domains, and each is encoded by different genes (Matsuoka *et al*, 2007).

1.8.2 Lipoxygenases (LOX) pathway

This pathway leads to the formation of linear eicosanoids containing conjugated double bond systems. LOXs produce fatty acid hydroperoxides that are rapidly reduced to the corresponding hydroxyl compounds by glutathione peroxidases. LOXs contain one mole non-heme iron per mole enzyme and this transition metal is involved in the rate-limiting step of the LOX reaction, the initial hydrogen abstraction. Following hydrogen abstraction LOXs catalyze the introduction of atmospheric oxygen into the fatty acid chain and it depends on the positional specificity of the enzyme, at which carbon atom oxygen is inserted. (Yamamoto *et al*, 1992; Kühn *et al*, 1986) (Figure 3).

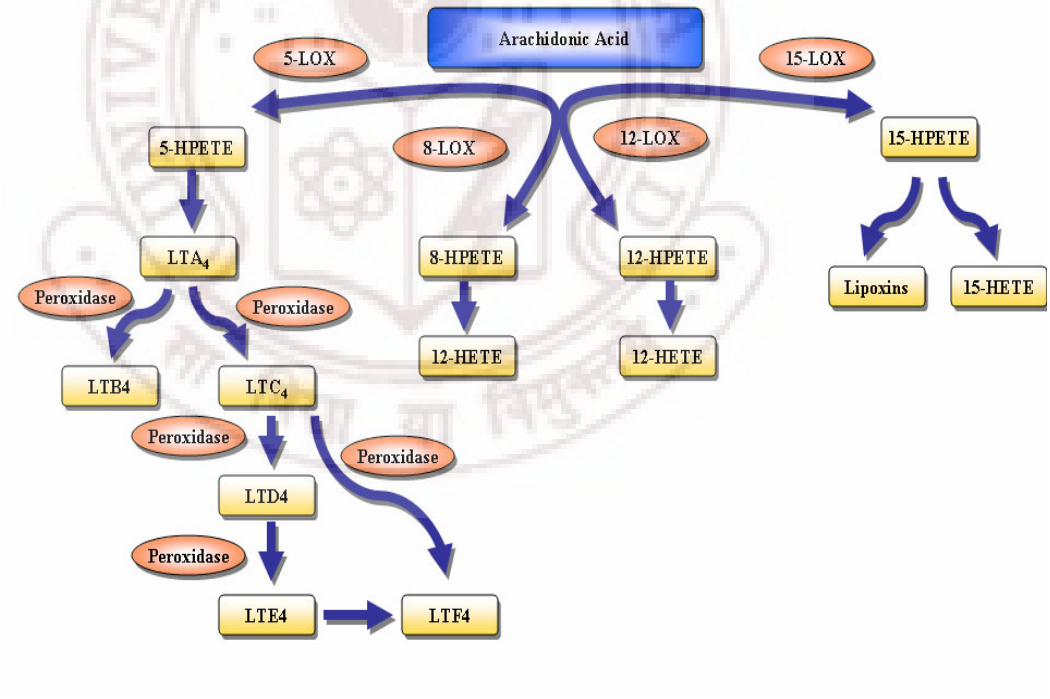


Figure 3: Lipoxygenase pathways

Hence, 5-LOXs generate 5-HPETE (5-hydroperoxy eicosa-6, 8, 11, 14-tetraenoic acid), 12-LOXs generate 12-HPETE (12-S-hydroperoxy eicosa-

tetraenoic acid) and 15-LOXs generate 15-HPETE (15- hydroperoxy eicosa-tetraenoic acid). 5-HPETEs and 15-HPETEs can be further metabolized to epoxy leukotrienes, 5, 6-leukotriene A_4 and 14, 15-LTA_4 respectively. 5, 6-epoxy leukotriene A4 (5, 6-LTA_4) constitutes the substrate for the cysteinyl leukotrienes (LTC_4, LTD_4, LTE_4), the mixture of which is known as slow-reacting substances of anaphylaxis (Lewis *et al*, 1980). Within the mammalian LOX family, a distinct subclass of epidermis-type LOX has been characterized that are preferentially expressed in skin and few other epithelial tissues (Krieg *et al*, 1998). They include the human 15-LOX-2 and its mouse orthologue 8-LOX, 12-R-LOX, and eLOX-3. The epidermal 12-R-LOX and eLOX-3 differ from all other mammalian LOXs in their unique structural and enzymatic features (Krieg *et al*, 1999; Kinzig *et al*, 1999; Boeglin *et al*, 1998). Both proteins contain an extra domain located at the surface of the catalytic subunit. 12-R-LOX represents the only mammalian LOX that forms products with R-chirality (Epp *et al*, 2007).

1.8.3 Cytochrome P-450 pathway

This pathway leads to the formation of two principle metabolites: i) Monohydroxylated eicosanoids, most of which do not contain conjugated double bond systems (20-HETE, 19-HETE, 18-HETE etc), ii) Epoxy fatty acids, which originate from epoxidation of double bonds to generate epoxyeicosatrieoic acids (EETs)(5, 6-EET, 8, 9-EET, 11, 12-EET, 14, 15-EET). In contrast to the LOX reaction cytochrome P-450-catalysed oxygenation involves insertion of atomic oxygen. In fact, molecular dioxygen is split into two oxygen atoms, one of which is introduced into the fatty acid substrate and the other one is reduced to water (Capdevila *et al*, 1992). Epoxy eicosanoids are rapidly hydrolyzed to dihydroxylated fatty acids. In tissues like kidney and cornea, cytochrome P- 450 mediated oxygenation of fatty acids results in the formation of biologically active products and plays an important role in the physiology of these tissues.

1.9 Arachidonic acid metabolism in skin

Skin, the largest human body organ, provides a major interface between the environment and the body and is constantly exposed to an array of chemical and physical environmental pollutants (Athar *et al*, 2002). In addition, a large number of dietary contaminants and drugs can manifest their toxicity in skin (Sander *et al*, 2004). These environmental toxicants or their metabolites are inherent oxidants and/or directly or indirectly drive the production of a variety of reactive oxidants also known as reactive oxygen species (ROS) (Cerutti *et al*, 1992). Skin exposure to ionizing and UV radiation and/or xenobiotics/ drugs generates ROS in excessive quantities that quickly overwhelm tissue antioxidants and other oxidant-degrading pathways. Uncontrolled release of ROS is involved in the pathogenesis of a number of human skin disorders including cutaneous neoplasia (Black *et al*, 2004; Briganti *et al*, 2003). One of the most important metabolites driving cutaneous inflammation is the eicosanoids (Bickers *et al*, 2006). Cancer results from disturbances of cellular signal transduction and data processing at the genetic and epigenetic level. Among these metabolic reactions becoming dys-regulated in the course of tumorigenesis, eicosanoid biosynthesis from arachidonic acid seems to play a pivotal role. A steadily increasing body of evidence indicates a causal relationship between cancer development and an abnormal overexpression of eicosanoid-forming enzymes, i.e. cyclooxygenases and lipoxygenases, in a wide variety of human and animal tumors. Besides their role as indicators of neoplastic development, eicosanoids also act as reporters of skin irritation (Marks *et al*, 2000). Many of these AA metabolites are clastogenic and act as tumor promoters in murine models of skin carcinogenesis and are induced following UVB irradiation (Bickers *et al*, 2006). Elevated levels of eicosanoids (prostaglandins and leukotrienes) have been shown to be associated

with a wide array of dermatological diseases, such as psoriasis, UV-induced erythema, and contact sensitivity (Wang *et al*, 2001).

1.10 COX pathway in skin health and disease

COX is the key enzyme generating prostaglandins from AA. In humans, prostaglandins are involved in diverse physiological functions. At least two isoforms of COX have been cloned and sequenced. COX-1 is a housekeeping isoform constitutively expressed in most tissues, whereas COX-2 is induced by a variety of proinflammatory agents and mitogens. It is known that COX-2 is upregulated following acute UVB exposure, and is increased in human actinic keratoses/ papillomas and in both murine and human SCCs (Bickers *et al*, 2006). In general, COX-1 regulates prostaglandin synthesis associated with cellular homeostasis whereas COX-2 is up regulated in inflammatory conditions and associated with synthesis of proinflammatory prostaglandins (Vane *et al*, 1994). Much attention has therefore been paid to develop specific inhibitors of COX-2. Recently, normal murine epidermis was found to express COX-1 but not COX-2. However, COX-2 could be induced either by acetone treatment or by topical application of the phorbol ester, TPA (Scholz *et al*, 1995). This is in contrast to normal human epidermis where COX-2 has been associated with keratinocyte differentiation (Leong *et al*, 1996). In normal human skin, COX-1 immunostaining is observed throughout the epidermis whereas COX-2 immunostaining was more in differentiated, suprabasilar keratinocytes (Leong *et al*, 1996). PGE_2 is the main AA cyclooxygenase product in human epidermal homogenates (Iversen *et al*, 2000).

1.11 Celecoxib

Celecoxib, 4-[5-(4-methylphenyl)-3-(trifluoromethyl) pyrazol-1-yl] benzene sulfonamide, C17H14F3N3O2S (MW 381.38) (Figure 4), is a highly specific inhibitor of COX-2 with 7.6 times more selectivity for COX-2 inhibition over COX-1.

This was the first COX-2 inhibitor approved for the use in U.S, for relief of signs and symptoms of rheumatoid arthritis and osteoporosis in adults. In addition to analgesic, antipyretic and anti-inflammatory activity, it has chemopreventive properties against colon cancer. Celecoxib is the only NSAID that has been approved by the FDA (in December 1999) for adjuvant treatment of patients with familial adenomatous polyposis. Since the introduction of celecoxib in 1998, several studies have investigated the molecular targets and clinical effects of the drug. Anticarcinogenic effects of celecoxib have been demonstrated in many types of cancers including non-small-cell lung cancer (Chen *et al*, 2007), gastric cancer (Zhu *et al*, 2007), lung cancer (Harris *et al*, 2007), prostate cancer (Anai *et al*, 2007), bladder cancer (Zhang *et al*, 2007), chronic myeloid leukemia (Arunasree *et al*, 2008; Zhang *et al*, 2006; Subhashini *et al*, 2005).

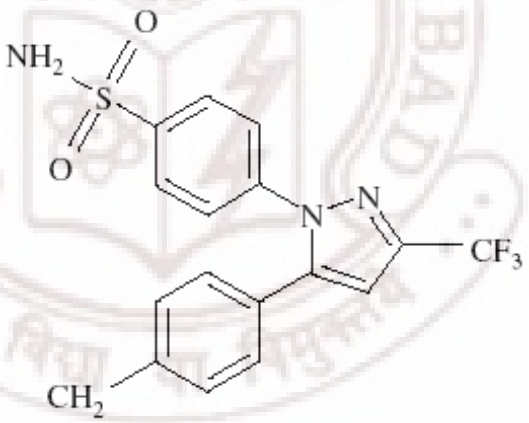


Figure 4: Chemical structure of Celecoxib

Celecoxib exerts its anti-carcinogenic effects in both COX-2-dependent and independent mechanisms. In general, the anticarcinogenic mechanisms of celecoxib involve blocking cell cycle progression, angiogenesis, metastasis and inducing apoptosis.

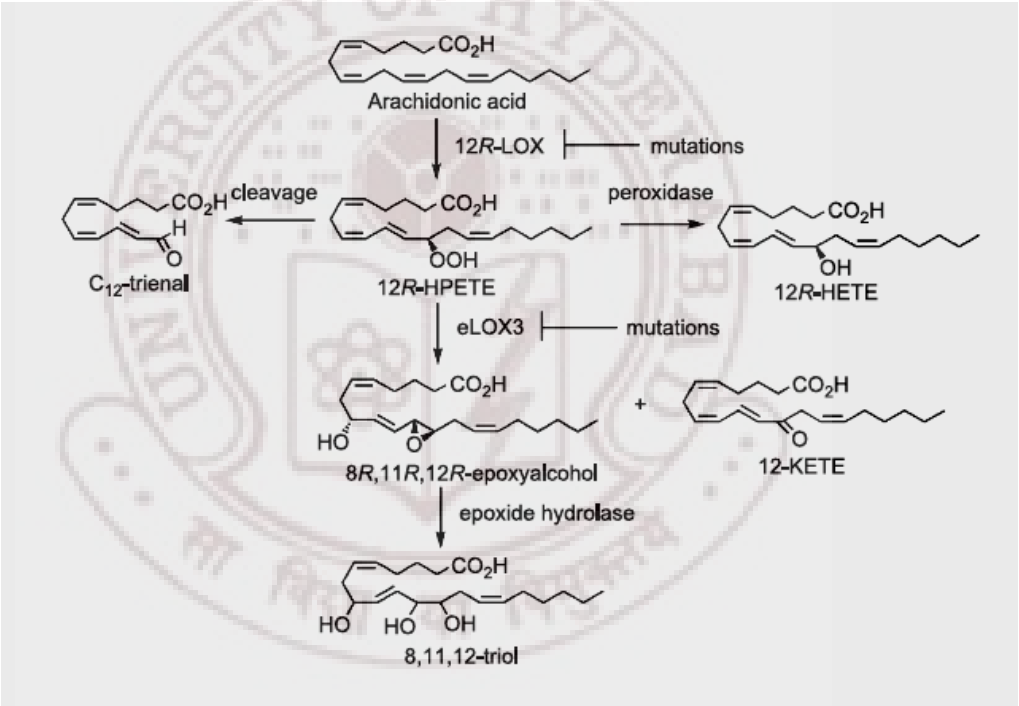
1.12 LOX pathway in skin health and disease

Within the mammalian LOX family, a distinct subclass of epidermis-type LOX has been characterized that are preferentially expressed in skin and few other epithelial tissues (Krieg *et al*, 1998). They include the human 15-LOX-2 and its mouse orthologue 8-LOX, 12-R-LOX, and eLOX-3. Their differentiation-dependent expression pattern in epithelial tissues suggests a common physiological role in the regulation of proliferation and differentiation of epithelial cells, especially keratinocytes. In humans, the metabolism of arachidonic acid in psoriasis and other proliferative dermatoses is characterized by the accumulation of the unusual metabolite, 12-R-hydroxyeicosatetraenoic acid (12-(R)-HETE) (Baer *et al*, 1991) (Figure 5A). Formation of 12-(R)-HETE results from the activity of 12-R-lipoxygenase (LOX) in the psoriatic lesions (Boeglin *et al*, 1998). In normal human skin, 12-R-LOX activity is almost undetectable (Schneider *et al*, 2004). 12-R-LOX represents the only mammalian LOX that forms products with R-chirality. Unlike all other LOXs, eLOX-3 does not exhibit dioxygenase activity, but functions as a hydroperoxide isomerase (Yu *et al*, 2003). Both enzymes act in sequence to convert arachidonic acid is a 12-R-hydroperoxyeicosatetraenoic acid (12-R-HPETE) to the corresponding hepoxilin like epoxyalcohol, 8R-hydroxy-11R and 12-R-epoxy eicosatrienoic acid (Figure 5C). This sequence has been hypothesized to be part of a novel LOX pathway in skin that plays an important role in terminal differentiation (Yu *et al*, 2003; Jobard *et al*, 2002). Recent genetic studies have identified mutations in the coding regions of 12-R-LOX and eLOX-3 genes in patients with autosomal recessive congenital ichthyosis (ARCI) (Figure 5B), linking for the first time mutations of a LOX gene to the development of a disease (Eckl *et al*, 2005; Jobard *et al*, 2002). ARCI is a clinically and genetically heterogeneous group of skin disorders that is associated with hyp-ERKeratosis and impaired skin barrier functions (Traupe, 1989). It was reported that point mutations found in the

LOX genes of the ARCI patients completely eliminated the catalytic activity of the LOX enzymes, indicating that mutational inactivation of either 12-RLOX or eLOX-3 is causally linked to the ARCI phenotype (Eckl *et al*, 2005; Yu *et al*, 2005). The implication of 12-R-LOX and eLOX-3 in ARCI has brought forth the concept that both enzymes function in the same metabolic pathway to convert arachidonic acid via 12-R-HPETE to hepoxilin and trioxilin-like metabolites that are critically involved in keratinocyte differentiation (Lefevre *et al*, 2006; Yu *et al*, 2005; Eckl *et al*, 2005; Jobard *et al*, 2002) (Figure 5C).

Moreover, very recent studies have shown that the creation of 12-R-LOX-deficient mice results in a severe impairment in barrier function, with the mice dying soon after birth from a defective barrier (Epp *et al*, 2007) (Figure 6). Among the diHETEs of the 5-LOX pathway are the biologically active leukotrienes (LTs) which have been ascribed an important role in inflammatory skin diseases like psoriasis and atopic dermatitis (Iverson *et al*, 2000; Sampson *et al*, 1992; Brain *et al*, 1985; Grabbe *et al*, 1984). Overexpression of the LOX isoforms- 8S-LOX and p12-S-LOX occurs in papillomas and SCCs, leading to accumulation of the corresponding metabolites 8S- and 12-(S)-HETE. Both LOX products are known to induce chromosomal damage in primary basal murine keratinocytes.

(A) (B)



(C)

Figure 5: (A) Patient with psoriasis (B) Patient with Non Bullous Congenital Ichthiosis (NCIE) (C) Metabolic pathway of 12-R-LOX in the maintainence of epidermal lipid barrier (Yu *et al*, 2005).

However, the amounts of these metabolites formed in tumors are sufficient for the formation of etheno adducts of DNA. Therefore, 8S- and 12-(S)-HETE may generate endogenous mutagens. Nordihydroguaretic acid (NDGA), a potent

common inhibitor of these enzymes, suppresses skin tumor induction in murine models (Muller *et al*, 2002).

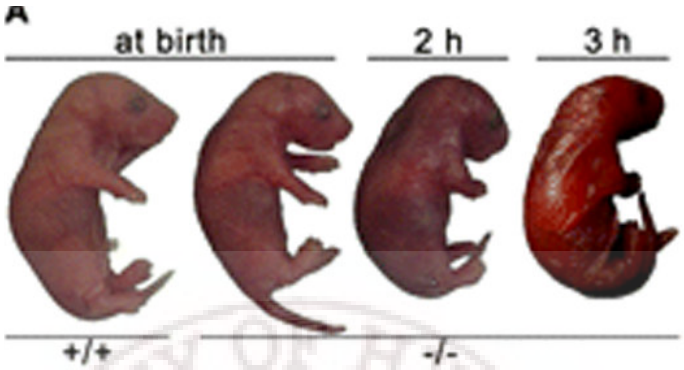


Figure 6: Macroscopic appearance of wild-type and 12-R-LOX–/– mice at birth, and 2 and 3 h after birth. Note the red, shiny skin and the dehydrated appearance of 12-R-LOX–/– mice (Epp *et al*, 2007).

1.13 Baicalein

The IUPAC name of baicalein is 5, 6, 7-Trihydroxyflavone (Figure 7). Baicalein (BE) is one of the major flavonoids in Scutellaria baicalensis, which has long been extensively used in Chinese herbal medicine. Baicalein is a highly

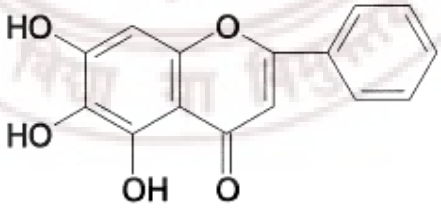


Figure 7: Chemical structure of Baicalein

specific inhibitor of platelet-12-LOX. Platelet 12-lipoxygenase is inhibited by baicalein with an IC_{50} value of 0.12 µM, with minimal inhibition of platelet cyclooxygenase-1 (IC_{50} = 0.83 mM). Several biological effects of BE such as antiviral, anti-inflammation, anti-hepatotoxicity, and anti-tumor properties have

been reported (Chen *et al*, 2006; Hwang *et al*, 2005; Huang *et al*, 2005; Ahn *et al*, 2001).

In MCF-7 cells, BE suppressed 17β-estradiol-induced transactivation, and induced apoptosis (Po *et al*, 2002). In lung squamous carcinoma CH27 cells, BE induced cell cycle arrest at the S-phase, followed by the induction of apoptosis (Chow *et al*, 2006; Lee *et al*, 2005). It has been reported that baicalein could make an S-phase arrest in cell cycle of lung squamous carcinoma CH27 cells (Lee *et al*, 2005) and induce apoptosis in many human cancer cell lines, e.g. hepatoma cells - Hep3B and HepG2 (Chen *et al*, 2005), pancreatic cancer cells- MiaPaCa-2 and AsPC-1 (Tong *et al* , 2002), breast cancer cells- MCF-7 (Tong *et al* , 2002) and prostate cancer cell lines (Chen *et al*, 2001). Although several biological activities of BE have been reported, intracellular molecules involved in modulation of apoptosis induced by BE are still undefined (Chow *et al*, 2006). A recent report demonstrated that baicalein induced a mitochondria-dependent caspase-3 and caspase-9 activation, and consequently led to apoptotic cell death in human myeloma cells (Ma *et al*, 2005).

