"STUDIES ON PHARMACO-TOXICOLOGICAL PROFILE OF ERLOTINIB, MELOXICAM AND METFORMIN FOLLOWING MONO AND COMBINATION THERAPY IN CANCER MODEL OF MICE"

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PATEL SATISHKUMAR DAHYALAL
B.V. Sc. & A.H., M.V.Sc.
(Reg. No.: 04-1113-2009)

DEPARTMENT OF PHARMACOLOGY & TOXICOLOGY
COLLEGE OF VETERINARY SCIENCE & ANIMAL HUSBANDRY
ANAND AGRICULTURAL UNIVERSITY
ANAND-388 001
2 0 12

Department of Veterinary Pharmacology & Toxicology
College of Veterinary Science and Animal Husbandry
Anand Agricultural University
Anand – 388 001

Dr. A.M.Thaker **email: amthaker@aau.in**
M.V.Sc., Ph. D.
Professor and Head **Ph:** (02692) 261486

CERTIFICATE

This is to certified that **Dr. Satishkumar Dahyalal Patel** registered for Ph.D. Degree course (Reg. No. 04-1113-2009) in the subject of **Veterinary Pharmacology and Toxicology** at College of Veterinary Science and Animal Husbandry, Anand Agricultural University, Anand has completed all the courses and submitted KACHCHA BOUND THESIS entitled **"Studies on pharmaco-toxicological profile of erlotinib, meloxicam and metformin following mono and combination therapy in cancer model of mice"** on 9th August, 2012.

Date: 09/08/2012 (A.M.Thaker)
Place: Anand Major Advisor

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Place: Anand
Date: / /2012

Dr. A. M. Thaker
(Major Advisor)

ABSTRACT

"STUDIES ON PHARMACO-TOXICOLOGICAL PROFILE OF ERLOTINIB, MELOXICAM AND METFORMIN FOLLOWING MONO AND COMBINATION THERAPY IN CANCER MODEL OF MICE"

Name of Student **Name of Major Advisor**
Satish D. Patel **Dr. A. M. Thaker**

Department of Pharmacology & Toxicology
College of Veterinary Science and A. H., AAU,
Anand 388 001, Gujarat, INDIA.

More effective and less toxic therapy to prevent tumor progression and recurrence is the requirement of present scenario and current therapeutic approach. Preliminary preclinical and clinical data suggest that targeting multiple pathways in cancer cells might be an effective anti-tumor treatment strategy in non small cell lung cancer (NSCLC). A tyrosine kinase inhibitors (e.g erlotinib), COX-2 inhibitors (e.g meloxicam) and mTOR inhibitors(e.g metformin) are widely used alone or with other drugs in their respective therapeutic area and sometime in complex conditions like cancer in diabetic and arthritic patients, where there are enormous potential for drug interaction and synergistic beneficial/adverse effect of combinational therapy. Drug interactions may occur when two or more drugs are concurrently administered and one drug (or more) may influence the time course of the other in the body. The pharmacokinetic of erlotinib (30 mg/kg, p.o), meloxicam (20 mg/kg, i.p) and metformin (100 mg/kg, p.o) as single drug and in combination with each other and all three drugs together were investigated in severe combined immunodeficient (SCID) male mice. Erlotinib, meloxicam and metformin concentration in plasma were assayed by LC-MS/MS. Pharmacokinetic parameters were calculated by non-compartmental technique using computer software (WinNonlin, version 5.0.1). The present study also evaluated pharmaco-toxicological effect of erlotinib (30 mg/kg, p.o), meloxicam (20 mg/kg, i.p) and metformin (100 mg/kg, p.o) after repeated administration at 24 h interval for 28 days in SCID male mice.

