Modulating Cyclooxygenase And Lipoxygenase Pathway In Cancer Chemoprevention

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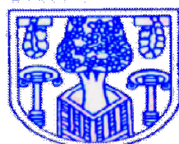

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DECLARATION

I hereby declare that the thesis entitled **"Modulating Cyclooxygenase And Lipoxygenase Pathway In Cancer Chemoprevention"** has been prepared by me under the supervision of **Dr. Gaurav Kaithwas** at Department of Pharmaceutical Sciences, School of Biomedical and Pharmaceutical Sciences, Babasaheb Bhimrao Ambedkar University, Lucknow (U.P.).

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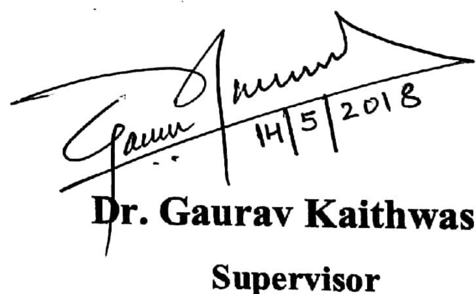

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CERTIFICATE

This is to certify that the thesis entitled **"Modulating Cyclooxygenase And Lipoxygenase Pathway In Cancer Chemoprevention"** submitted by **Ms. Swetlana** is an original research work and has not been previously submitted in part or full, for the award of any other degree or diploma to this or any other university.

The thesis submitted to Babasaheb Bhimrao Ambedkar University, Lucknow India, satisfies all the requirements as stipulated in the Doctor of Philosophy (Ph.D.) regulations -1999 as amended in 2008/2010/2013 and it is fit for submission and evaluation for the award of the degree of Doctor of Philosophy of the University.

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ABBREVIATIONS

• AA	Arachidonic acid
• AB	Alveolar bud
• ATP	Adenosine triphosphate
• Bcl-2	B-cell chronic lymphocytic leukaemia-2
• BSA	Bovine serum albumin
• COX	Cyclooxygenase
• CEC	Cuboidal epithelial cell
• DCT	Dense connective tissue
• ECG	Electrocardiogram
• ERK	Extracellular signal regulated kinase
• FLAP	5-LOX activating protein
• GSH	Glutathione
• GDP	Guanosine diphosphate
• GTP	Guanosine triphosphate
• GPC	Glycerophosphocholine
• H&E	Hematoxylin & eosin
• HETE	Hydroeicosa-tetraenoic acid
• HPETE	Hydroperoxyeicosa-tetraenoic acid
• 5-HETE	5-hydroxytetraenoic acid
• HRV	Heart rate variability
• HR	Heart rate
• HF	High frequency
• LF	Low frequency
• LCT	Loose connective tissue
• LDL	Low density lipoprotein
• LOX	Lipoxygenase
• LT's	Leukotrienes
• LXs	Lipoxins
• MAPK	Mitogen activated protein kinase
• MNU	N-methyl-N-nitrosourea
• MEC	Mypepithelial cell

- NAG N-acetyl glycoprotein
- NF-Kb Nuclear factor-kappa B
- NSAIDs Non-steroidal anti-inflammatory drugs
- OAG O-acetyl glycoprotein
- OPLS-DA Orthogonal projection to latent structure with discriminant analysis
- PC Phosphocholine
- PCA Principal component analysis
- PGI_2 Prostacyclin
- PG's Prostaglandins
- PI3K/AKT Phosphoinositide-3 Kinase/AKT
- PIP_2 Phosphatidylinositol 4, 5-bisphosphate
- PIP_3 Phosphatidylinositol-3,4,5-triphosphate
- PKC Protein Kinase C
- PLA_2 Phospholipase A_2
- PLS-DA Partial least squares discriminant analysis
- PPAR Peroxisome Proliferator Activated Receptor
- PUFA Polyunsaturated fatty acids
- PS Phosphatidylserine
- ROS Reactive oxygen species
- RTKs Receptor tyrosine kinase
- RXR Retinoid X receptor
- SOD Superoxide dismutase
- TBARs Thiobarbituric acid reactive substances
- TX's Thromboxane
- VGEF Vascular endothelial growth factor
- VLDL Very low density lipoprotein

Chapter 1

Introduction and Review of Literature

1. Introduction

Among the vast number of factors involved in tumor progression, arachidonic acid (AA) and its metabolites arewell perceived for their convincing role in cancer biology.Affirmations for the role of inflammation in cancer comesfrom a large number of epidemiological findings illustrating that prolonged treatment with a number of anti-inflammatory drugs, including non-steroidal anti-inflammatory drugs (NSAIDs) can scale down the incidence of several human cancers[Dubois, 2004; Green et al., 2011; Hammamieh et al., 2007; Jänne and Mayer, 2000;].

AA (5, 8, 11, 14-eicosatetraenoic acid) is an essentialfatty acid and a member of the omega-6 (x-6) polyunsaturated fatty acids (PUFA) [Astorg, 2004]. AA is required by themajority of mammals and plays a key role in metabolism,cell signaling and inflammation. The impetus of theenzyme phospholipase A2 releases AA from membranephospholipids, which is further metabolized by the two keyenzymes, namely cyclooxygenase (COX) and lipoxygenase(LOX). The metabolites of the AA are biologically activelipids' mediators and are termed as eicosanoids. Eicosanoids have a strategic role in diverse biological processes,including the regulation of immune response, development,reproduction and cancer likewise [Kawai et al., 2002]. As a result, inhibitionof AA metabolism has been aforethought as target area forchemotherapeutic manipulation of discrete types of cancer[Harris et al., 2014].

The COX and LOX are overexpressed in several cancersincluding the colon, breast, lung, pancreas, and esophaguswhich established the link between cancer and inflammation [Harris et al., 2014; Harris, 2009]. Two isoforms of COX (COX 1 and 2) catalyzethe rate-limiting steps in the biosynthesis of prostaglandins(PGs). The

biological functions and pattern of expressiondiffer betwixt these two isoforms. COX-1 is ubiquitouslypresent in all cells, whereas COX-2 is perused expeditiously in pathological conditions, for instance, in malignant and inflamed cells [Dempke et al., 2001]. COX-1 is expressed in mosttissues and is accredited to mediate normal physiologicalfunctions. COX-2 is overexpressed in tumor cells andresembles to have an important role in tumorigenesis [Ristimäki et al., 2001].COX-2 enzyme is induced by cytokines, growth factors,peroxisomal proliferator, tumor promoter and oncogenes[Sinicrope and Gill, 2004], and hence, the metabolic products of COX-2 areanticipated to have a far-reaching prospect in oncologyresearch [Lyons et al., 2014; Zha et al., 2004]. The mechanism by which COX-2 contributes to tumorigenesis through regulating the apoptosis,upsurged angiogenesis, invasiveness, modulation ofinflammation/immunosuppression and conversion of procarcinogen to carcinogen. Liu and colleagues have validated the inflated levels of COX-2 mRNA transcript, proteins and enzymatic activity in mammary glands' cancer[Liu et al., 2001].

The LOX and its metabolic products impersonate adecisive role in inflammatory pathways. MammalianLOX is majorly allocated into 5-, 12-, 15-LOX, whichproduces 5S-, 12S- or 15S hydroperoxyeicosa-tetraenoicacid (5-, 12-, 15-HPETE) and hydroeicosatetraenoic acid(5-, 12-, 15-HETE). It is the only 5-HPETE that can befurther metabolized to leukotrienes (LTs). Activation of5-LOX is dependent upon a second factor termed as5-LOX activating protein (FLAP) [Qu et al., 2000; Hatzelmann et al., 1994]. Theseenzymes impede the normal physiological function of the cells and take part in the apoptosis, angiogenesis, invasiveness, proliferation and

conversion of pro-carcinogento carcinogens [Nie et al., 2000; Nie et al., 2001; Ye et al., 2005]. Both the enzymes, COX-2 and5-LOX, have been implicated to be associated withcancer amelioration in one way or other by a large set ofresearch groups.

The association between the cancer progression, 5-LOX,and COX-2 is also sustained by the reports of escalatedproduction of LTB4 (5-LOX derived product) and PGE2(COX-2 derived) in malignant cells [Ye et al., 2005]. Similarly, the dualinhibitors of AA metabolism (Licfelone, flavocoxid andpsoralidin) [Altavilla., 2012; Tavolari et al., 2008; Ye et al., 2005] have been proclaimed to haveabrogating effect of the cancer progression. Owing to theabove and several other reports, a group of researchers hasparticularized favorable amicable effects of MK886 (5-LOXinhibitor) and celecoxib (COX-2 inhibitor) against pancreatic cancer [Ding et al., 2011]. Thereby, a large set of data has emerged inthe recent past commending the importance of dual COX-2/5-LOX (DuCLOX-2/5) inhibition in cancer progression.However, no explicit physiological/molecular mechanismhas been contemplated till date, except a review on the biochemical aspects of DuCLOX-2/5 inhibition [Schneider and Pozzi, 2011]. Considering the important physiological importance of DuCLOX-2/5 inhibition in cancer progression and lack of a suitable review to elaborate the after effects of DuCLOX-2/5inhibition, the present review was undertaken to amplify theeffects of DuCLOX-2/5 inhibition on various stages ofcancer progression. Henceforth, the present review attemptsto epitomize the new panorama towards defining the roles ofthe enzymes COX-2 and 5-LOX in

carcinogenesis andemphasizes the potential of DuCLOX-2/5 inhibition as atarget for cancer chemotherapy.

1.1.AA metabolism

AA-derived PUFA are the precursors for diversified imperative signaling molecules including the prostanoids, LTs, and lipoxins (LXs). AA is metabolized to PGE2, prostacyclins (PGI2) and thromboxanes (TXs) by COX [Hoque et al., 2005; Larsson et al., 2004; Needleman et al., 1986] and LTs by LOX. Oxygenation of AA forms PGG2 by COX-1 and 2 which get further reduced to PGH2. The PGH2 is highly unstable and is catabolized to the series of PGs by their respective synthase and isomerase enzymes. PG synthase forms a series of PGs named E2, F2a and D2, and PG isomerase forms PGI2 and TXA2 (an unstable compound) [Laesson et al., 2004]. The TXA2 is hydrolyzed rapidly to TXB2 an almost metabolically inactive compound) [Holtzman, 1991]. In the LOX pathways, 5-LOX catalyzes both the first step in oxygenation of AA to produce 5-HPETE with the consequent production of 5-HETE and LTs including LTA4, LTB4, LTC4, LTD4, and LTE4. The enzyme 12-LOX produces 12-HPEPE to 12-HEPE and 15-LOX produces 15-HPEPE TO 15-HEPE to LXs [Holtzman, 1991] (Fig. 1).

1.2.COX products and cancer progression

The interconnection between the inflammation, cancer and COX products has invariably been of interest. Published reports endorse the upsurge of COX-2 and its products in cancerous cells [Saukkonen et al., 2004; Shiota et al., 1998]. Since COX-2 products could get slightly increased in normal tissues but COX-2 is highly

expressed in tissue showing characteristics of inflammation and tumor-like properties [Sano et al., 1995]. Apart from the fact that the origin of COX-2 overexpression in tumor cells is in dilemma, the COX-2 and its metabolic products have been convoluted in discrete aspects in tumor progression as particularized in the forthcoming section.

1.2.1.Proliferation and apoptosis

Imbalance between cell proliferation and cell death leads to the generation of cancer. Indeed, COX-2 and PGE2 are known to galvanize the production of several growth factors, for instance, vascular endothelial growth factor (VEGF) [Hatazawa et al., 2007; Williams et al., 2000]. Apoptosis is a key event of signal-controlled suicide of cells harmonised by distinct series of cellular changes. The prime executors of apoptosis are several proteases termed as caspases [Greene and Reed, 1998]. Two classes of caspases have been identified as initiator and effector caspases. Initiator caspases are caspase-2, 8, 9 and 10 that trigger or activate theeffector caspase-3, 6 and 7 [Tavolari et al., 2008]. In regulation of apoptoticsignaling pathway, the B-cell chronic lymphocytic leukaemia-2 (Bcl-2) protein family plays an important role byacting as a checkpoint at mitochondria, governing theactivation of caspases and cytochrome c release [Bossy-Wetze and Green, 1999; Daniel, 2007].The Bcl-2 protein family includes both anti-apoptotic andpro-apoptotic members. They can homodimerize orheterodimerize to oversee each other's function. Otherproteins of Bcl-2 family constitute death proteins such asBax, Bak, Bad, Bid, Bim, Noxa, and Puma stimulatesapoptosis [Daniel, 2007; Gross et al., 1999; Hrdwick and Soane,

2013]. On activation of the apoptotic signal,interconnected series of caspases cascade starts by provoking each other [Elmore, 2007]. The proteins cleaved by theeffector caspases function as a vital protein to hold thenormal physiology of the cellular processes [Ly et al., 2003] (Fig. 2).

The COX-2 expression and inhibition of apoptosis resultin altered balance betwixt pro- and anti-apoptotic factors incancer cells [Hunter et al., 2007]. Pro-apoptotic protease caspase-3 isrecorded to be downregulated with increase in COX-2 and5-LOX expression [McGinty et al., 2000]. The COX-2 overexpression/PGE2upregulation and inhibition of apoptosis have been established to be affiliated with an upsurged expression of theanti-death protein Bcl-2 and caspase-3 [Li et al., 2005 Wang and DuBois, 2004]. Four- tofive-fold escalation of Bcl-2 protein levels but not Bax andBcl-xl was reported with augmented levels of PGE2[Li et al., 2005; Sheng et al., 1998].PGE2 also governs the apoptosis through phosphoinositide-3 kinase/AKT (PI3K/AKT) signaling pathway. On binding of the ligand to the receptor tyrosine kinase(RTKs), the active PI3K migrates to the inner side of thecell membrane and binds to the regular component of thecell membrane, i.e. phosphatidylinositol 4, 5-bisphosphate(PIP2). PI3K phosphorylates PIP2 to phosphatidylinositol(3, 4, and 5) triphosphate (PIP3) and actuates AKT, alsoknown as protein kinase B. Consequentially, AKT bindsto the Bax and hinders the caspases' mediated cellapoptosis. The metabolic product of eicosanoids, PGE2,augments the expression of the PI3K/AKT signalingpathway (Fig. 3).

The COX-2 metabolic product, PGI2, persuades apoptosis and damages the DNA by the peroxisome proliferatoractivated receptor (PPAR) signaling pathway [Wu and

Liou, 2009]. PPARis a transcriptional factor belonging to the ligand-activatednuclear hormone receptor super family. The three isotypesof this receptor family are PPAR-a, -c and -d, each encoded by an autonomous gene, of which PPAR-c is the isotype on which the current research is focused (Table 2).PPAR-c is also expressed in manifold cancers includingbreast, colon and lung [Michalic et al., 2004]. PPAR-cplays an extensivemajor role in allocating cellular signaling during tumorigenesis [Avis et al., 2005;Inoueet al., 2000]. By diversified signaling cascades, activated PPAR-c and retinoid X receptor (RXR) dimerimpede tumor cell growth and tumor metastasis. ActivatedPPAR-c and RXR dimer lead to the inflated formation ofthe cadherin protein that involves in cell-cell adhesionresulting in increased cell adhesion and decreased cell migration. Activated PPAR-c and RXR dimer also down regulate the production of various cytokines and curtail the angiogenesis [Panigraphy et al., 2005] (Fig. 4).

During cellular mitogen activated protein kinase (MAPK) pathway, binding of the ligand to the RTKs prompts Ras-bound guanosine diphosphate (GDP) protein in exchange of guanosine triphosphate (GTP). This change leads to the activation of the Ras protein bound to GTP. In its active state, it phosphorylates and activates MEK 1 and 2 which again in turn phosphorylate and actuate extracellular signal regulated kinase-1 and 2 (ERK 1 and 2). Eventually, via discrete machinery cascades, it leads to the expression of many genes and coding for the growth factors, cyclins and cytokines entangled in cell proliferation and apoptosis. Active GTP-bound Ras overexpressed in tumor cell which normally get inactivated shortly after activation. The overexpression of active Ras continuously phosphorylates and activates ERK 1 and 2 leads to the

uncontrolled proliferation of the cells. Eicosanoids derived PGs series such as PGE2 are the potent ligand for the MAPK and prompts the over-activation of the MAPK pathway in tumor cells and thus trigger cell proliferation (Fig. 5).

1.2.2.Angiogenesis and metastasis

The growth of solid tumor and the metastasis are dependent on the evolution of the new blood vessels [Basu et al., 2004; Tsujii et al., 1998]. Growthfactors, i.e. vascular endothelial growth factor (VEGF),basic fibroblast growth factor (bFGF), platelet derivedgrowth factor (PDGF) and transforming growth factor(TGF-a) stimulate angiogenesis and are secreted by thetumor cells. These growth factors are known to play a keyrole in vascularization and afterwards initiate the metastasis process. The metabolic product of the COX-2, inparticular PGE2, contributes to metastasis by neovascularization, by promoting excess secretion of several metastatic growth factors [Chang et al., 2004;Hatazawa et al., 2007;Karavitis et al., 2012; Tsujii et al., 1998]. The COX-2inhibitors are known to inhibit the VEGF and have beenproclaimed to regulate metastasis in evidenced literature.The PGE2 works in a way to impede the growth factors,VEGF and TGF-a [Kuwano et al., 2004].

In terms of angiogenesis, COX-2 activity was found toincrease the stimulation of VEGF and in activating the PPAR pathway [Dempke et al., 2001]. Likewise, COX metabolites, TXA2 and TXB2, are known to involve in angiogenesis and decreased apoptosis in cancerous cells, respectively [Michel et al., 2006]. TXA2 and LOX metabolite LTA4 activate VEGF [Romano et al., 2001]. TXA2 and TXB2 also vitalize the cancer cell proliferation by regulating diversified cyclins, involved in cell

cycle via PPAR-c pathway. In this pathway, activated PPAR-c and RXR complex downregulates cyclin E mediated cell cycle arrest and upregulates cyclin kinase inhibitor p21 decreases cell proliferation.

1.3.LOX products and cancer progression

In the 5-LOX pathway, LOX catalyzes the oxygenation of AA to produce 5-hydroxytetraenoic acid followed by production of 5-HETE and LTs. 5-LOX requires cofactors such as calcium, ATP (adenosine triphosphate) and 18 kDa protein termed as 5-LOX activating protein (FLAP) for its full activity to use AA as substrate [Brown et al., 1999; Pouliot et al., 1996; Qu et al., 2000]. Pathophysiologically, 5-LOX's metabolic products are recognized in regulation of inflammation and immune response. A considerable body of literature advocates the carcinogenic potential of 5-LOX's products. Various LOX products have been linked to tumorigenesis in experimental models and consecutive inhibition of LOX metabolism has been targeted as anticarcinogenicintervention [Pidgeon et al., 2007]. Experimental models of cancer progression studies have displayed upsurge in expression of LTs in cancer tissues in resemblance to normal tissues [Ihara et al.,2007; Pidgeon et al., 2007].

1.3.1.Proliferation and apoptosis

The significant role of 5-LOX in cellular proliferation and apoptosis has been documented in foregoing literature and the same is found to be overexpressed in tumor cells with profound cellular proliferation. Upregulated expression of LTB4 (5-LOX derived) in chronic inflamed cells wrecks the balance between the normal growth of the cells and apoptosis [Ye et al., 2005]. LTB4 is a potent activator of

neutrophils chemotaxis and transendothelial migrator, whereas rest of the LTs are key mediators of allergic inflammation. LTB4has a major role in disrupting the normal physiologicalprocess of NF-kB (nuclear factor-kappa B) pathway whichsubsequently results in augmented proliferation. In anearlier study, LTB4 has been divulged as a stimulator forescalated DNA synthesis and proliferation in vitro inhuman pancreatic cells PANC-1 and AsPc1, where thereceptor antagonists for LTB4 blocked the growth of thecells and induced apoptosis in pancreatic cells both in vitroand in vivo [Tong et al., 2002]. The pathways of MAPK, NF-kB andPI3K/AKT majorly augment the disruption of normalgrowth of the cells, leading to the uncontrolled cell growthand upturned resistance to the cell death. LTB4 inducesphosphorylation of ERK through MAPK pathway andupraised cancer cell proliferation. As discussed earlier,death protein, Ras, on overexpression, continuously phosphorylates and activates ERK-1 and -2 leading to theuncontrolled proliferation of the cells.

A dramatic increase in the level of anti-apoptotic proteinsBcl-2 and marked subsidence in the pro-apoptotic proteinBax is seen on LOX overexpression [Pidgeon et al., 2007; Tong et al., 2002; Wong et al., 2001] (Fig. 2).

Metabolic products of 5-LOX like 5-HETE and LTA4,LTC4, LTD4 and LTE4 increase tumor cell proliferationand decrease apoptosis in cancer cells and produceinflammatory effects in host cells [Wallace, 2002]. However, LTD4 andLTE4, and 5-HETE also activate ERK through PPAR-cpathway and induce normal cell proliferation [Lee et al., 2002; Paruchuri et al., 2002].

The cysteinyl LTs, LTC4 and LTD4 augment cell proliferation induced by mitogen epidermal growth factors.LTC4 induces cancer cells by PKC alpha (protein kinase

C)mediated phosphorylation and LTD4 also induces proliferation by activating ERK through MAPK pathway [Mahshid, 2006].

1.3.2.Neo-angiogenesis and metastasis

A number of studies accustomed the anticancer activity of 5-LOX inhibitors by modulation of cancer cell proliferation and metastasis and advocate the crucial role of 5-LOX in tumorigenesis and its progression [Romano ad Claria, 2003]. Indeed, 5-LOX and LTB4 (metabolic product of LOX enzyme) are known to stimulate the production of several growth factors and inhibit the apoptotic machinery, promote cell proliferation and growth [Nieves D, Moreno, 2006; Tong et al., 2002; Wada et al., 2006; Zhou et al., 2007].