2. Scope and Objectives

Cancer results from disturbances of cellular signal transduction and data processing at the genetic and epigenetic level. Among these metabolic reactions becoming dys-regulated in the course of tumorigenesis, eicosanoid biosynthesis from arachidonic acid seems to play a critical role (Marks *et al*, 2000). A large body of evidence indicates that the metabolism of polyunsaturated fatty acids is critically involved in epithelial cancer development. This holds true, in particular, for the COX and the LOX pathways of arachidonic and linoleic acid metabolism as supported by the accumulation of prostaglandins and related products in human and experimentally induced epithelial tumors. Suppression of these pathways has been found to inhibit tumor formation in animal models such as the initiation-promotion approach of mouse skin carcinogenesis (Muller *et al*, 2002; Marks *et al*, 2000; Marks *et al*, 1999). Considerable evidence suggests that LOXs are involved in epidermal tumor development. Compared with normal epidermis, large quantities of 12(S)-HETE (50–60-fold greater) were found in papillomas and carcinomas induced by 7, 12-dimethylbenz anthracene (DMBA) and 12-O-tetradecanoylphorbol-13-acetate (TPA) in a mouse skin tumor model (Krieg *et al*, 1995). In the same study, 12-LOX enzyme activity was elevated 6-fold in papillomas and 3-fold in carcinomas compared with normal tissue (Chang *et al*, 1993). Recent studies found that epidermal growth factor (EGF) and TPA up-regulate expression of 12-LOX mRNA in the human A431 cell line (Chang *et al*, 1993; Chang *et al*, 1992; Tsai *et al*, 1989). Also, 12(S)-HETE is the predominant metabolic product of metastatic B16 melanoma cells (Liu *et al*, 1994). 12(S)-HETE overproduction in papillomas may be a mechanism for progression to malignant carcinoma (Steele *et al*, 1999).Studies on skin cancer in humans are often limited to epidemiological data for self-evident reasons (Buckman *et al*, 1998). Squamous

cell carcinomas (SCCs) can be very aggressive and metastatic. Currently the primary form of treatment for these types of skin tumors is excision. However, excision of the initial lesion may not be curative because almost 50% of patients with one non melanoma skin cancer lesion develop another tumor within the next 5 yr at the site or adjacent to the site of excision (Wilgus *et al*, 2003). Standard chemo therapeutic agents used for the treatment of pre-cancerous skin lesions and non-melanoma skin cancers are not completely effective. Data in a variety of cancer types suggest greater efficacy in treating tumors with combination chemotherapies targeting genes which are overexpressed in the tumors (Wilgus *et al*, 2004).

It was demonstrated that skin tumor promotion caused by ultraviolet B radiation can be decreased up to 89% by celecoxib, a selective inhibitor of COX-2) in hairless mice (Fischer *et al*, 1999). A similar study showed that Celecoxib can decrease new tumor formation by 44% in mice that already have tumors (Thompson *et al*, 2001). In a subsequent study, celecoxib in combination with difluoromethylornithine, caused regression of UV-induced skin tumors to a much greater extent than did each compound alone (Fischer *et al*, 2003; Chun *et al*, 2004). In animal models, the p12-LOX expression is also found in xenografts of melanoma (Fischer *et al*, 2002) and in skin tumors developed by an initiation/ promotion protocol (Akunda *et al* 2007; Fischer *et al*, 1999).

The foregoing studies on the skin cancers are mainly focused on 12-R-LOX, but no comprehensive study was undertaken on the metabolism of arachidonic acid by various LOX and COX pathways. Hence it would be interesting to identify the specific LOX and COX pathways and understand their role in skin carcinogenesis. With this, the present study was undertaken to study the metabolism of AA in human squamous (epidermoid) cancer cell line A431. Based on these studies the specific inhibitors of these pathways, either alone or in

combination, was undertaken to evaluate their effectiveness in regulating the growth and proliferation of A431 cells. Further, studies were undertaken on the detailed molecular mechanism involved in the regulation of growth of A431 cells by the COX/LOX inhibition.

Thus the specific objectives of the present study were:

- To study the expression pattern of LOX/COX genes in Human epidermoid carcinoma cell line (A431) and compare it with normal skin fibroblast cell line (NIH3T3),
- To study the effect of metabolites and inhibitors of specific LOX/COX enzymes on A431 cells,
- To study the detailed molecular mechanisms involved in the regulation of growth of A431 cells by LOX/COX inhibition,
- To evaluate the effects of COX/LOX inhibition in regulating the growth of A431 xenografts in Swiss mice.

3. Materials and Methods

3.1 Materials

Cell lines used in this study, A431 (Human epidermoid carcinoma) and NIH3T3 (Mouse Embryonic Fibroblast) were obtained from the National Center for Cell Science, India. Phosphate buffered saline (PBS), DMEM-F12 medium and fetal bovine serum (FBS) were purchased from GIBCO Ltd (BRL Life Technologies, Inc., USA). Takara in Situ Apoptosis Detection Kit was procured from Takara Bio Inc., Shiga, Japan. RT-PCR kit was procured from MBI Fermentas, Maryland, USA. Ac-DEVD-AFC was procured from BD Biosciences. MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide], proteinase K, RNase A, propidium iodide, phenylmethylsulfonyl fluoride (PMSF), leupeptin, aprotinin, pepstatin A, trypsin, tween-20, triton X-100, ponceau S, sodium orthovanadate, sodium bicarbonate, EDTA and calcium chloride were purchased from Sigma Chemical Company (St.Louis, USA). Low fat milk powder was purchased from E-Merck. Nitrocellulose membranes and the enhanced chemiluminescence (ECL) kit were purchased from Amersham Life Science (Amersham, Bucks, UK). X-ray film and development solutions were from Kodak. Mouse monoclonal antibodies against cytochrome c, ERK, p-ERK, Akt, p-Akt, β-tubulin and Bax were purchased from Santa Cruz, CA, USA. Polyclonal antibodies of Bcl-2 and PARP were purchased from R&D systems Inc, USA. Acrylamide, N, N'-Methylene-bis-acrlylamide, sodium dodecyl sulfate (SDS), ammonium persulfate, β-mercaptoethanol and bromophenol blue were purchased from Bio-Rad Laboratories (Richmond, USA).

The LOX/COX metabolites (12-(R)-HETE, 12-(S)-HETE, PGE_2) and baicalein used in this study were purchased from Cayman Chemicals, USA. DAPI,

U0126 (ERK specific inhibitor) and wortmanin (PI3 Kinase inhibitor) was procured from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Celecoxib was a kind gift from Dr. Reddy's Laboratories. All the other chemicals and reagents were purchased from local companies and are of molecular biology grade.

3.2 Cell culture and treatment

The cell lines A431 and NIH3T3 were grown in DMEM-F-12 medium supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 IU/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine in a humidified atmosphere with 5% CO2 at 37 ºC. Cells were treated with 12-LOX or COX-2 metabolites (Cayman Chemicals, USA) or inhibitors (Cayman Chemicals, USA) for 48 h. A stock solution of 10^{-2} M celecoxib and baicalein was prepared in DMSO freshly for each experiment. The final concentration of the vehicle never exceeded 0.1%.

3.3 RNA isolation and RT-PCR analysis

Total RNA was isolated from A431 and NIH3T3 cells using TRizol (Life Technologies, Inc., Grand Island, NY) according to manufacturer's instructions. RNA was estimated using the ratio of absorbencies, 260/280, measured on Hitachi UV/Vis spectrophotometer. RT-PCR analysis was performed using M-MuLV reverse transcriptase (MBI fermentas, USA). All the steps were performed on MJ Research thermal cycler. The reaction mixture was prepared according to manufacturer's instructions. First strand synthesis was performed by incubating the mixture at 47 ℃ for 30 min. The reverse transcriptase inactivation and initial denaturation was performed at 94℃ for 2 min. Then the mix was allowed to undergo 28 PCR cycles with temperature profile of denaturation at 94 ℃ for 20 sec, annealing at 55-60ºC for 30 sec and extension at 72 ℃ for 1 min.The primer sequences and the annealing temperatures used are given in Table 1&2. The GAPDH primers served as control.

.Primers (5' → 3')	A (°C)	D (°C)	E (°C)	No. of Cycles	Size (bp)
5LOX-F CCCGGGGCATGGAGAGCA	60	94	72	28	416
5LOX-R GCGGTCGGGCAGCGTGTC					
FLAP-F GCTGCGTTTGCTGGACTGATGTA	60	94	72	28	224
FLAP-R TAGAGGGGAGATGGTGGTGGAGAT					
12(S) LOX-F GGCCCGGACCCAACTCATCTC	60	94	72	28	465
12(S)LOX-R GCATTAGGGACCCAGGCATACCAG					
15LOX-F GCTGCGGCTCTGGGAAATCATCT	60	94	72	28	459
15LOX-R GGGCCCGAAAAATACTCCTCCTCA					
COX-1-F CACTCACG-GGCGCTGGTTCTGG	60	94	72	28	574
COX-1-R CTGGCTCTGGGGCGGGATGC					
COX-2-F TGTGGGGCAGGAGGTCTTTGGTCT	60	94	72	28	690
COX-2-R GCATCTGGCCGAGGCTTTTCTAC					
12-(R) LOX-F CAACTTCCCAGCGTCCATGCGTAATC CA	54	94	72	28	431
12-(R) LOX-R TGGTGTTTTGGTCTCTGAGGTTTTTGT GTT	54	94	72	28	272
GAPDH-F CTCATGACCACAGTCCATGCCATC					
GAPDH-R CTGCTTCACCACCTTCTTGATGTC					

A = Annealing Temperature, D = Denaturation Temperature, E = Extension Temperature

Table 1: Primer sequences and conditions used for the RT-PCR analysis of LOX/COX genes in A431 cells

Primers (5' → 3')	A (°C)	D (°C)	E (°C)	No. of Cycles	Size (bp)
5LOX-F CTAGAGCGGCAGCTCAGTTT **5LOX-R** AACCTCACATGGGCTACCAG	60	94	72	28	410
FLAP-F AGGTGGAGCATGAAAGCAAG **FLAP-R** CTCCCAGATAGCCGACAAAG	60	94	72	28	225
12(S) LOX-F GCTACCCATGGCTATCCAGA **12(S)LOX-R** AAGCATCCCGAGCATAGAGA	60	94	72	28	461
15LOX-F CAGGGATCGGAGTACACGTT **15LOX-R** GGGGTTAGCACCATTGAGAA	60	94	72	28	453
COX-1-F GTACTCACAGTGCGGTCCAA **COX-1-R** AAGCCAGATCGTGGAGAAGA	60	94	72	28	573
COX-2-F CCCCCACAGTCAAAGACACT **COX-2-R** CAATTCTGCAGCCATTTCCT	60	94	72	28	687
12-(R)- LOX-F AGAGTATGTGGCCGAGCACT **12-(R)- LOX-R** GCCTCGTGGCTGTAGAACTC	54	94	72	28	432
GAPDH-F CTCATGACCACAGTCCATGCCATC **GAPDH-R** CTGCTTCACCACCTTCTTGATGTC	54	94	72	28	272

A = Annealing Temperature, D = Denaturation Temperature, E = Extension Temperature

Table 2: Primer sequences and conditions used for the RT-PCR analysis of LOX/COX genes in NIH3T3 cells.

3.4 Cell viability and cytotoxicity

Growth inhibitory effects of baicalein and celecoxib were assessed using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay as described by Mosmann (Mosmann, 1983). For the MTT assay, $5x10^3$ exponentially growing cells were plated in 100 µl of the growth medium in the presence or absence of 1–200 µM of inhibitors in 96 well plates and cultured at 37 ºC in 5% CO2 for 48 h. The cells were then incubated with 20 µl of MTT (5 mg/ml) at 37 ºC for 4 h. After dissolving the crystals in a lysis buffer containing 20% SDS, 50% dimethyl formamide, the plates were read in a micro titer plate reader at 570/630 dual nm. Each concentration was tested in three independent experiments run in four replicates. Standard errors of means were calculated and data were presented as the % growth vs. control. The concentration of the compound that inhibited cell growth by 50 % (IC_{50}) was determined from these cell survival plots.

3.5 Morphological differentiation and analysis of nuclear morphology

A431 cells were incubated with baicalein (100 µM) or celecoxib (60 µM) or both baicalein (100 µM) and celecoxib (60 µM) for 48 h and examined for morphological differentiation by phase contrast microscopy and photographed with Nikon F-601 AF Camera. The treated cells were harvested, washed with ice cold PBS, fixed in a solution of methanol: acetic acid (3:1) for 30 min and then stained with DAPI (1 µg/ml) to study their nuclear morphology by fluorescence microscopy (Olympus BH2RFC). Apoptotic cells were defined based on morphological changes like membrane blebbing, formation of apoptotic bodies and nuclear changes such as chromatin condensation and fragmentation. Apoptotic cells were observed and photographed under high magnification (400X). Each experiment was repeated at least three times and photographs included in the results are representative of one of those experiments.

3.6 ^{3}[H] Thymidine Incorporation Assay

A431 cells ($1x10^4$) were grown in 96 well plates and treated with 12(S)-HETE (30 nM and 300 nM), 12-(R)-HETE (30 nM and 300 nM), PGE_2 (3 µM and 15 µM), baicalein (100 µM), celecoxib (60 µM) and combination of baicalein (100 µM) and celecoxib (60 µM) for 48 h. After 48 h, [^{3}H] thymidine (BARC, Mumbai, India) was added to each well (0.5 µCi /well) and the incubation was continued for another 24 h. The culture medium was then removed, washed twice with PBS, and the proteins were precipitated with 5% trichloroacetic acid. The supernatant was removed and after washing with ethanol, the cells were solubilized with 0.2 N NaOH, and transfered into scintillation vials. The radioactivity was counted using a liquid scintillation counter.

3.7 *In situ* Apoptosis Detection (TUNEL) Assay

A431 cells were treated with baicalein (100 µM), celecoxib (60 µM) and combination for 48 h. Detection of in situ apoptosis in these cells was performed using the TdT-mediated dUTP-biotin nick labeling (TUNEL) method. The cells were stained according to the manufacturer's protocol (Takara *in situ* Apoptosis Detection Kit; Takara Bio Inc., Shiga, Japan). The green fluorescence of individual cells was measured with a FACS Calibur flowcytometer (Becton Dickinson, San Jose, USA). A minimum of 10,000 events were counted per sample.

3.8 Quantification of apoptosis by flow cytometry

To quantitate apoptosis, flow cytometric analysis using propidium iodide was performed. Cells that were less intensely stained than G1 cells (sub- G0/G1 cells) in flow cytometric histograms were considered apoptotic cells. The method for DNA labeling was done as described previously (Madhava Reddy *et al*, 2003) with minor modifications. After treatments, cells were prepared as single cell suspension in 200 µl PBS, fixed with 2 ml of ice-cold 70 % ethanol, and maintained at 4 ^{o}C overnight. The cells were harvested by centrifugation at 500xg for 10 min,

resuspended in 500 µl PBS supplemented with 0.1 % Triton X-100 and RNase A (50 µg/ml), incubated at 37 ^{o}C for 30 min, and stained with 50 µg/ml propidium iodide (PI) in the dark at 4 ^{o}C for 30 min. The red fluorescence of individual cells was measured with a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA). A minimum of 10,000 events were counted per sample.

3.9 Flow cytometric analysis of mitochondrial membrane potential

A431 cells were treated with baicalein (100 µM) or celecoxib (60 µM) or both baicalein (100 µM) and celecoxib (60 µM) for 48 h. The cells were harvested and changes in the mitochondrial membrane potentials were measured by the uptake of cation Rhodamine 123 into mitochondria (Seuduto *et al*, 1999). Untreated control cells were used to determine the normal uptake of this cation and the percentage of treated cells with low membrane potentials were then calculated. Briefly, the cells were centrifuged at 800 x g for 10 min and resuspended in 1 ml of Rhodamine 123 (10 µg/ml) for 30 min at room temperature, and washed once in PBS. The cells were resuspended in PBS. The samples (10000 events) were analyzed for fluorescence (FL-1 detector, filter 430/30 nm band pass) using a FACscan (Becton Dickson, San Jose, CA).

3.10 Preparation of whole cell extracts and immunoblot analysis

The cell lysis was carried out based on a method described earlier (Sambrook *et al*, 1989). To prepare the whole cell extract, cells were washed with PBS and suspended in a lysis buffer (20 mM Tris, 1 mM EDTA, 150 mM NaCl, 1% NP-40, 0.5 % sodium deoxy cholate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM PMSF, 10 µg/ml leupeptin, 20 µg/ml aprotinin). After 30 min of shaking at 4 ^{o}C, the mixtures were centrifuged (10,000 x g) for 10 min, and the supernatants were collected as the whole-cell extracts. The protein content was determined according to the Bradford method (Bradford, 1976). An equal amount

of total cell lysate (80 µg) was resolved on 8-12 % SDS-PAGE gels along with protein molecular weight standards, and then transferred onto nitrocellulose membranes. Membranes were stained with 0.5 % Ponceau S in 1 % acetic acid to check the transfer. The membranes were blocked with 5 % w/v nonfat dry milk and then incubated with the primary antibodies in 10 ml of antibody-diluted buffer (1:5,000 dilution) (1X Tris-buffered saline and 0.05 % Tween-20 with 5 % milk) with gentle shaking at 4 ^{o}C for 8-12 h and then incubated with peroxidase conjugated secondary antibodies. Signals were detected by using ECL Western blotting detection kit according to manufacturer's recommendations (Amersham Biosciences, Indianapolis, IN). The blots were stripped and reprobed with β-tubulin antibodies to confirm equal loading.

3.11 Detection of cytochrome c release using Western blot analysis

After exposure to 100 µM baicalein or 60 µM celecoxib for various time periods, cells were collected and washed once with PBS and subsequently with buffer A (0.25 M sucrose, 30 mM Tris-HCl, pH 7.9, 1 mM EDTA). Cells were then resuspended in buffer A containing 1 mM PMSF, 1 mg/ml leupeptin, 1 mg/ml pepstatin, 1 mg/ml aprotinin and homogenized with a glass dounce homogeniser. After centrifugation for 10 min at 21,000x g, protein concentration of the supernatant was determined using the Bradford method. 50 µg of cytosolic protein extract was then used for Western blot analysis as described above. Cytochrome c was detected using the mouse monoclonal antibody directed against human cytochrome c.

3.12 Caspase-3 activity assay

After the stipulated treatments, 2x10^{6} cells were lysed in 100 µl of CHAPS lysis buffer by three to four freeze thaw cycles. The extracts were centrifuged at 12,000xg and resulting supernatants were used for the assay. The assay was performed according to the manufacturer's protocol (BD biosciences, USA). The

assay buffer contained 20 mM PIPES, 100 mM NaCl, 10 mM DTT, 1 mM EDTA, 0 .1 % (w/ v) CHAPS, 10 % sucrose, pH-7.2. 50 µg of the protein and 8 µM of the substrate (Ac-DEVD-AFC) were added to 1 ml of the assay buffer and incubated for 1 h at 37 °C. Measurements were done on spectrofluorimeter with an excitation wavelength of 400 nm and an emission wavelength of 480- 520 nm.

3.13 Electrophoretic Mobility Shift Assay (EMSA)

The EMSA analysis was carried out according to the method described elsewhere (Chaturvedi *et al*, 1997). After the treatment, $3x10^6$ cells were used for the preparation of nuclear extraction. The cells were washed with PBS (1X) and harvested by centrifugation at 2000 rpm for 5 min in 200 µl of ice cold lysis buffer (20 mM Tris-HCl (pH 7.5) 10 mM magnesium acetate,1% NP-40, 1mM PMSF, and incubated for 5 min on ice with 3-4 vortexings of 10 sec each. The nuclei were then harvested by centrifugation at 1600 rpm. The nuclear pellet was resuspended in 50 µl of nuclear extraction buffer (420 mM NaCl, 10 mM HEPES, 10 mM MgCl2, 1 mM EDTA, 0.1 mM DTT and 25% glycerol) and incubated on ice for 30 min with intermittent vortexing of 10 sec each. The sample was then centrifuged at 13,000 rpm for 30 min at 4 °C. The supernatant collected was used for the assay after protein estimation using Bradford assay. 8 µg of nuclear extracts were incubated with ^{32}P labeled double stranded oligonucleotides [AP-1 (5' CTGAATCAACTGCTTCAA 3') or NF-κB (5' AGT TGA GGG GAC TTT CCC AGG C 3')] for 30 min at 37 ^oC and the DNA-protein complex formed was separated from free oligonucleotides on 6.6 % native acrylamide gel. The specificity of binding was also examined by competition with unlabeled oligonucleotide.

3.14 A431 xenograft animal studies

Swiss mice (4-6 weeks old) were obtained from National Institute of Nutrition, Hyderabad, and were allowed to acclimatize for 3-5 days period. 10 $x10^6$ cells were suspended in 300 µL PBS and subcutaneously injected into the

mice and tumors were allowed to develop for 10 days. After the development of visible tumors the mice were divided into 4 groups (n=5). In the first group vehicle was injected, second group was injected with baicalein at the daily dose of 20 mg/kg/day I.P, third group was injected with celecoxib at the daily dose of 10 mg/kg/day I.P and fourth group was injected with both baicalein and celecoxib at the daily dose of 20 mg/kg/day I.P and 10 mg/kg/day I.P respectively. The treatment with inhibitors was done for 1 week and after that the animals were sacrificed and the tumor weights were documented.