The mean peak plasma concentration of erlotinib following its administration as single drug and in combination with meloxicam and metformin, separately as well as both meloxicam and metformin together in SCID male mice were 16372.63 ± 846.59, 17741.60 ± 2918.54, 13400.85 ± 882.54 and 13600.97 ± 1176.92 ng/ml, respectively, which were observed at 0.167 h. The drug was detected in plasma upto 24 h. The mean peak plasma concentration of meloxicam following its administration as single drug and in combination with erlotinib and metformin, separately as well as both erlotinib and metformin together in SCID male mice were 44858.46 ± 3486.54, 49994.17 ± 2928.14, 46540.83 ± 3879.53 and 39648.94 ± 2190.42 ng/ml, respectively, which were observed at 0.33 h. The drug was detected in plasma upto 24 h. The mean peak plasma concentration of metformin following its administration as single drug and in combination with erlotinib and meloxicam, separately as well as both erlotinib and meloxicam together in SCID male mice were 5782.29 ± 441.77, 6307.71 ± 577.57, 7724.44 ± 384.36 and 9490.58 ± 963.64 ng/ml, respectively, which were observed at 0.67 hr after administration of drug in case of metformin along with meloxicam whereas 0.33 h in all other treatment group. The drug was detected in plasma upto 12 h.

Following oral administration of erlotinib with metformin and both meloxicam and metformin together in SCID male mice the value of $AUC_{0-\infty}$ (66493.10 ± 3418.82 h.ng/ml and 62452.70 ± 2400.15 h.ng/ml) was significantly ($p< 0.05$) decreased and the value of clearance (456.74 ± 21.69 ml/h and 483.92 ± 18.55 ml/h) was significantly ($p< 0.05$) increased in compared to the mean $AUC_{0-\infty}$ (84784.69 ± 3209.24 h.ng/ml) and mean clearance Cl (356.48 ± 14.03 ml/h) of erlotinib alone treatment , respectively. Following intra peritoneal administration of meloxicam with erlotinib in SCID male mice the value of $AUC0_{-\infty}$ (196948.88 ± 6206.02 h.ng/ml) and MRT (4.33 ± 0.19 h) of meloxicam were significantly ($p<0.01$) decreased whereas Vz (524.79 ± 75.33 ml) and Cl (102.10 ± 3.52 ml/h) were significantly ($p<0.05$) increased. The Vz (1184.20 ± 87.65 ml) of meloxicam was significantly ($p<0.05$) increased when given in combination with metformin and clearance (146.10± 3.02 ml/h) of meloxicam was significantly ($p<0.05$) decreased in combination with both metformin and meloxicam together. Following oral administration of metformin along with both erlotinib and meloxicam the $AUC_{0-\infty}$ (24658.04 ± 1303.61 h.ng/ml) were significantly increased whereas the Vz (15338.18 ± 2005.80 ml) and Cl (4124.21 ± 261.29 ml/h) was significantly ($p<0.01$) decreased. The $AUC_{0-\infty}$ (23339.74 ± 1314.84 h.ng/ml) of metformin was significantly increased where as the $T_{1/2}$ (1.79 ± 0.21 h), Vz (11329.62 ± 1581.91 ml) and Cl (4356.26 ± 255.94 ml) was significantly ($p<0.01$) decreased when given in combination with meloxicam. The Vz (18957.40 ± 2289.64 ml) of metformin was also significantly ($p<0.05$) decreased when given in combination with erlotinib.

Abstract

Repeated administration of erlotinib (30 mg/kg, p.o), meloxicam (20 mg/kg, i.p) and metformin (100 mg/kg, p.o) at 24 h interval for 28 days in SCID male mice were found safe based on evaluation of haematological and blood biochemical parameters. Moreover, no gross or microscopic changes were found in the heart, lung, liver and kidney in SCID male mice. Few animals treated with erlotinib along with meloxicam and in combination with both meloxicam and metformin together revealed symptoms like piloerection, staring eyes, hunched posture, incordinating gait etc and mortality was observed in treatment group of erlotinib with meloxicam and metformin as well as in all three drugs together. The body weight was significantly reduced ($p < 0.05$) in animal group treated by erlotinib along with metformin during all 4 weeks of study. The animal group treated with all three drugs together shown marked percentage reduction in tumor volume as well as mean relative tumor volume (RTV) through out the study period and more than 50 % reduction in tumor volume on 4^{th} week of treatment in comparison to tumor control. A substantial reduction in mean relative tumor volume was also observed in all other treatment groups in comparison to tumor control group. The RTV was observed in descending pattern in animal group treated with metformin > meloxicam > erlotinib > erlotinib with metformin > meloxicam with metformin > erlotinib with meloxicam > all three drugs together. The lowest RTV was observed in animal group treated with all three drugs together.