1.4.DuCLOX-2/5 inhibition

Large number of evidences can be summed up to the conclusion that the AA pathway has a distinct combination of metabolites which could be promising, and some amalgamations could be disastrous. Some metabolites of AA like PGD2, PGI2 and PGF2a have protective effects on cell undergoing cell damage and upsurge alterations [Paruchuri et al., 2002]. Whereas, manifold AA derivatives including COX-2 metabolites PGE2, TXA2 and TXB2; and 5-LOX metabolites LTA4, LTB4, LTC4, LTD4 and LTE4 are known to wreck the normal physiology of cell thus presuming them towards the distorted cancerous pathway through variable mechanisms.

The products like TXA2 and TXB2 are entangled in angiogenesis and decrease apoptosis in cancer cells [Gross et al., 1999; Nie et al., 2000]. TXA2 stimulates VEGF and promotes angiogenesis [Daniel, 2007;Michel et al., 2006]. Alongside,

PGE2 uplifts proliferation in cancer and host cell by the activation of MAPK and also impedes apoptosis by elevating Bcl-2 protein level in cells.

The LTB4, LTC4, LTD4 and LTE4 (end products of 5-LOX enzymes) are deliberated as cancer-enhancing agents divulged in several data in the recent times. LTD4 and LTE4 activate the ERK which augment the cell proliferation through PPAR-c pathway and subsequently results in cancer progression [Paruchuri et al., 2002]. Another metabolite of the LOX enzymes is LTA4, which can abolish tumor growth by inhibiting VGEF and that could be beneficial in the cancer chemoprevention. The LTA4 is a first metabolite which is further converted to LTB4, LTC4, LTD4 and LTE4 and hence, its role in suppression of tumor growth could not be neglected. LTC4 also induces cancer cell by inhibiting apoptosis via PKC-a mediated phosphorylation [Mahshid, 2006]. Various HETEs are formed by virtue of distinct LOX enzymes such as 5-HETE, 12-HETE and 15-HETE. 5-HETE formed through 5-LOX has a significant role in tumor cell proliferation and is known to induce cancer by upregulating the PPAR-d pathway. From the above, it became conspicuous that the metabolic products of AA, in particular, derived through COX-2 and 5 LOX have a momentous role to play. Conspicuously, COX-2 derived PGE2, PGI2, TXA2 and TXB2; and 5-LOX arbitrated LTA4, LTB4, LTC4, LTD4 and LTE4 have a major saytowards the cellular proliferation, angiogenesis andmetastasis.

Hence, it appears indisputable that the COX-2 and5-LOX derived products can exploit diversified pathwaysto exert their procarcinogenic effects and inhibiting thesame could provide wider prospects to the current cancermanagement strategies. Authors would also like to add thatdual inhibition can be more advantageous in

comparison toinhibition of individual pathways and can be summarizedas follows: a) Dual inhibitors act upon the two majormetabolic acid metabolic pathway and possess a widerange of anti-inflammatory activity, b) Dual inhibitors areexempted from gastric toxicity, which is most troublesomeside effect of COX inhibitor [Wallace et al., 2000], c) Recent studies haveimplicated selective COX-2 inhibitors to be associated withcardiotoxic effects [Brophy, 2007], however dual inhibition will helpto counteract the cardiotoxic effects associated with COX-2 inhibitors and one of the most associated deleteriouseffects of the anticancer therapies, and d) COX and LOXderivatives (prostanoids and leukotrienes, respectively) areinvolved in other diseases than inflammation such as proliferation where the use of dual inhibitors could provide anadditive benefit [Zhang et al., 2005].

1.5.DuCLOX-2/5 inhibition: gaps and lacunae

Results from the preclinical studies and clinical trials have elucidated the role of inhibition of arachidonic acid metabolism in cancer progression [Goossens et al., 2007]. Combined effect of DuCLOX-2/5 inhibition in carcinogenesis represents various advantages over individual inhibition of COX or LOX. However, the lacunae in chemotherapeutic usage of DuCLOX-2/5 inhibitors rely on the lack of sufficient literature in various human cancers.

Numbers of evidences now indicate inhibition of DuCLOX-2/5 leads to the alteration of AA metabolism with carcinogenesis and now has been considered as potential anticancer agents. Reports have investigated that COX-2 and 5-LOX being primarily involved not only in producing inflammation but also in cell proliferation and neo-angiogenesis [Claria and Romano, 2005]. Thus, it evokes the researchers to

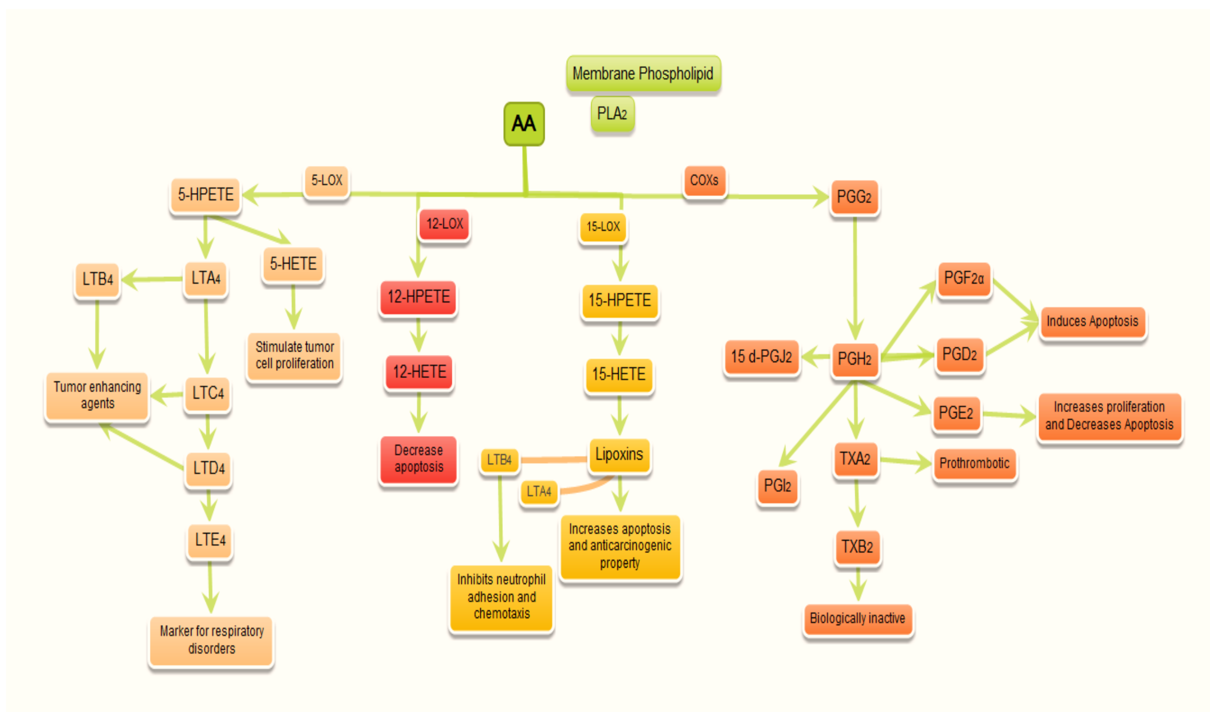


Figure 1: Metabolism of Arachidonic acid.Arachidonic acid is metabolized by one of two distinct pathways. Cyclooxygenase converts arachidonic acid to the unstable cyclic endoperoxides, PGG2 and PGH2, which are converted to prostaglandins and thromboxanes by respective synthases and isomerase enzymes. The lipoxygenase metabolizes arachidonic acid to cyclic hydroperoxides (HPETEs), which are then further converted to HETE and leukotriene A4. Leukotriene A4 is then further metabolized to LTB4, LTC4, LTD4 and LTE4. PLA2 Phospholipase A2, AA arachidonic acid, 5-LOX 5-lipoxygenase, 5-HETE 5-hydroxytetraenoic acid, 12-HPETE hydroperoxyeicosa-tetraenoic acid, LTs leukotrienes, LTA4 leukotriene A4, PGE2 prostaglandin E2, PGF2a prostaglandin F2a, PGD2 prostaglandin D2, PGI2 prostacyclin, PGH2 prostaglandin H2, TXA2 thromboxane A2, TXB2 thromboxane B2

design new drugs of DuCLOX-2/5 inhibitors to direct the chemotherapy of human cancers. One study demonstrated the combined treatment with COX-2 inhibitor celecoxib and 5-LOX inhibitor MK886 had additive effects on inhibiting tumor cell proliferation, inducing cell apoptosis, decreasing Bcl-2 expression, increasing Bax expression in human colon cancer cells [Cianchi et al., 2006]. Another study evaluated the effect of dual inhibitor of COX-2 and 5-LOX, flavocoxid, in experimental benign prostatic hyperplasia (BPH) and was found to reduce BPH

through modulation of eicosanoid production [Altavilla et al., 2012]. Large set of data is available to define the role DuCLOX-2/5 inhibition in cancer chemoprevention; however authors could find some scope for further research with few gaps to be filled. The same could be majorly summarized as: (1) adequate number of investigation in various human cancers using fixed dose combination could be of scientific interest and is a matter of investigation, (2) role of LOX in non-solid tumors needs to be investigated as there is lack of sufficient scientific literature, (3) there is scope and need to develop a single pharmacological agent with COX-2 and 5-LOX inhibiting potential, and (4) variability in effect of COX and LOX on various aspects of tumorigenesis stand still as research question. DuCLOX-2/5 inhibition: breast cancer Estrogen (ER) is a major regulator of the endothelial cell growth and angiogenesis in physiological and pathological process. A study on the breast cancer cell lines has demonstrated that COX-2 is overexpressed in the estrogenindependent, highly invasive metastatic cell line. More recently, high levels of COX-2 proteins were reported in breast cancers cases with overexpression of HER-2/neu, in comparison to HER-2/neu-negative breast cancers [Howe et al., 2002]. Additionally, PGs (the metabolite of COX), and estrogen are the key molecules linked together for the angiogenesis [Tamura et al., 2004].

Clinical studies failed to establish any significant correlation between COX-2 overexpression and estrogen (ER), and progesterone (PR) receptors in the clinical cases of endometrial and mammary gland carcinoma. There was no difference in the distribution of COX-2 positive cases according to ER or PR positivity in the endometrial cancer cases. It would be appropriate to remark that increased COX 2 expression has been reported to be associated with larger tumor size and advanced

clinical stage, although lymph node status, ER/PR expression, nuclear and histologic grade were not significantly correlated in the clinical cases of mammary gland carcinoma [Ferrandina et al., 2005].

In corroboration to COX-2, researchers have revealed significant positive correlation between 5-LOX expression and clinical cases of mammary gland carcinoma. As observed for COX, significant correlation was established between the LOX expression and TNM staging in the mammary gland cancer; however, it was nonsignificant in case of ER and PR. COX-2 overexpression was positively correlated with the HER2/neu (oncogene) expression, which was non-significantly associated with ER and PR expression [Simeonete al., 2004]. Although LOX expression was positively correlated with the cancer progression researchers failed to find any correlation between HER2/neu and subsequently ER and PR.

1.6.DuCLOX inhibition as target chemotherapy for cancer

The interconnection between inflammation, cancer progression, COX-2 and 5-LOX products has invariably been of interest. Published reports have endorsed the over expression of COX and LOX in several cancers who depicts the link between cancer and inflammation. Emerging evidences proclaimed upsurge production of COX-2 and 5-LOX products in malignant cells [Harris, 2009; Harris et al., 2014]. These enzymes interfere with normal physiological function within the cells and take part in the apoptosis, angiogenesis and invasiveness, proliferation and conversion of pro-carcinogen to carcinogens [Nie et al., 2000, 2001; Ye et al., 2005].While the expression of individual enzyme, the dual inhibition of AA metabolism has also been increasingly appreciated for their importance in cancer progression. Since no

experimental data has been exercised in the direction of dual COX and LOX inhibition impeding to cancer, suggesting plethora of biochemical/physiological element needs to be understood and performed [Dempke et al., 2001; Ristimäki et al., 2002; Schneider and Pozzi, 2011]. Authors considered it worth elaborating the effect of dual inhibition of AA metabolism on cancer progression. The proposed study presents the insight of differing roles of the enzymes COX-2 and 5-LOX in mammary gland carcinogenesis and emphasizes the potential of DuCLOX inhibition as target chemotherapy for cancer.

1.7.Literature Review

Pender et al, 2013: Study was undertaken to investigate whether 6-Desaturase and PGE_2 level was affected during Arachidonic Acid synthetic pathway. The study was conducted on 69 breast cancer patients. The results showed that the endogenous Arachidonic Acid synthetic pathway, 6-Desaturase activity, and PGE_2 levels are increased in breast tumors, particularly those of the ER- genotype. These findings suggest that the AA synthetic pathway and the 6-Desaturase enzyme in particular may be involved in the pathogenesis of breast cancer. The development of drugs and nutritional interventions to alter this pathway may provide new strategies for breast cancer prevention and treatment.

Askari et al, 2013: p21 (Waf-1) is a cyclin-dependent kinase inhibitor that plays essential roles in cell growth arrest, terminal differentiation, and apoptosis. Statistically significant difference in the level of methylation of p21/CIP1 between the patients with breast cancer and the healthy controls was observed. Risk of breast cancer was increased in patients with hypermethylated p21/CIP1 promoter by 2.31-fold. The downregulation of p21/CIP1 mRNA expression was statistically significant in patients with methylated promoter in comparison to patients with unmethylated genes.

Sensorn et al, 2013: The aim of this study was to investigate the impact of CYP3A4/5, ABCB1, and ABCC2 polymorphisms on the risk of recurrence in Thai patients who received tamoxifen adjuvant therapy. Patients with early-stage breast cancer who received tamoxifen adjuvant therapy were recruited in this study. Patients

with heterozygous ABCB1 3435 CT genotype showed significantly shorter DFS than those with homozygous 3435 CC genotype (P = 0.041). In contrast, patients who carried homozygous 3435 TT genotype showed no difference in DFS from wild-type 3435 CC patients. Cox regression analysis showed that the relative risk of recurrence was increased by five times (P = 0.043; hazard ratio = 5.11; 95% confidence interval: 1.05-24.74) in those patients carrying ABCB1 3435 CT genotype compared to those with ABCB1 3435 CC.ABCB1 3435 C>T is likely to have a clinically significant impact on recurrence risk in Thai patients with breast cancer who receive tamoxifen adjuvant therapy.

Michael et al, 2003: One in ten of all new cancers diagnosed worldwide each year is a cancer of the female breast, and it is the most common cancer in women in both developing and developed areas. It is also the principal cause of death from cancer among women globally. Worldwide, breast cancer accounts for 22.9% of all cancers (excluding non-melanoma skin cancers) in women. In 2008, breast cancer caused 458,503 deaths worldwide (13.7% of cancer deaths in women).Breast cancer is more than 100 times more common in women than in men, although men tend to have poorer outcomes due to delays in diagnosis.Few of the important work contributing towards breast cancer research are enumerated below.

Greene et al., 2011: Inflammation in the tumor microenvironment is now recognized as one of the hallmarks of cancer. Endogenously produced lipid autacoids, locally acting small molecule lipid mediators, play a central role in inflammation and tissue homeostasis, and have recently been implicated in cancer. A well-studied group of

autacoid mediators that are the products of arachidonic acid metabolism include: the prostaglandins, leukotrienes, lipoxins and cytochrome P450 (CYP) derived bioactive products. Novel experimental approaches that demonstrate the anti-tumor effects of inhibiting cancer-associated inflammation currently include: eicosanoid receptor antagonism, overexpression of eicosanoid metabolizing enzymes, and the use of endogenous anti-inflammatory lipid mediators. Here they reviewed the actions of eicosanoids on inflammation in the context of tumorigenesis. Eicosanoids may represent a missing link between inflammation and cancer and thus could serve as therapeutic target(s) for inhibiting tumor growth.

Liu et al., 2010: The cyclooxygenase (*COX*)*-2* gene encodes an inducible prostaglandin synthase enzyme that is overexpressed in adenocarcinomas and other tumors. Deletion of the murine *Cox-2* gene in *Min* mice reduced the incidence of intestinal tumors, suggesting that it is required for tumorigenesis. They have derived transgenic mice overexpressd the human *COX-2* gene in the mammary glands using the murine mammary tumor virus promoter. Cox-2-induced tumor tissue expressed reduced levels of the proapoptotic proteins Bax and Bcl-xL and an increase in the anti-apoptotic protein Bcl-2, suggesting that decreased apoptosis of mammary epithelial cells contributes to tumorigenesis. These data indicate that enhanced Cox-2 expression is sufficient to induce mammary gland tumorigenesis. Therefore, inhibition of Cox-2 may represent a mechanism-based chemopreventive approach for carcinogenesis.

Melstron et al., 2008: The purpose of this study was to evaluate the expression of 5-LOX in colonic polyps and cancer and the effect of 5-LOX inhibition on colon

cancer cellproliferation.Colonic polyps, cancer, and normal mucosa were evaluated for 5-LOXexpression by immunohistochemistry. Reverse transcription-PCR was used to establish 5-LOXexpression in colon cancer cells.Thymidine incorporation and cell counts were used to determinethe effect of the nonspecific LOX inhibitor Nordihydroguaiaretic Acid and the 5-LOX inhibitor Rev5901on DNA synthesisThis study showed that 5 LOX is up-regulated in adenomatous colon polyps andcancer compared with normal colonic mucosa. The blockade of 5-LOX inhibits colon cancer cellproliferation both in vitro and in vivo and may prove a beneficial chemopreventive therapy in coloncancer.

Lawrence, 2007: They have reviewed a clear links between chronic inflammation andcancer; strong epidemiological and genetic evidenceindicates that inflammation can drive tumour progression, and more-recent evidence indicates that the disruption of endogenous anti-inflammatory mechanisms inmice can lead to tumour development.The resolution ofinflammation is an active coordinated process thatrequires the production of anti-inflammatory mediators,the termination of proinflammatorysignalling pathwaysand the appropriate clearance or migration of inflammatory cells.Disruption of any of these processes can lead tochronic persistent inflammation and tumour growth.Although the mediators and mechanisms that driveinflammation have become increasingly well characterized, the endogenous mechanisms that limitthe inflammatory response, and particularly their role in cancer, are unclear. There are clear opportunities for drug discoveryand the development of new therapeutic approaches thattarget tumour-associated inflammation and the mechanisms of chronic inflammation.

Sinha et al., 2007: the study reported that the proinflammatory cytokine, interleukin-1B, induces the accumulation and retention of myeloid-derived suppressor cells (MDSC), which are commonly found in many patients and experimental animals with cancer and are potent suppressors of adaptive and innate immunity. This finding led to hypothesize that inflammation leads to cancer through the induction of MDSC, which inhibit immune surveillance and thereby allow the unchecked persistence and proliferation of premalignant and malignant cells. They further reported that host MDSC have receptors for prostaglandin E2 (PGE2) and that E-prostanoid receptor agonists, including PGE2, induce the differentiation of Gr1+CD11b+ MDSC from bone marrow stem cells, whereas receptor antagonists block differentiation. BALB/c EP2 knockout mice inoculated with the spontaneously metastatic BALB/c-derived 4T1 mammary carcinoma have delayed tumor growth and reduced numbers of MDSC relative to wild-type mice, suggesting that PGE2 partially mediates MDSC induction through the EP2 receptor. Treatment of 4T1- tumor–bearing wild-type mice with the cyclooxygenase 2 inhibitor, SC58236, delays primary tumor growth and reduces MDSC accumulation, further showing that PGE2 induces MDSC and providing a therapeutic approach for reducing this tumor-promoting cell population.

Avis et al., 2005: Arachidonic acid (AA) metabolizing enzymes and peroxisomeproliferator–activated receptors (PPARs) have been shown to regulate the growth of epithelial cells. We have previouslyreported that exposure to the 5-lipoxygenase activatingprotein–directed inhibitor MK886 but not the cyclooxygenaseinhibitor, indomethacin, reduced growth, increased apoptosis,and up-regulated PPARA and ; expression in breast cancercell lines. In the present study, we explore approaches to

maximizing the proapoptotic effects of PPAR; on lung cancercell lines. Non–small-cell cancer cell line A549 revealed dosedependent PPAR; reporter activity after treatment withMK886. The addition of indomethacin in combination withMK886 further increases reporter activity. We also showincreased growth inhibition and up regulation of apoptosisafter exposure to MK886 alone, or in combination withindomethacin and the PPAR ligand, 15-deoxy-#12,14-prostaglandin J2 compared with single drug exposures on theadenocarcinoma cell line A549 and small-cell cancer cell linesH345, N417, and H510. Real-time PCR analyses showedincreased PPAR mRNA and retinoid X receptor (RXR)A mRNAexpression after exposure to MK886 and indomethacin in atime-dependent fashion. The results suggest that the principalproapoptotic effect of these drugs may be mediated throughthe known antiproliferative effects of the PPAR;-RXR interaction. We therefore explored a three-drug approach toattempt to maximize this effect. The combination of low-doseMK886, ciglitazone, and 13-cis-retinoic acid interacted at least in a superadditivefashion to inhibit the growth of lungcancer cell lines A549 and H1299, suggesting that targetingPPAR; and AA action is a promising approach to lung cancergrowth with a favorable therapeutic index.

Ning et al., 2005: 5-Lox expression was examined in 7,12-dimethylbenz[a]anthracene (DMBA)–induced hamster and human oral cancer tissues by immunohistochemistry, and Cox2 expression was investigated in hamster oral tissues using in situ hybridization. Zileuton (a specific 5-Lox inhibitor) and celecoxib (a specific Cox2 inhibitor), either alone or in combination, were investigated for their chemopreventive effects on the DMBA-induced hamster model at the post-initiation

stage through topical application. The results clearly showed that both 5-Lox and Cox2 played important roles in oral carcinogenesis. Zileuton and celecoxib prevented oral carcinogenesis at the post-initiation stage through their inhibitory effects on arachidonic acid metabolism.

Ye et al., 2005: The study investigated whether there exists a relationship between COX-2 and 5-LOX, and whether dual inhibition of COX-2 and 5-LOX has an anticarcinogenic effect in the colonic tumorigenesis promoted by cigarette smoke. Results showed that pretreating colon cancer cells with cigarette smoke extract (CSE) promoted colon cancer growth in the nude mouse xenograft model. In an in vitro study, they found that the action of CSE on colon cancer cells was mediated by 5-LOX DNA demethylation. In summary, these results indicate that inhibition of COX-2 may lead to a shunt of arachidonic acid metabolism towards the leukotriene pathway during colonic tumorigenesis promoted by CSE. Suppression of 5-LOX did not induce such a shunt and produced a better response. Therefore, 5-LOX inhibitor is more effective than COX-2 inhibitor, and blocker of both COX-2 and 5-LOX may present a superior anticancer profile in cigarette smokers.