3.15 Immunohistochemistry of tumor tissues (HE staining)

Tumor tissues isolated from control and experimental animals were rinsed in PBS and fixed in formalin overnight followed by thorough washing with distilled water. Tissues were then dehydrated sequentially in 70%, 80%, 90% alcohol and finally in absolute alcohol for 10 minutes 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 tissues were placed in molten paraffin for 2-3 h to allow infiltration of paraffin into the tissue and then allowed to harden. Thin sections (5µm) were taken on Leitz microtome and mounted on polylysine-coated slides. Sections were deparaffinised by incubating in xylene for 10 min, rehydrated by sequential incubations in 90, 80, and 70 % alcohol for 10 min each. The tissue sections were stained with haematoxylin and eosin and were observed under light microscope at 400X magnification and photographs were taken (Lloyd *et al*, 1985).

3.16 TUNEL staining of tumor tissues

Apoptotic cells in the tumor sections were visualized by the terminal deoxynucleotidyl transferase mediated d-UTP nick end labeling (TUNEL) technique according to the manufacturer's instruction (Takara). Sections were deparaffinised by incubating in xylene for 10 min, rehydrated by sequential

incubations in 90, 80, and 70 % alcohol for 10 min each. Tissue sections were treated with 10 µg/ml proteinase K for 10 min at room temperature. The slides were immersed in 2% H_2O_2 solution to block endogeneous peroxidase activity. TdT was used to catalyze the addition of biotin-conjugated d-UTP to the 3′-OH ends of DNA fragments. The incorporated biotin was detected by streptavidin conjugated to FITC. The sections were mounted and observed under fluorescent microscope.

3.17 Statistical analysis

Data were presented as the mean ± SE of three independent experiments. Statistical analysis of differences was carried out by one-way analysis of variance (ANOVA) using Student-Newman-Keul's method. A P-value of less than 0.05 was considered as significant.

4. Results

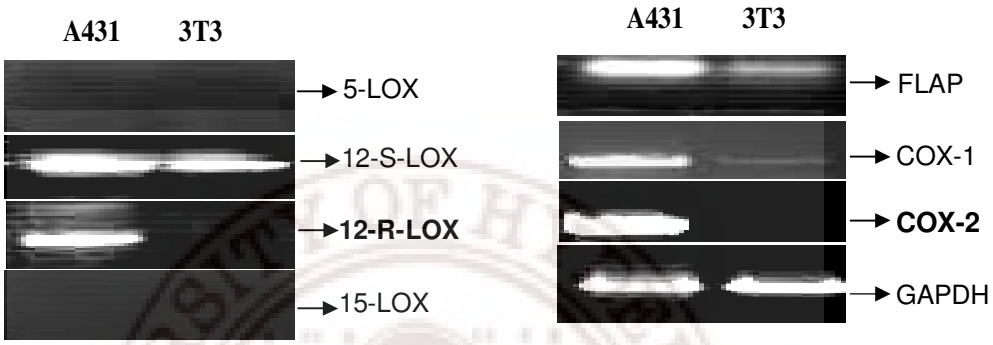
4.1 To study the expression pattern of LOX/COX genes in A431 cells and compare with normal cells

Eicosanoids are a diverse group of small–molecular weight lipids derived from arachidonic acid that act as local signaling molecules (Edelman *et al*, 2008). They include prostaglandins, prostacyclin, leukotrienes, thromboxanes and lipoxins. Earlier evidence strongly supports a fundamental role for dysregulation of these molecules in carcinogenesis (Steele *et al*, 1999). Most tumor cells produce AA metabolites and these compounds have been found to modulate a wide range of biological factors that induce growth and invasiveness of tumors (Hong *et al*, 1999). Recent investigations have shown that tumors of different histogenesis considerably differ in the metabolism of AA (Kudryavtsev *et al*, 2005). Thus, the expression and role of AA metabolizing enzymes would be different in different cancers and thus it is very important to know the expression profile of AA metabolizing enzymes first. The role of LOX/COX genes in epidermoid cancer is not fully understood. To know the role of these genes it is very important to know the expression profile of LOX/COX genes in Human epidermoid carcinoma. Therefore, our first question was to investigate the expression of various AA metabolizing enzymes in A431 cells.Thus the first objective of the present study is to analyze the expression profile of the LOX/COX genes in A431 cell line and compare it with normal skin fibroblast cell line, NIH3T3.

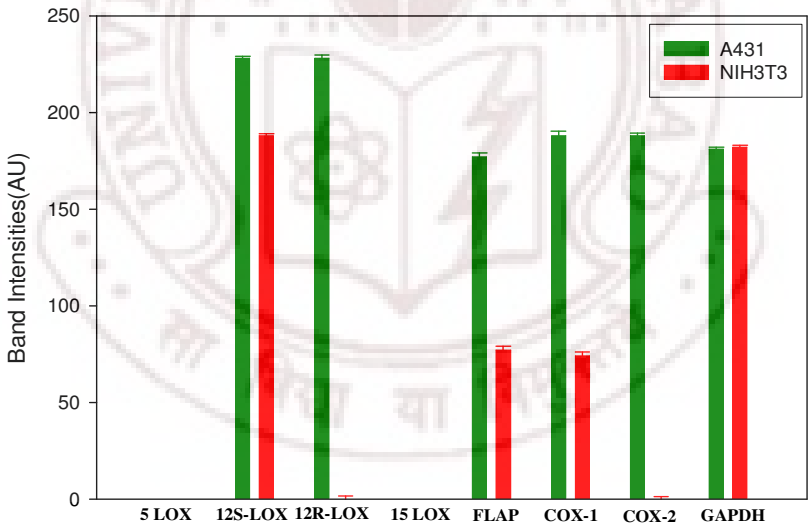
4.1.1 Overexpression of 12-R-LOX and COX-2 in A431 cells

In order to study the expression profile of lipoxygenases and cyclooxygenases in A431 cells, RT-PCR analysis was carried out. NIH3T3 cell line was used as normal control cells. As shown in Figure 8A, very high expression of 12-R-LOX and COX-2 was observed in A431 cells whereas the expression levels

were undetectable in NIH3T3 cells. There was slightly higher expression of 12-S-LOX, and very high expression of 5-LOX activating protein (FLAP) and COX-1 in skin cancer (A431) cells compared to normal cells. 5-LOX and 15-LOX, however, were not detected in both the cell lines.



(A)



(B)

Figure 8: (A) RT-PCR analysis of LOX/COX genes in Human epidermoid carcinoma cell line (A431) and its comparison with normal mouse skin fibroblast cell line (NIH3T3). (B) Bar diagram showing the intensities of the RT-PCR bands measured by Scion Image Analysis.

In the light of the expression of 12-R-LOX and COX-2 only in A431 cells but not in NIH3T3 cells, further studies were undertaken to analyze the growth of A431 cells in the presence of 12-LOX and COX-2 metabolites and their inhibitors.

4.2 To study the effects of metabolites and inhibitors of specific COX/LOX enzymes on A431 cells

After getting the overexpression of 12-R-LOX and COX-2 several queries came into our mind like whether the metabolites of COX-2 and 12-LOX increase the proliferation of A431 cells and whether the inhibitors of 12-LOX and COX-2 inhibit the growth of A431 cells.So the next objectives were:

- To study the effect of 12-LOX specific inhibitor (baicalein) and COX-2 specific inhibitor (celecoxib) on the growth of A431 cells.
- To study the effect of metabolites of 12-LOX and COX-2 on the growth of A431 cells.

Baicalein, a 12-LOX specific inhibitor and celecoxib, a COX-2 specific inhibitor were used in the present study. The metabolites used in the present study were PGE_2 (COX-2 metabolite), 12-(R)-HETE (12-R-LOX metabolite) and 12-(S)-HETE (12-S-LOX metabolite).

4.2.1 Baicalein and celecoxib inhibited the proliferation of A431 cells

A431 cells were treated with a specific 12-LOX inhibitor baicalein ranging from 0-200 µM for 48 h and specific COX-2 inhibitor celecoxib ranging from 0-100 µM for 48 h and cell viability was determined by the MTT assay. Under these experimental conditions, a dose-dependent decrease in the proliferation was observed in baicalein and celecoxib treated A431 cells with a GI50 value of 60 µM for celecoxib (Figure 9A) and 100 µM for baicalein (Figure 9B). Since 50% inhibition of cell proliferation was observed at 60 µM for celecoxib and 100 µM for baicalein, further experiments were carried out on A431 cells at these concentrations.

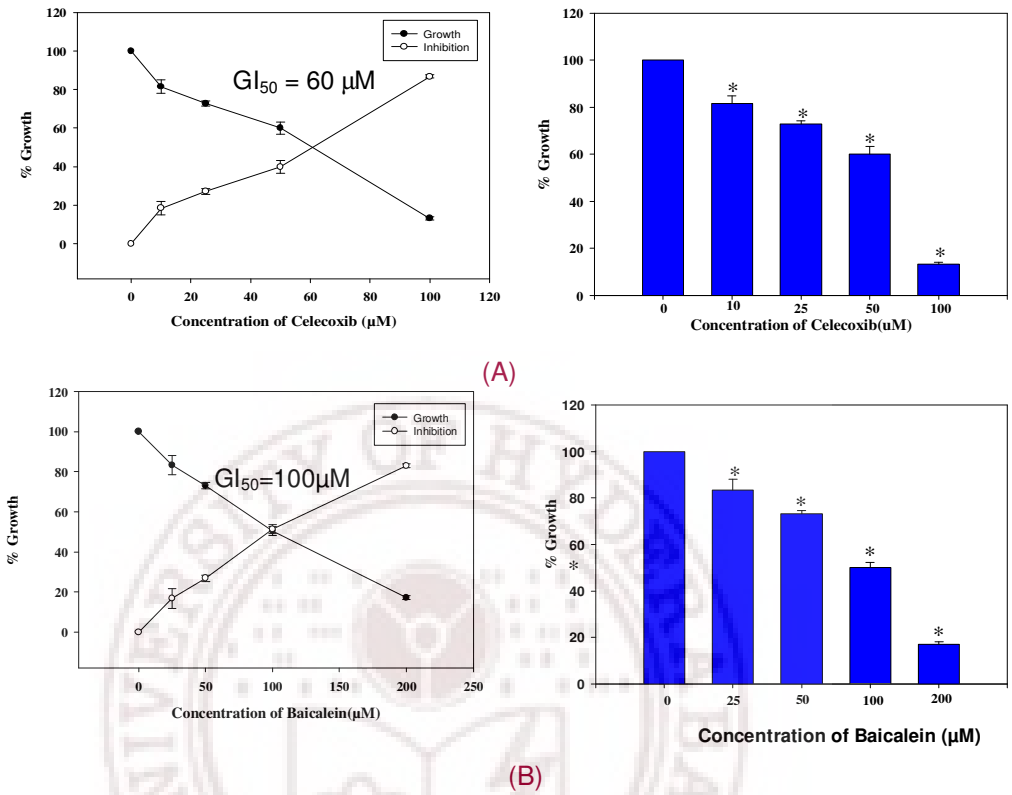


Figure 9: Effect of celecoxib (A) and baicalein (B) on the growth of A431 cell line. Cells ($5x10^3$) were treated with various concentrations of baicalein and celecoxib and the cell viability was measured by MTT assay at 48 h post treatment. The percent viable cells were calculated in comparison to untreated cells. The number of cells in the control was taken as 100%. The values represent the mean ± S.E.M from three independent experiments.* Indicates statistical significance with $P<0.05$.

4.2.2 12-LOX and COX-2 regulate the growth of A431 cells

In the light of observed inhibition in the growth of A431 cells by baicalein and celecoxib, further studies were undertaken to evaluate the effects of 12- LOX and COX-2 metabolites on the growth of A431 cells and to understand the molecular mechanisms. Thymidine incorporation assay was used to measure the effects of inhibitors and metabolites of 12-R-LOX, 12-S-LOX and COX-2 on proliferation of A431 cells. After treatment of cells with 100 µM baicalein, 60 µM celecoxib or both for 48 h, there was significant decrease in the thymidine incorporation (Figure 10). The incubation of A431 cells with 300 nM of 12-(S)-HETE, 300 nM of 12-(R)-HETE and 15 µM of PGE_2, on the other hand,

significantly increased the thymidine incorporation in comparison to that of the control group whereas 30nM 12-(R)-HETE and 30nM 12-(S)-HETE did not have significant effect on cell proliferation.

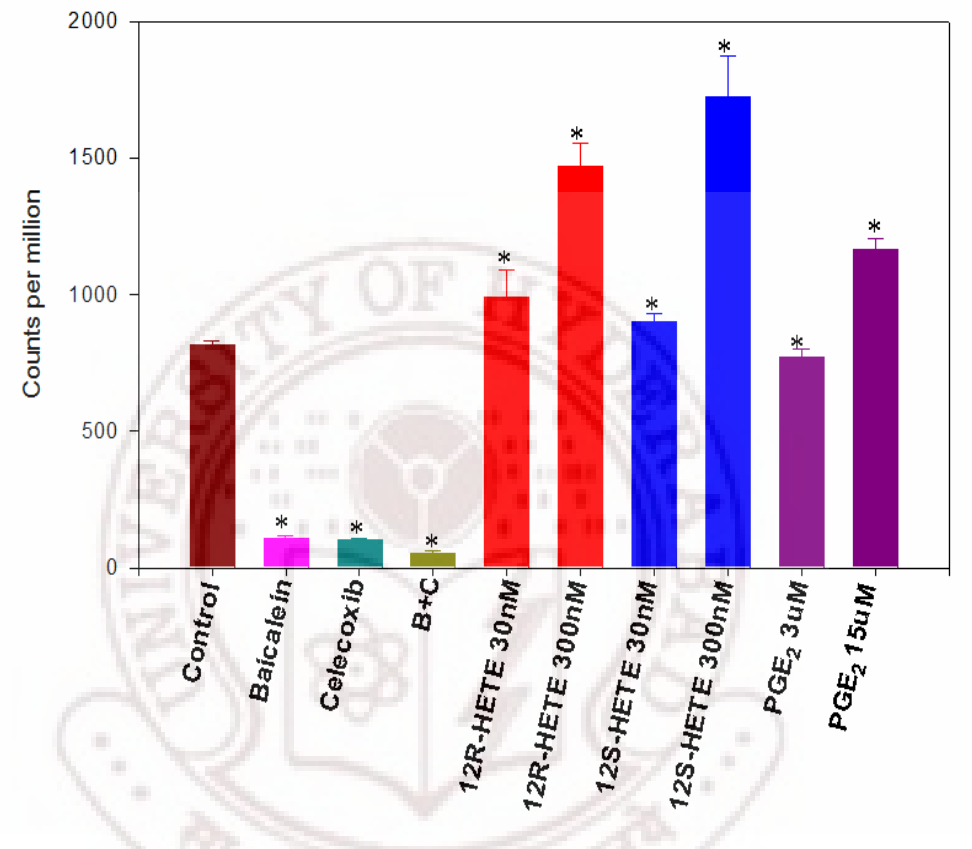


Figure 10: Effect on metabolites and inhibitors of 12-LOX and COX-2 on A431 cells. [3H]Thymidine Incorporation Assay was done to check the effect of inhibitors and metabolites of 12-R-LOX, 12-S-LOX and COX-2 on A431 cells. 1x104 cells were seeded in 96 well plate and then metabolites or drug were added and after 48 h thymidine was added and after 24 h the radioactivity was measured in the scintillation counter. Bar 1: Untreated cells, Bar 2: Baicalein (100 µM), Bar 3: Celecoxib (60 µM), Bar 4: Baicalein (100µM) + Celecoxib (60 µM), Bar 5 :12-(R)-HETE (30 nM), Bar 6: 12-(R)-HETE (300 nM), Bar 7: 12-(S)-HETE (30 nM), Bar 8: 12-(S)-HETE (300 nM), Bar 9: PGE_2 (3 µM), Bar 10: PGE_2 (15 µM).∗ Indicates statistical significance with $P<0.05$.

4.2.3 Phase contrast microscopic studies

Phase-contrast microscopic pictures of A431 cells, treated with baicalein (100µM), celecoxib (60µM) and both baicalein (100µM) and celecoxib (60µM) for 48 h were taken to observe the altered morphological features. Cells grown in the

absence of inhibitors were very healthy with no vacuolation and well defined boundaries (Figure 11). However, treatments with baicalein (100µM), celecoxib (60µM) and both baicalein (100µM) and celecoxib (60µM) showed extensive vacuolization and cytoplasmic shrinkage in the cells.

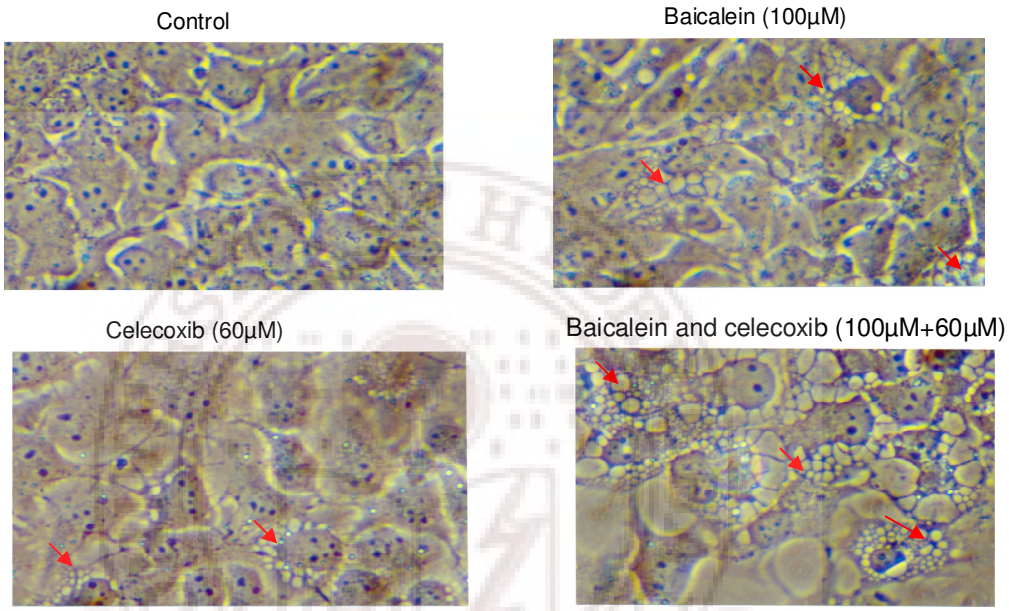


Figure 11: Phase-contrast microphotographs of A431 cells treated with baicalein (100µM), celecoxib (60µM) and both baicalein (100µM) and celecoxib (60µM) for 48h .Arrows indicate vacuoles formed in the cell (Magnification - 400X)

4.2.4 Confocal microscopic studies

A distinguishing feature of apoptosis is the condensation and fragmentation of nuclear chromatin, which can be monitored by fluorescence microscope when stained with specific DNA binding fluorescent dyes like DAPI. A431 cells were treated with baicalein (100µM), celecoxib (60µM) and both baicalein (100µM) and celecoxib (60µM) for 48 h and then assessed for morphological signs of apoptosis by staining with DAPI. Nuclear condensation and fragmentation, hallmarks of apoptosis, were clearly observed in cells treated with

baicalein (100µM), celecoxib (60µM) and both baicalein (100µM) and celecoxib (60µM) where as control cells have shown intact nuclei (Figure 12).

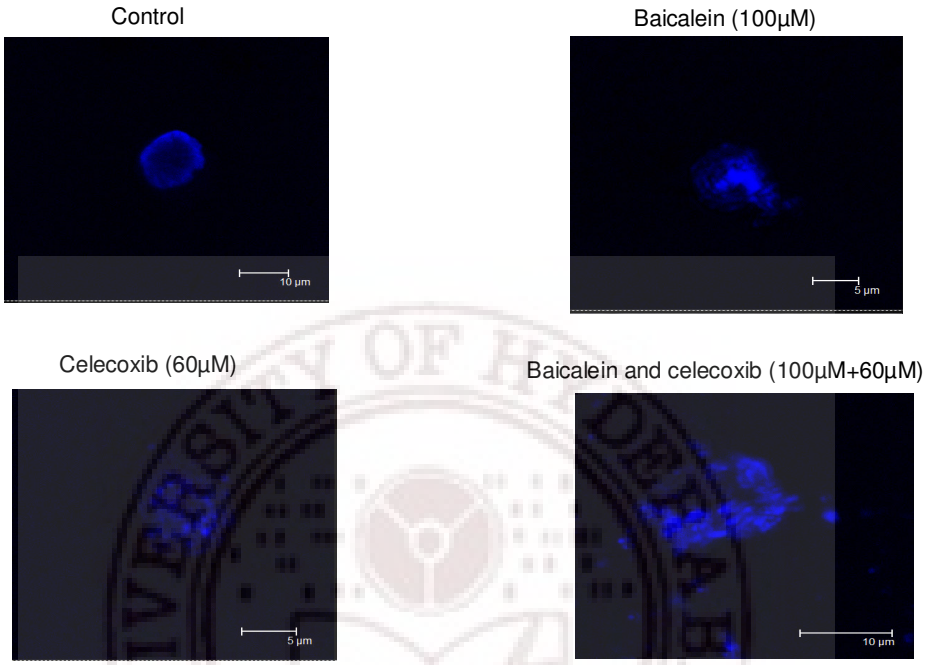


Figure 12: Confocal microscopic analysis of A431 cells treated with baicalein (100µM), celecoxib (60µM) and both baicalein (100µM) and celecoxib (60µM) for 48h. Nuclear morphology of A431 cells was observed under a confocal microscope after treatment and stained with DAPI.