The mean value of creatinine kinase (CK) of animal group treated with meloxicam alone (210.90 ± 28.10 IU/L) was significantly decreased whereas animal group treated with all three drugs together (1916.10 ± 335.93 IU/L) was significantly increased in comparison to untreated tumor control group (1155.02 ± 143.24 IU/L). The mean value of blood urea nitrogen (BUN) in animal group treated by erlotinib with metformin (24.3 ± 0.76 mg/dL) was significantly increased in comparison to untreated tumor control group (21.17 ± 0.40 mg/dL). The absolute organ weights were obtained for tumor mass, heart, lungs, liver and kidneys of SCID male mice of all groups on termination day of experiment at the time of necropsy. The results showed that there was a considerable reduction in tumor weight of treatment groups compared to tumor control group. The mean kidney weight of animal group treated by metformin with meloxicam was significantly reduced in comparison to the untreated tumor control group.

The gene expression change in tumor tissue was analyzed for AKT, AMPK, P070S6K, PTEN and RAF was markedly increased in all treatment groups in comparison to the tumor control group. The expression of PTEN gene was significantly increased in animal group treated with erlotinib along with meloxicam as well as metforimn compared to tumor control group whereas in other treated group also, it was markedly increased. The expression of AKT is considerably decreased in animal groups treated with erlotinib, meloxicam and metformin as a single drug as well as in combination with meloxicam and metformin and all three drugs together in comparison to the tumor control group. The AMPK expression is considerably increased in animal group treated with metformin alone and in combination with erlotinib or meloxicam as well as erlotinib and meloxicam treatment group in comparison to tumor control. The expression of P070S6K gene was considerably reduced in animal group treated by erlotinib along with metformin and metformin along with meloxicam in comparison to tumor control group. The expression of RAF gene was considerably reduced in animal group treated by meloxicam along with metformin in comparison to tumor control group. The histopathological changes were observed in tissues like tumor mass, heart, lungs, liver, and kidneys of all the animals of treated and control groups. The efficacy of drugs was evaluated by considering necrotic core in tumor mass. The necrotic core was graded as minimal, mild, moderate and severe. The necrotic core of tumor mass was severe and moderate in animals treated with all three drugs together and erlotinib with meloxicam, respectively. The mild to moderate necrotic core of tumor mass was observed in animal treated by erlotinib with metformin and meloxicam with metformin. The minimal to mild necrotic core in tumor mass was seen in animals treated with metformin or meloxicam or erlotinib alone.

The present study revealed that administration of erlotinib, meloxicam and metformin in combination with each other or all three together altered pharmacokinetics of each other and raised the awareness about the potential drug interaction between erlotinib, meloxicam and metformin as well as also exhibit the significant therapeutic effect as a treatment agent and combinational therapy in SCID male mice. The COX-2 inhibitors and mTOR inhibitors showed their noticeable effect in prevention of tumor growth. Therefore, concomitant use of these drugs requires close therapeutic monitoring for potential outcome. However, clinical experience in this field is still limited. More research is needed to assess whether the efficacy of target agent combinations or of multi-target drugs is superior to current chemotherapy or biological approaches.