Chang et al., 2003: The study showed that COX-2 expressed in theepithelial cell compartment regulates angiogenesis in the stromaltissues of the mammary gland.Microvessel density increasedbefore visible tumor growth and exponentially during tumorprogression. Inhibition of prostanoid synthesis with indomethacinstrongly decreased microvessel density and inhibited tumor progression. Up-regulation of angiogenic regulatory genes in COX-2transgenic mammary tissue was also potently inhibited by indomethacin treatment, suggesting that prostanoids

released from COX 2-expressing mammary epithelial cells induce angiogenesis. PGE2stimulated the expression angiogenic regulatory genes in mammary tumor cells isolated from COX-2 transgenic mice. Such cellsare tumorigenic in nudemice; however, treatment with Celecoxib,a COX-2-specific inhibitor, reduced tumor growth and microvesseldensity. These results define COX-2-derived PGE2 as a potentinducer of angiogenic switch during mammary cancer progression.

Ristimaki et al., 2000: They analyzed the expression of Cox-2 protein by immunohistochemistry in tissue array specimens of 1576 invasive breast cancers. Elevated Cox-2 expression was associated with a large tumor size, a high histological grade, a negative hormone receptor status, a high proliferation rate (identified by Ki-67), high p53 expression, and the presence of *HER-2* oncogene amplification ($P < 0.0001$ for all comparisons), along with axillary node metastases and a ductal type of histology. Interestingly, association with the unfavorable outcome was especially apparent in the subgroups defined by estrogen receptor positivity, low p53 expression, and no *HER-2* amplification ($P < 0.0001$ for all comparisons). These results indicate that elevated Cox-2 expression is more common in breast cancers with poor prognostic characteristics and is associated with an unfavorable outcome. The present findings support efforts to initiate clinical trials on the efficacy of Cox-2 inhibitors in adjuvant treatment of breast cancer.

Tsujii et al., 1998: Study explored the role of cyclooxygenase (COX) in endothelial cell migration and angiogenesis. They have used two in vitro model systems involving coculture of endothelial cells with colon carcinoma cells. COX-2-overexpressing cells produce prostaglandins, proangiogenic factors, and stimulate

both endothelial migration and tube formation, while control cells have little activity. The effect is inhibited by antibodies to combinations of angiogenic factors, by NS-398 (a selective COX-2 inhibitor), and by aspirin. NS-398 does not inhibit production of angiogenic factors or angiogenesis induced by COX-2-negative cells. Treatment of endothelial cells with aspirin or a COX-1 antisense oligonucleotide inhibits COX-1 activity/expression and suppresses tube formation.

Chapter 2

Aim and Objectives

2. Aim and Objectives

2.1. Aim

The study is aimed to investigate the possible link between dual inhibition of Arachidonic acid and its correlation with the suppression of tumorigenesis of mammary gland.

2.2. Objectives

Objective 1: To study the effect of a COX-2 inhibitor, 5 LOX inhibitor and a dual inhibition of AA metabolism on N-Nitroso- N-methylurea (MNU) induced mammary gland carcinogenesis.

Objective 2: To elaborate the effect of COX-2 inhibitor, 5 LOX inhibitor and a dual inhibition of AA metabolism on cellular proliferation and angiogenesis.

Objective 3: To study the effect of COX-2 inhibitor, 5 LOX inhibitor and a dual inhibition of AA metabolism on mitochondrial mediated apoptotic pathway.

Objective 4: To study the effect of COX-2 inhibitor, 5 LOX inhibitor and a dual inhibition of AA metabolism on metabolic alterations using quantitative NMR.

2.3. Plan of work

Assay protocols for the study are as follows:

2.3.1. Haemodynamic studies:

Heart Rate variability (HRV)

Electrocardiogram analysis (ECG)

2.3.2. Morphological evaluation of mammary gland

Carmine staining of whole mount mammary gland

Histopathology of mammary gland tissue

2.3.3. Antioxidant markers:

Thiobarbituric acid reactive substances (TBARs)

Superoxide dismutase (SOD)

Catalase

Protein carbonyl (PC)

Glutathione (GSH)

2.3.4. Enzymatic activity of COX-2 and 5-LOX through immunoblotting and qRT-PCR.

2.3.5. Evaluation of Caspase-3 and Caspase-8

2.3.6. Immunoblotting assay for quantification of mitochondrial apoptotic proteins.

2.3.7. Densitometry quantification of mitochondrial apoptotic proteins:

BCL-2, BCL-xl, BAX,BAD, VDAC, Cytochrome-c, APAF-1, Procaspase-9.

2.3.8. qRT-PCR analysis for quantification of genomic contributors of mitochondrial apoptotic proteins.

2.3.9. NMR based serum metabolomics analysis.

2.4. Compilation of data and statistical analysis.

Chapter 3

Drug profile

3.Drug profiles

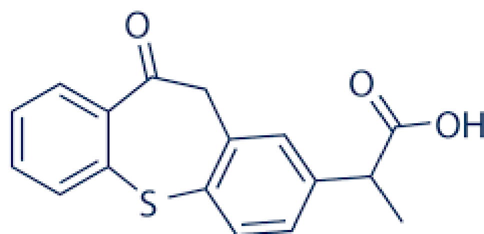
The drugs selected for this study were Zaltoprofen (COX-2 inhibitor) and Zileuton (5-LOX inhibitor), which are anti-inflammatory and antiasthmatic respectively.

3.1.The drug: Zaltoprofen

Zaltoprofen was procured from the local market under the brand name Zaltokin from IPCA laboratories Ltd, India.

3.1.1.Chemical structure

The structural formula of Zaltoprofen:



74711-43-6; 2-(10-oxo-10,11-dihydrodibenzo[b,f]thiepin-2-yl)propanoic acid

3.1.2.Molecular formula: $C_{17}H_{14}O_3S$

3.1.3.Molecular weight: 298.356

3.1.4.Category: Anti-inflammatory Agent.

3.1.5. Pharmacodynamics: Zaltoprofen is a preferential COX-2 inhibitor. The drug selectively inhibits PGE2 (Prostaglandin E2) that mediates the pain pathway. Zaltoprofen inhibits bradykinin-induced pain responses without interfering with the bradykinin receptors.

3.1.6.Pharmacokinetics: After oral administration, Zaltoprofen is well absorbed (82%) in the GIT. About 98% of the administered drug is bound to plasma proteins. The drug is mostly metabolized by hepatic UGT2B7 and CYP2C9. The drug is

metabolized into conjugates and S-oxide forms. No systemic accumulation has been reported. About 62% of the administered drug is excreted via the urine as drug conjugates.

3.1.7.Mechanism of action: Zaltoprofen is a nonsteroidal anti-inflammatory drug that exhibits anti-inflammatory, analgesic and antipyretic activities. It is a COX-2 preferential inhibitor. The main mechanism of zaltoprofen is prostaglandin biosynthesis inhibitory action due to the COX inhibition in the arachidonic acid metabolism system. Besides this, membrane stabilizing action such as leukocyte migration inhibitory action and lysosomal enzyme inhibitory action are also observed with zaltoprofen. Experimental studies have shown that Prostaglandin biosynthesis inhibitory action in the stomach tissue is weaker with Zaltoprofen than in case of indomethacin.Zaltoprofen was shown to have more powerful inhibitory effect to bradykinin-nociceptor than other NSAIDs.

3.1.8.Administration: Zaltoprofen is given by mouth in a usual initial dose of 80 mg in the evening. An initial dose of 70 mg may be used in patients who are at high cardiovascular risk.

3.1.9.Physicochemical properties: According to official monographs (USP) zaltoprofen occurs as white to light yellow, crystalline powder, tasteless, odourless. It is freely soluble in acetone, chloroform, soluble in methanol, slightly soluble in ethanol, benzene, and practically insoluble in water, and cyclohexane

3.1.10.Sotrage: According to monographs, it should be stored in well-closed containers and between 15° and 30°C.

3.1.11. Drug Interactions

Quinolone antibacterial- Concomitant use of antibacterials with zaltoprofen may trigger convulsion. The dose may have to be adjusted in such cases.

Coumarin anticoagulant agent- the dose may have to be adjusted as there may be intensification in the anticoagulant action.

Sulfonylurea antidiabetic agents- The dose may have to be adjusted as there may be an intensification in the hypoglycaemic action.

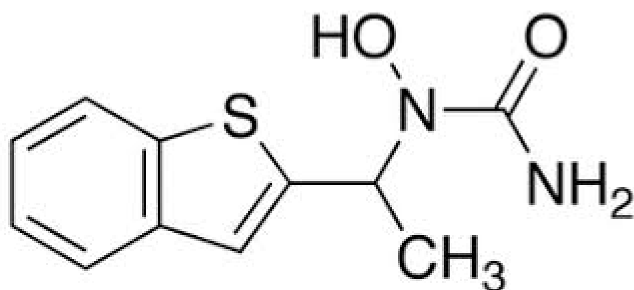
Lithium- The dose of lithium may have to be adjusted as there may be intensification in the lithium action.

3.2. The drug: Zileuton

Zileuton was solicited as API from Shanghai worldyang chemical co., Ltd, China.

3.2.1. Chemical structure

The structural formula of Zileuton:



1-[1-(1-benzothiophen-2-yl)ethyl]-1-hydroxyurea

3.2.2. Molecular formula: $C_{11}H_{12}N_2O_2S$

3.2.3. Molecular weight: 236.289

3.2.4. Category: Anti-inflammatory Agent, Anti-asthmatic.

3.2.5. Pharmacodynamics: Zileuton is an asthma drug that differs chemically and pharmacologically from other antiasthmatic agents. It blocks leukotriene synthesis by inhibiting 5-lipoxygenase, an enzyme of the eicosanoid synthesis pathway.In humans, pretreatment with zileuton attenuated bronchoconstriction caused by cold air challenge in patients with asthma.

3.2.6. Pharmacokinetics: After oral administration, zileuton israpidly and almost completely absorbed. The absolute bioavailability is unknown.The drug is mostly metabolized by hepatic. Zileuton and its N-dehydroxylated metabolite are oxidatively metabolized by the cytochrome P450 isoenzymes 1A2, 2C9 and 3A4. Elimination of zileuton is predominantly via metabolism with a mean terminal half-life of 2.5

hours.The urinary excretion of the inactive N-dehydroxylated metabolite and unchanged zileuton each accounted for less than 0.5% of the dose.

3.2.7. Mechanism of action: Leukotrienes are substances that induce numerous biological effects including augmentation of neutrophil and eosinophil migration, neutrophil and monocyte aggregation, leukocyte adhesion, increased capillary permeability, and smooth muscle contraction. These effects contribute to inflammation, edema, mucus secretion, and bronchoconstriction in the airways of asthmatic patients. Zileuton relieves such symptoms through its selective inhibition of 5-lipoxygenase, the enzyme that catalyzes the formation of leukotrienes from arachidonic acid. Specifically, it inhibits leukotriene LTB4, LTC4, LTD4, and LTE4 formation. Both the R(+) and S(-) enantiomers are pharmacologically active as 5-lipoxygenase inhibitors in *in vitro* systems. Due to the role of leukotrienes in the pathogenesis of asthma, modulation of leukotriene formation by interruption of 5-lipoxygenase activity may reduce airway symptoms, decrease bronchial smooth muscle tone, and improve asthma control.

3.2.9. Physicochemical properties: zileuton occurs as white to off white powder.It is freely soluble in acetone, chloroform, soluble in methanol, slightly soluble in ethanol, benzene, and partially soluble in water.

3.2.10. Sotrage: According to monographs, it should be stored in well-closedcontainers and between 15° and 30°C.

3.2.11. Drug Interactions

Aminophylline: Zileuton may increase the serum concentration of Aminophylline.Management.Reduce aminophylline dose by 50% upon initiation of zileuton therapy. If aminophylline is added to existing zileuton therapy, use a lower starting dose. Monitor for increased theophylline serum concentrations and effects.

Clozapine: CYP1A2 Inhibitors (Weak) may increase the serum concentration of Clozapine.

Loxapine: Agents to Treat Airway Disease may enhance the adverse/toxic effect of Loxapine. More specifically, the use of Agents to Treat Airway Disease is likely a marker of patients who are likely at a greater risk for experiencing significant bronchospasm from use of inhaled loxapine. Management: This is specific to the Adasuve brand of loxapine, which is an inhaled formulation. This does not apply to non-inhaled formulations of loxapine.

Pimozide: Zileuton may increase the serum concentration of Pimozide.

Propranolol: Zileuton may increase the serum concentration of Propranolol.

Theophylline: Zileuton may increase the serum concentration of Theophylline. Management: Reduce theophylline dose by 50% upon initiation of zileuton therapy. If theophylline is added to existing zileuton therapy, use a lower starting dose. Monitor for increased serum concentrations and effects of theophylline.

Tizanidine: CYP1A2 Inhibitors (Weak) may increase the serum concentration of Tizanidine. Management: Avoid these combinations when possible. If combined use is necessary, initiate tizanidine at an adult dose of 2 mg and increase in 2 to 4 mg increments based on patient response. Monitor for increased effects of tizanidine, including adverse reactions.

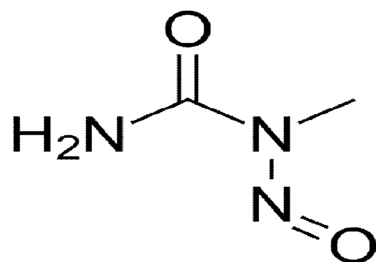
Warfarin: Zileuton may increase the serum concentration of Warfarin.

3.3.Toxicant

3.3.1. MNU

MNU (N1517) was purchase from Sigma Aldrich, India.

3.3.2. Chemical Structure



3.3.3. Molecular formula: $C_2H_5N_3O_2$

3.3.4. Molecular weight: 103.08

3.3.5. Pharmacology: The mammary carcinoma induced by the application of MNU to female albino wistar rats is one of the most frequently used animal models for the investigation of breast carcinogenesis and mammary tumour treatments.MNU is a N-Nitro compound and direct- acting alkylating agent which work by transferring its methyl group to nucleobases in nuclic acid, can leads to AT:GC transition mutations. Generally, MNU induced mammary carcimomas are aggressive and locally invasive, they are capable metastasis [Tsubura et al., 2011].

3.3.6. Preparation: The toxicant was prepared in glacial acetic acid and water with pH 4.5-5. The MNU was given in a dose of 47mg/kg, i.v. once in the whole study.

Chapter 4

Materials and Methods

4. Materials and Methods

4.1. Drugs and chemicals

Zaltoprofen was procured from the local market under the brand-namezaltokin from IPCA laboratory's Ltd, India; and zileuton was solicited as API from Shanghai worldyang chemical co., Ltd, China RNase (SRL, 58895); dimethyl sulfoxide (DMSO) (Merck,1.16743.0521); MNU (Sigma Aldrich,N1517); ponceauS (Himedia,ML045); hematoxylin (Himedia,S058); eosin (Himedia,S007); RIPA lysis buffer (Amresco, N653); protein assay kit (Amresco,M173); bovine serum albumin (BSA) (Genetix,PG-2330); transfer buffer (Genetix, GX-9411AR), trizol reagent (Sigma-T9424), cDNA synthesis kit (Genetix-K1612). Caspase 3 (SC-4263) and caspase 8 (SC-4267) assay kits were procured from Santacruz Biotechnology Inc., California, Delaware. All others chemicals were of molecular biology grade and purchased from Genetix Biotech Asia Pvt. Ltd, New Delhi.

4.1.2 Equipment used

Table 1: Lists of equipments used

Serial no.	Equipment	Manufacturer and Model
1	Homogenizer	Remi, Mumbai RQT-127A
2	Weighing balance	Sartorius, Mumbai BSA224S-CW
3	pH meter	Labman Scientific Instruments, Lucknow LMPH-10
4	Vortex shaker	Remi Mumbai CM101
5	Homogenizer	Remi, Mumbai RQT-127A
6	Cooling centrifuge	Eppendorf India Limited, Chennai 5418R

7	Micropipette	Genetix Biotech Asia Pvt. Ltd. New Delhi
8	Refrigerator	Godrej, Lucknow
9	Deep freezer	Celfrost, BFS150, Lucknow
10	Microvolume Spectrophotometer	Agilent Technologies, Mumbai Carry 500
11	Microplate Reader	Bio-Rad Laboratories Inc. Model 680XR
12	SDS-PAGE	GX-SCZ2, Genetix Biotech Asia Pvt. Ltd. New Delhi
13	Semidry transfer unit	GX-ZY3, Genetix Biotech Asia Pvt. Ltd. New Delhi
14	Bio-amplifier (ML-136) and channel power lab (ML-826)	AD Instruments, Australia

4.2.Animals

Wistar strain of female albino rats of (100–120 g) wereprocured from the central animal house. Animals were housedunder standard condition (23◦C, 12 h light/dark cycle), witha free access to a standard pellet diet and water *ad libitum*.Animals were acclimatized for a period of 2 weeks priorto the commencement of the experiment, and the studywas performed according to the standard ethical guidelinesand approved by the Institutional Animal Ethics Committee(BBDNIIT/IAEC/020/2014).

4.3.Experimental design

Animals were randomized and divided into 5 groups of 6 animalseach. Group I (control 0.9% normal saline, 3 ml/kg, p.o.); GroupII (toxic control, MNU 47 mg/kg,

i.v.); Group III (Zaltoprofen, 10mg/kg, p.o.); Group IV (Zileuton, 10 mg/kg, p.o.); and Group V(Zaltoprofen, 5 mg/kg, p.o+ Zileuton, 5 mg/kg, p.o.). Mammarygland carcinoma was induced by single i.v. injection of MNUon 7th day after commencing the treatment. The animals wererecorded for the autonomic control though electrocardiogram(ECG) and Heart rate variability (HRV) paradigms on 119th day.The animals were sacrificed by using light ether anesthesia on120th day and mammary gland tissue was collected. The wholemount tissue was assessed for their morphological changes usingcarmine staining, and the rest was further evaluated for otherparameters.

Table 2: Experimental Design:

S.No	Groups	Treatment (n=10)
1	**Group I**	Normal control (0.9% normal saline, 3ml/kg, p.o.)
2	**Group II**	Toxic control, (MNU 47 mg/kg, i.v.)
3	**Group III**	Zaltoprofen (10 mg/kg, p.o) + MNU (47 mg/kg, i.v.)
4	**Group IV**	Zileuton (10 mg/kg, p.o.) + MNU (47 mg/kg, i.v.)
5	**Group V**	Zaltoprofen+Zileuton (5 mg/kg+5mg/kg, p.o + MNU 47 mg/kg, i.v.)

Mammary gland carcinoma was induced by single i.v. injection of MNU on 7th day after commencing the treatment. Zaltoprofen, Zileuton and their combination was administered daily for 120 days.

4.4.Antioxidant markers

The mammary gland tissues (10% w/v) were homogenized in0.15 M KCl and centrifuged at 10,000 rpm. The supernatants werescrutinized for biochemical

parameters, including thiobarbituricacid reactive substances (TBARs), superoxide dismutase (SOD),catalase, protein carbonyl (PC) and glutathione (GSH) using themethods established at our laboratory (Kaithwas et al., 2007,2011; Kaithwas and Majumdar, 2012).

4.5.Hemodynamic studies

Animals were anesthetized using ketamine hydrochloride (100mg/kg, i.m.) and diazepam (5 mg/kg, i.m.) in combinationand mounted on a wax tray. The ECG signals were recordedusing platinum hook electrodes connected to bio amplifier (ML-136) and single channel PowerLab (ML-826) (AD Instruments,Australia). The ECG signals were used to perform HRV analysis(Labchard PRO-8, AD Instruments, Australia) using the methoddescribed previously (Roy et al., 2018).

4.6.Carmine staining of whole mountmammary gland

Whole mount of the mammary gland was prepared using cornoy'sfixative solution using the methods previously established at ourlaboratory. The slides were examined under the microscope toassess the number of terminal end buds (TEBs), alveolar buds(ABs) type 1 and 2, and differentiation (DF) score. Detailedprocedure for the same has been described by us previously (Manral et al., 2016; Rani et al., 2016).

4.7.Histopathology of mammary gland tissue

Mammary gland tissues were appraised histopathologically using haematoxyline and eosin staining (H&E). 5 µm sections of mammary gland tissue were prepared and stained with H&E.The sections were visualized and photographed

at 40X using digital biological microscope (N120, BR-Biochem Life Sciences, New Delhi, India)(Belur et al., 1990; Murray et al., 2009; Rani et al., 2018).

4.8.Evaluation of caspase-3 and caspase-8

The serum samples were assayed for the caspase-3 and caspase-8 levels using DEVD-AFC complex and IETD-AFC complex principles respectively, following the instruction manual.To 10 µl of serum sample used for the assay, 100 µl of 10 mM DTT in 1x Reaction buffer was added. To the reaction mixture, 2.5 µl DEVD-AFC complex (in Caspase 3) and 2.5 µl IETD-AFC complex (in Caspase 8) was added. The reaction mixture was then incubated at 37°C for 1 hour which was then read at 400 nm excitation filter and 505 emission filter in Spectrofluorimeter (Bustamante et al.,2002; Martinez et al., 2004; Gautam et al., 2018).