4.2.5 Quantification of apoptosis by Flow cytometry

The induction of apoptosis in treated cells was further verified and quantified by flow cytometric analysis of DNA content. Loss of DNA is a typical feature of apoptotic cells. In the present study, A431 cells treated with baicalein (100µM), celecoxib (60µM) and both baicalein (100µM) and celecoxib (60µM) for 48 h were taken for FACS analysis. Figure 13, illustrates the DNA content histograms obtained after PI staining of permeabilized cells after treatments. Typical sub-diploid apoptotic peaks were observed in A431 cells treated with baicalein (100µM), celecoxib (60µM) and both baicalein (100µM) and celecoxib (60µM) for 48h. The FACS analysis of control cells, on the other hand, showed prominent G1, followed by S and G2/M phases.

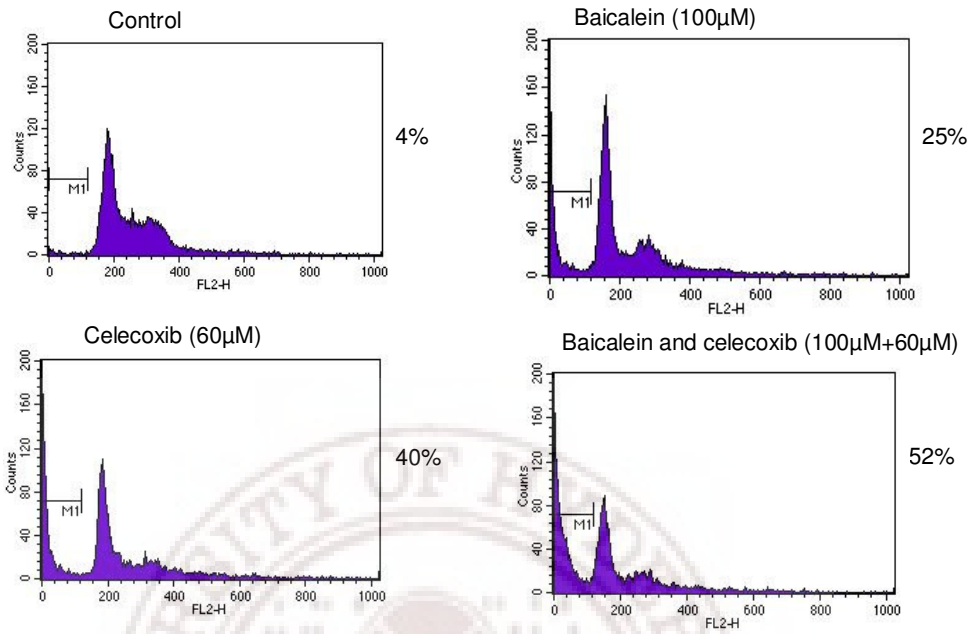


Figure 13: Flow cytometric analysis of DNA content in A431 cells treated with baicalein (100µM), celecoxib (60µM) and both baicalein (100µM) and celecoxib (60µM) for 48h showing induction of apoptosis. A431 cells (1.3 x 10^6 cells) treated with baicalein (100µM), celecoxib (60µM) and both baicalein (100µM) and celecoxib (60µM) for 48h were fixed in 1 ml of 70 % ethanol with 0.5 % Tween-20 at 4° C for 30 min and suspended in PBS. The cells were then stained with propidium iodide (PI) solution for 1 h and analyzed for DNA content by flow cytometry. Data represent the percentage of cells in the sub G0/G1 phase. Results from one of three similar experiments.

Only around 4 % of these cells showed hypodiploid DNA (sub G0/G1 peak) (Figure 13).This value of hypodiploid DNA was increased to 25% in baicalein treated cells, 40% in celecoxib treated cells and to 52% in both baicalein and celecoxib treated cells. These studies thus reveal increase of hypodiploid apoptotic cells in response to treatment in a concentration-dependent manner and the decrease of cells in other phases of cell cycle.

4.2.6 *In situ* apoptosis detection (TUNEL Assay)

Apoptosis or programmed cell death is frequently determined by the terminal transferase–mediated nick end labeling technique (TUNEL). In the present study, A431 cells treated with baicalein (100µM), celecoxib (60µM) and both baicalein (100µM) and celecoxib (60µM) for 48h were fixed in formaldehyde,

then permeabilized and DNA strand breaks were labeled with biotin dUTP using exogenous terminal transferase (end labeling). The incorporated nucleotides were visualized with streptoavidin FITC. Only 5% of control cells showed fluorescence, when compared to 23% in 100 µM baicalein treated cells and 26% in 60 µM celecoxib treated cells and 33% in both 100 µM baicalein and 60 µM celecoxib treated cells (Figure 14).

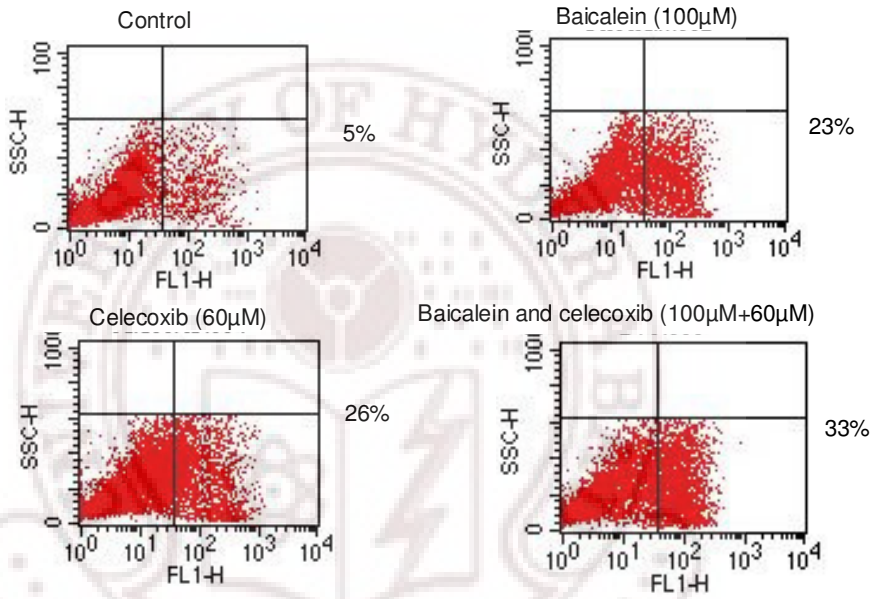


Figure 14: Detection of *in situ* apoptosis in A431 cells was performed using the TdT-mediated dUTP-biotin nick labeling (TUNEL) method. The green fluorescence of individual cells showed that the cells are undergoing apoptosis. Only around 5% of control cells showed fluorescence. The number of flourescent cells increased to 23% in baicalein treated cells and 26% in celecoxib treated cells and to 33% in both baicalein and celecoxib treated cells. Increase of flourescent cells in response to baicalein and celecoxib shows that the cells are undergoing apoptosis.

4.2.7 Baicalein and celecoxib decreased the mitochondrial membrane potential

A change in the mitochondrial membrane potential is a critical step in the induction of apoptosis. In the present study, mitochondrial membrane depolarization was studied by flow cytometric analysis using Rhodamine 123. These studies showed a decrease in mitochondrial membrane depolarization in A431 cells treated with baicalein (100µM), celecoxib (60µM) and both baicalein

(100µM) and celecoxib (60µM) for 48h. Membrane depolarization was shown by the shift in peak towards the lower median values of the FL1-H scale (Figure 15). These studies reveal the induction of apoptosis in A431 cells by baicalein and celecoxib.

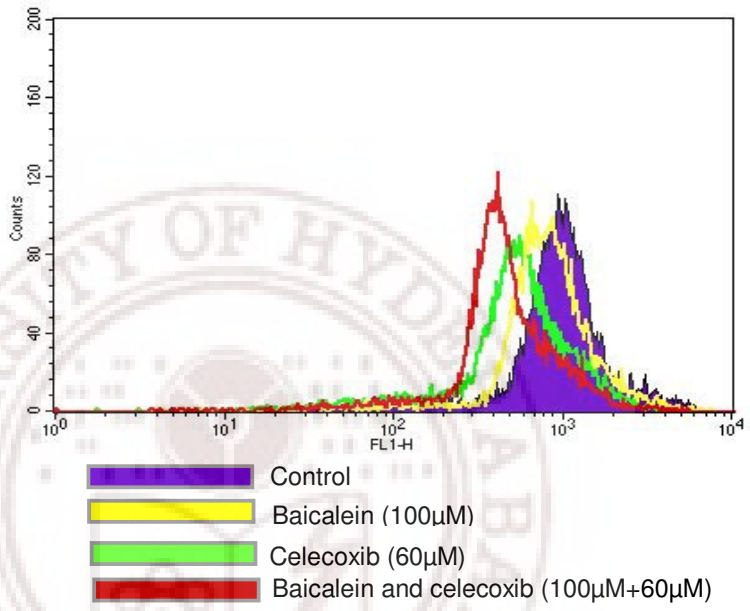


Figure 15: Mitochondrial membrane potential of A431 cells treated with baicalein (100µM), celecoxib (60µM) and both baicalein (100µM) and celecoxib (60µM) for 48h as estimated by Rhodamine 123 staining and FACS analysis.

4.2.8 Baicalein and celecoxib treatment evoke cytochrome c release

One of the major apoptotic pathways is activated by the release of apoptogenic protein, cytochrome c from mitochondria into the cytosol. The release of cytochrome c, one of the most important respiratory-chain proteins, from the mitochondria into the cytosol is the hallmark of cells undergoing apoptosis (Martinou *et al*, 2000; Liu *et al*, 1996). To specify the molecular basis of apoptosis, the release of cytochrome c into the cytosol was analysed in A431 cells treated with baicalein (100µM), celecoxib (60µM) and both baicalein (100µM) and celecoxib (60µM) for 48 h by Western blot analysis employing cytochrome c antibodies.

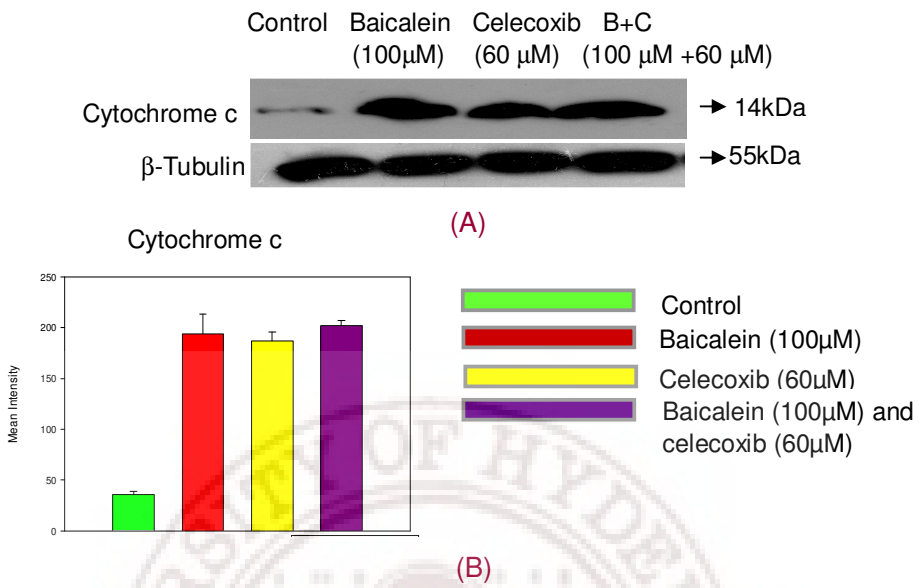


Figure 16: (A) Release of cytochrome c from the mitochondria measured using western blot analysis (B) Bar diagrams showing the intensities of cytochrome c blots, as measured by Scion Image Analysis.

The levels of cytochrome c in the cytosol were elevated within 48 h of treatment with 100 µM baicalein (lane 2) or 60 µM celecoxib (lane 3) or both (lane 4) (Figure 16A).

4.2.9 Decreased bcl-2/bax ratio in A431 cells after baicalein and celecoxib treatments.

Different proteins of the Bcl-2 family have been implicated in triggering or preventing apoptosis. Bax and Bcl-2 are the proteins associated with the mitochondrial membrane and their ratio is crucial for cell survival. Studies were undertaken to test whether Bcl-2 expression is affected after baicalein/celecoxib treatment in A431 cells. Changes in the expression of cellular anti-apoptotic proteins, Bcl-2, and of the pro-apoptotic protein, Bax, following baicalein and celecoxib treatment for 48 h were examined by Westen blotting. An appreciable decrease in the expression of Bcl-2 with no change in the Bax expression was observed in A431 cells treated with both celecoxib (60 µM) and baicalein (100 µM) as compared to cells treated with baicalein (100 µM) or celecoxib (60µM) alone

(Figure 17A).This would result in the decrease of Bcl-2/Bax ratio, which is a marker of apoptosis (Yin *et al*, 2000).

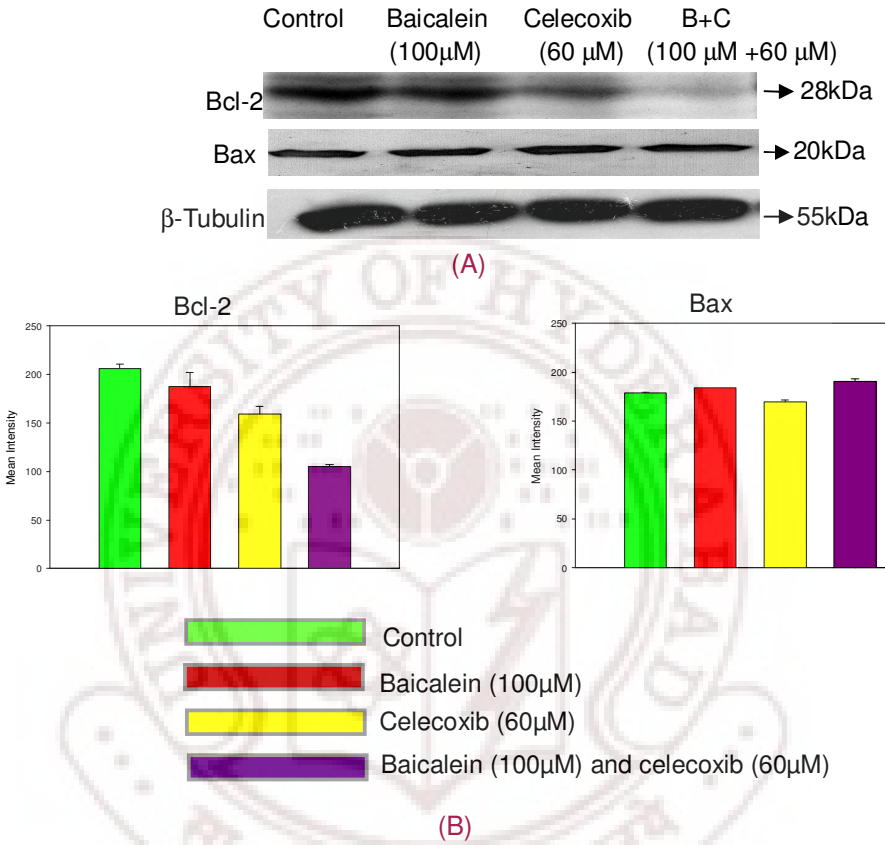


Figure 17: (A) Estimation of the Bcl-2 and Bax was done using Western blot analysis (B) Bar diagrams showing the intensities of the bands as measured by Scion Image Analysis.

4.2.10 Increased caspase-3 activity levels in A431 cells after treatment with baicalein or celecoxib

Cytochrome c leakage into the cytosol results in the activation of caspases (Li *et al*, 1997), a family of cysteine aspartate proteases, which cleave the cellular proteins and eventually steer a cell to undergo apoptosis. As treatment with baicalein or celecoxib resulted in the leakage of cytochrome c, we examined for the activation of caspase-3. The caspase-3 activity assay was performed as described in the materials and methods. Indeed caspase-3 was activated during

baicalein and celecoxib treatments as shown by the fold increase in caspase-3 activity (Figure 18).

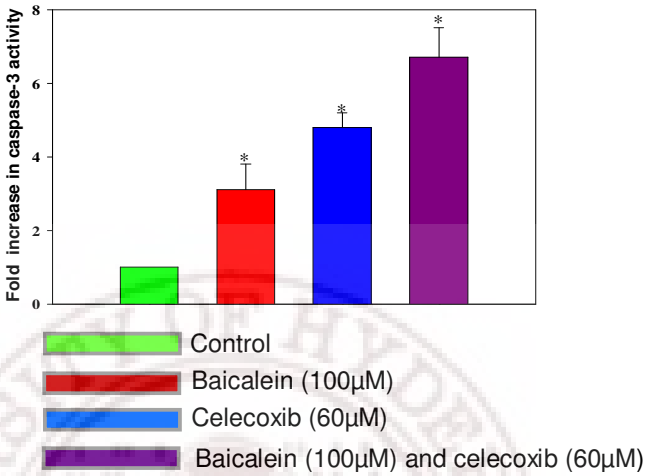


Figure 18: Fold Increase in the caspase 3 activity of A431 cells after treatment with baicalein (100µM), celecoxib (60µM) and both baicalein (100µM) and celecoxib (60µM) for 48 h. After treatments the cells were lysed and assayed for caspase-3 activity with a fluorescence caspase–3 substrate, Ac-DEVD-AFC. Spectro-fluorimetric analysis was done and the graph depicts the results from three independent experiments. ∗ Indicates statistical significance with $P<0.05$.

4.2.11 PARP cleavage in response to baicalein and celecoxib

Activated caspase-3 cleaves many vital cellular proteins including nuclear poly (ADP-ribose) polymerase (PARP), which has been implicated in many cellular processes such as apoptosis and DNA repair (Casciola-Rosen *et al*, 1996). PARP is a 116 kDa protein and is cleaved by caspases to generate 85 and 23 kDa fragments during apoptosis.

To determine whether PARP is cleaved in baicalein and celecoxib induced cell death, we treated A431 cells with baicalein (100µM), celecoxib (60µM) and both baicalein (100µM) and celecoxib (60µM) for 48h and PARP cleavage was monitored with PARP antibodies, which specifically recognize the 85 kDa fragment of the cleaved PARP and uncleaved 116 kDa PARP. Figure 19A illustrates the gradual increase in the proportion of the 85 kDa cleavage product. In the control

cells, however, no 85 kDa fragment of PARP was detected, except the uncleaved 116-kDa protein (lane 1).

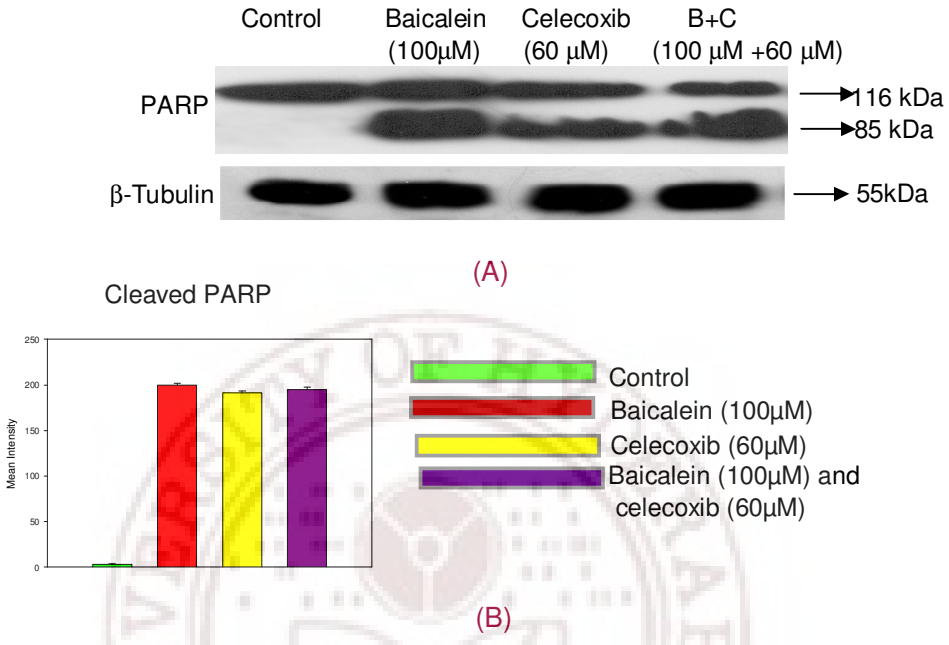


Figure 19: (A) Western Blot analysis showing the PARP cleavage leading to the generation of 85 kDa protein as a result of caspase-3 activity (B) Bar graph showing the intensity of the bands as measured by Scion Image Analysis.