Key Words: Pharmaco-Toxicological Profile, Erlotinib, Meloxicam, Metformin, Cancer model of mice

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ABBREVIATIONS

%	Per cent
µ	Micron
µl	Microliter
<	Less than
>	Greater than
±	Plus or minus
≤	Less or equal
≥	Greater or equal
µg.h/mL	Microgram hour per milliliter
$\mu g.h^2/mL$	Microgram hour square per milliliter
µg/ml	Microgram per milliliter
4E-BPs	4E-binding proteins
ACE	Angiotensin-Converting Enzyme
ACN	Acetonitrile
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AMPK	Adenosine Monophosphate Kinase AMP-activated protein kinase
ARE	Antioxidant Response Element
AST	Aspartate aminotransferase
AUC	Area under curve
$AUC_{(0-\infty)}$	Area under the plasma concentration-time curve from time 0.0 hour to infinity
B.wt.	Body Weight
CAL	Calibration point
Cl	Total body clearance
C_{max}	Maximum drug concentration
COX	Cyclooxygenase
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
CV%	Interindividual Variability

CYP2C9	Cytochrome P450 2C9
CYP3A4	Cytochrome P450 3A4
DLC	Differential leukocyte count
DLT	Dose Limiting Toxicity
DMSO	Dimethyl sulphoxide
e.g.	Exempli gratia (for example)
EDTA	Ethylene diamine tetra acetate
EGFR	Epidermal Growth Factor Receptor
ErbB1	Erythroblast 1
et al.	et alibi (and others)
F	Bioavailability
FDA	Food and Drug Administration
fL	Femtolitre
g	Gram
G	Gauge
h	Hour
H & E	Hematoxylin and Eosin
h^{-1}	Per hour
Hb	Haemoglobin
HCT	Hematocrit
HER-1	Human epidermal growth factor receptor-1
HPLC	High performance liquid chromatography
i.e.	id est (that is)
I.M.	Intramuscular
IAEC	Institutional animal ethics committee
IL	Interleukin
IL1β	Interleukin 1 Beta
IL6	Interleukin 6
ILD	Interstitial Lung Disease
IS	Internal standard
IU	International unit

IV	Intra-venous
IVC	Individually ventilated cage
K_{el}	Elimination rate constant
kg	Kilogram
L or l	Liter
L/h	Liter per hour
L/h/kg	Liter per hour per kilogram
L/kg	Liter per kilogram
LC/MS/MS	Liquid Chromatography tandem mass spectrometry
LLOQ	Lower limit of quantitation
LQC	Low quality control
MCH	Mean Corpuscular Hemoglobin
MCHC	Mean Corpuscular Hemoglobin Concentration
MCV	Mean Corpuscular Volume
mg	Milligram
mg .h/L	Milligram hour per liter
mg/day	Milligram per day
mg/kg	Milligram per kilogram
mg/kg/h	Milligram per kilogram per hour
mg/L	Milligram per liter
MIC	Minimum inhibitory concentration
min	Minute
mL	Milliliter
mL/h/kg	Milliliter per hour per kilogram
mL/hr	Milligram per hours
mL/kg/h	Milliliter per kilogram per hour
mL/min/kg	Milliliter per minute per kilogram
MQC	Medium quality control
MRT	Mean Residence Time
NCCSC	National Cancer Chemotherapy Service Center
NCI	National Cancer Institute

NF-kB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
ng	Nanogram
ng.h/mL	Nanogram hour per milliliter
ng/mL	Nanogram per milliliter
No.	Number
NSAIDs	Non Steroidal Anti-inflammatory Drugs
NSCLC	Non-Small Cell Lung Cancer
OA	osteoarthritis
^{o}C	Degree Centigrade
OS	Overall Survival
P	Level of Significance
PBM	Pancreaticobiliary maljunction
PCV	Packed cell volume
PFS	Progression-Free Survival
PGE2	Prostaglandin E2
PGH2	Prostaglandin H2
PI3-K	Phosphatidylinositol 3-kinase
pKa	Dissociation constant
PKB	Protein Kinase B
PO	Per Oral
PR	Partial Response
RA	rheumatoid arthritis
RBCs	Red Blood Corpuscles
RCC	Renal Cell Carcinoma
rpm	Revolution per minute
RSD	Relative standard deviation
RT	Radiation Therapy
S.C.	Subcuteneous
S6Ks	Ribosomal Protein S6 Kinases
SD	Stable Disease
SEM	Standard Error of Mean