4.9.Western blotting

Protein samples were prepared from the mammary gland tissue through acetone precipitation and quantified by using the Bradford reagent (Ahmad and Sharma, 2009). SDS-PAGE analysis was performed following the principles of Laemmli with slight modifications. Briefly, protein samples were mixed with sample buffer (125 mM Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 0.05% bromophenol blue, 10% 2- mercaptoethanol). A 30 µg of protein sample was allowed to resolve through 12% polyacrylamide gel using SDS-PAGE (GXSCZ2+, Genetix Biotech Asia Pvt. Ltd. New Delhi). The proteins as resolved through SDS-PAGE were transferred to a PVDF membrane (IPVH 00010 Millipore, Bedford, MA USA) using semidry transfer (GX-ZY3), Genetix Biotech Asia Pvt. Ltd., New Delhi. Subsequently, membrane was

blocked with 3% BSA and 3% non-fat milk in TBST for 2 h and incubated overnight with primary antibody against B cell lymphoma-2 (BCL-2) (SC-7382), B cell lymphoma-xl (BCL-XL) (MA-5-15142), BAX (SC-23959), BAD (SC-8044), Voltage dependent anion channels (VDAC) (390996), Apoptotic protease activating factor-1 (APAF-1) (SC- 65891), Procaspase-9 (SC-73548), COX-2 (MA5-14568), 5-LOX (PA1-16953), and β-actin (MA5-15739 HRP) (Pierce, Thermo scientific) (1:3000 dilution). The membrane was washed with TBST thrice and incubated with HRP conjugated rat antimousesecondary antibody (31430, 1:5000 dilutions) (Pierce Thermo Scientific) at room temperature for 2 h. The signals were detected using an enhanced chemiluminescence substrate (Western Bright ECL HRP substrate, Advansta, Melanopark, California, US). The Quantification of protein was done through densiometeric digital analysis of protein bands using ImageJ software (Laemmli, 1970; Towbin et al., 1979).

4.10.Quantitative RT-PCR

Primers for real time were designed online using the primer quest tool from the IDT DNA technologies' website (www.idtdna.com). The amplicon size was kept between 100 and 200 base pairs, GC% was kept above 50% and melting temperature was kept between 58 and 62◦C. The specific sequences of the forward and reverse primers are specified in **Table 2**.

Total RNA was extracted from mammary gland tissue using trizol reagent (Invitrogen, Life Technologies) according to the manufacturer‘s instructions. Briefly, tissues were washed off treatment plates using 0.1% DEPC water. The tissues were crushed in 250 µl trizol reagent using micro pestles. Another 750 µl of trizol reagent

was added to make the final volume to 1 ml, followed by addition of 200 µl of chloroform and mixing for 2– 5 min on a vortex mixer. The suspension was then centrifuged at 14,000 rpm, 4◦C for 15 min and upper aqueous phase was gently pipette out in the fresh vials. RNA was precipitated by addition of 500 µl chilled isopropanol. The vials were kept at room temperature for 10 min and were centrifuged at 14,000 rpm, 4◦C for 10 min and RNA pellet so obtained was washed twice with 75% ethanol (chilled) at 7,500 rpm, 4◦C for 5 min. The RNA pellet was finally dissolved in 15 µl of 0.1% DEPC water. To quantify RNA absorbance was read using nano drop (Qua Well Q5000). cDNA synthesis was done from 1 µg of total mammary gland RNA in a 96 well thermal cycler (BioRad, C1000) with steps including, incubation at 25◦C for 10 min, 37◦C for 120 min, 85◦C for 5 min and 4◦C forever RNA using high capacity cDNA synthesis Kit (Applied Biosystems).cDNA sample were quantified using nanodrop and were stored at -80◦C until use. 125 ng of cDNA was used as a template for each reaction of qRT-PCR with β-actin as housekeeping control using light cycler 480 machine (Roche Diagnostics, Germany). For each primer pair, a melting curve analysis was performed according to instrument. The program in brief was an initial incubation of 50◦C for 2 min hold (UDG incubation) and 95◦C for 10 min followed by 40 cycles at 95◦C for 15 s (denaturation), 58◦C for 30 s (annealing) and final extension at 72◦C for 20 s. Differential expression was calculated by 2-11CT method.β-actin was used as internal control and used to normalize ratios between samples (Giulietti et al., 2001; Kaithwas et al., 2007; Roy et al., 2017).

4.11.NMR Based Serum Metabolomics Analysis

At the time of NMR measurement, the stored serum samples at -80 ^{0}C were thawed, vortexed, and centrifuged at 10,000 rpm on room temperature. Then, the aliquots of 250µL of serum were transferred into 250µL of 0.9% saline phosphate buffer (20 mM, pH 7.4) prepared in 100% D_2O, to minimize the variation in pH[1].The samples were centrifuged for 5 min at 6164 rcf and 450µL of each sample supernatant was subsequently moved into a 5 mm NMR tube (Wilmad Glass, USA). Before starting the NMR experiments, a co-axial insert containing 0.01 mM TSP (Sodium salt of 3-trimethylsilyl-(2,2,3,3-d4)-propionic acid) dissolved in deuterium oxide (D2O) was inserted separately both for the purpose of locking and chemical shift referencing. Deuterium oxide (D2O) and sodium salt of trimethylsilylpropionic acid-d4 (TSP) used for NMR spectroscopy were purchased from Sigma-Aldrich (Rhode Island, USA).One dimensional^{1}H CPMG (Carr–Purcell–Meiboom–Gill) NMR spectra were acquired for all the serum samples using the standard Bruker's pulse program library sequence (cpmgpr1d) on 800 MHz NMR spectrometer (Bruker Avance-III) equipped with Cryoprobe at 300 Kelvin (K). The raw ^{1}H-NMR data were processed, phased, and baseline corrected using Topspin 3.5 (Bruker Biospin, GmbH, Rheinstetten, Germany). All spectra were referenced to the lactate chemical shift (δ=1.3102) to minimize inter-spectral chemical shift variation between the spectra.

For NMR based metabolomics analysis, the serum samples stored at -80◦C were thawed, vortexed, and centrifuged at 10,000 rpm on room temperature. Next, the serum samples were analyzed by high resolution 1D 1H NMR spectroscopy on a

Bruker AVANCE III 800 MHz NMR spectrometer equipped with TXI Cryoprobe. For small metabolite profiling, the standard 1D 1H Carr-Purcell-Meiboom-Gill (CPMG) NMR spectra was recorded on all serum samples after processing the sera samples as described previously (Rawat et al., 2016a,b). The NMR spectra obtained from different study groups was afterwards imported into the Topspin AMIX software (Bruker GmbH, Rheinstetten, Germany) and segmented into 0.02-ppm bins (buckets) in the spectral region 0.7–8.5 ppm, excluding the residual water signal ranging from (4.65–5.01) ppm. The CPMG data matrix was Pareto scaled for each NMR variable (i.e., for each bin) and subsequently subjected to multivariate/univariate analysis using MetaboAnalyst 3.0 (Xia et al., 2009, 2015). First, we performed the multivariate analysis based on standard algorithms–PCA (principal component analysis), PLS-DA (partial least-squares discriminant analysis), and OPLS-DA (Orthogonal Projection to Latent Structure with Discriminant Analysis)-to evaluate the metabolic differences between the control and treated groups. The unsupervised PCA was performed initially to get an overview of the grouping trends and to separate the effective treatmentdose. Next, we performed the supervised PLS-DA modelling (pair wise as well as combined) to discriminate the groups based on metabolic differences. The metabolites responsible for group discrimination were evaluated based on the variable importance on projection scores (i.e., VIPs) >1. The goodnessof-fit parameters R2 and Q2, which relate to the explained and predicted variance, respectively, were used to evaluate the PLSDA model performance. Further, univariate analysis was applied to assess the significance for the change in the metabolic profile for the pair wise and combined analysis (*t*-test and ANOVA

respectively), $p << 0.05$ was used as the criterion for statistical significance (Guleria et al., 2014; Kumar et al., 2016;Wishart et al., 2007)

4.12.Statistical analysis

All data were presented as mean ± SD and analyzed by oneway ANOVA followed by Bonferroni test and for the possible significance identification between the various groups. $^{c/*}p < 0.05$, $^{b/**}p < 0.01$, and $^{a/***}p < 0.001$ were considered as statistically significant. Statistical analysis was performed using Graph Pad Prism software (5.02).

Table 3: Sequence of forward and reverse primers used for quantitative RT-PCR

Primer	Sequence
Bcl-2 F	GTG GAT GAC TGA GTA CCT GAA
Bcl-2 R	GAG ACA GCC AGG AGA AAT CAA
Bcl-xl F	CCC TCG TAT CTG GAA GCC AC
Bcl-xl R	CAG CGG AGA CCT CGT TTT CT
Bad F	CTC CGA AGA ATG AGC GAT GAA
Bad R	ATC CCA CCA GGA CTG GAT AA
Bax F	TGC TAC AGG GTT TCA TCC AG
Bax R	GAC ACT CGC TCA GCT TCT T
Apaf-1 F	GAA CAT AGA CTC CCG GGT AAA G
Apaf-1 R	CTT GTC TCC CAG ACC CTT ATT G
Cas- 9 F	GGC TCT CTG GCT TCA TTC TT
Cas- 9 R	GGG TCC AGC TTC ACT ACT TTC
*Vdac*F	GGA GTT TGG TGG CTC CAT TTA
*Vdac*R	GAC CTG ATA CTT GGC TGC TAT TC
Cyto-c F	TCC ATT TCC CTT CCT TGG GC
Cyto-c R	ATC GGG GCT GTC CAA CAA AA
COX-2 F	CCT TCG GGC ACA TGG TAA GT
COX-2 R	CAG CCC ACT CCA TAC TGC AA
5-LOX F	CTA CAA GTA CTC CGA CGA CA
5-LOX R	AAG TAA CCG GTG CCA TAT CC

Chapter 5

Results and Discussion

5.Results and Discussion

5.1.Change in oxidative stress markers in MNU-induced mammary gland carcinogenesis

The precedent studies have suggested the participationof the reactive oxygen species (ROS) in conjunction withthe AA mediated inflammatory pathway in carcinogenesis.Considering the same, we scrutinized theenzymatic (SOD/catalase/GSH) and peroxidative biomarkers(TBARs and PC) in mammary gland tissue.There was an upsurge (0.23 ± 0.07 nM of MDA/µg of protein) in TBAR's level after MNU administration in comparison with normal control (0.09 ± 0.02) rats. A significant dose dependent decrease in the TBAR's level was observed subsequent to zaltoprofen (0.15 ± 0.05), zileuton (0.09 ± 0.01) and in combination treatment (0.08 ± 0.00).

Decrease in the enzymatic activity of SOD (0.22 ± 0.02 units of SOD/mg of protein) and catalase (1.51 ± 0.06 nM of H_2O_2/min/mg of protein) was scrutinized in the animals treated with MNU.Moreover, antioxidantenzyme's SOD, catalase, and GSH constitute the major supportivedefense against free radicals and all work in tandem. SODscavenges the superoxide radicals to form hydrogen peroxide(H_2O_2), which is further catalyzed to H_2O and O_2.Similar patterns of results were scrutinized for the GSH.All in all, the combination treatment helped to restore the levels of SOD and catalase more efficiently in comparison to test drugs alone (**Table 4**).

5.2.Effects of zaltoprofen and zileuton on ECG &HRV

Administration of MNU demonstrated aberration in the ECG profile characterized by QT prolongation; QRS prolongation; marginal decrease in HR; increased dispersion of P wave amplitude in comparison to control (**Figure 2&3**).QRS complex indicatesthe ventricular depolarization, and the prolongation of theQT interval reflects the patients risk for the cardiac damage.Treatment with monotherapy and combination therapy ofzaltoprofen and zileuton demarcated a significant effect towardrestoration of QRS, QT, and QTc interval.

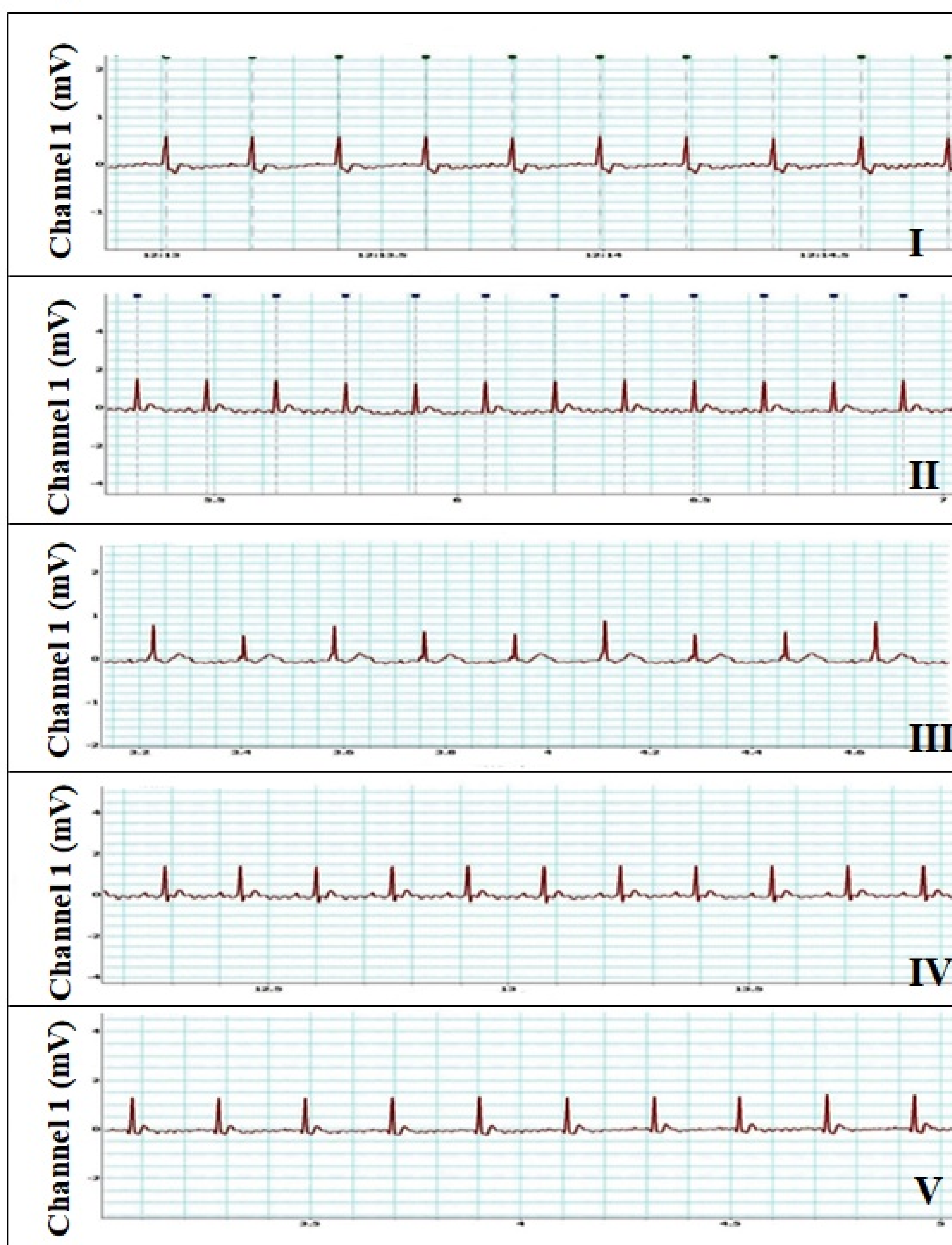
Inconsideration to the cardinal damages and deterioration ofthe autonomic physiology, we also scrutinized the same usingtime and frequency domain in HRV parameters. It would beappropriate to mention that HRV is the most utilized noninvasive marker to study post chemotherapeutic autonomicdysfunction.Distorted HRV profile was recorded for the time domain (Average RR, Median RR, SDRR, SDARR and CVRR) and frequency domain (LF, HF, and LF/HF) parameters after MNU treatment.Treatment with zaltoprofen and zileuton exerted favorable effects toward restoring the HRV paradigms toward normal with more profound effects by combination regime (**Figure 4 & 5**).

Table 4: Effect of Zaltoprofen and Zileuton on oxidative stress markers against MNU induced mammary gland carcinoma

Groups	TBARS (Nm of MDA/mg of protein)	GSH*10^{-4} (mg %)	SOD (unit of SOD/mg of protein)	Catalase (nM of H_2O_2/min/mg of protein)	Protein Carbonyl (nM/ml)
Group I Control (Normal Saline, 3ml/kg, i.p.)	0.088±0.02	0.076± 0.00	0.256 ± 0.02	1.66±0.09	55.98±13.06
Group II Toxic Control (MNU 47mg/kg, i.v.)	0.229±0.07***	0.065±0.00	0.224±0.02	1.51±0.06	78.10±9.04
Group III Zaltoprofen (10mg/kg, p.o.)	0.150±0.05^{a}	0.061±0.00	0.205±0.01^{a}	1.28±0.01***	72.87±5.47
Group IV Zaltoprofen (10mg/kg, p.o.)	0.089±0.01^{a}	0.077±0.01	0.305± 0.00^{b}	1.53±0.15	66.59±31.52
Group V Zaltoprofen+ Zileuton (5mg/kg+5mg/kg, p.o.)	0.086±0.00***	0.066±0.00	0.607± 0.29***	1.32±0.13***	62.04±3.05

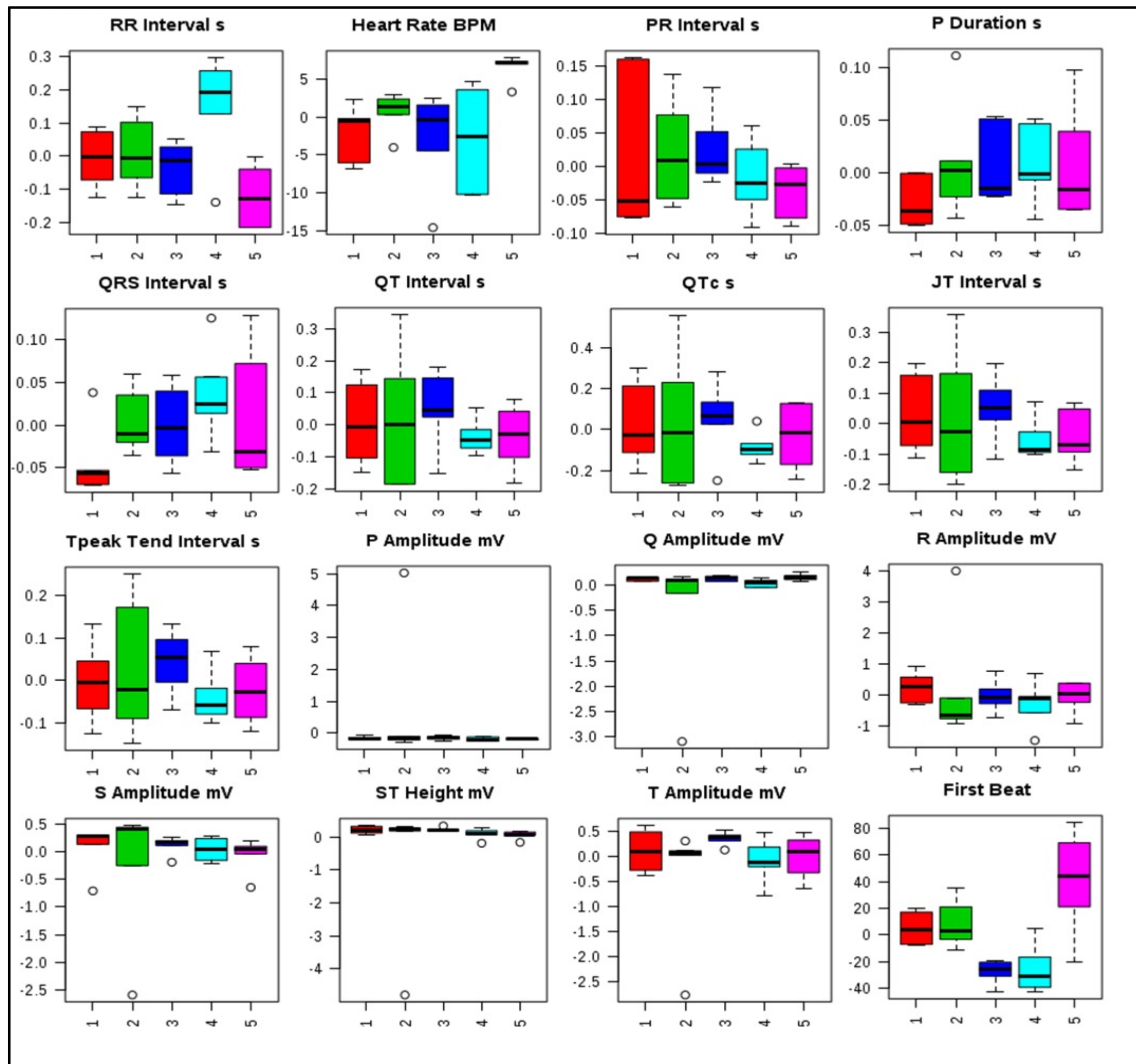
(Values are Mean ± SD), each group contains six animals. Comparisons were made on the basis of the one-way Anova followed by Bonferroni test. All groups were compared to the MNU treated group (*p<0.05, **p<0.01, ***p<0.001). Group V were compared to the III and IV treated group (^{c}p<0.05, ^{b}p<0.01, ^{a}p<0.001).