4.2.12 RTPCR analysis of LOX/COX genes after treatment with baicalein and celecoxib

The transcript levels of LOX/COX genes were determined by RTPCR analysis after treatment with baicalein and celecoxib. There was a decrease in the transcript level of 12-R-LOX and COX-2 with celecoxib and both baicalein and celecoxib treatments. 12-S-LOX levels decreased with baicalein and combination treatments but did not change with celecoxib. The level of COX-1, however, was not altered in any treatment (Figure 20). Thus the levels of 12-R-LOX, 12-S-LOX and COX-2 were decreased in the combination treatments. GAPDH was used as a loading control.

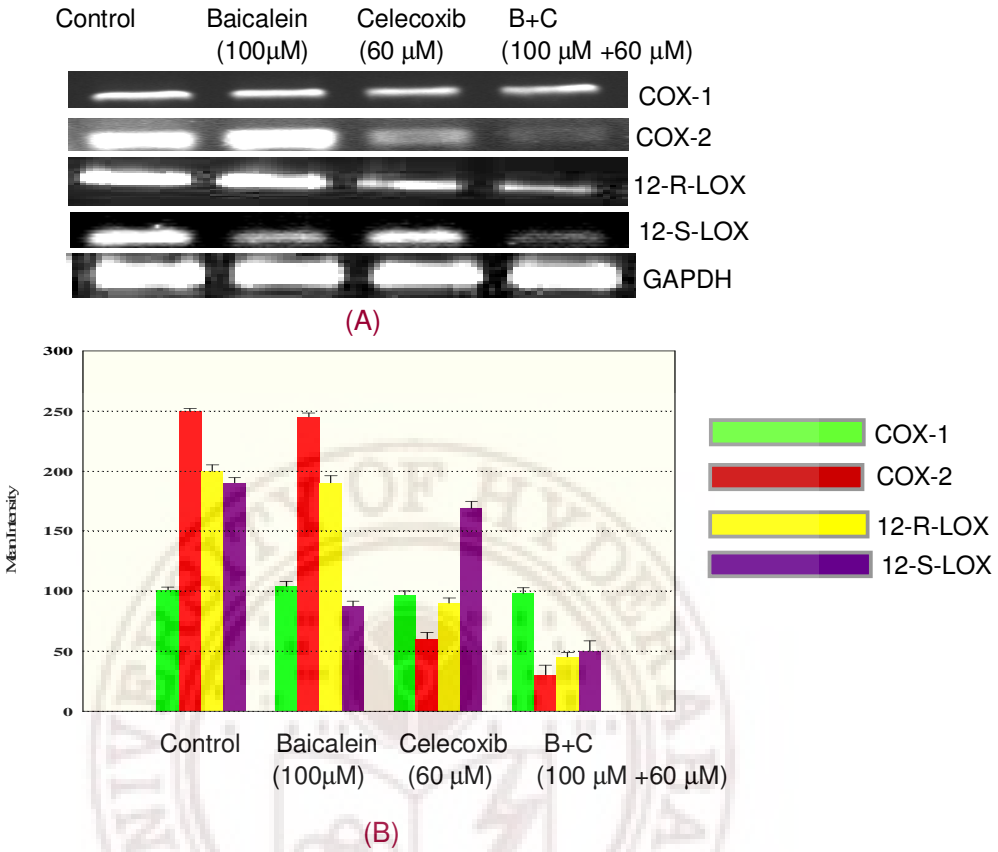


Figure 20: (A) RTPCR analysis of COX-1, COX-2, 12-S-LOX, 12-R-LOX and GAPDH after treatment with baicalein (100µM), celecoxib (60µM) and both baicalein (100µM) and celecoxib (60µM) for 48h. (B) Band Intensities measured by Scion Image Analysis and graph drawn using Sigma plot 9.0

4.3 To study the detailed molecular mechanisms involved in the regulation of growth of A431 cells by LOX/COX inhibition

The A431 cells are known to overexpress EGF receptor. The cell survival-signaling pathway present in cells over expressing EGF receptor is mainly PI3-Akt and ERK dependent. In view of the observed induction of apoptosis with baicalein and celecoxib, it would be interesting to check whether PI3-Akt and ERK survival pathways are getting altered. Also it would be interesting to check the effect of 12-LOX and COX-2 metabolites on the above two cell survival signaling pathways in A431 cells. In the light of the above further studies were undertaken with the following specific objectives:

- To study the effect of baicalein and celecoxib on the levels of phospho-ERK and phospho-Akt,
- To study the effect of inhibitors and metabolites of 12-LOX and COX-2 on the levels of phospho-Akt and phospho ERK along with specific inhibitors of ERK (U0126) and PI3 kinase (Wortmanin),
- To check the effects of metabolites and inhibitors of 12-LOX and COX-2 on the translocation of NF-κB which is downstream of the PI3-Akt pathway and
- To check the effects of metabolites and inhibitors of 12-LOX and COX-2 on the translocation of AP-1, downstream of ERK pathway.

4.3.1 Baicalein and celecoxib inhibited the activation of Akt

Akt, a cell survival protein is activated by various growth and survival factors. A431 cells were treated with baicalein (100µM), celecoxib (60µM) and both baicalein (100µM) and celecoxib (60µM) for 48h and the levels of phospho Akt and Akt were measured by Western blot analysis. The levels of phospho-Akt were decreased in the cells treated with baicalein (100µM) and celecoxib (60µM) and were minimum in the combination treatment with no change in Akt levels.

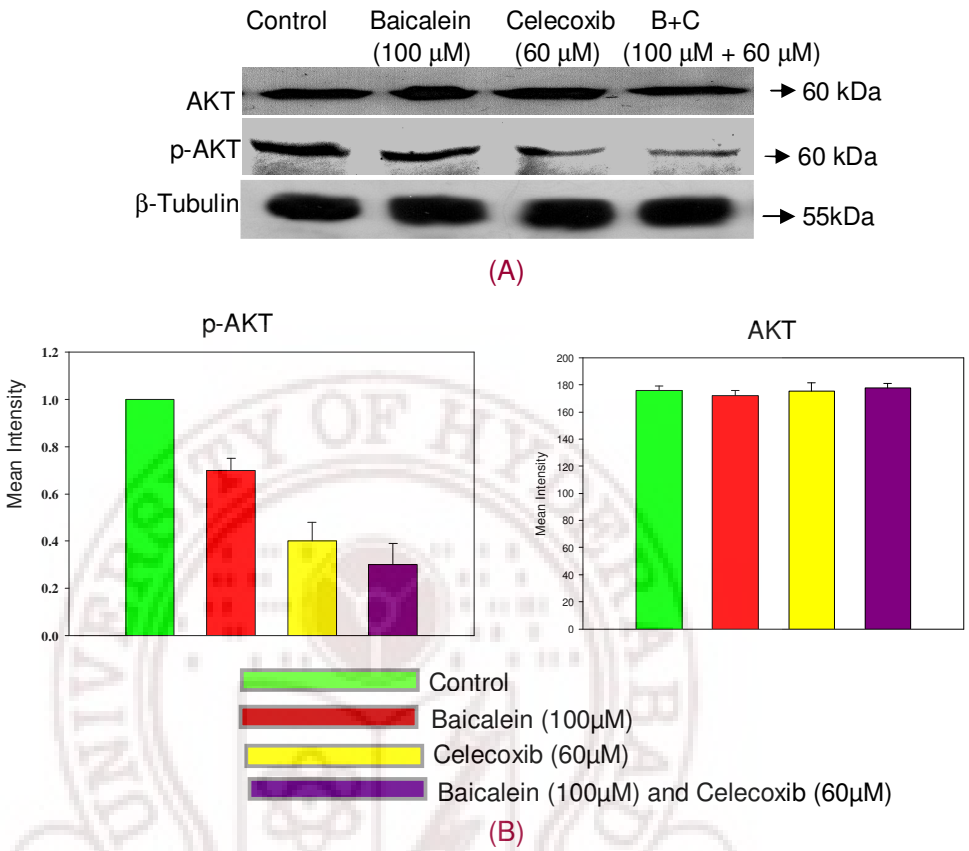


Figure 21: (A)Western Blot analysis of p-Akt and Akt levels of A431 cells treated with baicalein (100µM),celecoxib (60µM) and both baicalein (100µM) and celecoxib (60µM) for 48 h. (B) Bar graph showing the intensity of the bands as measured by Scion Image Analysis.

4.3.2 Baicalein and celecoxib inhibited the activation of ERK

Most of the signals for cell survival trigger growth factor receptors which activate extra cellular signal-regulated kinase (ERK) and promote cell growth. A431 cells were treated with baicalein (100µM), celecoxib (60µM) and both baicalein (100µM) and celecoxib (60µM) for 48h and the levels of phospho ERK and ERK were measured by western blot analysis. The level of phospho-ERK was decreased in the cells treated with baicalein (100µM) and celecoxib (60µM) and the decrease was maximum in the combination treatment with no change in ERK levels.

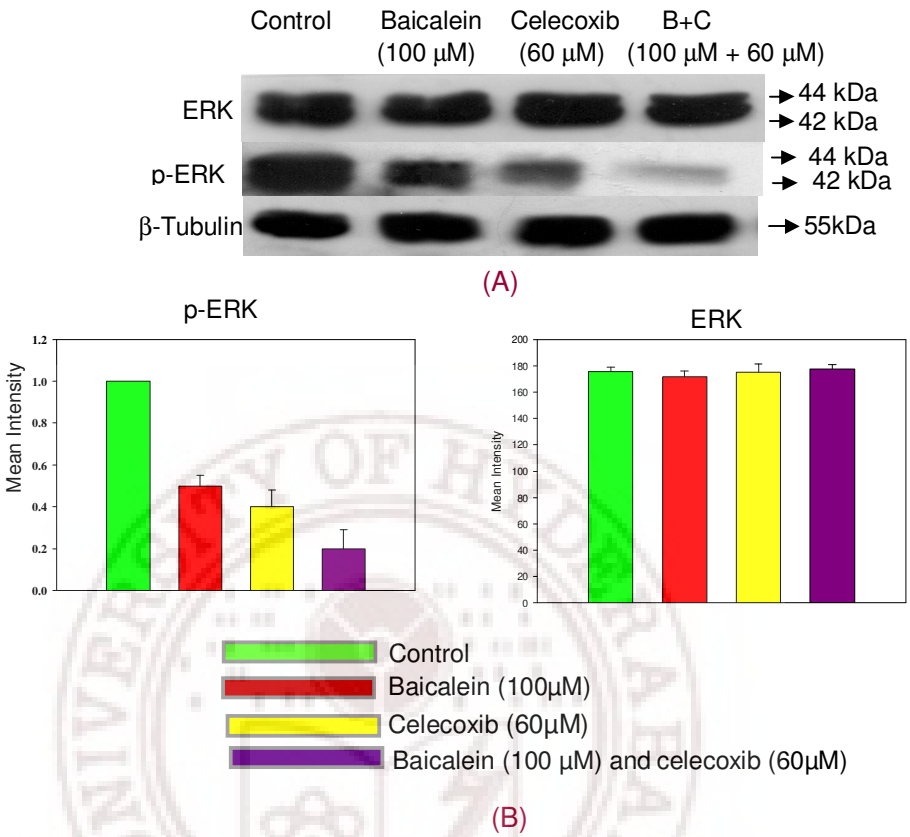
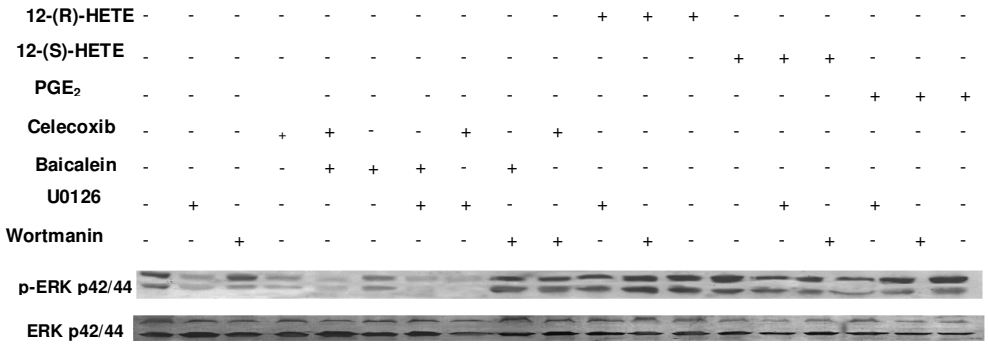


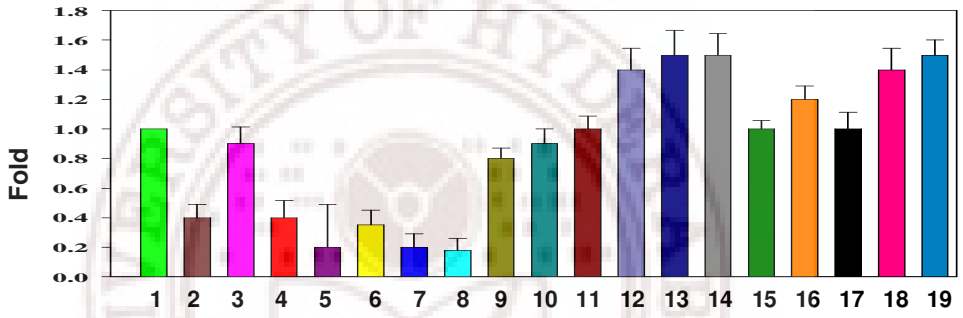
Figure 22: (A) Western Blot analysis of p-ERK and ERK levels of A431 cells treated with baicalein (100µM), celecoxib (60µM) and both baicalein (100µM) and celecoxib (60µM) for 48 h. (B) Bar diagram showing the intensity of the bands as measured by Scion Image analysis.

4.3.3 Effect of metabolites and inhibitors of 12-LOX and COX-2 on ERK signaling in A431 cells

The use of ERK specific inhibitor U0126 (10 µM) (Bar2) also decreased the phospho ERK levels in A431 cells. The levels of p-ERK were further decreased when U0126 was used in combination with baicalein or celecoxib.



(A)



(B)

Figure 23: (A) Effects of metabolites and inhibitors of 12-LOX and COX-2 on the ERK and phospho ERK levels (B) Bar diagram showing the intensity of the bands as measured by Scion Image analysis. 1-Control, 2-U0126, 3- Wortmanin, 4-baicalein, 5-baicalein+celecoxib, 6-celecoxib, 7-baicalein+U0126, 8-celecoxib+ U0126, 9-baicalein+wortmanin, 10-celecoxib+wortmanin, 11-12-(R)-HETE +U0126, 12-12-(R)-HETE+wortmanin,13-12-(R)-HETE, 14-12-(S)-HETE, 15-12-(S)-HETE+U0126, 16-12-(S)-HETE+wortmanin, 17-PGE_2+U0126, 18- PGE_2 + wortmanin, 19-PGE_2

However, the levels of phospho ERK were increased with the metabolites of 12-LOX (12-(R)-HETE, 12-(S)-HETE) and COX-2 (PGE_2) and their effect was slightly decreased when the cells were preincubated with U0126 (Figure 23A). The preincubation of cells with PI3 kinase inhibitor wortmanin (200nM) for 4 h also decreased the phospho Akt levels in A431 cells whereas the levels were further decreased when wortmanin was used in combination with baicalein or celecoxib.

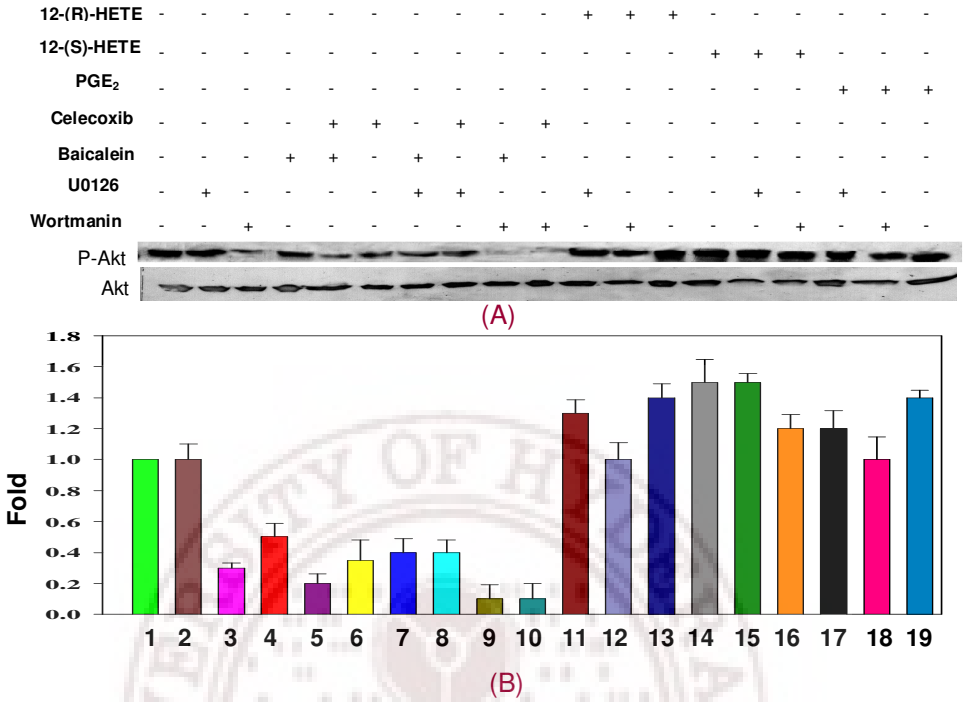


Figure 24: (A) Effects of metabolites and inhibitors of 12-LOX and COX-2 on the Akt and phospho Akt levels. (B) Bar diagram showing the intensity of the bands as measured by Scion Image analysis. 1-Control, 2-U0126, 3- Wortmanin, 4-baicalein, 5-baicalein+celecoxib, 6-celecoxib, 7-baicalein+U0126, 8-celecoxib+ U0126, 9-baicalein+wortmanin, 10-celecoxib+wortmanin, 11-12-(R)-HETE +U0126, 12-12-(R)-HETE+wortmanin,13-12-(R)-HETE, 14-12-(S)-HETE, 15-12-(S)-HETE+U0126, 16-12-(S)-HETE+wortmanin, 17-PGE_2+U0126, 18-PGE_2 + wortmanin, 19-PGE_2

The levels of phospho Akt were increased with the metabolites of 12-LOX (12-(R)-HETE, 12-(S)-HETE) and COX-2 (PGE_2) and their effect was slightly decreased when the cells were preincubated with wortmanin (Figure 24).

4.3.4 Effect of metabolites and inhibitors of 12-LOX and COX-2 on Caspase-3 activity in A431 cells

Caspase-3 plays a central role in inducing apoptosis by the intrinsic pathway. In the present study, the estimation of the caspase 3 activity was taken up in A431 cells, after the treatments with metabolites and inhibitors of 12-LOX and COX-2 pathways. There was an increase in the caspase 3 activity when the A431 cells were incubated with U0126 (Bar 2) or wortmanin (Bar 3). Pretreatment

with wortmanin or U0126 sensitized the cells to baicalein (Bar 7&9) or celecoxib (Bar 8&10) which was shown by a tremendous increase in the caspase 3 activity.

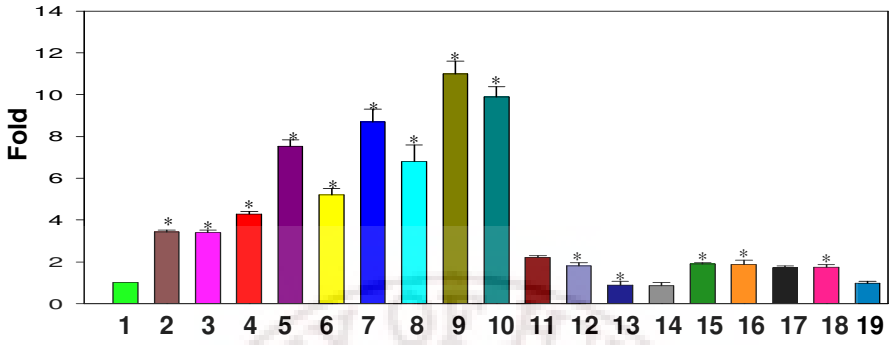


Figure 25: Bar diagram showing the fold differences in caspase 3 activity in A431 cells in response to different treatments. 1-Control, 2-U0126, 3- Wortmanin, 4-baicalein, 5-baicalein+celecoxib, 6-celecoxib, 7-baicalein+U0126, 8-celecoxib+ U0126, 9-baicalein+wortmanin, 10-celecoxib+wortmanin, 11-12-(R)-HETE + U0126, 12-12-(R)-HETE+wortmanin, 13-12-(R)-HETE, 14-12-(S)-HETE, 15-12-(S)-HETE+U0126, 16-12-(S)-HETE+wortmanin, 17-PGE_2+U0126, 18-PGE_2+ wortmanin, 19-PGE_2. ∗ Indicates statistical significance with $P<0.05$.

The caspase 3 activity in the cells treated with metabolites 12-(R)-HETE (Bar13), 12-(S)-HETE (Bar 14) and PGE_2 (Bar 19) was not much altered from that of untreated control cells (Bar 1) whereas the effect of U0126 and wortmanin was compensated in the cells treated with the metabolites as shown by very small increase in the caspase-3 activity (Bar 11, 12, 15, 16, 17, 18) (Figure 25).