SGOT	Serum glutamic-oxaloacetic transaminase
SGPT	Serum glutamic-pyruvic transaminase
$t_{1/2}$	Half life
TAFs	Tumor Angiogenic Factors
TB	Total bilirubin
TGI	Tumor Growth Inhibitor
TK	Tyrosine Kinase
TKI	Tyrosine Kinase Inhibitor
TLC	Total leukocyte count
T_{max}	Time of maximum observed drug concentration
TNFα	Tumor Necrosis Factor Alpha
TP	Total protein
TSC2	Tuberous Sclerosis Complex 2
TZDs	Thiazolidinedione
U/L	Unit per liter
UV	Ultra violet
v/v	Volume in volume
Vd/Vz	Volume of Distribution
VEGF	Vascular Endothelial Growth Factor
viz.	Videlicet (Namely)
Vss	Volume of distribution at steady state
w/v	Weight in volume
WBC	White blood cells

CHAPTER – I

INTRODUCTION

Millions of fatality caused by cancer all over the world (Brambilla, 2010) has forced the researchers to come forward in the field of cancer research and anti-cancer drug development. Lack of early diagnostic tools, presentation of diseases at a late stage and the modest effect of chemotherapy are prime of all factors responsible for depressing prognosis in case of cancer in man, dog (Castellano *et al.*, 2006), cat (Clements *et al*., 2004) and other species of animals. Amongst various types of carcinoma, adenocarcinoma is the most common histological subtype of lung cancer in most countries, accounting for almost half of all lung cancers in human. Most lung neoplasms in dog and cattle are categorized as adenocarcinomas (Kraegel *et al*., 1992; Baba and catoi, 2007).

Over the past two decades, adenocarcinoma has replaced squamous cell carcinoma as the most common subtype of non-small cell lung cancer (NSCLC). Adenocarcinomas account for approximately 40% of lung cancers (Subramanian and Govindan, 2007) in human. As a class, NSCLCs are relatively insensitive to chemotherapy, compared to small cell carcinoma. The most common types of NSCLC are squamous cell carcinoma, large cell carcinoma and adenocarcinoma with several other types that occur less frequently.

Several malignancies in human and animals are associated with aberrant or overexpressed epidermal growth factor receptor (EGFR) and HER-1/ErbB1 (Modijtahedi and Dean 1994). EGFR tyrosine kinase (TK) serves as a potential target for therapeutic intervention in tumors including ovarian, head and neck, breast, bladder, lung and other squamous cell carcinomas (Lei *et al.,* 1999). The intracellular

signaling pathways like PI3K/AKT and RAS/RAF/MAPK are activated upon phosphorylation of EGFR, which regulate key cellular processes such as proliferation and apoptosis (Ladanyi and Pao, 2008). This concept leads to screening of new EGFR-targeted drugs into clinical trials of small molecule EGFR TK inhibitors (e.g. erlotinib or gefitinib) during last one decade. EGFR and its ligands are involved in carcinogenesis, which also regulate the growth and progression of cancer and the therapies targeting EGFR inhibit the growth of tumor (Hattori *et al.,* 1998; Perez-Soler *et al.,* 2004; Bencardino *et al.,* 2007).