Figure 2: Representative of ECG tracing



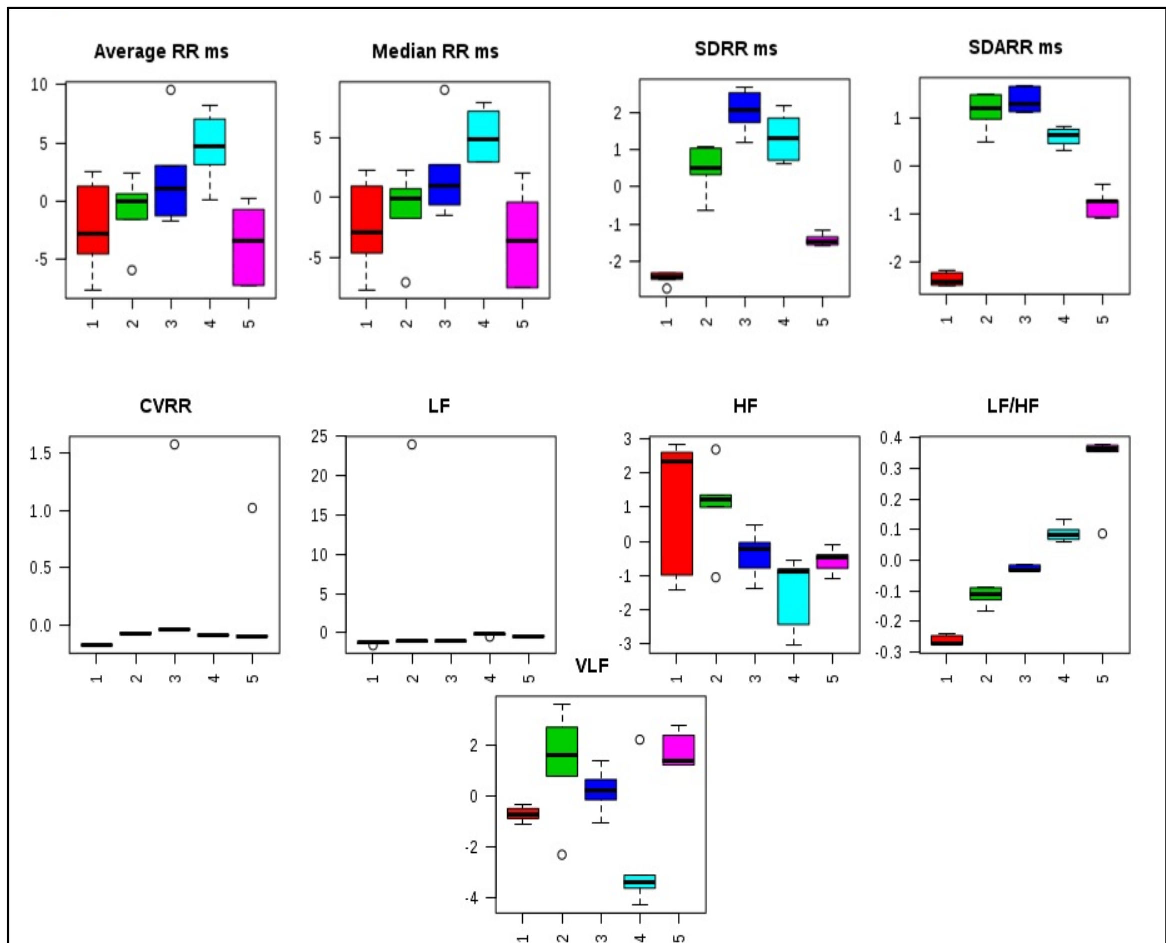
Group I: Control (Normal saline, 3ml/kg, p.o.), Group II: Toxic control (47 mg/kg MNU, i.v.), Group III: Zaltoprofen (10 mg/kg, p.o. + MNU 47mg/kg, i.v.), Group IV: Zileuton (10 mg/kg, p.o. + MNU 47mg/kg, i.v.), Group V: Zaltoprofen + Zileuton (5mg/kg+5mg/kg, p.o. + MNU 47mg/kg, i.v.).

Figure 3:Effect of DuCLOX-2/5 inhibition treatment on ECG recording.



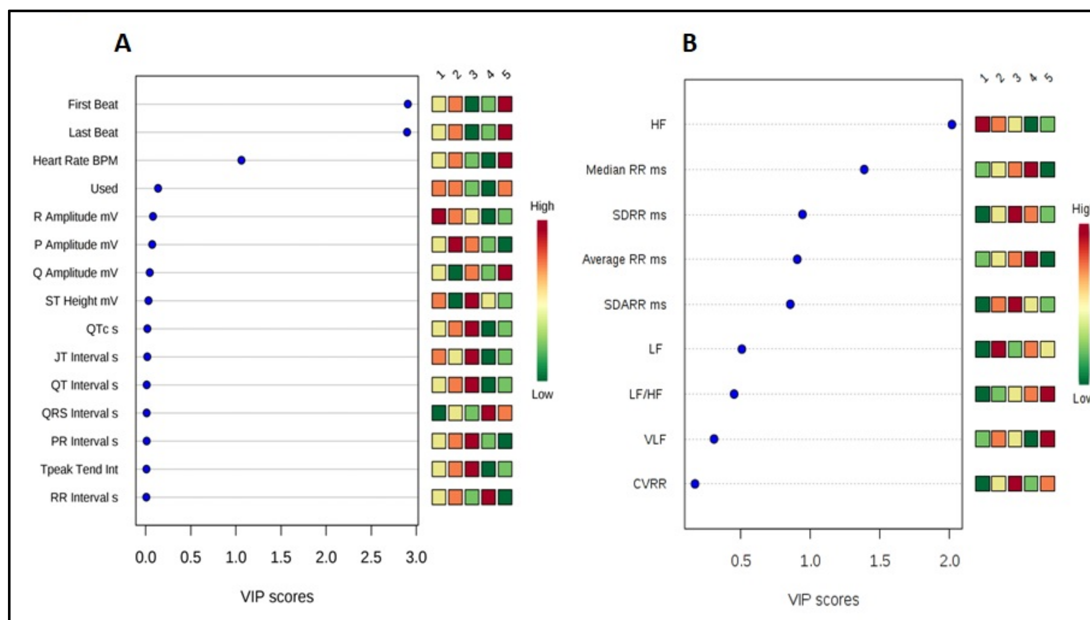
Representative box-cum-whisker plots showing quantitative variations of relative signal integrals for autonomic dysfunction relevant in the context of pathophysiology of mammary gland cancer. Groups were differentiated as: 1- Control (Normal saline, 3ml/kg, p.o.), 2-Toxic control (MNU 47mg/kg, i.v.), 3- Zaltoprofen (10 mg/kg, p.o. + MNU 47mg/kg, i.v.), 4- Zileuton (10 mg/kg, p.o. + MNU 47mg/kg, i.v.) and 5-Zaltoprofen+Zileuton-(5mg/kg+5mg/kg, p.o. + MNU 47mg/kg, i.v.). For presented ECG recordings, the VIP score >1 and statistical significance is at the level of $p \le 0.05$. In the box plots, the boxes denote interquartile ranges, horizontal line inside the box denote the median, and bottom and top boundaries of boxes are 25th and 75th percentiles, respectively. Lower and upper whiskers are 5th and 95th percentiles, respectively.

Figure 4: Effect of DuCLOX-2/5 inhibition treatment on HRV



Representative box-cum-whisker plots showing quantitative variations of relative signal integrals for HRV parameters relevant in the context of pathophysiology of mammary gland cancer. Groups were differentiated as: 1- Control (Normal saline, 3ml/kg, p.o.), 2-Toxic control (MNU 47mg/kg, i.v.), 3- Zaltoprofen (10 mg/kg, p.o. + MNU 47mg/kg, i.v.), 4- Zileuton (10 mg/kg, p.o. + MNU 47mg/kg, i.v.) and 5-Zaltoprofen+Zileuton-(5mg/kg+5mg/kg, p.o. + MNU 47mg/kg, i.v.). For presented heart rate variablity, the VIP score >1 and statistical significance is at the level of p□≤□0.05. In the box plots, the boxes denote interquartile ranges, horizontal line inside the box denote the median, and bottom and top boundaries of boxes are 25th and 75th percentiles, respectively. Lower and upper whiskers are 5th and 95th percentiles, respectively.

Figure 5: The ECG and HRV biomarker entities identified from PLS-DA analysis and are listed in decreasing order of VIP score to highlight their discriminatory potential.



In **(A and B)**, the complete HRV was used to PLS-DA modeling and resulted VIP scores for time and frequency domain are shown. Group 1: Control (Normal saline, 3ml/kg, p.o.), Group 2: Toxic control (47 mg/kg MNU, i.v.), Group 3: Zaltoprofen (10 mg/kg, p.o. + MNU 47mg/kg, i.v.), Group 4: Zileuton (10 mg/kg, p.o. + MNU 47mg/kg, i.v.), Group 5: Zaltoprofen + Zileuton (5mg/kg+5mg/kg, p.o. + MNU 47mg/kg, i.v.).

5.3.Carmine staining of whole mount's mammary gland

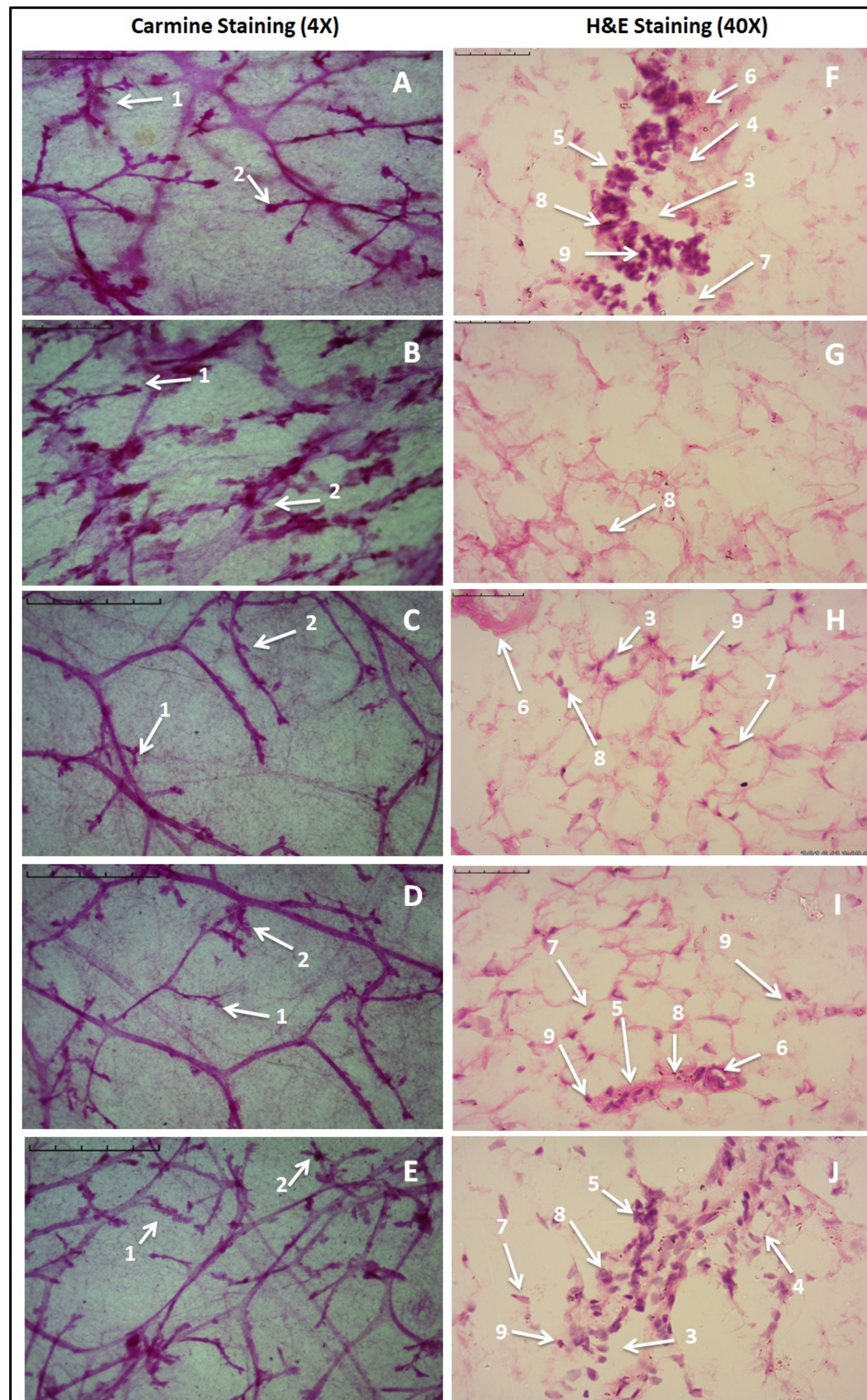
The whole mount preparations are often used as anappropriate method for the examination of small proliferativelesions as represented as an increase in the number of AB/TEBsand lobules.Zaltoprofen and zileuton treatment, and in combination therapy (**Figures 6C–E**), offered significant protection similar to control group (**Figure 6A**), against MNU induced degeneration of cellular morphology (**Figure 6B**).Persistent TEBs implies the deregulation of apoptosis. Theundifferentiated AB/TEBs are the sites for the malignanttransformations and growth in AB/TEBs

number is a directindicator of proliferative breast tissue.MNU treatment was marked increase in the lobules and AB count, representing cellular proliferation, and combination therapy afforded a marked protection against the same**(Table 5).**

5.4.Effects of zaltoprofen and zileuton on mammary gland morphology

H&E staining of the mammary gland tissue revealed the presence of duct, lymphocytes, adipocytes, loose connective tissue (LCT), dense connective tissue (DCT), cuboidal epithelial cells (CEC), and myoepithelial cells (MEC) in case of control animal (**Figure 6F**). MNU treated groups were observed for the loss of LCT, DCT and adipocytes, and scattered cuboidal epithelial cells (**Figure 6G**). Treatment with zaltoprofen and zileuton restored the cellular architecture as evident through presence of lymphocytes, adipocytes, LCT, DCT, and MEC (**Figures 6H,I**). It would be appropriate to remark that the combination therapy embarked a more profound effect in comparison to monotherapy (**Figure 6J**).

Figure 6: Microscopic evaluation of mammary gland tissue of the animal treated with Zaltoprofen, Zileuton and their combination through carmine and H&E staining.



Whole mount carmine alum staining of ductal epithelium reveals the presence of lobules (1) and AB (2) (A, B, C, D and E). The extent of AB and lobules formation was excessive in the MNU treated group (B) which was subsided through respective treatment zaltoprofen, zileuton and a combination (C, D & E). The images were captured under microscope with 4X

magnification. H&E staining of respective groups (F, G, H, I and J) revealed duct (3), adipocytes (4), LCT (5), DCT (6), MEC (7), lymphocytes (8) and CEC (9) in control (F) as well as treated groups zaltoprofen, zileuton and a combination treatment respectively (H, I and J). In MNU treated group (G), the cell morphology was distorted and cell organelles were absent. The images were captured under microscope with 40X magnification.

5.5.Effects on caspase-3 and caspase-8

Treatment with monotherapy and combination therapy of zaltoprofen and zileuton upregulated the apoptotic markers caspase-3 and caspase-8, when compared with MNU treatment (**Figure 7**).In death inducing signaling pathway, caspase-8 from theextrinsic pathway directly activates caspase-3, and facilitates therelease of cytochrome-c, which both proteins are imperative partof the intrinsic pathway (Zhuang et al., 1999; Steward and Brown,2013).From above, it became conspicuous that DuCLOX-2/5 inhibition treatment curtailed down the proliferative andanti-apoptotic effects of MNU when affirmed through themitochondrial mediated apoptotic pathway.

5.6.Western blot

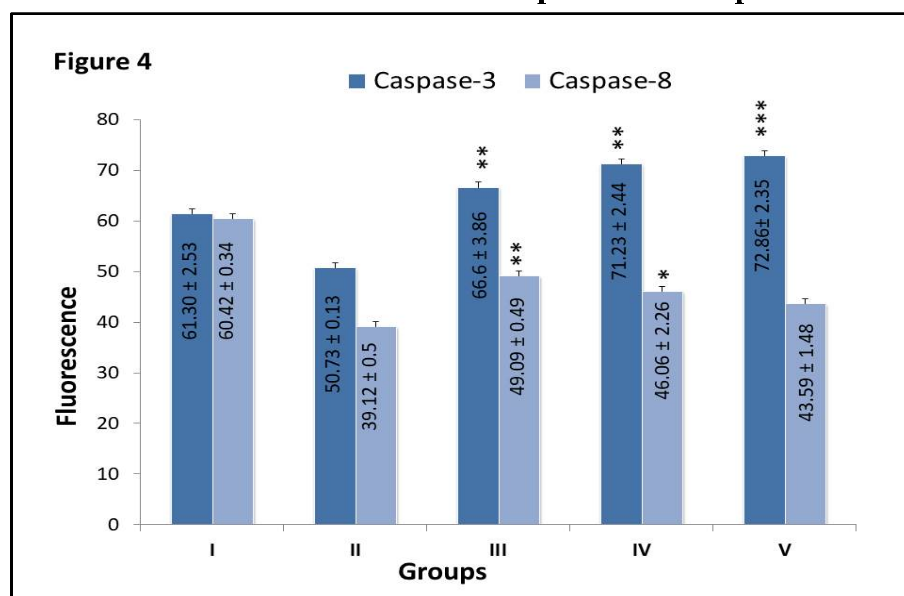
When ascertained on the grounds of protein expression of mitochondrial apoptotic pathway, MNU administration was recorded for the upregulated expression of BCL-2, BCL-xl, VDAC, Apaf-1 and procaspase-9, and downregulation of BAD, BAX and cytochrome-c. Zaltoprofen and zileuton, alone and in combination, demonstrated significant restoration of the apoptotic markers (**Figure 8**).

Table 5: Effect of Zaltoprofen and Zileuton on differentiation of mammary gland against MNU induced mammary gland carcinoma

Groups	AB1	AB2	AB1 +AB2	Lobules	DF.SCORE 1 (AB1+AB2+ Lobules)	DF.SCORE 2 (Lobules/AB1+AB2)
Control (NS, 3 ml/kg, i.p.)	14.5±0.70	0.5±0.70	15±1.41	1±0.00	16±1.41	0.66±0.00
MNU (47 mg/kg, i.v.)	21.5±6.36	11±2.8	32.5±9.19	6.5±2.12	39±11.31	0.19±0.00
Zaltoprofen (10 mg/kg, p.o.)	15.5±3.53	8±0.00	23.5±3.53	1±1.41***[c]	27.5±2.12**	0.17±0.08**[a]
Zileuton (10 mg/kg, p.o.)	17±2.82	8.5±0.70	25.5±2.12	5±2.82	30.5±0.70	0.20±0.12**
Zaltoprofen+Zileuton (5 mg/kg+ 5 mg/kg, p.o.)	14±2.82*	6.5±3.53**	20.5±6.36**	4.5±2.12	25±8.48**	0.21±0.03*

(Values are Mean ± SD), each group contains six animals. Comparisons were made on the basis of the one-way Anova followed by Bonferroni test. All groups were compared to the MNU treated group (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Group V were compared to the III and IV treated group ($^{c}p<0.05$, $^{b}p<0.01$, $^{a}p<0.001$)

Figure 7: Effect of DuCLOX-2/5 inhibition on caspase-3 and caspase-8



The activity of caspase was detected by commercial fluorescence based assay in Group I- Control (Normal saline, 3ml/kg, p.o.), II- Toxic control (MNU 47mg/kg, i.v.), III- Zaltoprofen (10 mg/kg, p.o. + MNU 47mg/kg, i.v.), IV- Zileuton (10 mg/kg, p.o. + MNU 47mg/kg, i.v.) and V- Zaltoprofen + Zileuton (5mg/kg+5mg/kg, p.o. + MNU 47mg/kg, i.v.). Data are expressed as mean+ SD of individual groups. Comparisons were made by the one-way ANOVA followed by Bonferroni multiple test. All groups were compared to the MNU treated group (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

Previous reports endorsed the combined inhibition of COX-2 and 5-LOX leads to apoptotic signaling in the malignant cellsvia modulating balance between death and anti-death proteins(Schroeder et al., 2007). Therefore, we scrutinized the markers of mitochondrial apoptosis to validate the efficacy of DuCLOX-2/5inhibition. Test groups alone and combination group decreasedanti-apoptotic BCL-2 and BCL-xl protein levels. In a similarline, the expression of pro-apoptotic proteins BAD and BAXwas found to be diminished in MNU toxic group and againnormalized in the test groups. The same was justified significantlywith the RT-PCR when contemplated

against genomic expressionof proteins of apoptosis. The monotherapy and combinationtherapy showed the significant impact on expression of VDAC,cytochrome-c, Apaf-1, and procaspase-9 toward normal. Inmitochondrial apoptosis pathway, cytochrome-c (acts as anintracellular death signal) combines with a cytosolic proteincalled Apaf-1 (apoptotic protein activating factor-1) to form acomplex called the apoptosome. Apaf-1 is a scaffold chaperonlike protein which when activated by cytochrome-c recruitsand activates procaspase-9 (Bratton and Salvesen, 2010). Onactivation of procaspase-9 to caspase-9, it activates downstreameffector caspase-3/7, and promotes apoptosis (Ghosh andKarin, 2002; McIlwain et al., 2013). However, Benedict et al.performed the analysis of normal tissue mRNAs to examinethe relative expression of the Apaf-1 isoforms in activationof procaspase-9. Experiment demonstrated the expression of multiple Apaf-1 isoforms in cancer cells, and specific isoformactivate procaspase- 9 in response to cytochrome-c and dATP,and form apoptosomes (Benedict et al., 2000). Hence, one cancommensurate that the increase in free APAF-1 expression,despite the overexpression of cytochrome c, in MNU treatedexperimental groups directly reflect its unbound form presentin cells. The same could be justified while conferring levels ofprocaspase-9 in toxic treatment. Apaf-1 and procaspase-9 werefound to be overexpressed reflecting its inactivated forms, whichare unable to form a complex to induce apoptosis after MNUtreatment.