4.3.5 Effects of metabolites and inhibitors of 12-LOX and COX-2 on the translocation of NF-κB in A431 cells

As NF-κB is a downstream target of Akt and also regulates the expression of several cell survival proteins, we examined the effect of metabolites (12-(R)-HETE/12-(S)-HETE/PGE_2) and inhibitors (baicalein/ celecoxib) of 12-LOX and COX-2 on the nuclear translocation of NF-κB. A431 cells were treated with 12-(R)-HETE/12-(S)-HETE (300 nM), PGE_2 (15μM), baicalein (100μM), celecoxib (60 μM) or both baicalein and celecoxib for 48 h and the nuclear proteins were analyzed on 6% native polyacrylamide gel electrophoresis (PAGE) to detect NF-κB activity by

gel shift assay. The results shown in Figure 26A indicate that baicalein (lane 3), celecoxib (lane 4) and both (lane 5) significantly inhibit NF-κB activation when compared to control (lane 2). Incubation of the cells with 12-RHETE/12-(S)-HETE/ PGE_2, on the other hand, up regulate NF-κB activity. 12-(R)-HETE (lane 6) and PGE_2 (lane 8) were more potent in up regulating the activity of NF-κB compared to 12-(S)-HETE (lane 7).

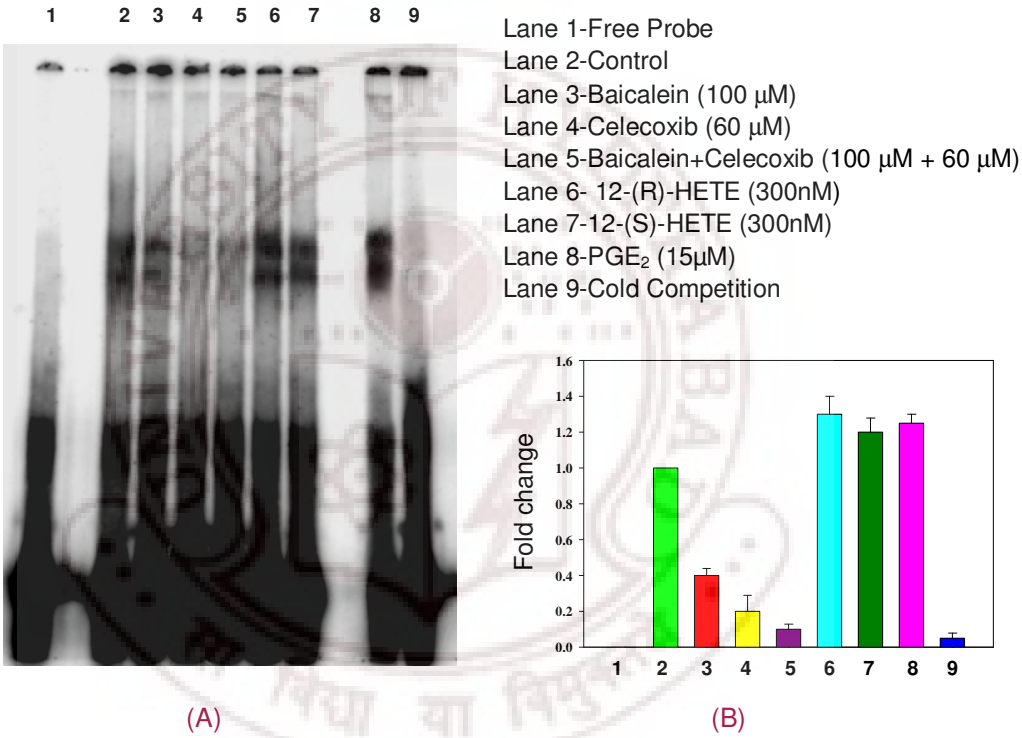
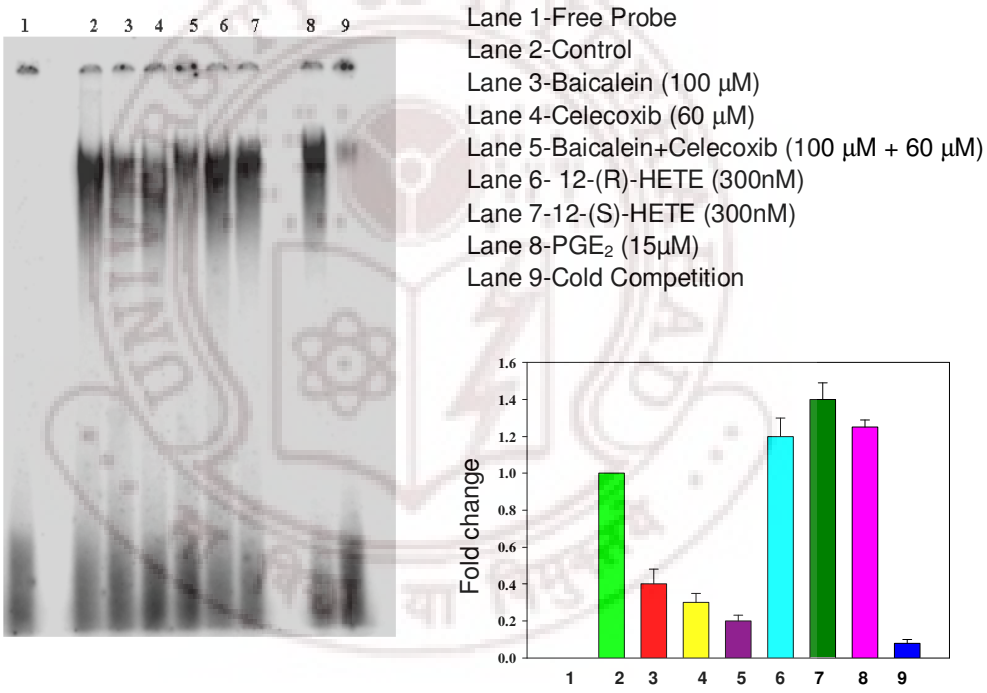


Figure 26: (A) Effect of 12-LOX and COX-2 metabolites and inhibitors on nuclear translocation of NF-κB in A431 cells. Electrophoretic mobility shift assay of nuclear extracts using specific NF-κB binding probe in cells exposed to 12-(R)-HETE (300 nM), 12-(S)-HETE (300 nM), PGE_2 (15 µM), baicalein (100 µM), celecoxib (60 µM) and baicalein (100 µM) and celecoxib (60 µM) for 48 h. Lane 1: Free probe; Lane 2: control cells; lane 3: baicalein (100 µM); lane 4: celecoxib (60 µM); lane 5: baicalein (100 µM) + celecoxib (60µM); lane 6:12-(R)-HETE (300 nM); lane 7:12-(S)-HETE (300 nM); lane 8: PGE_2 (15µM); lane 9: cold competition (B) Band Intensities measured by Scion Image Analysis and graph drawn using Sigma plot 9.0

4.3.6 Effects of metabolites and inhibitors on the AP-1 in A431 cells

As AP-1 is the downstream target of ERK pathway, we examined the effect of metabolites (12-(R)-HETE/12-(S)-HETE/PGE_2) and inhibitors of 12-LOX and COX-2 (baicalein/celecoxib) on the transcriptional activity of AP-1. A431 cells were incubated with 12-(R)-HETE/12-(S)-HETE (300 nM), PGE_2 (15 µM), baicalein (100 µM), celecoxib (60 µM) or both baicalein and celecoxib for 48 h and the nuclear proteins were analyzed on 6% native polyacrylamide gel electrophoresis (PAGE) to detect AP-1 activity by gel shift assay.



(A) (B)

Figure 27: (A) Effect of 12-LOX and COX-2 metabolites and inhibitors on nuclear translocation of AP-1 in A431 cells. Electrophoretic mobility shift assay of nuclear extracts using specific AP-1 binding probe in cells exposed to 12-(R)-HETE (300 nM), 12-(S)-HETE (300 nM), PGE_2 (15 µM), baicalein (100 µM), celecoxib (60 µM) and baicalein (100 µM) + celecoxib(60 µM) for 48 h. Lane 1: Free probe; Lane 2: control cells; lane 3: baicalein (100 µM); lane 4: celecoxib (60 µM); lane 5: baicalein (100 µM) + celecoxib (60µM); lane 6:12-(R)-HETE (300 nM); lane 7:12-(S)-HETE (300 nM); lane 8: PGE_2 (15µM); lane 9: cold competition. (B) Bar graphs showing the band intensities as measured by Scion Image Analysis.

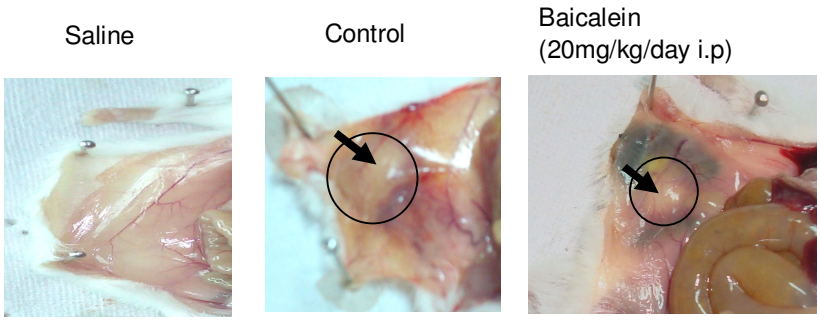
The results shown in Figure 27A indicate that baicalein (lane 3), celecoxib (lane 4) and both (lane 5) significantly inhibit AP-1 activation when compared to control (lane 2) while 12-RHETE/12-(S)-HETE/ PGE_2 up regulate AP-1 activity. 12-(R)-HETE (lane 6) and PGE_2 (lane 8) were more potent in up regulating the activity of AP-1 compared to 12-(S)-HETE (lane 7).Thus we see that 12-(R)-HETE, 12-(S)-HETE and PGE_2 promote the activity of NF-κB and AP-1 which are important transcription factors promoting cell growth and the inhibitors baicalein and celecoxib counteract these effects. These studies clearly demonstrate the role of ERK/AP-1 and Akt/NF-κB in the regulation of growth of A431 cells.

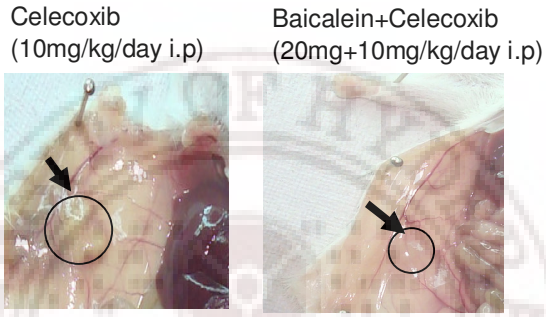
4.4 To evaluate the efficiency of COX/LOX inhibition in regulating the growth of A431 xenografts in Swiss mice.

The foregoing studies clearly demonstrate the role of 12-LOX and COX-2 inhibitors in regulating the growth of A431 cells *in vitro* by inducing apoptosis. In the light of these findings in cell lines, it would be interesting to evaluate the efficiency of baicalein and celecoxib in regulating the growth of tumor cells *in vivo*. Hence, further studies were undertaken to study the effect of baicalein and celecoxib on A431 xenografts in Swiss mice.

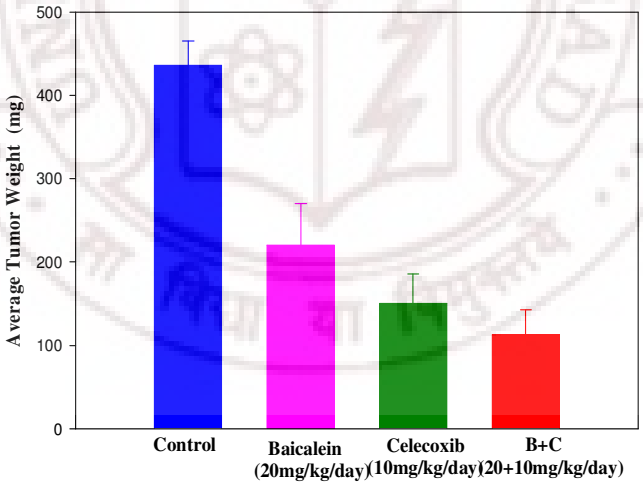
4.4.1 Baicalein and celecoxib reduced the tumor weight

10 x 10^6 A431 cells were xenografted in Swiss mice and tumors were allowed to develop. These studies resulted in the development of tumors in 80% animals in 10 days. Baicalein treatment for 1 week at the dose of 20mg/kg/day I.P effectively reduced the tumor weight by 45% whereas celecoxib at the dose of 10mg/kg/day I.P reduced the tumor weight by 65%. The combination of baicalein and celecoxib for 1 week at the dose of 20+10 mg/kg/day I.P was most effective in the reduction of tumor weight by 80%. Figure 28A shows the development of tumor in control followed by the significant reduction in treated animals.





(A)



(B)

Figure 28: (A) Photographs taken on dissected animals showing the tumor. (B) Bar graphs showing the reduction in the tumor weight drawn using sigma plot 9. Values are mean ± of 5 different animals.

4.4.2 Haematoxylin and eosin (HE) staining of tumor sections from control and treated animals

The histological sections of tumors were stained with haematoxylin and eosin dye and were observed under the microscope. The tissue sections of the tumors showed highly proliferating cells in the control and apoptotic cells in the treated tissues.

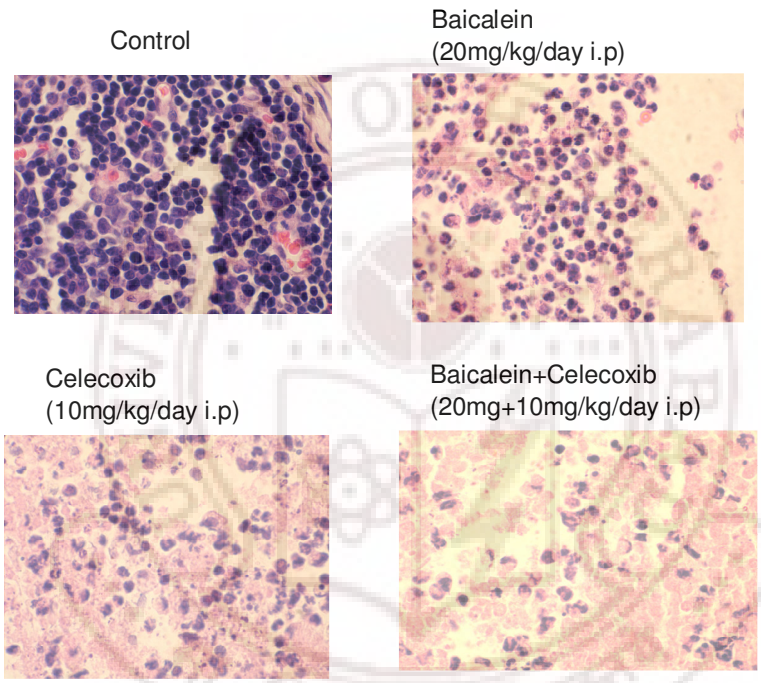


Figure 29: Haematoxylin and Eosin (H&E) staining of sections taken on tumor xenografts in Swiss mice.

The combination treatment showed the maximum cell death as seen by the lowest number of cells present in the tumor sections from those tumors when compared to either baicalein or celecoxib (Figure 29).

4.4.3 *Insitu* Apoptosis Detection (TUNEL) on Tumor sections

The tumor tissue sections (5µm) were taken and stained for in situ apoptosis by TUNEL method according to manufacturer's protocol. The TUNEL staining showed high fluorescence in the treated tissue sections when compared

to the control. The combination treatment showed the maximum cell death which is proportional to the intensity of flourescence when compared to either baicalein or celecoxib (Figure 30).

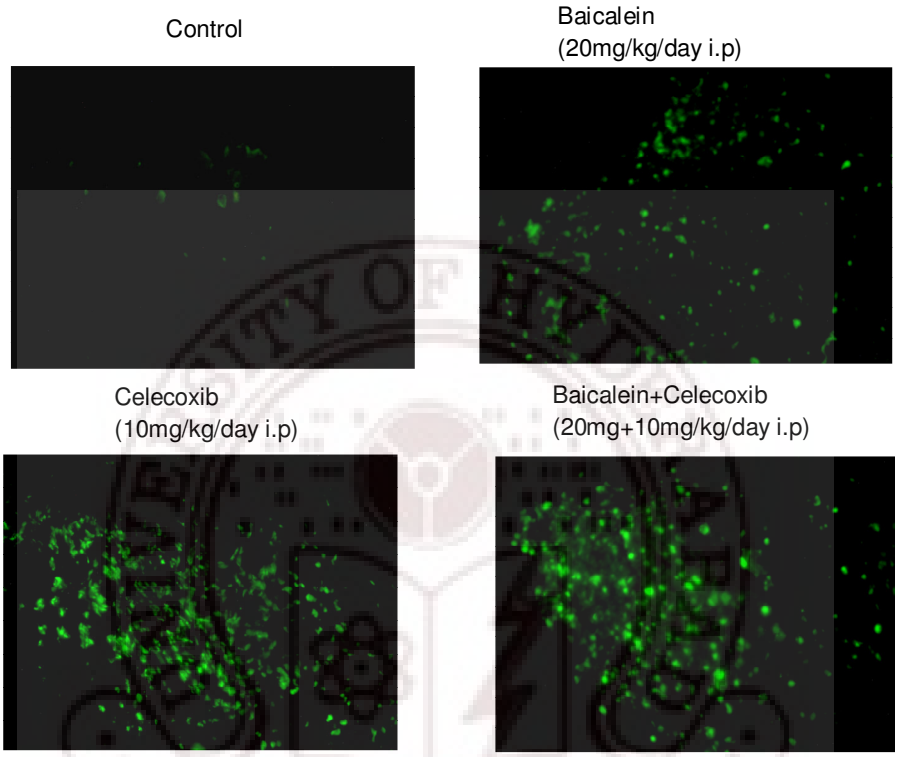


Figure 30: *In situ* apoptosis detection (TUNEL) assay of tumor sections from xenografts in Swiss mice treated with baicalein, celecoxib, and both baicalein and celecoxib.

5. Discussion

Eicosanoids serve as intermediates in growth factor signaling pathways. Most tumor cells produce AA metabolites and these compounds have been found to modulate a wide range of biological factors that induce growth and invasiveness of tumors (Hong *et al*, 1999). It is well-established that human malignancies frequently overexpress COX-2 and produce high levels of the COX-2 metabolite, PGE_2 (Arunasree *et al*, 2008; Krysan *et al*, 2005; Subbaramaiah *et al*, 2002; Kirschenbaum *et al*, 2001; Fujita *et al*, 1998; Soslow *et al* ,2000; Huang *et al*, 1998; Wolff *et al*, 1998; Kutchera *et al*, 1996). COX-2 overexpression has since been found in many human cancers including breast (Half *et al*, 2002; Soslow *et al*, 2000), esophageal (Li *et al*, 2000; Zimmermann *et al*, 1999), lung (Hosomi *et al*, 2000; Hida *et al*, 1998), prostate (Uotila *et al*, 2001; Yoshimura *et al*, 2000), bladder (Ristimaki *et al*, 2001; Mohammed *et al*, 1999), skin (Tang *et al* ,2001; Neufang *et al*, 2001) and pancreas (Basu *et al*, 2004; Tucker *et al*, 1999; Molina *et al*, 1999). LOX activation may be involved in both pro- and antitumorigenic effects (Muller *et al*, 2002; Shureiqui *et al*, 2001). Several line of evidences indicate 12-lipoxygenase (12-LOX) as a key regulator of human cancer development. Overexpression of 12-LOX has been detected in a variety of tumors including breast, renal, pancreatic, and prostate cancers (Nie *et al*, 2006; Yoshimura *et al*, 2004; Natarajan *et al*, 1998; Gao *et al*, 1995).

Many of the AA metabolites are clastogenic and act as tumor promoters in murine models of skin carcinogenesis and are induced following UVB irradiation (Bickers *et al*, 2006). Elevated levels of eicosanoids (prostaglandins and leukotrienes) have been shown to be associated with a wide array of dermatological diseases, such as psoriasis, UV-induced erythema, and contact sensitivity (Wang *et al*, 2001). Besides their role as indicators of neoplastic

development, eicosanoids also act as reporters of skin irritation (Marks *et al*, 2000). A number of studies indicate overexpression of 12-R-LOX in skin cancers. However, a comprehensive study on the metabolism of AA via the LOX and COX pathways on epidermoid carcinoma are lacking. In the present study the expression of various genes involved in LOX and COX pathways were analysed in human eptdermoid carcinoma cell line, A431.

5.1 12-R-LOX and COX-2 overexpressed in A431 cells compared to NIH3T3 cells

Recent investigations have shown that tumors of different histogenesis considerably differ in the metabolism of AA (Kudryavtsev *et al*, 2005). Thus, the expression and role of AA metabolizing enzymes would be different in different cancers and thus it is very important to know the expression profile of AA metabolizing enzymes first. Therefore, our first question was to investigate the expression of various AA metabolizing enzymes in A431 cells. This study clearly showed the overexpression of 12-R-LOX and COX-2 in A431 cells with no expression in the normal skin fibroblasts suggesting their significance in the pathology of skin cancer. The expression of 12-S-LOX on the other hand, was slightly higher in A431 cells when compared to the normal 3T3 cells. This forms the first report on the overexpression of 12-R-LOX in epidermoid carcinoma cell line, A431.