Prostanoids produced by the arachidonic acid pathway play an important role in multiple stages of carcinogenesis and progression of cancer. Cyclooxygenase (COX) enzymes are known to be involved in carcinogenesis at several organ sites. COX exists in two isoforms, COX-1 is constitutively expressed in normal tissues and is essential for several important physiologic functions as well. COX-2 is selectively over expressed in neoplastic and inflammatory tissues (Ramalingam and Belani, 2004). Non–small-cell lung cancer (NSCLC) especially adenocarcinoma, overexpresses COX-2, which contributes to the progression of malignancy by several mechanisms. Emerging genetic evidence suggests that COX-2 is important in carcinogenesis and expression of COX-2 is up-regulated in both human cancers and chemically induced rodent and murine cancers (Abou-Issa *et al.,* 2001; Ristimaki *et al*., 2001; Suzuki *et al.,* 2009). COX-2 inhibitors exhibit anticancer activity by several mechanisms including induction of apoptosis, inhibition of angiogenesis, and decreased invasiveness and metastatic potential. This represents the basis of therapy with COX-2 inhibitors for which clinical efficacy has also been evaluated as chemopreventive in cancers and also for treatment of NSCLC (Abou-Issa *et al.,* 2001; Ramalingam and Belani, 2004).

Over the last decade, extensive studies have been made to understand the role of the mammalian target of rapamycin (mTOR) in cancer. In cancer, unrestricted cellular proliferation and decreased sensitivity to apoptotic inducing agents are typically associated with activation of Ras/Raf/MEK/ERK and Ras/PI3K/PTEN/Akt/ mTOR signaling pathways (Steelman *et al*., 2011). Adenosine monophosphate kinase (AMPK) activation leads to decrease in mTOR activity (Kimura *et al.,* 2003). The mTOR plays important role in cell growth, proliferation, survival and is at the crossroads of different signaling pathways that are frequently mutated in various types of cancer (Courtney *et al*., 2010). Over-activation of mTOR play role in resistance to cancer cell death, invasion, metastasis and cell metabolism in cancer (Zoncu *et al*., 2011). The mTOR inhibitors also play role in angiogenesis and the immunological responses at the microenvironment of a tumor (Guba *et al*., 2002)

Erlotinib or gefitinib, a selective EGFR- TK inhibitor has been tested and used for anticancer effect in animal xenograft models and clinical trials (Pérez-Soler *et al*., 2004; Bencardino *et al.*, 2007). Many clinical development studies show that erlotinib (Tarceva) is a highly potent, orally active inhibitor of HER1/EGFR -TK thereby, prevents phosphorylation of the receptors and the subsequent cascade of signaling events (Friess *et al*., 2006).

Celecoxib, a highly selective COX-2 inhibitor has shown antitumor effect in tumor xenograft models of colon (Masferrer *et al.*, 2000), breast (Blumenthal *et al.*, 2001), prostate (Liu *et al.*, 2000) and lewis lung carcinoma (Masferrer *et al.*, 2000). Singh *et al*. (2006) have reported that celecoxib enhances the antitumor effect of docetaxel in lung tumors (xenograft mice model by A549 lung cancer cell line) in mice. Meloxicam is also a selective COX-2 inhibitor, which has been tested for its

chemopreventive effects in bladder cancer but not in other types of cancer (Hattori *et al.,* 2006).

Metformin is the most widely used antidiabetic drug in the world and there is increasing evidence of a potential efficacy of this agent as an anticancer drug. Epidemiological studies show a decrease in cancer incidence in metformin treated patients; metformin also decreases insulin resistance and indirectly reduces insulin level, a beneficial effect because insulin promotes cancer cell growth. Many reports represent direct inhibitory effect of metformin on cancer cell growth and an anti tumoral action. Metformin activates the AMP activated protein kinase (AMPK) pathway, a major sensor of the energetic status of the cell, which has been proposed as a promising therapeutic target in cancer (Sahra *et al*., 2010).

Xenograft mice model is widely used test system for cancer research (Tomayko and Reynolds, 1989). A549 cell line is developed through removal and culturing of cancerous lung tissue (Giard *et al.,* 1973). Xenograft can be developed in immunocompromised mice using A549 cell and further studies for drug efficacy and probable pathway can be carried out.