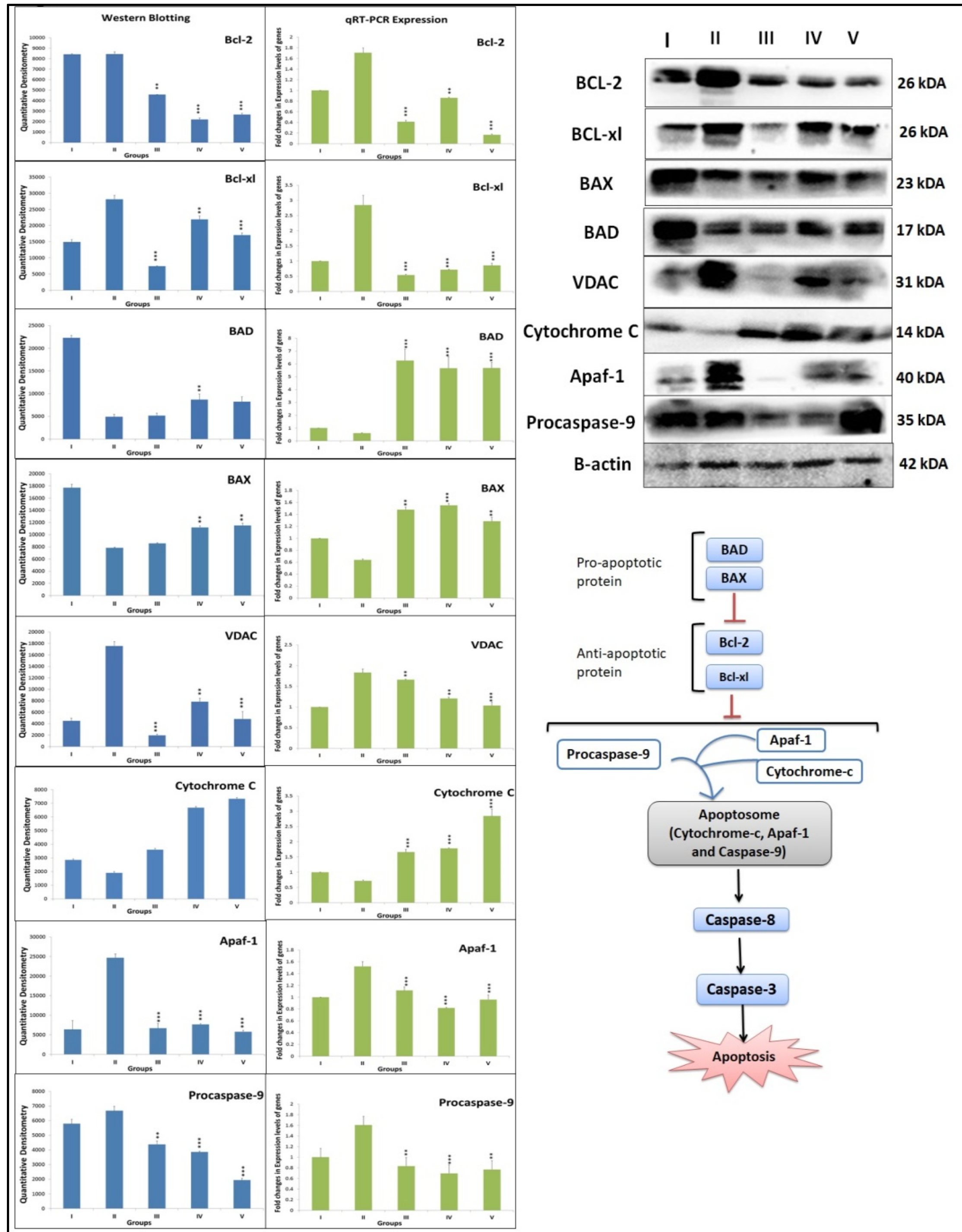
While ascertaining the expression of COX-2 and 5-LOX proteins in the inflammatory pathway, the same were found to be overexpressed in MNU treated animals. Test drug’s treatment, in particular, combination regime, modulated the

expression for COX and LOX proteins favorably (**Figure 9**).Since, the test drugs were well reportedto be specific inhibitors of COX-2 and 5-LOX, RT-PCR andimmunoblotting confirmed the expression of these enzymesconcomitantly curtailed down with the test drug treatment.

5.7.Quantitative RT-PCR

After ascertaining that Zaltoprofen and zileuton, alone and in combination, treatment was leading to elevated death protein and inflammatory markers, our next objective was to identifythe genomic contributors for the observed phenotype, which was achieved through quantitative PCR for genes BCL-2, BCL-xl, BAD, BAX, VDAC, Apaf-1, procaspase-9, cytochrome-c, COX-2, and 5-LOX. In comparison to the toxic, we observed a significant downregulated expression of gene BCL-2 (0.41, 0.86, and 0.16), BCL-xl (0.51, 0.71, and 0.85) and upregulation of BAD (6.26, 5.67, and 5.68), BAX (1.47, 1.55, and 1.28) in treatment groups of zaltoprofen, zileuton and combination treatment respectively. The fold change in relative expression of the genes of the cell death pathways, i.e., VDAC, Apaf-1, procaspase-9, cytochrome-c,(**Figure 8**) and COX-2 and 5-LOX of the inflammatory pathway was, however, close to normal (**Figure 9**).

Figure 8: DuCLOX-2/5 mediated activation of mitochondrial associated protein signaling in mammary gland cells



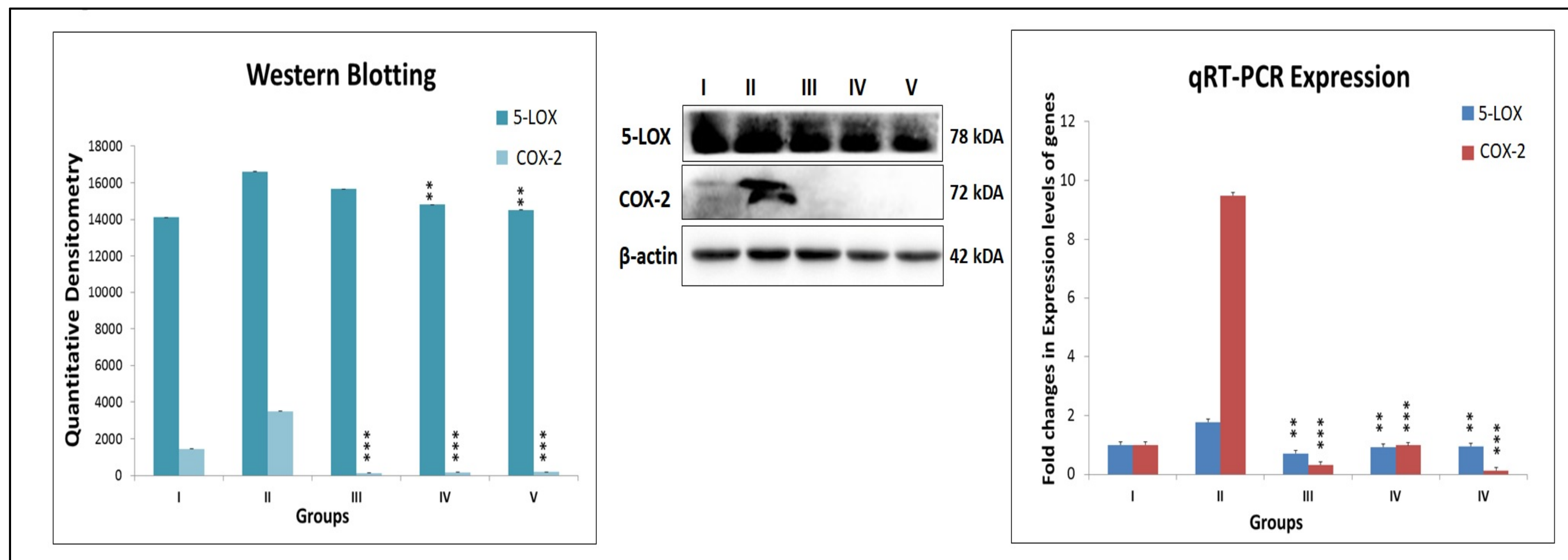
Protein extracted from individual groups [I-Control (Normal saline, 3ml/kg, p.o.), II-Toxic control (MNU 47mg/kg, i.v.), III- Zaltoprofen (10 mg/kg, p.o. + MNU 47mg/kg, i.v.), IV- Zileuton (10 mg/kg, p.o. + MNU 47mg/kg, i.v.) and V-Zaltoprofen + Zileuton (5mg/kg+5mg/kg, p.o. + MNU 47mg/kg, i.v.)] were subjected

to immunoblotting of proapoptotic (BAX and BAD) and anti-apoptotic (Bcl-2 and Bcl-xl) protein with downstream apoptotic markers (VDAC, cytochrome-c, Apaf-1 and procaspase-9) of respective pathway. mRNA expression of above mentioned protein were also in line with the findings of immunoblotting assay. β-actin was used as loading control. Each experiment was performed in triplicate. Values are presented as Mean ± SD. Comparisons were made by the one-way ANOVA followed by Bonferroni multiple test. All groups were compared to the MNU treated group (*p<0.05, **p<0.01, ***p<0.001).

5.8.1H-NMR method for serum metabolites profiling

The quantitative NMR profiles of sera were subjected to multivariate discriminaotry analysis to screen the different metabolites between the controls and tumor-bearing rats. The combined PCA score plot showed a clear pattern of clustering in different groups, and no outlier sample was detected (**Figure 10A**). As evident from **Figure 10A**, the PCA itself was able to give an excellent separation between the groups, where the treated group with dose zaltoprofen found to be closer to the normal control (NC) group compared to those treated with zileuton or combination.The supervised PLS-DA was used as a discriminatory model to distinguish between the groups and to identify the marker metabolites that differentiate the groups. The combined PLS-DA score plot for all the groups (**Figure 10B**) showed that the samples in various groups are well clustered; Toxic control (TC) being the farthest and zaltoprofen group being the closest to NC group. Additionally, OPLS-DA was also employed for combined analysis of spectral data which further support for the trends as observed in PCA and PLSDA (**Figure 10C**).

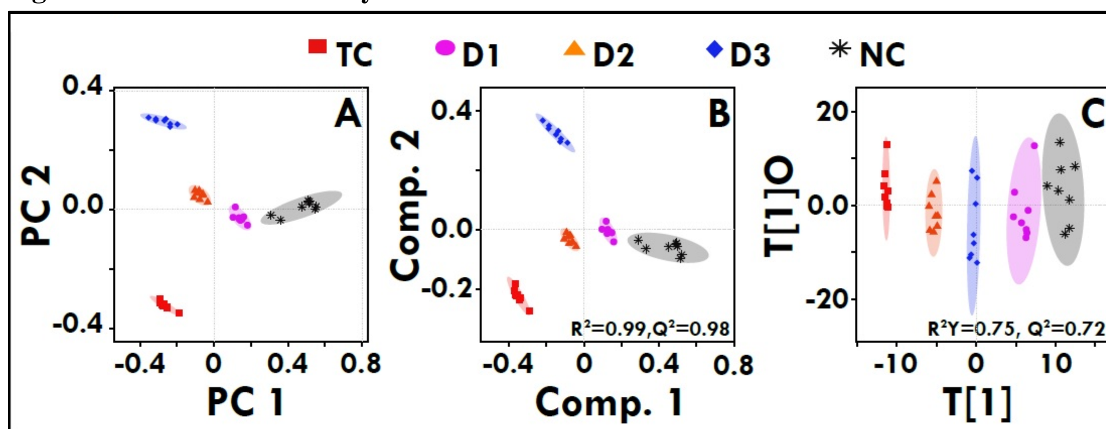
Figure 9: Expression level of protein of COX-2 and 5-LOX through western blot and levels of gene contributor through quantitative RT-PCR on DuCLOX-2/5 inhibition treatment.



Immunoblotting of respective individual group [I-Control (Normal saline, 3ml/kg, p.o.), II- Toxic control (MNU 47mg/kg, i.v.), III- Zaltoprofen (10 mg/kg, p.o. + MNU 47mg/kg, i.v.), IV- Zileuton (10 mg/kg, p.o. + MNU 47mg/kg, i.v.) and V- Zaltoprofen + Zileuton (5mg/kg+5mg/kg, p.o. + MNU 47mg/kg, i.v.)] for COX-2 and 5-LOX. Excised mammary gland tissue sample lysed in trizol for RNA extraction and analyzed for the mRNA expression of COX-2 and 5-LOX by qRT-PCR. β-actin was used as loading control. Each experiment was performed in triplicate. Values are presented as Mean ± SD. Comparisons were made by the one-way ANOVA followed by Bonferroni multiple test. All groups were compared to the MNU treated group (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

Similarly, the pairwise PCA, PLS-DA and OPLS-DA analyses were also performed comparing all the study groups toxic control, zaltoprofen, zileuton and combination therapy, with respect to the NC (**Figure 11**). Each pairwise model revealed that there are significant metabolic differences in treated rats compared to TC rats as evident from the model cross-validation parameters R2 and Q2, representingthe explained variance and predictive capability of the model, respectively.

Figure 10: Multivariate analysis



The combined 2D PCA **(a)** and 2D PLS-DA **(b)** 2D OPLS-DA **(c)** score plots derived from cumulative analysis of 1D ^{1}H CPMG NMR spectra comprising of all the groups: NC- Normal control (Normal saline, 3ml/kg, p.o.), TC-Toxic control (MNU 47mg/kg, i.v.), D1-Zaltoprofen (10 mg/kg, p.o. + MNU 47mg/kg, i.v.), D2- Zileuton (10 mg/kg, p.o. + MNU 47mg/kg, i.v.) and D3-Zaltoprofen+Zileuton-(5mg/kg+5mg/kg, p.o. + MNU 47mg/kg, i.v.). Colour circles indicate the 95% confidence interval for each class (**9B**). Colour circles indicate the 95% confidence interval for each class.

Further, significant metabolic features responsible for theseparation between the groups were identified based on theirvariable influence on the projection (VIP) scores in the PLSDA model. In general, significant metabolites were selected based on VIP scores of more ≥1; however, a stringent VIPscore of 2 has been selected in the

present study to screen outthe metabolites of discriminatory significance. The metabolitesranked according to their VIP scores were identified using theassignment shown in **Figure 12**and then labelled on the corresponding VIP score plots as shown in **Figures 13& 14**. The pair wise analysis between NC and MNU treatment, showed a clear differentiation indicating significant metabolicalterations in MNU group. Overall, we identified 16 metabolitessignificantly perturbed in the sera of MNU treated animalscompared to NC. These markers metabolic entities alongwith their chemical shifts, variable importance on projection(VIP) score and *p*-value are listed in **Table 6**. Compared withcontrol group, MNU treatment had a significant elevation ofVLDL/LDL, PUFA, choline, isoleucine, leucine, valine, alanine,proline, tyrosine, phenylalanine, NAG, OAG whereas, they werehaving decreased levels of glucose, lactate, creatine, trimethylamine-N-oxide (TMAO), andformate. These metabolic changes observed in MNU treated rats might be related to multiple tumor-related metabolicpathways, involving energy metabolism, amino acid metabolism,fatty acid metabolism and choline phospholipid metabolism.As evident from **Table 6**, these metabolic alterations werefound to get ameliorated partially after the treatment, however,different treatments resulted in a different metabolic response(**Table 6**). The treatment effect is also pictorially depicted throughrepresentative box plots shown in **Figure 15**. To summarize, thezaltoprofen treatment was effective in resetting the elevatedserum levels of proline, alanine, valine, lipid metabolites (VLDL/LDL,PUFA), NAG and decreased serum levels of formate, lactate, TMAO, and creatine. Zileuton was well in range to reset theelevated serum levels of isoleucine, leucine, valine, alanine,proline, phenylalanine, lipid metabolites

(VLDL/LDL, PUFA), anddecreased serum levels of formate, lactate, and creatine.All in all, the combination therapy improved the serum metabolic profiles of branched chain and aromatic amino acids along with choline metabolism.

We observed a significant increase in the levelsof PUFAs, lipoproteins (LDL/VLDL), and choline in theTC rats compared to NC, suggesting altered phospholipidmetabolism (choline/GPC) and fatty acid metabolism (PUFAs,LDL/VLDL) in TC rats. The lipoproteins (VLDL/LDL) mainlytransports cholesterols, oxysterols and triglycerides from theliver to rapidly proliferating cancer cells where it is used inmembrane biogenesis, protein modifications, and steroidhormone production (Flote et al., 2016). Further fatty acids andlipids are also consumed through β-oxidation to meet the energyrequirement for cell membrane synthesis, rapid proliferationand cancer cell survival (Zhang and Du, 2012). On the contrary,PUFAs and choline are important intermediates of membranemetabolism and inflammatory mediators (Raphael and Sordillo, 2013). Therefore, the elevated levels might be related to their augmented utilization to repair the damaged cells and dampen the inflammation associated with TC induced injury to mammary gland cells (Zhang and Spite, 2012). Chronicinflammation is a common clinical manifestation of variouscancer types. Consistent with this the increased levels of Nand O -acetyl glycoproteins (NAG and OAG) were presentin the rat sera. The NAG and OAG are acute phase proteinsand are expressed more during infection, trauma, surgery,and inflammation (Saldova et al., 2007) and are consistentwith various types of cancers (Jobard et al., 2014). Both cancerand inflammation are known to trigger a hyper-catabolicstate, resulting in increased energy requirements and

proteinmetabolism.Consistent with this phenomenon, the decreasedserum levels of glucose and lactate indicate the increased energydemand in these rats to sustain the active inflammatory processesand cell proliferation activities. The decreased levels of serumglucose in the animals imply that the glucose is being rapidlyconsumed by aerobic glycolysis for tumor cell proliferationand growth, which is consistent with the “Warburg effect”(Vander Heiden et al., 2009). The significant increase in serumlactate levels has been instituted in many cancer’s studies.

Nevertheless, in our study the serum lactate levels were foundto be depleted, as cancer cells take up the lactate and use itto feed cancer cell mitochondrial energy production and togenerate mitochondrial precursors for cancer cell biogenesis alsocalled as “reverse Warburg’s effect” (Wallace, 2012). To maintainphysiological homeostasis and meet the energy requirementsof cancerous cells, there is an increased reliance on alternateenergy substrates preferably amino acids. Amino acids serve asa major source of energy, especially during conditions in whichglucose availability is limited. Muscles along with the liver releasehigh quantity of amino acids present in the body to maintainthe cellular homeostasis in conditions of energy deprivation(Schutz, 2011). Consistent with this, increased levels of severalamino acids in the sera such as alanine, proline, branched chainamino acid (isoleucine, leucine, valine-BCAA) and aromaticamino acid (phenylalanine, tyrosine-AAA) suggests aberrantamino acid metabolism. The amino acids are broken down intopyruvate, alpha-ketoglutarate, succinyl-CoA, fumarate, and/oroxaloacetate that can be predominantly converted into glucoseor glycogen via TCA cycle or gluconeogenesis to generate energyduring stress (DeBerardinis et al., 2008). The

decreased level ofcreatine is supposed to compensate for the lower efficiency ofATP production and act as an alternate energy source for cancerproliferation. TMAO is a product of gut microflora activity;decreased levels might be the consequence of adaptation to thedisease state (Hartiala et al., 2016). Formate is primarily derivedfrom mitochondrial metabolism and is the precursor moleculerequired to make DNA and other critical compounds within thecell (Ahn and Metallo, 2015; Newman and Maddocks, 2017) andmight be responsible for their decreased level in the serum.

Asevident from our study the up and down regulated metabolites,suggest perturbed glycolysis, beta-oxidation pathway, andderanged mitochondrial activity. Henceforth, authors would liketo submit that zaltoprofen, zileuton and a combination dose canfavorably regulate the metabolic alterations induced by MNU.

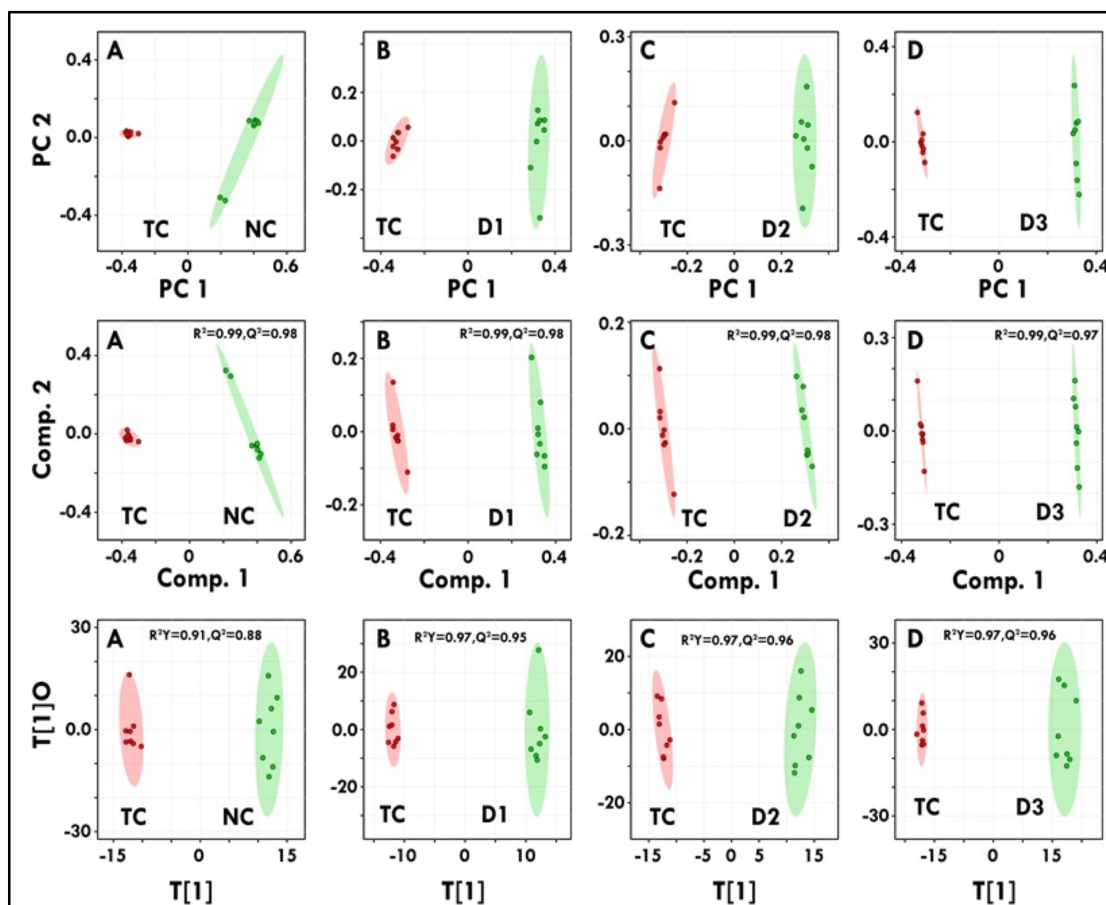
Table 6: Metabolic variability's among the groups treated with MNU, Zaltoprofen and Zileuton when compared to toxic control.