In recent years, it has become clear that multiple forms of lipoxygenases are expressed in skin of mice and humans (Virmania *et al*, 2002; Krieg *et al*, 1998; Boeglin *et al*, 1998; Sun *et al*, 1998; Jisaka *et al*, 1997; Krieg *et al*, 1995; Chen *et al*, 1994). In mouse skin, there is a TPA-inducible 8-lipoxygenase and at least three isoforms of 12-lipoxygenase with various designations; a `platelet type' 12-lipoxygenase (P-12LO), epidermal-type 12- lipoxygenase (e-12LOX-1), and a 12-(R)-lipoxygenase (e-12LOX-2). P-12 LOX expression has been demonstrated in epidermis *in vivo* and in cultured keratinocytes (Krieg *et al*, 1995; Chen *et al*,

1994). In neoplastic epidermal preparations, the predominant expression of P-12 LOX was reported (Krieg *et al*, 1995) and in human skin immunohistochemical evidence was provided for increased expression in germinal layer keratinocytes in psoriatic scales (Hussain *et al*, 1994). A constitutive expression of p12-S-LOX was previously demonstrated both in mouse and human epidermis (Heidt *et al*, 2000; Krieg *et al*, 1995; Chen *et al*, 1994). In addition, p12-S-LOX-deficient mice have been shown to be less sensitive for tumor induction according to the initiation-promotion protocol (Muller *et al*, 2002; Virmani *et al*, 2001). The discrete expression of 12-R-LOX on day 16.5 of fetal life of mouse embryos indicates a critical function during embryonic development of skin (Sun *et al*, 1998). A preferential expression of 12-R-LOX and e-LOX-3, in stratifying epithelia was observed, which exhibited only weak signals in all other tissues (Heidt *et al*, 2000). 8-LOX is also constitutively up regulated in skin tumors (Muga *et al*, 2000; Furstenberger *et al*, 1991). It is a murine homolog of human 15S-LOX-2 and metabolizes AA and LA to 8-HETE and 9-HODE, respectively (Burger *et al*, 1999; Jisaka *et al*, 1997; Furstenberger *et al*, 1991; Gschwendt *et al*, 1986). 8S-LOX plays a role as a pro-differentiating, anti-tumorigenic, and tumor suppressing gene in mouse skin carcinogenesis (Kim *et al*, 2005).

While COX-2 expression in normal skin was usually very low and restricted to regions of differentiated epidermis (Muller-Decker *et al*, 1999; Buckman *et al*, 1998), studies on mouse and human skin carcinogenesis revealed that overexpression of COX-2 contributes to the development of skin cancers (Higashi *et al*, 2000; Muller Decker *et al*, 1999, 1998; Buckman *et al*, 1998). COX-2 overexpression occurs not only in the tumor cells but also in the tumor vasculature (Edelman *et al*, 2008; Masferrer *et al*, 2000). COX-1 is constitutively expressed in most tissues including mouse and human skin (Neumann *et al*, 2007; Muller-Decker *et al*, 1999, 1998). COX-1 and COX-2 can play very different roles in skin

tumor development, depending on the nature of the agents used to induce tumors. While COX-1 is important in phorbol ester promotion (Tiano *et al*, 2002), it does not contribute to UV carcinogenesis (Pentland *et al*, 1999). COX-2, on the other hand, is a prerequisite for skin tumor development with both protocols. COX-2 thus remains a viable target for topical skin cancer prevention (Fischer *et al*, 2007). There is also evidence of the aberrant expression of COX-2 in tumors of non-epithelial origin, such as malignant melanomas (Muller Decker *et al*, 2007; Denkert *et al*, 2001). The targeted disruptions of the genes encoding either COX-1 or COX-2 (Langenbach *et al*, 1995; Morham *et al*, 1995) lead to altered epidermal differentiation and reduced skin tumorigenesis (Tiano *et al*, 2002). The constitutive expression of COX-2 in murine epidermis confers both alopecia and a skin tumor resistance phenotype. These unexpected findings suggest that the role of PGs in normal skin physiology and skin tumor development is complex and not well understood (Bol *et al*, 2000).

In the present study also predominant expression of COX-2 was observed in human epidermoid carcinoma cell line, A431 but no expression in the normal 3T3 cells, suggesting a possible role for COX-2 in regulating the growth. In the light of dominant expression of 12-R-LOX and COX-2 in skin cancer cells, (A431), but not in normal 3T3 cells, further studies were undertaken to analyse the effect of metabolites of 12-LOX (12-(R)-HETE and 12-(S)-HETE) and COX-2 (PGE_2) as well as inhibitors of 12-LOX (Baicalein) and COX-2 (Celecoxib).

5.2 12-LOX and COX-2 regulate the growth of A431 cells

The specific 12-LOX inhibitor baicalein and COX-2 inhibitor, celecoxib, alone or in combination, effectively suppressed the growth of A431 cells as indicated by the lowered incorporation of thymidine into DNA in comparison to that in untreated cells. 12-(R)-HETE and PGE_2 increased the incorporation of thymidine in a dose dependent manner. The addition of exogenous 12-(S)-HETE,

on the other hand, increased the incorporation of thymidine into DNA in a dose dependent manner which was in accordance with the earlier report (Kudryavtsev *et al*, 2005). Similar increase in the proliferation of prostate (Pidgeon *et al*, 2002) and gastric cancer cells (Hong *et al*, 2001) was reported in response to 12-(S)-HETE. Very limited data exist regarding 12-(R)-HETE and tumorigenesis. 12-(R)-HETE was shown to induce the proliferation of colon cancer cells *in vitro* (Bortuzzo *et al*, 1996). PGE_2 is known to promote tumor-cell proliferation (Ye *et al*, 2005). 12(S)-HETE has been found as a determinant of metastastic potential of B16a melanoma cells and other tumor cells such as Clone A and Walker 256 (Chen *et al*, 1994; Liu *et al*, 1994). The above studies clearly demonstrate the role of 12-R-LOX and COX-2 in the regulation of A431 cell proliferation. However, the molecular mechanisms underlying the regulation of A431 cell proliferation and the mode of cell death by 12-LOX and COX-2 pathways are not clear. So our next step was to elucidate the molecular mechanisms of apoptosis induced by baicalein and celecoxib.

Apoptosis or programmed cell death is a physiological cell suicide program that is critical for the development and maintenance of healthy tissues. Deregulation of this pathway occurs in cancer, autoimmune diseases, and neurodegenerative disorders. Two major apoptotic pathways have been identified thus far, the death receptor-mediated (extrinsic) and mitochondria-mediated (intrinsic) pathways (Green *et al*, 2000; Strasser *et al*, 2000). The former is initiated by triggering of cell surface death receptors of the tumor necrosis factor receptor superfamily by their ligands and the activation of caspase-8. The latter is initiated by specific chemicals, growth factor deprivation, or irradiation. It involves cytochrome c release from mitochondria and activation of capase-9. The Bcl-2 protein family plays an important role in regulating apoptosis. This family includes bad and bax proteins, which are presumed to form pores in the outer mitochondrial

membrane through which cytochrome c can be released into the cytosol (Kluck *et al*, 1997). Anti apoptotic Bcl-2 family members such as Bcl-2 and Mcl-1 appear to provide negative regulation of apoptosis by impeding bad/bax induced pore formation and cytochrome c release in response to death-inducing stimuli, thereby preventing apoptosis. In contrast, the proapoptotic Bcl-2 family members such as bax, bad, bik and bak promote cytochrome c release and enhance apoptosis (Yin *et al*, 2000; Marzo *et al*, 1998). Once caspase-8 and caspase-9 are activated, they can further activate the downstream effector caspases, including caspase-3, caspase-6, and caspase-7, which can then cleave their respective substrates and induce characteristic apoptotic changes. Induction of apoptosis is the characteristic of chemotherapy and irradiation therapy of human cancers. The ability to induce apoptosis in tumor cells is a very attractive feature of antitumor agents (Houghton *et al*, 1999; Rioux *et al*, 1998). Caspases exist in cells in an inactive zymogen form called procaspases and can be activated by proteolytic cleavage to produce active caspases. Activated caspases can cleave their substrates after specific aspartic acid residues. Caspase-3 is the most important among these proteases. The substrates for caspase-3 include PARP, retinoblastoma protein, actin, and laminin (Tong *et al*, 2002; Rosen *et al*, 1997).

5.3 12-LOX and COX-2 inhibition induce apoptosis in A431 cells via the intrinsic pathway

The mode of cell death induced by the baicalein (12-LOX inhibitor) and celecoxib (COX-2 inhibitor) was evaluated by analyzing cell and nuclear morphology, tunnel assay and Propidium iodide staining. These findings demonstrate that baicalein and celecoxib induce apoptosis by activating the intrinsic death pathway involving alterations in the mitochondrial membrane potential, decrease in the Bcl-2/Bax ratio, release of cytochrome c, activation of caspase 3 and PARP cleavage. Baicalein (BE) has been reported to induce apoptosis and inhibit proliferation in several cell types including gastric, pancreatic,

and prostate cancer cells via the inhibition of 12-LOX (Tong *et al*, 2002; Pidgeon *et al*, 2002; Wong *et al*, 2001). In MCF-7 cells, BE suppressed 17β-estradiol-induced transactivation, and induced apoptosis (Po *et al*, 2002. It has been reported that baicalein could make an S-phase arrest in cell cycle of lung squamous carcinoma CH27 cells (Lee *et al*, 2005) and induce apoptosis in many human cancer cell lines, e.g. hepatoma cells Hep3B and HepG2 (Chang *et al*, 2002), pancreatic cancer cells MiaPaCa-2 and AsPC-1 (Tong *et al*, 2002), breast cancer cells MCF-7 (Tong *et al*, 2002) and prostate cancer cell lines (Chen *et al*, 2001). Although several biological activities of BE have been reported, intracellular molecules involved in modulation of apoptosis induced by baicalein are still undefined (Chow *et al*, 2006). A recent report demonstrated that baicalein induced a mitochondria-dependent caspase-3 and caspase-9 activation, and consequently led to apoptotic cell death in human myeloma cells (Ma *et al*, 2005). Treatment with highly selective COX-2 inhibitors results in a reduction in the size and number of colonic lesions in several animal models (Kawamori *et al*, 1998) and suppression of carcinogenesis of human colon, prostate and esophageal cancers (Zimmermann *et al*, 1999). The mechanism by which COX-2 inhibitors suppress carcinogenesis is attributed to its modulation of prostanoid production which affects cell proliferation, tumor growth and immune responsiveness. PGE_2, a major product of COX-2, induces bcl-2 expression and inhibits apoptosis and, conversely, that COX-2 inhibitors induce apoptosis (Zimmermann *et al*, 1999; Sheng *et al*, 1998a). While the involvement of prostanoids in carcinogenesis is clear, another study showed that selective inhibition of COX-2 activity suppresses the production of PGE_2 but does not alter the progress of the morphological transformation of rat fibroblasts (Sheng *et al*, 1998b), suggesting the existence of distinct signal pathways, PGE_2-dependent and PGE_2- independent, in the regulation of carcinogenesis (Higashi *et al*, 2000). Diclofenac induced apoptosis in three of four

cutaneous SCC cell lines as shown by DNA fragmentation and caspase activation (Fecker *et al*, 2007). Similarly inhibition of COX-2 was shown to inhibit growth of a human cutaneous squamous cell carcinoma cell line (Thompson *et al*, 2001) and induce apoptosis in K562 cell line (Subhasini *et al*, 2005). In addition to the induction of apoptosis, celecoxib has the potential to inhibit tumor angiogenesis and metastasis and hence might serve as an ideal agent for long-term maintenance therapy. COX/LOX dual inhibition has been evaluated in several experimental models and found to be potentially beneficial (Edelman *et al*, 2008; Cianchi *et al*, 2006; Ye *et al*, 2005; Wenger *et al*, 2002). In the present study we show that both 12-LOX and COX-2 inhibition in the combination treatment is more effective in inducing apoptosis when compared to either baicalein or celecoxib alone. Interestingly celecoxib could inhibit the RNA expression of 12-R-LOX which might be one of the reasons for the additive effect of baicalein and celecoxib observed in the present study. This forms the first report for the combination effect of LOX and COX inhibitors on epidermoid carcinoma.

The proposed mechanism of action of baicalein and celecoxib in inducing apoptosis in A431 cells is outlined below (Figure 31).

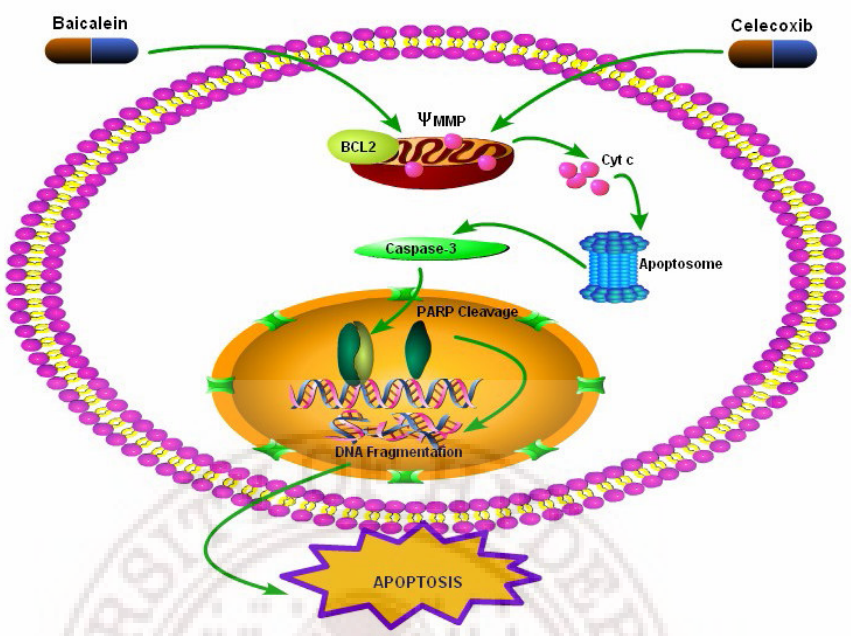


Figure 31: Proposed mechanism of induction of apoptosis in A431 cells by baicalein and celecoxib.

5.4 12-LOX and COX-2 inhibition induce apoptosis via the inactivation of ERK/AP-1 and Akt/NF-κB pathway

The foregoing studies clearly demonstrate the induction of apoptosis by the inhibition of either 12-LOX by baicalein or COX-2 by celecoxib or by their combined inhibition in A431 cells by intrinsic death pathway involving alterations in mitochondrial membrane potential. However, it is not clear how these effects are mediated by baicalein and celecoxib.The signaling pathways that govern cell proliferation, survival and oncogenesis are of prime interest in cancer biology (Rayet *et al*, 1999). Most of the signals for survival trigger growth factor receptors that activate the ERK and PI3K/Akt pathways and promote cell growth (Jin *et al*, 2007; Kennedy *et al*, 1997; Xia *et al*, 1995). The mitogen-activated protein kinase (MAPK) family consists of extra cellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38MAPK, which are involved in mediating the processes associated with cell growth, survival and death (McCawley *et al*, 1999; Wada *et al*, 2004). JNK and p38 MAPK pathways are activated in response to

chemicals and environmental stress (Davis *et al*, 2000; Nagata *et al*, 1999; Roulston *et al*. 1998; Minden *et al*, 1997), while the ERK cascade is activated by mitogenic stimuli, such as growth factors, cytokines, and phorbol esters, and is critical for proliferation and survival (Johnson *et al*, 2002; Chang *et al*, 2001). However, ERK signaling has been suggested to be proapoptotic in cells undergoing apoptosis (Choi *et al*, 2003; Xiao *et al*, 2002; Bacus *et al*, 2001; Wang *et al*, 2000). Recent evidence indicates that the MAPK family protein kinases are important mediators of apoptosis induced by stressful stimuli (Johnson *et al*, 2002; Chang *et al*, 2001). Among MAPK subfamilies, ERK is controversial in its role in cell death. While some studies showed that the ERK activation mediates survival response that counteracts cell death, other studies reported that the ERK activation is associated with apoptotic signaling pathways (Yang *et al*, 2007; Choi *et al*, 2003; Xiao *et al*, 2002; Bacus *et al*, 2001; Wang *et al*, 2000).

In highly specialized cells, which participate in the constant renewal of the skin epithelium, a basal level of ERK activity is absolutely indispensable for normal homeostasis (Bourcier *et al*, 2006). Given the role of ERK in the development of hyperproliferative skin lesions (Hasse *et al*, 2002), we asked whether alteration in ERK activity could play a role in apoptosis mediated by baicalein and celecoxib. The data presented here show that baicalein and celecoxib induce apoptosis in parallel with the inactivation of ERK in A431 cells. Similar down regulation of ERK activity associated with the inhibition of proliferation and induction of apoptosis was reported in gastric cancer (Chen *et al*, 2008) and primary AML blasts (Lunghi *et al*, 2003).The metabolites 12-(R)-HETE, 12-(S)-HETE and PGE_2 on the other hand increased the p-ERK levels and prevented apoptosis. Also, PGE_2 was shown to stimulate ERK kinase pathway in NSCLC cells (Krysan *et al*, 2005) and 12-(S)-HETE to promote phosphorylation of ERK in A431 cells (Szekeres *et al*, 2000). There are no reports on 12-(R)-HETE and ERK activation. Since it has been

reported that down regulation of phosphorylated ERK protein expression level is consistent with inhibition of ERK activation (Vial *et al*, 2005), blockage of the function of ERK via the inhibition of 12-LOX and COX-2 pathways might be associated with baicalein and celecoxib induced apoptosis.

The phosphoinositide 3-kinase (PI3K)/Akt pathway is activated in a wide variety of cancers and results in enhanced resistance to apoptosis through multiple mechanisms. Inhibition of PI3K decreases cell survival and enhances the effects of chemotherapeutic drugs in many types of cancer cells (Jin *et al*, 2007; Wang *et al*, 2002; Hu *et al*, 2002; Asselin *et al*, 2001). Akt, also known as PKB (protein kinase B) (Bellacosa *et al*, 1991; Coffer *et al*, 1991) is a serine/threonine protein kinase that has been shown to regulate cell survival signals in response to growth factors, cytokines, and oncogenic Ras (Downward *et al*, 1998; Franke *et al*, 1997; Marte *et al*, 1997). Akt becomes activated via the phosphoinositide-3-OH kinase (PI3K) pathway (Downward *et al*, 1998; Fruman *et al*, 1998; Hawkins *et al*, 1997) and by other upstream kinases, including the Ca21- and calmodulin-dependent protein kinase (Yano *et al*, 1998). Akt inhibits cell death pathways by directly phosphorylating and inactivating proteins involved in apoptosis, including Bad, procaspase 9, and members of the Forkhead transcription factor family (Madrid *et al*, 2000; Brunet *et al*, 1999; Tang *et al*, 1999; Kops *et al*, 1999; Cardone *et al*, 1998; Datta *et al*, 1997; del Peso *et al*, 1997). The Akt signaling pathway, however, has been increasingly documented as a prime determinant of tumor promotion and progression in several cell types, including skin (Segrelles *et al*, 2002). Consequently, Akt-dependent deregulation of the apoptotic response to UVB could be a key event in the multistep process of skin carcinogenesis (Decraene *et al*, 2004).

In the present study the level of phosphorylated Akt was decreased in A431 cells after treatment with celecoxib.These findings are similar to earlier

reports where celecoxib treatments showed antiproliferative, antiangiogenic, and proapoptotic effects by decreasing PI3K/Akt phosphorylation in metastatic breast cancer cells (Basu *et al*, 2004). The regulation of Akt with baicalein treatment is not well understood. The results were further validated by the use of ERK specific inhibitor (U0126) and PI3 Kinase inhibitor (Wortmanin) and the metabolites of 12-LOX and COX-2. U0126 and Wortmanin pretreatment sensitized the cells to baicalein and celecoxib resulting in increase in caspase-3 activity and massive apoptosis suggesting the role of these two survival pathways in A431 cells. The metabolites 12-(S)-HETE, 12-(R)-HETE and PGE_2, on the other hand, increased the phospho ERK and phospho Akt levels suggesting that they can stimulate cancer growth through activation of p44/42 mitogen-activated protein kinase and PI3/Akt kinase pathways in A431 cells. There are earlier reports where 12-(S)-HETE stimulates cancer growth through activation of p44/42 mitogen-activated protein kinase and PI3/Akt kinase pathways in human pancreatic cancer cells (Ding *et al*, 1999a; Ding *et al*, 1999b; Tong *et al*, 2002). PI 3-kinase pathway is an important signaling pathway for 12-LOX to increase VEGF expression in human prostate cancer cells. Increased 12-LOX expression or activity leads to the increased formation of 12(S)-HETE, which subsequently activates PI 3-kinase (Szekeres *et al*, 2000; Nie *et al*, 2006).