Mono and combination therapeutic approaches have also been studied and employed to treat or inhibit growth of tumor (adenocarcinoma) in animal models (eg. Xenograft mice model) and clinical cases of human cancer. There is a possible interaction of EGFR and COX-2; EGFR activation results in the induction of COX-2 expression through nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) (Richardson *et al.,* 2003). A combination of sulindac, a COX-2 inhibitor, and EKI-569, an EGFR-TK inhibitor, resulted in a synergistic effect in preventing mice intestinal neoplasia (Torrance *et al.,* 2000).

Therefore, a combination of COX-2 and EGFR inhibitors would be a reasonable and promising strategy for the chemoprevention of cancer. Hattori *et al*. (2006) reported that both meloxicam and gefitinib, when given as single agent, significantly inhibited rat bladder carcinogenesis, with no adverse effects. They concluded that a combination of these drugs would be worth studying to test their synergistic effects. Tsuchida *et al*. (2005) reported that meloxicam suppresses carcinogenesis in hamster pancreaticobiliary malfunction models. Meloxicam in combination with carboplatin and weekly paclitaxel chemotherapy showed promising activity in many malignancies including lung cancer (Suzuki *et al.,* 2009).

A combination therapy of anti-diabetic drugs along with chemotherapeutic drugs is routinely employed to alleviate suffering in diabetic patients having carcinoma. The effect of metformin in case of carcinoma has also been evaluated and it has been found that metformin may have promising effect in cancer patients. Hirsch *et al*. (2009) reported that metformin inhibits cellular transformation and selectively kills cancer stem cells in four genetically different types of breast cancer. Gagnon *et al*. (2009) reported that the use of metformin may be associated with better survival of lung cancer patients. Mazzone *et al*. (2010) studied the effect of metformin and thiazolidinedione on lung cancer and found that diabetic patients with lung cancer who were previously exposed to metformin and/or thiazolidinedione are less likely to present with metastatic disease and may survive longer. A potential mechanism of this effect is through the activation of AMP-activated protein kinase. Metformin directly activates the AMPK pathway. When cells are faced with energy stresses, AMPK functions to restore energy balance by inhibiting synthetic pathways and stimulating catabolic pathways. This function may serve to protect against lung cancer development or progression.

More effective and less toxic therapy to prevent tumor progression and recurrence is the requirement of advanced therapeutic approach of present scenario. Erlotinib, meloxicam and metformin are widely used alone or with other drugs in their respective therapeutic area and sometime in complex conditions like cancer in diabetic and arthritic patients, where benefit of combination therapy is to be taken up for betterment and survival of patients. Based on the studies already done in the field of adenocarcinoma and effectiveness of combination therapy, the present study on evaluation of pharmaco-toxicological profile of erlotinib, meloxicam and metformin following mono and combination treatment in cancer has been planned using cancer model of mice with following objectives.

OBJECTIVES

1. To study the pharmacokinetic profile of erlotinib (ERT), meloxicam (MEX) and metformin (MET) in immunodeficient mice.
2. To study the pharmacokinetic profile of erlotinib in combination of either one (meloxicam and/or metformin) or both drugs in immunodeficient mice.
3. Development of xenograft cancer model of mice.
4. To evaluate the pharmacological effect of monotherapy of erlotinib, meloxicam and metformin in xenograft cancer model of mice.
5. To evaluate the pharmacological effect of combinational therapy with erlotinib, meloxicam and metformin in xenograft cancer model of mice.
6. To study toxicological profile of mono and combinational therapy with erlotinib, meloxicam and metformin in xenograft cancer model of mice.