S No	Metabolites	^{1}H (ppm)	Control vs. Toxic Control	Zaltoprofen vs. Toxic Control	Zileuton vs. Toxic Control	Zaltoprofen +Zileuton vs. Toxic Control
1	LDL/VLDL	0.89/1.27	↓↓↓↓	↓↓↓	↓↓↓	↑
2	Iso/Leucine	0.95	↓↓↓↓	↓↓	↓↓↓	↓↓↓
3	Valine	0.97	↓↓↓↓	↓↓↓	↓↓↓	↓↓↓
4	Lactate	1.31	↑↑↑↑	↑↑↑	↑↑	↑↑↑
5	Alanine	1.45	↓↓↓↓	↓↓	↓↓	↓↓↓
6	Proline	1.99	↓↓↓↓	↓↓↓	↓↓	↓
7	NAG	2.01	↓↓↓↓	↓↓↓	↓↓	↓↓
8	OAG	2.11	↓↓↓↓	↓↓↓	↑↑↑	↑↑↑
9	Creatine	3.01	↑↑↑↑	↑↑	↑↑↑	↑↑
10	Choline	3.19	↓↓↓↓	↑↑↑	↑↑	↓↓↓
11	TMAO	3.25	↑↑↑↑	--	↑↑↑	↑↑
12	Glucose	3.39	↑↑↑↑	↓↓↓	↓↓	↓
13	PUFA	5.31	↓↓↓↓	↓↓↓	↓↓	↑↑
14	Tyrosine	7.17	↓↓↓↓	↑↑	↓	--
15	Phenylalanine	7.31	↓↓↓↓	↓↓↓	↓↓	--
16	Formate	8.43	↑↑↑↑	↑#	↑#	↑#

The up and down arrows represent, respectively, increased and decreased metabolite levels. A ↑↑↑/↓↓↓ or ↑↑/↓↓ score was given to the metabolites of the treatment dose which showed ameliorating effects from MNU towards control. Abbreviations used are as follows: LDL (low density lipoproteins); VLDL (very low density lipoproteins); NAG (N-acetyl glycoprotein); OAG (O-acetyl glycoprotein); PUFA (poly unsaturated fatty acids).

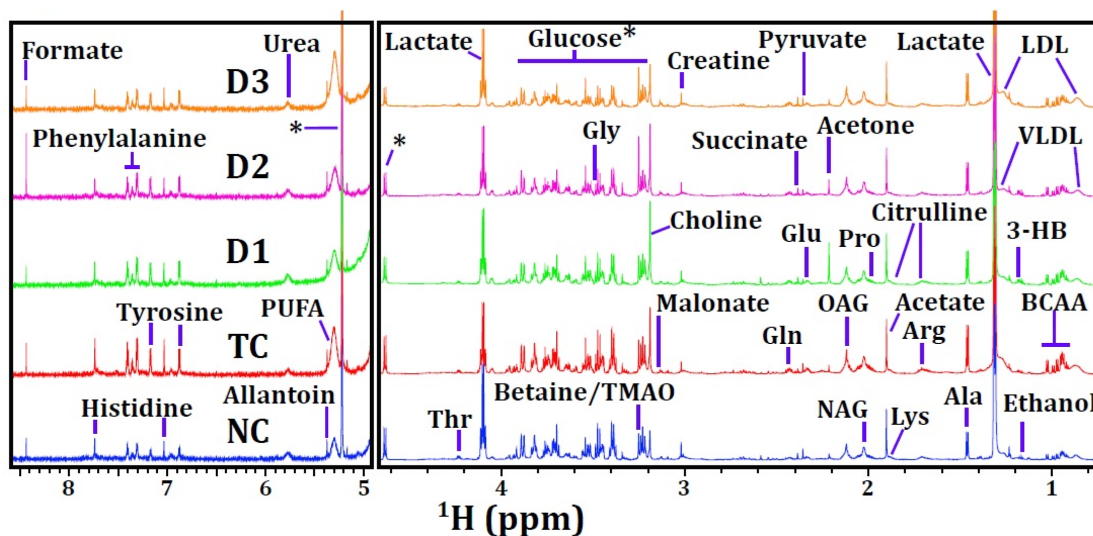
Note-: Symbols * = p-value < 0.05; # = VIP Score < 1; -- = Level similar to control.

Figure 11: The 2D PCA score plots for pairwise analysis.



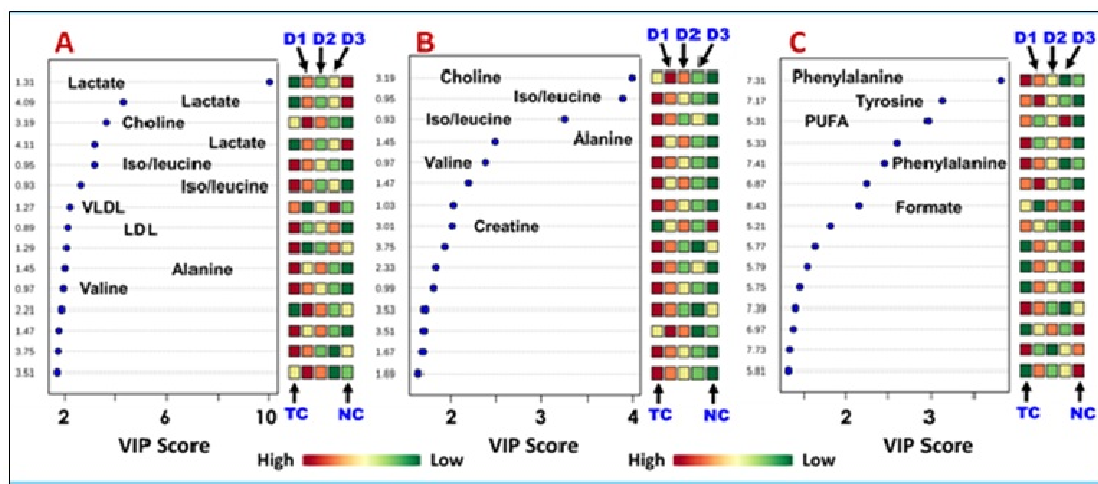
The pair wise 2D PCA, PLS-DA, and OPLS-DA score plots with their respective R^2 and Q^2 values derived from 1D ^{1}H CPMG NMR spectra. Groups were differentiated as: NC- Normal control (Normal saline, 3ml/kg, p.o.), TC-Toxic control (MNU 47mg/kg, i.v.), D1- Zaltoprofen (10 mg/kg, p.o. + MNU 47mg/kg, i.v.), D2- Zileuton (10 mg/kg, p.o. + MNU 47mg/kg, i.v.) and D3-Zaltoprofen+Zileuton-(5mg/kg+5mg/kg, p.o. + MNU 47mg/kg, i.v.). (**A**) NC vs. TC, (**B**) NC vs. D1, (**C**) NC vs. D2, (**D**) NC vs. D3. Coloured circles indicate the 95% confidence interval for each class.

Figure 12: Stack plot of representative 1D ^{1}H NMR spectra of rat sera obtained from different groups



The representative1D ^{1}H CPMG NMR spectra of rat serum obtained from different groups. The peaks annotated in the Figure show the assignments of serum metabolites. Groups were differentiated as: NC- Normal control (Normal saline, 3ml/kg, p.o.), TC-Toxic control (MNU 47mg/kg, i.v.), D1- Zaltoprofen (10 mg/kg, p.o. + MNU 47mg/kg, i.v.), D2- Zileuton (10 mg/kg, p.o. + MNU 47mg/kg, i.v.) and D3-Zaltoprofen+Zileuton-(5mg/kg+5mg/kg, p.o. + MNU 47mg/kg, i.v.).The abbreviations used are: LDL/VLDL: Low/very-low density lipoproteins; PUFA: polyunsaturated fatty acids; BCAA: Isoleucine, Leucine, Valine, 3-HB: 3-hydroxy-butyrate; Ala: Alanine; Arg: Arginine; Lys: Lysine; NAG: N-acetyl glycoproteins; OAG: O-acetyl glycoprotein; Pro: Proline; Glu: Glutamate; Gln: Glutamine; TMAO: Trimethylamine-N-oxide; Gly: Glycine; Thr: Threonine.

Figure 13: The potential biomarker metabolite entities identified from PLS-DA analysis and are listed in decreasing order of VIP score to highlight their discriminatory potential.



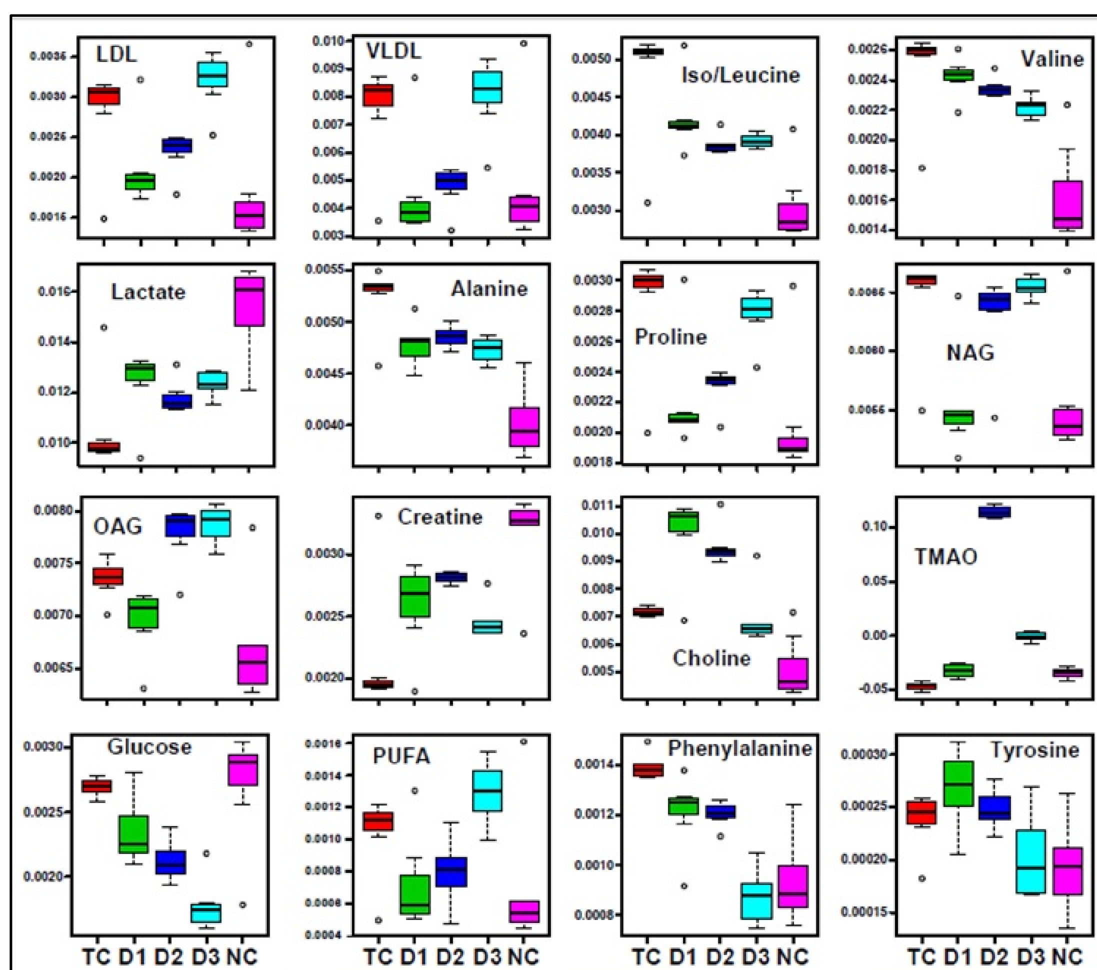
In **(A)**, the complete NMR data matrix was used to PLS-DA modeling and resulted VIP scores for top 35 metabolite entities are shown. In **(B)**, the down-field spectral region from 5.4 to 9.5 ppm was used for PLS-DA modeling and revealed the discriminatory importance of aromatic amino acids like Histidine, Tyrosine and phenylalanine. In **(C)**, the up-field spectral region from 0.9 to 4.5 ppm was used for PLS-DA modeling and revealed the discriminatory importance of other serum metabolites mainly amino acids and metabolites of tricarboxylic acid cycle. Groups were differentiated as: NC- Normal control (Normal saline, 3ml/kg, p.o.), TC-Toxic control (MNU 47mg/kg, i.v.), D1- Zaltoprofen (10 mg/kg, p.o. + MNU 47mg/kg, i.v.), D2- Zileuton (10 mg/kg, p.o. + MNU 47mg/kg, i.v.) and D3- Zaltoprofen+Zileuton-(5mg/kg+5mg/kg, p.o. + MNU 47mg/kg, i.v.).

Figure 14: The potential biomarker metabolite entities identified from PLS-DA analysis and are listed in decreasing order of VIP score to highlight their discriminatory potential.

In **(A)**, the complete NMR data matrix was used to PLS-DA modeling and resulted VIP scores for top 35 metabolite entities are shown. In **(B)**, the down-field spectral region from 5.4 to 9.5 ppm was used for PLS-DA modeling and revealed the

discriminatory importance of aromatic amino acids like Histidine, Tyrosine and phenylalanine. In **(C)**, the up-field spectral region from 0.9 to 4.5 ppm was used for PLS-DA modeling and revealed the discriminatory importance of other serum metabolites mainly amino acids and metabolites of tricarboxylic acid cycle.Groups were differentiated as: NC- Normal control (Normal saline, 3ml/kg, p.o.), TC-Toxic control (MNU 47mg/kg, i.v.), D1- Zaltoprofen (10 mg/kg, p.o. + MNU 47mg/kg, i.v.), D2- Zileuton (10 mg/kg, p.o. + MNU 47mg/kg, i.v.) and D3- Zaltoprofen+Zileuton-(5mg/kg+5mg/kg, p.o. + MNU 47mg/kg, i.v.).

Figure 15: Metabolic variability on DuCLOX-2/5 inhibition treatment



Representative box-cum-whisker plots showing quantitative variations of relative signal integrals for serum metabolites relevant in the context of pathophysiology of mammary gland cancer. Groups were differentiated as: NC- Normal control (Normal saline, 3ml/kg, p.o.), TC-Toxic

control (MNU 47mg/kg, i.v.), D1- Zaltoprofen (10 mg/kg, p.o. + MNU 47mg/kg, i.v.), D2-Zileuton (10 mg/kg, p.o. + MNU 47mg/kg, i.v.) and D3-Zaltoprofen+Zileuton-(5mg/kg+5mg/kg, p.o. + MNU 47mg/kg, i.v.). For presented metabolite entities, the VIP score >1 and statistical significance is at the level of $p \leq 0.05$. In the box plots, the boxes denote interquartile ranges, horizontal line inside the box denote the median, and bottom and top boundaries of boxes are 25th and 75th percentiles, respectively. Lower and upper whiskers are 5th and 95th percentiles, respectively.

Chapter 6

Summary and Conclusion

6.Summary and Conclusion

The present study was inquested to elucidate the effect ofDuCLOX-2/5 inhibition against MNU induced mammary glandcarcinogenesis. Zaltoprofen (a specific COX-2 inhibitor) andzileuton (a specific 5-LOX inhibitor) were scrutinized alone andas a combination regime against MNU induced mammary glandcarcinogenesis.

The precedent studies have suggested the participationof the reactive oxygen species (ROS) in conjunction withthe AA mediated inflammatory pathway in carcinogenesis.The authors evaluated the oxidativestress biomarkers primarily relying on inflammation andcarcinogenesis.We observeda decrease in SOD, catalase and GSH, and increase in TBARs and PC in MNU treated groups affirming ROS attack, and the same could be attributed to celltoxicity.Collectively, this can be stated that the proposed regimeof DuCLOX-2/5 inhibition can impart favorable regulation ofoxidative stress markers in MNU induced carcinogenesis.

In the emergence of the risk factors associated with thechemotherapeutic agents, autonomic dysfunction and associatedcardiovascular abnormalities are among the most prevailingcomplications (Albini et al., 2010; Ewer and Ewer, 2010).In ECG profiling, MNU administration was found to be evident forprolongation of the QRS, QT, and QTc interval reflecting thecardiac risks with the carcinogenic which was restored back to normal after treatment regime.The time andfrequency-domain parameters for HRV were also favourablyregulated by the proposed regime, implicating considerableregulation of autonomic control during cancer progression.

The MNU treatment wasevident as the increase in AB/TEB count and DF score, which isin corroboration with the previous findings (Manral et al., 2016;Rani et

al., 2016). Treatment with monotherapy and combinationtherapy significantly curtailed down the AB/TEBs count andDF score. For validation of the morphology observed with thecarmine staining, mammary gland tissues were further examinedhistopathologically. Mammary gland tissue from the controlanimals revealed the presence of duct, adipocytes, LCT, DCT,lymphocytes, CEC, and MEC. MNU treatment was recordedto have distorted cellular architecture, with scattered cuboidalepithelial cells and loss of adipocytes, which is in line with theprevious reports and same, has been restored with treatmentgroups.

Reports have endorsed the combined inhibition of COX-2 and 5-LOX leads to apoptotic signaling in the malignant cellsvia modulating balance between death and anti-death proteins(Schroeder et al., 2007). Therefore, we scrutinized the markers of mitochondrial apoptosis to validate the efficacy of DuCLOX-2/5inhibition. Test groups alone and combination group decreasedanti-apoptotic BCL-2 and BCL-xl protein levels. In a similarline, the expression of pro-apoptotic proteins BAD and BAXwas found to be diminished in MNU toxic group and againnormalized in the test groups. The monotherapy and combinationtherapy showed the significant impact on expression of VDAC,cytochrome-c, Apaf-1, and procaspase-9 toward normal.Considering the findings from the preceding paragraph,authors find it more justifiable to extend the dimensionof evidence through genomic expression for the observedphenotype. The genomic contributors' expression throughquantitative RT-PCR mirrors the immunoblotting studies, andconfirms the effective treatment of DuCLOX inhibitor.

The current study also established the apoptotic potentialof zaltoprofen and zileuton when evaluated for caspase activitybiochemically.Subsequential significant upsurge of caspase-8and caspase-3 levels were found in test compounds, alone orin combination, against MNU administration (Cheung et al.,2006).

To make the study more defensible for the role of DuCLOX-2/5 inhibition in cancer progression, the COX-2 and 5-LOXgene expression were assessed along with the mitochondrialapoptotic proteins. Since, the test drugs were well reportedto be specific inhibitors of COX-2 and 5-LOX, RT-PCR andimmunoblotting confirmed the expression of these enzymesconcomitantly curtailed down with the test drug treatment.

NMR-based serum metabolomics in conjunction withmultivariate data analysis revealed the metabolic profile ofMNU treated rats, and those treated with dose zaltoprofen andzileuton alone, and in combination compared to toxic control.The treatment groups zaltoprofen, zileuton and a combination were shifted relativelyclose toward control, showing the improving effects fromthe treatment

In summary, this study identified DuCLOX-2/5 inhibition aschemopreventive targets of mammary gland cancer. Theirspecific inhibitors prevented MNU-induced mammarygland carcinogenesis through their inhibitory effects on AAmetabolism. Considering the lack of pharmaceutical agents withthe potential to inhibit DuCLOX-2/5 in particular, we believefurther studies are needed to be implemented in a safe and efficacious strategy for prevention of human mammary glandcancer.

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Appendix -I

Animal Approval Certificate

Animal Approval Certificate

INSTITUTIONAL ANIMAL ETHICAL COMMITTEE (IAEC)
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Ref : **BBDNIIT/IAEC/020/2014** Date : May 19, 2014

CERTIFICATE

This is to certify that Mr./Ms. Swetlana is permitted to carry out experiments on animals for the research work entitled **Modulating Cyclooxygenase and Lipooxygenase Pathway in Cancer Chemoprevention** as per the details mentioned and after observing the usual formalities laid down by IAEC as per the provisions made by CPCSEA.

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Appendix -II

Plagiarism Report

Plagiarism report of thesis

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1.

Introduction Among the vast number of factors involved in tumor progression, arachidonic acid (AA) and its metabolites are well perceived for their convincing role in cancer biology. Affirmations for the role of inflammation in cancer comes from a large number of epidemiological findings illustrating that prolonged treatment with a number of anti-inflammatory drugs, including non-steroidal anti-

inflammatory drugs (NSAIDs) can scale down the incidence of several human cancers [

Dubois, 2004; Green et al., 2011; Hammamieh et al., 2007; Jänne and Mayer, 2000;].

AA (5, 8, 11, 14-eicosatetraenoic acid) is an essential fatty acid and a member of the omega-6 (x-6) polyunsaturated fatty acids (PUFA) [Astorg, 2004]. AA is required by the majority of mammals and

plays a key role in metabolism, cell signaling and inflammation. The impetus of the enzyme phospholipase A2 releases AA from membrane phospholipids, which is further metabolized by the two key enzymes, namely cyclooxygenase (COX) and lipoxygenase (LOX).