Akt is frequently activated in a wide variety of human cancers and has been shown to confer resistance to conventional cancer therapies. Hence, there is considerable rationale for targeting the Akt pathway to develop anti-cancer drugs. Interest in the role of Akt in cancer has increased enormously over the past decade, and it is now evident that activation of the Akt pathway is one of the most common molecular alterations in human malignancy. Importantly, many consequences of hyperactive Akt signaling are considered as hallmarks of cancer (Altomare *et al*, 2005). 12-(S)-HETE, 12-(R)-HETE and PGE_2 activate the ERK

and Akt levels whereas baicalein and celecoxib treatment results in their decrease. Baicalein or celecoxib or their combination with U0126 may become powerful anticancer agents for the treatment of human squamous carcinoma.

Nuclear factor κB (NF-κB) and activator protein 1 (AP-1) are key transcription factors that orchestrate expression of many genes involved in inflammation, embryonic development, lymphoid differentiation, oncogenesis, and apoptosis (Fujioka *et al*, 2004; Li *et al*, 2002; Shaulin *et al*, 2002). Nuclear factor kappa B (NF-κB) is a pleiotropic transcriptional factor involved in the inducible expression of a wide variety of genes, particularly those that promote cell growth and survival and that protect cells from apoptotic death stimuli (Woods *et al*, 2002; Chen *et al*, 1999). NFκB might be involved in keratinocyte transformation and skin carcinogenesis (Budunova *et al*, 1999; Pandolfi *et al*, 1992). It has been demonstrated that epidermal inflammation and hyperplasia play a critical role in skin tumor promotion and NF-κB is one of the well-known mediators of these effects (Ouyang *et al*, 2006; Budunova *et al*, 1999). Continuous activation of NF-κB factors is also emerging as a hallmark of various types of solid tumors, including breast, ovarian, colon, pancreatic, thyroid, bladder and prostate carcinomas as well as melanomas (Chinenov *et al*, 2001). The present study clearly demonstrates a regulatory role for COX-2 and 12-LOX in the activation of NF-κB as evidenced by its down-regulation with baicalein and celecoxib and up regulation with 12-(R)-HETE, 12-(S)-HETE and PGE_2 treatments. Baicalein inhibited the survival of primary myeloma cells, especially MPC-1 immature myeloma cells *in vitro*, and induced apoptosis in myeloma cell lines through the down regulation of IkB- α phosphorylation (Ma *et al*, 2005). NF-κB activation has been also identified in squamous cell carcinomas of the head and neck. Inhibition of NF-κB activity in these tumors inhibits cell survival and tumor growth. In solid tumors, high levels of c-Rel have been found in non-small cell lung carcinoma

(Mukhopadhyay *et al*, 1995) and breast cancer (Dolcet *et al*, 2005; Sovak *et al*, 1997). PI3K/Akt signaling has been proposed to induce NF-κB-mediated upregulation of COX-2 following IκB phosphorylation and degradation (Wu *et al*, 2005; Sheu *et al*, 2005; St-Germain *et al*, 2004; Chang *et al*, 2002).

The activator protein 1 (AP-1) and MAPK signaling pathways are believed to play an important role in cancer chemoprevention and chemotherapy due to their involvement in tumor cell growth, proliferation, apoptosis, and survival. The transcriptional factor activator protein 1 (AP-1) has been proposed to play important roles in carcinogenesis and cancer development (Amit *et al* , 2003; Oya *et al* , 2003; Bremner *et al* , 2002; Shaulian *et al*, 2002; Heiss *et al*, 2001; Hsu *et al*, 2000). AP-1 activity can be regulated by several mechanisms including the activation of the mitogen-activated protein kinase (MAPK) pathways (Xu *et al*, 2005). ERK activation usually leads to elevated AP-1 activity via c-fos induction. This results in increased synthesis of c-fos, which upon translocation to the nucleus dimerizes with the pre-existing Jun proteins to form AP-1 dimers (Gopalakrishnan *et al*, 2006; Karin *et al*, 1996; Karin *et al*, 1992). AP-1 is one of the major eukaryotic transcription factors involved in regulating COX-2 expression (Chun *et al*, 2004; Subbaramaiah *et al*, 2002, 2001; Guo *et al*, 2001). It was recently reported that the stimulation of young human keratinocytes with EGF increases the AP-1 DNA-binding activity mediated through ERK activation, whereas blocking ERK activation by PD098059 inhibited the AP-1 DNA binding activity (Kim *et al*, 2006; Shi *et al*, 2005).

We observed a decrease in AP-1 levels in the nuclear fractions of the baicalein and celecoxib treated cells and up regulation in cells treated with 12-(R)-HETE, 12-(S)-HETE and PGE_2. The present study, thus clearly demonstrates a regulatory role for COX-2 and 12-LOX in the activation of AP-1. The proposed

signaling pathways involved in the activation of AP-1 by baicalein and celecoxib are presented in the Fig. 32.

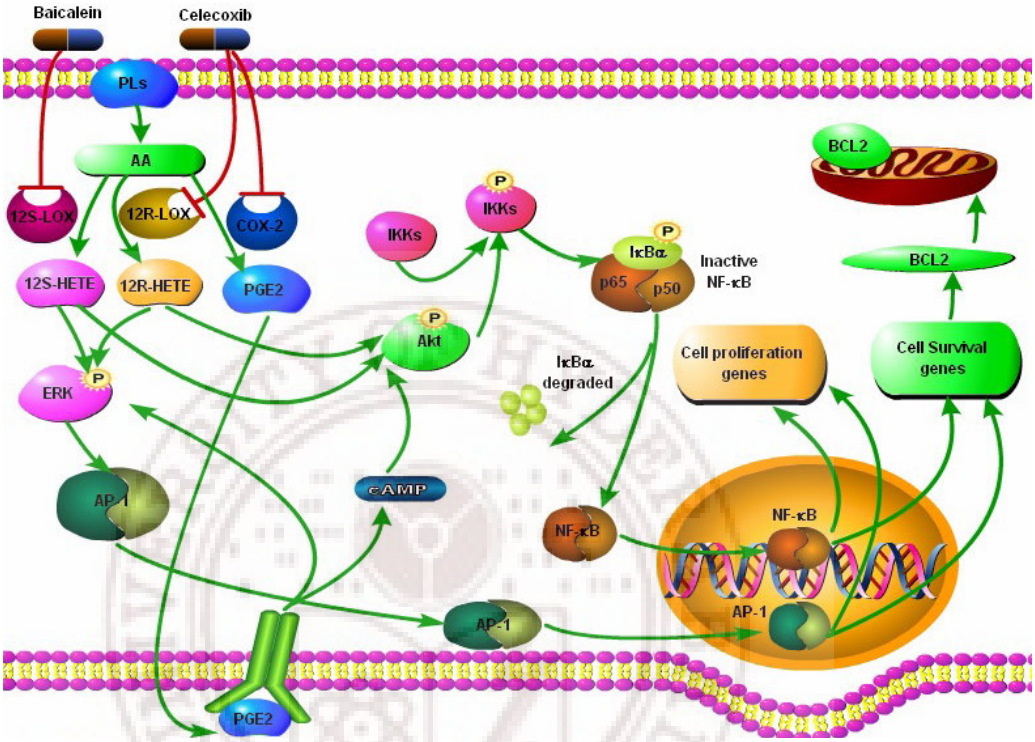


Figure 32: Schematic overview of the proposed signaling pathways involved in the survival and apoptosis in A431 cells mediated by 12-LOX and COX-2 pathways.

The molecular mechanisms underlying anticancer activities of inhibitors of COX and LOX are very complex, and manifest differentially in different cell types. The antitumor effects of baicalein and celecoxib may not only be observed in cells which express 12-LOX and COX-2 but also in cells which do not express these enzymes suggesting that their effects may be independent of inhibitors of COX-LOX pathways. Indeed, many *in vitro* cell culture and animal experiments have shown that the anticancer activity exerted by certain NSAIDs is independent of their COX-2 inhibitory properties (Kim *et al*, 2004; Tegeder *et al*, 2001). In the present study the observed effects of baicalein and celecoxib appear to be mediated through the respective metabolites 12-(S)-HETE, 12-(R)-HETE and PGE_2.

5.5 Baicalein and Celecoxib reduce the tumor weight of A431 xenografts in Swiss mice

In vitro studies on A431 cells have revealed the overexpression of 12-LOX and COX-2 suggesting the possible role in the regulation of cell growth. Further studies have clearly demonstrated the induction of apoptosis in A431 cells by baicalein (12-LOX inhibitor) and/or celecoxib (COX-2 inhibitor). These encouraging results under *in vitro* conditions prompted us to evaluate the efficacy of baicalein and celecoxib in reducing/arresting the growth of A431 xenografts in Swiss mice. The *in vivo* studies confirm the inhibitory effect of LOX/COX inhibitors on human epidermoid cancer xenografts in Swiss mice. The decrease in the tumor weight in animals treated with both baicalein and celecoxib was maximum suggesting that the treatment of skin cancer by dual inhibition of 12-LOX and COX-2 is highly effective. Baicalein and celecoxib induced apoptosis is A431 xenografts as seen in the TUNEL assay. It was demonstrated that skin tumor promotion caused by ultraviolet B radiation can be decreased by up to 89% by inhibiting blocking cyclooxygenase-2 (COX-2) with celecoxib (Thompson *et al*, 2001). A similar study showed that Celecoxib can decrease new tumor formation by 44% in mice that already have tumors (Thompson *et al*, 2001). Baicalein may be acting through the inhibition of 12-LOX activity and expression, as 12-LOX was reported in xenografts of melanoma (Fischer *et al*, 2002) and in skin tumors developed by an initiation/promotion protocol (Akunda *et al*, 2007; Fischer *et al*, 1999). COX-2 inhibitors were also effective in retarding tumor progression and metastasis in mouse models of injected breast cancer cell lines and in xenograft models of human breast cancer cells in nude mice (Basu *et al*, 2004; Kundu *et al*, 2002; Rozic *et al*, 2001; Blumenthal *et al*, 2001). It was shown that dietary celecoxib had a significant chemopreventive activity against UV-induced skin carcinogenesis in SKH-HR-1 hairless mice (Fischer *et al*, 1999) and blocked additional tumor formation after the onset of photocarcinogenesis in hairless mice (Chun *et al*,

2004; Pentland *et al*, 1999). The inhibitory effect of LOX inhibitors on human pancreatic cancer xenografts in athymic mice was reported (Tong *et al*, 2002). The present study also confirms the anti-tumor effect of 12-LOX and COX-2 inhibition on A431 xenografts in Swiss mice.

In summary, the present study demonstrates the predominant expression of 12-R-LOX and COX-2 in human epidermoid carcinoma cell line, A431. Further studies reveal that baicalein (a 12-LOX inhibitor) and celecoxib (a COX-2 inhibitor) significantly reduce the cell proliferation and induce apoptosis in A431 cells via the intrinsic death pathway involving reduction in the Bcl-2/Bax ratio, release of cytochrome c, activation of caspase 3 and PARP cleavage. The apoptosis induced by baicalein and celecoxib was mediated by ERK/AP-1 and Akt/NF-κB pathway. The *in vivo* studies confirm the inhibitory effect of 12-LOX /COX-2 inhibitors on human epidermoid cancer xenografts in Swiss mice. Our findings suggest that 12-LOX and COX-2 have a critical role in the regulation of growth of epidermoid carcinoma and their inhibitors may be of potential therapeutic importance.

6. Summary

Lipids have long since been recognized as signaling molecules that have the capacity to trigger profound physiological responses. Arachidonic acid (AA) oxygenation via cyclooxygenase (COX) or lipoxygenase (LOX) pathways generates a number of bioactive eicosanoids like prostaglandins, leukotrienes, lipoxins and hydroxy eicosatetraenoic acids (HETEs), which regulate cell growth and proliferation as well as survival and apoptosis. Arachidonic acid released from membrane phospholipids plays a central role in tumor cell proliferation. The overexpression of COX and LOX has been reported in a variety of cancers, including colon, pancreas, skin, prostate, oral, epidermoid and liver, suggesting a role for the eicosanoids in carcinogenesis. Under physiological conditions, however, LOXs are known to regulate normal proliferation and differentiation of epithelial cells and kerotinocytes. 12-R-LOX is essential for the maintenance of epidermal barrier function and in the terminal differentiation of the skin. The role of COX is implicated in the maintenance of cell homeostasis and in the regulation of epidermal cell proliferation *in vitro*.

Non melanoma skin cancers, including basal cell carcinoma and squamous cell carcinomas, are the most common type of cancers and occur more frequently than any other type of malignancy in the human population. Skin irritation and injury lead to a rapid but transient activation of AA metabolism. An understanding of the enzymatic pathways involved in AA metabolism is therefore important. In recent years, it has become clear that there are multiple forms of AA metabolizing enzymes expressed in skin of mice and humans. Elevated levels of PGE_2 have been observed in squamous and basal cell carcinomas of the skin and may correlate with an increased propensity for metastatic and invasive behavior. 12-HETE has been shown as one of the main eicosanoids formed by the

epidermis and with the discovery of large quantities of 12-(R)-HETE in human psoriatic lesions, the epidermal 12-LOX has gained considerable interest.

Although the expression of LOX and COX has been reported in various cancer cell lines limited information is available as to whether LOX and COX metabolites are involved in the regulation of epidermoid cancer cell survival. In the present study we elucidate the role of LOX and COX pathways in epidermoid cancer. To evaluate the role of eicosanoids in epidermoid carcinoma, the expression of AA metabolizing enzymes such as lipoxygenases (LOX) and cyclooxygenases (COX) was analysed in human epidermoid carcinoma cell line (A431). These studies revealed the over expression of 12-R-LOX and COX-2 only in A431 cell but not in the normal NIH3T3 cell line suggesting their possible role in promoting the cell proliferation in cancer cells. Incubation of A431 cells with the metabolites of 12-LOX (12-(S)-HETE and 12-(R)-HETE) and COX-2 (PGE_2) promoted the growth, suggesting such a possibility. Further studies involving baicalein (a 12-LOX inhibitor) and celecoxib (a COX-2 inhibitor) showed significant reduction in the thymidine incorporation in A431 cells. These studies thus conclusively demonstrate a role for 12-R-LOX and COX-2 in the regulation of A431 cell proliferation. The mode of cell death induced by baicalein and celecoxib appears to be apoptosis, as revealed by reduction in the Bcl-2/Bax ratio, release of cytochrome c, activation of caspase-3 and PARP cleavage.

The signaling pathways that govern cell proliferation, survival and oncogenesis are of prime interest in cancer biology. Most of the signals associated with cell survival trigger growth factor receptors which activated extra cellular signal-regulated kinase (ERK) and PI3/Akt pathways and thuspromote cell growth. In the present study baicalein and celecoxib induced apoptosis in parallel with the inactivation of ERK in A431 cells. The metabolites 12-(R)-HETE, 12-(S)-HETE and

PGE_2, on the other hand, increased the p-ERK levels, which might be promoting cell proliferation by downregulating apoptosis.

The AKT signaling pathway has been increasingly documented as a prime determinant of tumor promotion and progression in several cell types, including skin. Recent evidences indicate that PI3K/Akt pathway plays a crucial role in tumorigenesis and tumor progression by promoting cell proliferation and inhibiting apoptosis. In the present study the level of phosphorylated Akt was increased in cells treated with the metabolites of 12-LOX and COX-2 and decreased after treatment with celecoxib and baicalein in A431 cells. The results were further validated by the use of ERK specific inhibitor (PD098059) and PI3 Kinase inhibitor (Wortmanin). PD098059 and Wortmanin pretreatment sensitized the cells to baicalein and celecoxib resulting in increase in caspase-3 activity and massive apoptosis suggesting a role for these two survival pathways in the regulation of cell growth in A431 cells. The metabolites of 12-LOX and COX-2, on the other hand increased the phospho ERK and phospho Akt levels, suggesting the involvement of ERK and Akt pathways in the 12-LOX and COX-2 mediated regulation of growth in A431 cells.

Nuclear factor-κB (NF-κB) and activator protein 1 (AP-1) are the key transcription factors that orchestrate expression of many genes involved in inflammation, embryonic development, lymphoid differentiation, oncogenesis, and apoptosis. The present study clearly demonstrates a regulatory role for COX-2 and 12-LOX in the activation of NF-κB as evidenced by its down-regulation with baicalein and celecoxib and up regulation with 12-(R)-HETE, 12-(S)-HETE and PGE_2 treatments. As Activator protein-1 (AP-1) is a downstream target of the ERK pathway and also regulates the expression of cell cycle proteins, we examined the effect of baicalein and celecoxib on the DNA binding and transcriptional activities of AP-1. The present study, clearly demonstrates a regulatory role for COX-2 and

12-LOX in the activation of AP-1 as evidenced by its down-regulation with baicalein and celecoxib and up regulation with 12-(R)-HETE, 12-(S)-HETE and PGE_2 treatments.

These *in vitro* studies on epidermoid carcinoma cell line were extended to animal models by monitoring the growth of A431 xenografts in Swiss mice. Both baicalein and celecoxib reduced the tumor weight in A431 xenografts in Swiss mice. The tumor sections of the animals treated with baicalein and celecoxib showed significant apoptosis as compared to tumors from untreated animals. Further studies on nude mice might provide conclusive evidence on the usefulness of COX-2 and 12-LOX inhibition in the control of epidermoid carcinoma. The molecular mechanisms and signaling pathways involved in the regulation of growth in epidermoid carcinoma by 12-LOX and COX-2 pathways are presented in Figure 33.

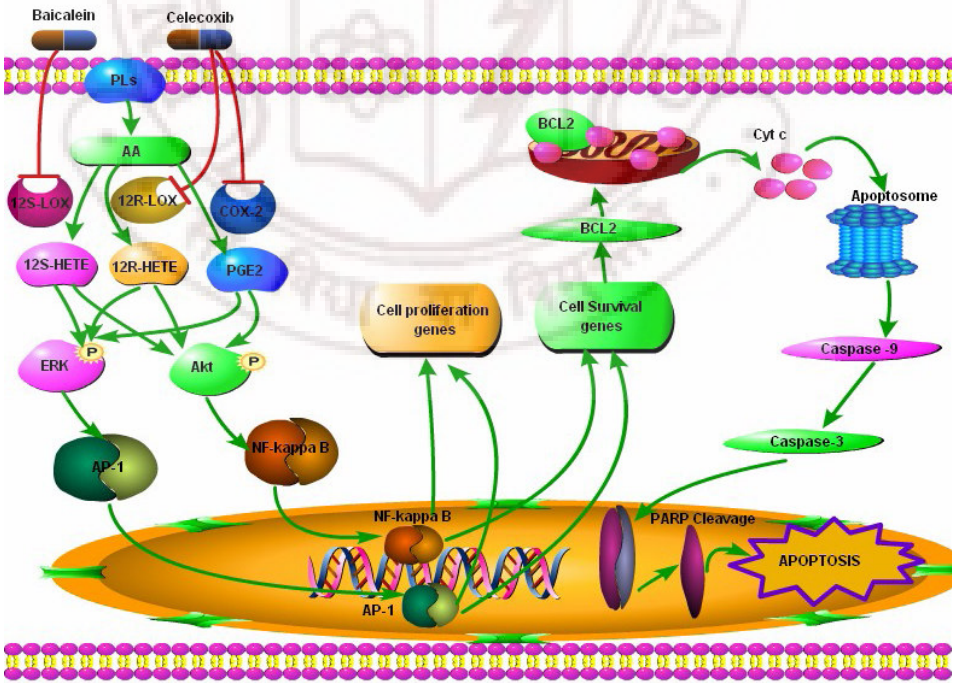


Figure 33: Schematic representation of the proposed mechanisms involved in the promotion of growth in A431 cells by 12-LOX and COX-2 pathways and the inhibition in the growth by baicalein and celecoxib.

In summary, the present study reveals that 12-LOX and COX-2 have a critical role in the regulation of growth of epidermoid carcinoma and the combined therapy of inhibitors of 12-LOX and COX-2 may be of potential importance.

7. References

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8. List of Publications

- **Smita Agarwal**, Chandrani Achari, D. Praveen, K. R. Roy, G. V. Reddy, P. Reddanna (2008). Inhibition of 12-LOX and COX-2 reduce the proliferation of Human epidermoid carcinoma cell (A431) by modulating the ERK and PI3-Akt signaling pathways. *Experimental Dermatology* (Revised)

- **Smita Agarwal,** G. V. Reddy, P. Reddanna (2008). Eicosanoids in Inflammation and Cancer: Role of COX-2. *Expert Reviews in Clinical Immunology (In press)*

- K. R. Roy, **Smita Agarwal**, M. Leela, G. V. Reddy, Shireen Vali, P. Reddanna (2008). Celecoxib inhibits MDR1 expression through COX-2 dependent mechanism in human hepatocellular carcinoma (HepG2) cell line. *Biochemica et Biophysica Acta* (Revised)

- MBCR Naidu and **Smita Agarwal** (2008). Anti-proliferative effect shudda guggul (*Commiphora wightii*) extract in comparison with one of its active principles guggulsterone on A431 cell line. *Apoptosis* (Under review)

- S. Padma, **Smita Agarwal**, D. B. Reddy, T. S. Prasad, P. Reddanna (2007) Metabolism of arachidonic acid in sheep uterus: in vitro studies. *Indian J Biochem Biophys*. 44(4): 216-222.