The metabolites of the AA are

biologically active lipids' mediators and are termed as eicosanoids. Eicosanoids have a strategic role in diverse biological processes, including the regulation of immune response, development, reproduction and cancer

Appendix -III

Publications

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 06 April 2018
doi: 10.3389/fphar.2018.00314

DuCLOX-2/5 Inhibition Attenuates Inflammatory Response and Induces Mitochondrial Apoptosis for Mammary Gland Chemoprevention

Swetlana Gautam[1], Atul K. Rawat[2], Shreesh R. Sammi[3], Subhadeep Roy[1], Manjari Singh[1], Uma Devi[4], Rajnish K. Yadav[1], Lakhveer Singh[1], Jitendra K. Rawat[1], Mohd N. Ansari[5], Abdulaziz S. Saeedan[5], Dinesh Kumar[2], Rakesh Pandey[3] and Gaurav Kaithwas[1]*

[1] Department of Pharmaceutical Sciences, School of Biosciences and Biotechnology, Babasaheb Bhimrao Ambedkar University (A Central University), Lucknow, India, [2] Center for Biomedical Research, Sanjay Gandhi Post Graduate Institute of Medical Sciences Campus, Lucknow, India, [3] Department of Microbial Technology and Nematology, CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow, India, [4] Department of Pharmaceutical Sciences, Faculty of Health and Medical Sciences, Sam Higginbottom Institute of Agricultural Sciences and Technology, Allahabad, India, [5] Department of Pharmacology, College of Pharmacy, Prince Sattam Bin Abdulaziz University, Al-Kharj, Saudi Arabia

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Gautam S, Rawat AK, Sammi SR, Roy S, Singh M, Devi U, Yadav RK, Singh L, Rawat JK, Ansari MN, Saeedan AS, Kumar D, Pandey R and Kaithwas G (2018) DuCLOX-2/5 Inhibition Attenuates Inflammatory Response and Induces Mitochondrial Apoptosis for Mammary Gland Chemoprevention. Front. Pharmacol. 9:314. doi: 10.3389/fphar.2018.00314

The present study is a pursuit to define implications of dual cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX) (DuCLOX-2/5) inhibition on various aspects of cancer augmentation and chemoprevention. The monotherapy and combination therapy of zaltoprofen (COX-2 inhibitor) and zileuton (5-LOX inhibitor) were validated for their effect against methyl nitrosourea (MNU) induced mammary gland carcinoma in albino wistar rats. The combination therapy demarcated significant effect upon the cellular proliferation as evidenced through decreased in alveolar bud count and restoration of the histopathological architecture when compared to toxic control. DuCLOX-2/5 inhibition also upregulated levels of caspase-3 and caspase-8, and restored oxidative stress markers (GSH, TBARs, protein carbonyl, SOD and catalase). The immunoblotting and qRT-PCR studies revealed the participation of the mitochondrial mediated death apoptosis pathway along with favorable regulation of COX-2, 5-LOX. Aforementioned combination restored the metabolic changes to normal when scrutinized through ^{1}H NMR studies. Henceforth, the DuCLOX-2/5 inhibition was recorded to import significant anticancer effects in comparison to either of the individual treatments.

Keywords: DuCLOX-2/5 inhibition, angiogenesis, apoptosis, cyclooxygenase, lipoxygenase, NMR, Zaltoprofen, Zileuton

INTRODUCTION

Accruing numbers of factors involved in tumorigenesis, mounting evidence indicates that the inflammatory microenvironment accounts for the tumor development. Arachidonic acid (AA) and its metabolites involve the presumed convincing role in cancer biology (Hammamieh et al., 2007; Greene et al., 2011). Prolonged treatment with the non-steroidal anti-inflammatory drugs (NSAIDs) was well proved evidence to be associated with a lower risk of the several cancers, including mammary gland cancer (Dubois, 2000; Jänne and Mayer, 2000; Joyce and Pollard, 2009; Qian and Pollard, 2010).

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Breast Cancer
DOI 10.1007/s12282-016-0723-2

REVIEW ARTICLE

DuCLOX-2/5 inhibition: a promising target for cancer chemoprevention

Swetlana Gautam[1] · Subhadeep Roy[1] · Mohd Nazam Ansari[2] · Abdulaziz S. Saeedan[2] · Shubhini A. Saraf[1] · Gaurav Kaithwas[1]

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Abstract Cancer is a leading cause of death and major health concern worldwide. The animal and human studies support the presumption that inflammation directs the cancer initiation and progression. Cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX) are the key players in the inflammatory cascade contributing towards the angiogenesis, tumor cell invasiveness, and disruption in the pathways of cellular proliferation/apoptosis. Contemporary studies have particularized a promising role of COX-2 and 5-LOX inhibitors in cancer chemoprevention. The present review is a pursuit to define implications of dual COX-2 and 5-LOX (DuCLOX-2/5) inhibition on various aspects of cancer augmentation and chemoprevention.

Keywords Angiogenesis · Apoptosis · Cyclooxygenase · Lipoxygenase · NSAIDs · Proliferation

Abbreviations

ATP	Adenosine triphosphate
AA	Arachidonic acid
Bcl-2	B cell chronic lymphocytic leukaemia-2
COX	Cyclooxygenase
ERK	Extracellular signal regulated kinase
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
HETE	Hydroeicosa-tetraenoic acid
HPETE	Hydroperoxyeicosa-tetraenoic acid
LXs	Lipoxins
LOX	Lipoxygenase
LTs	Leukotrienes
MAPK	Mitogen activated protein kinase
NSAIDs	Non-steroidal anti-inflammatory drugs
NF-Kb	Nuclear factor-kappa B
PPAR	Peroxisome proliferator activated receptor
PI3K/AKT	Phosphoinositide-3 kinase/AKT
PLA_2	Phospholipase A_2
PIP_2	Phosphatidylinositol 4, 5-bisphosphate
PIP_3	Phosphatidylinositol-3,4,5-triphosphate
PUFA	Polyunsaturated fatty acids
PKC	Protein kinase C
PGI_2	Prostacyclin
PGs	Prostaglandins
RTKs	Receptor tyrosine kinase
RXR	Retinoid X receptor
TXs	Thromboxane
VEGF	Vascular endothelial growth factor
5-HETE	5-Hydroxytetraenoic acid
FLAP	5-LOX activating protein

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Introduction

Among the vast number of factors involved in tumor progression, arachidonic acid (AA) and its metabolites are well perceived for their convincing role in cancer biology. Affirmations for the role of inflammation in cancer comes from a large number of epidemiological findings illustrating that prolonged treatment with a number of anti-inflammatory drugs, including non-steroidal anti-

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RSC Advances

PAPER

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Rifaximin, a pregnane X receptor (PXR) activator regulates apoptosis in a murine model of breast cancer

Swetlana Gautam,[a] Priyanka Singh,[a] Manjari Singh,[a] Subhadeep Roy,[a] Jitendra K. Rawat,[a] Rajnish K. Yadav,[a] Uma Devi,[b] Pushpraj S. Gupta,[b] Shubhini A. Saraf[a] and Gaurav Kaithwas *[a]

The present study was proposed to investigate the effect of rifaximin (RFX) on methyl nitrosourea (MNU) induced mammary gland carcinoma in albino wistar rats. Animals were randomized and divided among four groups of six animals each. Group I (control 0.9% normal saline, 3 ml kg^{-1}, p.o.); Group II (toxic control, MNU 47 mg kg^{-1}, i.v.); Group III (RFX, 25 mg kg^{-1}, p.o.); Group IV (RFX, 50 mg kg^{-1}, p.o.). Toxicity was induced by single i.v. injection of MNU. MNU treatment was evident with increased alveolar bud count, differentiation score, up-regulated inflammatory enzyme markers (COX, LOX, NO and H_2S) and oxidative stress markers (TBAR's, protein carbonyl, SOD, catalase and Ach). The mammary gland surface architecture was studied using SEM, carmine staining and H&E staining. The treatment with RFX elicited noticeable restoration of the overall histological architecture in the experimental animals similar to the control. In the MNU treated toxic group, the levels of oxidative stress markers significantly increased in comparison to the control, which was subsequently restored after RFX treatment. Furthermore, RFX up regulated the levels of caspase 3 and caspase 8, when compared to the MNU treated animals. MNU associated toxicity was also ascertained, when determined for UCHL-1, COX, NF-κBp65, BAD, and BCL-xl expression, while RFX demonstrated modulation of the same.

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Introduction

Rifaximin (RFX), is a nonsynthetic antibiotic of the rifamycin group of antibiotics, approved for treatment of traveller's diarrhoea and hepatic encephalopathy. Several clinical trials have revealed that RFX has efficacy toward irritable bowel disease (IBD), presumably as a result of alteration of intestinal microbia.[1] The mechanism contributing to the beneficial effects of RFX in IBD is not fully understood. Recently, it was reported that the susceptibility to IBD was strongly associated with genetic variation in the pregnane X receptor gene (PXR), a member of the nuclear receptor family.[2] Subsequently, RFX was investigated for its role in the activation of the PXR. PXR-humanized (hPXR), PXR-null, and wild-type mice were treated orally with RFX and rifampicin (a well-established human PXR ligand) and it was reported that RFX mediates activation of human PXR.[3]

PXR is a ligand dependent transcription factor known to regulate xenobiotic and cholesterol metabolism, energy homeostasis, gut mucosal defence and cancer development. Initially, the expression of PXR was reported to be highly tissue specific in liver and intestine.[4-7] Later, studies endorsed the PXR expression in mouse kidney, ovary, uterus, human brain and breast tissues.[7-10] PXR has also been reported to be highly expressed in certain cancers and promote cell proliferation and chemoresistance,[11-13] and potentially contributing to malignancy.[12] Moreover, PXR overexpression by stable transfection of hPXR or by pharmacological activation has been reported to inhibit apoptosis in HepG2 cells.[14] On the same line, over-expressing constitutively activated PXR or through pharmacological activation by rifampicin has been reported to pro-proliferative and anti-apoptotic in HCT116 (human colon cancer) and LS180 (intestinal human colon adenocarcinoma) cells.[15] The studies have given an overall impression of pro-proliferative and anti-apoptotic role of PXR. However, PXR has also been shown to favourably regulate apoptosis, particularly in the tissues that are outside the metabolic realm of the liver and intestine, including tumour tissues of endometrial and breast cancer.[16,17]

A recent *in vitro* study has also proposed PXR as a novel mediator of apoptosis *via* p53-dependent and independent pathways. Ectopic expression of human PXR *via* stable

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Appendix -IV

List of Publications

Publications

Publications related to the work

1. **Swetlana Gautam**, Atul Kumar Rawat, Shreesh Raj Sammi, Subhadeep Roy, Manjari Singh, Uma Devi, Rajnish Kumar Yadav, Lakhveer Singh, Jitendra Kumar Rawat, Mohd. Nazam Ansari, Abdulaziz S. Saeedan, Dinesh Kumar, Rakesh Pandey, Gaurav Kaithwas. DuCLOX-2/5 inhibition attenuates inflammatory response and induces mitochondrial apoptosis for mammary gland chemoprevention. **Frontiers in Pharmacology.**2018;9:314(**Impact Factor 4.400)**
2. **Swetlana Gautam**, Priyanka Singh, Manjari Singh, Subhadeep Roy, Jitendra K Rawat, Rajnish K Yadav, Uma Devi, Pushpraj S Gupta, Shubhini A Saraf, Gaurav Kaithwas. Rifaximin, a Pregnane X Receptor (PXR) Activator regulates apoptosis in Murine Model of Breast Cancer. **RSC Advances**2018**;**8**:**3512-3521. **(Impact Factor 3.108)**
3. **Swetlana Gautam**, Subhadeep Roy, Mohd Nazam Ansari, Abdulaziz S. Saeedan, Shubhini A. Saraf, and Gaurav Kaithwas. DuCLOX-2/5 inhibition: a promising target for cancer chemoprevention. **Breast Cancer**, 2016:1-11. **(Impact Factor 1.170).**

Publications related to other work

4. **Swetlana Gautam**, Soniya Rani, Sara A Aldossary, Abdulaziz S. Saeedan, Mohd. Nazam Ansari, Gaurav Kaithwas. Effects of Phenidone (DuCLOX-2/5 inhibitor) against N-Methyl-N-Nitrosourea induced mammary gland carcinoma in Albino rats. **Toxicology and Applied Pharmacology**2018. **(IF 3.705)**

5. Subhadeep Roy , Atul Rawat , Shreesish Sammi , Uma Devi , Manjari Singh , **Swetlana Gautam**, Rajnish Yadav , Jitendra Rawat , Lakhveer Singh , Md Nazam Ansari , Abdulaziz Saeedan , Rakesh Pandey , Dinesh Kumar and Gaurav Kaithwas. Alpha-linolenic acid arbitrates mitochondrial apoptosis, curbs the hypoxic microenvironment and de novo fatty acid synthesis to impart anticancer effects. **Oncotarget**, 2017; 8(41):70049. **(Impact Factor5.008).**

6. Subhadeep Roy, Manjari Singh, Atul Rawat, Uma Devi, **Swetlana Gautam**, Rajnish K Yadav, Jitendra K Rawat, Mohd. Nazam Ansari, Abdulaziz S. Saeedan, Dinesh Kumar, Gaurav Kaithwas. GLA supplementation regulates PHD2 mediated hypoxia

and mitochondrial apoptosis in DMBA induced mammary gland carcinoma. **The international journal of biochemistry & cell biology.** 2018;96:51-62. **(Impact Factor 4.240).**

7. Vidhata Rani, **Swetlana Gautam**, Jitendra K Rawat , Manjari Singh, Uma Devi, Rajnish K Yadav, Subhadeep Roy, Gaurav Kaithwas. Effects of minocycline and doxycycline against terbutaline induced early postnatal autistic changes in albino rats. **Physiology & behavior.** 2018;183:49-56. **(Impact Factor 3.033)**

8. Shreesh Raj Sammi, Jitendra K Rawat, Neetu Raghav, Ajay Kumar, Subhadeep Roy, Manjari Singh, **Swetlana Gautam**, Rajnish k Yadav, Uma Devi, Rakesh Pandey, Gaurav Kaithwas. Galantamine attenuates N, N-dimethyl hydrazine induced neoplastic colon damage by inhibiting acetylcholinesterase and bimodal regulation of nicotinic cholinergic neurotransmission. **European journal of pharmacology.** 2018;818:174-83. **(Impact Factor 2.896)**

9. Abdulaziz S. Saeedan, Varsha Gautam, Mohd. Nazam Ansari, Manjari Singh, Rajnish K Yadav, Jitendra K Rawat, Uma Devi, **Swetlana Gautam**, Subhadeep Roy, Gaurav Kaithwas. Revisiting the Systemic Lipopolysaccharide Mediated Neuroinflammation: Appraising the Effect of L-Cysteine Mediated Hydrogen Sulphide on it. **Saudi Pharmaceutical Journal.** 2018 Feb 6.

10. Uma Devi, Vikas Kumar, Pushpraj S. Gupta, Suchita Dubey, Manjari Singh, **Swetlana Gautam**, Jitendra K. Rawat, Subhadeep Roy, Rajnish K Yadav, Mohd nazam Ansari, Abdulaziz S Saeedan, Gaurav Kaithwas. Experimental Models for Autism Spectrum Disorder Follow-Up for the Validity. **Review Journal of Autism and Developmental Disorders,** 2016; 3(4):358-376. **(Impact Factor 3.493)**

11. Rakesh K Mishra, Shreesh Raj Sammi, Jitendra K. Rawat, Subhadeep Roy, Manjari Singh, **Swetlana Gautam**, Rajnish K. Yadav, Uma Devi, Mohd Nazam Ansari, Abdulaziz S Saeedan, Shubhini A Saraf, Rakesh Pandey, Gaurav Kaithwas. Palonosetron attenuates 1, 2-dimethyl hydrazine induced preneoplastic colon damage through downregulating acetylcholinesterase expression and up-regulating synaptic acetylcholine concentration. **RSC Advances,** 2016; 6;46::40527-40538. **(Impact Factor 3.708).**

12. Virendra Tiwari, Manjari Singh, Jitendra K. Rawat, Uma Devi, Rajnish K. Yadav, Subhadeep Roy, **Swetlana Gautam**, Shubhini A Saraf, Vikas Kumar, Mohd Nazam Ansari, Abdulaziz S Saeedan, Gaurav Kaithwas. Redefining the role of peripheral LPS as a neuroinflammatory agent and evaluating the role of hydrogen sulphide through metformin intervention. **Inflammopharmacology**, 2016; 24(5):253-264. **(Impact Factor 2.304).**

13. Sneha Yadav, Virendra Tiwari, Manjari Singh, Rajnish K. Yadav, Subhadeep Roy, Uma Devi, **Swetlana Gautam**, Jitendra K Rawat, Mohd Nazam Ansar, Abdulaziz S Saeedan, Anand Prakash, Shubhini A Saraf, Gaurav Kaithwas. Comparative efficacy of alpha-linolenic acid and gamma-linolenic acid to attenuate valproic acid-induced autism-like features. **Journal of Physiology and Biochemistry,** 2016; 1-12. **(Impact Factor 2.054)**.

14. Asha Rani, Subhadeep Roy, Manjari Singh, Uma Devi, Rajnish K. Yadav, **Swetlana Gautam**, Jitendra K. Rawat,Mohd Nazam Ansar, Abdulaziz S Saeedan, Anand Prakash, Gaurav Kaithwas. α-Chymotrypsin regulates free fatty acids and UCHL-1 to ameliorate N-methyl nitrosourea induced mammary gland carcinoma in albino wistar rats. **Inflammopharmacology**, 2016: 24(5):277-286. **(Impact Factor 2.304).**

15. Chetan Manral, Subhadeep Roy, Manjari Singh, **Swetlana Gautam**, Rajnish K. Yadav, Jitendra K. Rawat, Uma Devi, Md Nazam Ansari, Abdulaziz S. Saeedan, and Gaurav Kaithwas. Effect of β-sitosterol against methyl nitrosourea-induced mammary gland carcinoma in albino rats. **BMC Complementary and Alternative Medicine**, 2016; 16(1):260. **(Impact Factor 1.987).**

16. Raju Gautam, Manjari Singh, **Swetlana Gautam**, Jitendra Kumar Rawat, Shubhini A. Saraf, and Gaurav Kaithwas. Rutin attenuates intestinal toxicity induced by Methotrexate linked with anti-oxidative and anti-inflammatory effects. **BMC complementary and alternative medicine,** 2016;16(1):99. **(Impact Factor 1.987)**.

17. Neha Sharma, **Swetlana Gautam**, Uma Devi, Manjari Singh, Jitendra K. Rawat, Nikunj Sethi, Shubhini A. Saraf, and Gaurav Kaithwas. Correction: Preclinical appraisal of terbutaline analogues in precipitation of autism spectrum disorder. **RSC Advances**, 2015;5(127):104972-104972. **(Impact Factor 3.708)**

18. Neha Sharma, **Swetlana Gautam**, Uma Devi, Manjari Singh, Jitendra K. Rawat, Nikunj Sethi, Shubhini A. Saraf, and Gaurav Kaithwas. Preclinical appraisal of terbutaline analogues in precipitation of autism spectrum disorder. **RSC Advances**, 2015;5(49):39003-39011. **(Impact Factor 3.708).**

19. Arun Kumar, **Swetlana Gautam**, Jitendra K Rawat, Manjari Singh, Shubhini A Saraf, Gaurav Kaithwas. Effect of palonosetron (5HT-3 antagonist) and pantoprazole (PPI) against surgical esophagitis induced by forestomach and pylorus ligation in albino rats. **Human and Experimental Toxicology**, 2015 **(Impact Factor 1.407)**

20. Sukesh K Gupta, **Swetlana Gautam,** Jitendra K Rawat, Manjari Singh, Shubhini A Saraf and Gaurav Kaithwas. Efficacy of variable dosage of aspirin in combating methotrexate-induced intestinal toxicity. **RSC Advances,**2015;5**:**9354-9360. **(Impact Factor 3.708).**

21. Arvind Kumar Giri, Jitendra Kumar Rawat, Manjari Singh, **Swetlana Gautam**, and Gaurav Kaithwas. "Effect of lycopene against gastroesophageal reflux disease in experimental animals." BMC complementary and alternative medicine 15, no. 1 (2015): 110. (**IF 1.987**).

22. Prince Raj, Manjari Singh, Jitendra K Rawat, **Swetlana Gautam,** Shubhini A Saraf, Gaurav Kaithwas.Effect of enteral administration of α-linolenic acid and linoleic acid against methotrexate induced intestinal toxicity in albino rats. **RSC Advances,** 2014, 4**,** 60397-60403. **(Impact Factor 3.708).**

23. Sanjit Kumar, Manjari Singh, Jitendra K Rawat, **Swetlana Gautam,** Shubhini A Saraf, Gaurav Kaithwas. Effect of rutin against gastric esophageal reflux in experimental animals.**Toxicology Mechanisms and Method,** 2014; 24(9):666-671**(Impact Factor 1.548)**

24. Suchita Dubey, Swetlana Gautma, Gaurav Kaithwas. Comment on “Discruption of social approach by MK-801, amphetamine & fluoxetine in adolescent C57BL/6J mice”—by S. Moy et al.**Neurotoxicology & Teratology.** 2014; 41:96. **(Impact Factor 3.082).**

25. Danish Ahmed, Mohd. Ibrahim Khan, Gaurav Kaithwas, Subhadeep Roy, **Swetlana Gautam**, Manjari Singh, Uma Devi, Rajnish Yadav, Jitendra Kumar Rawat , Shubhini Saraf. Molecular Docking Analysis and Antidiabetic activity of Rifabutin against STZ-NA induced diabetes in albino wistar rats. **Beni-Suef University Journal of Basic and Applied Sciences,** 2017 Apr 25.

Book Chapter

1. **Swetlana Gautam,**Gaurav Kaithwas, Ram Naresh Bharagava, Gaurav Saxena. Pollutants in tannery wastewater, their pharmacological effects and bioremediation approaches for human health preotection and environmental safety. In book: Environmental Pollutants and their Bioremediation Approaches, Edition: Ist, Publisher: CRC Press, **Taylor & Francis Group, USA**.

Conferences

1. Presented a Poster and Attended IHPA Golden Jubilee Conference 2014 on "**Restructuring Pharmacy Curricula: Need of Health Sector**" at BBAU, Lucknow, in the year 2014.
2. Attended 1st Lucknow Science Congress on "**Innovations in Science for Better Tomorrow**", held at BBAU, Lucknow, in the year 2014.
3. Attended 15th Indo-US Flow Cytometry Workshop-2 on "**Application of Flow cytometry In Biomedical Research**", held at BBAU, Lucknow, in the year 2014.
4. Two days workshop organized by BBD, Lucknow & conducted by IIPTA, Delhi on "**Role of IPR in Pharmaceutical Industry**".
5. One day workshop organized by BBAU, Lucknow on "**Placements & Employments Prospects in Indian Patent Prospects in Indian Patent offices and Hands on Training for Patenting the Research Work**."
6. Participated in workshop on "**NMR/MRI from molecules to human behavior**" Sponsored by DST-India.
7. Presented poster in **2nd Lucknow Science Congress** organized by Babasaheb Bhimrao Ambedkar University, Lucknow.

8. Presented oral presentation in International Conference in Babasaheb Bhimrao Ambedkar University Lucknow 2015 under the theme "**Nanoformulations and Translational Research: Small getting Bigger**".
9. Presented oral presentation in International Conference in Babasaheb Bhimrao Ambedkar University Lucknow 2017 under the theme "**International conference on Updated in Cancer Prevention and research**".