CHAPTER – II

REVIEW OF LITERATURE

2.1 Introduction

Cancer is a disease of cells characterized by loss of regulatory mechanisms which control cell growth and maturation required for homeostasis in complex multicellular organisms. Cancer is the second leading cause of death among Americans and is responsible for one of every four deaths in the United States as per report of American Cancer Society. Chemotherapy is one of the most commonly used methods of cancer treatment, but most forms of chemotherapy are extremely toxic and take a heavy toll on the health of the patient. This is because many standard forms of chemotherapy kill target cells in the process of division that destroys cancerous cells as well as non-cancerous and healthy cells. Cancer patients undergoing chemotherapy suffer from hair-loss and compromised immune systems because both affect rapidly dividing cells. Still, the full effects of chemotherapy are not completely understood. By creating a better model of tumors, tumor growth and the effects of chemotherapy upon such tumors, new knowledge about tumors may be uncovered to improve treatments for patients with cancer.

With 1.3 million deaths annually (Rudin *et al*., 2009; Samet *et al*., 2009), lung cancer is the leading cause of cancer death worldwide (Parkin *et al*., 2002; Boyle and Levin, 2008). The dismal prognosis of lung cancer is due to lack of early diagnostic tools, presentation at a late stage and the modest effect of chemotherapy. Adenocarcinoma is the most common histological subtype of lung cancer in most countries, accounting for almost half of all lung cancers. Primary lung tumors are less common than metastatic lung lesions in dogs and cats, especially compared with their

frequency in humans. Adenocarcinomas account for 70% to 80% of primary pulmonary neoplasia in dogs and cats (Moulton *et al*., 1981).

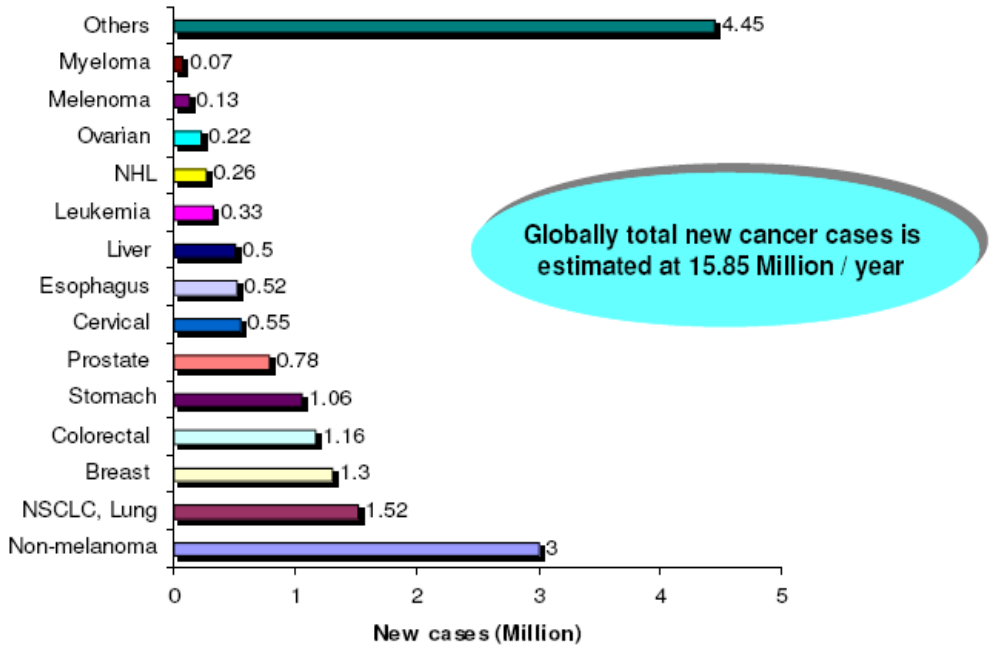

Figure-2.1: Global prevelences of various cancer

2.1.1 History of cancer chemotherapy

Although there have been remedies for the treatment of cancer from the earliest time mostly in the form of herbal preparations (Hajdu, 2005), the era of modern chemotherapy began with the start of the 20th century when Paul Ehrlich published the first book on chemotherapy in 1909 (Ehrlich, 1909). He was the first to apply the Paracelsian idea of specific remedies for specific diseases to cancerous cells that can be damaged by chemicals without harm to the healthy host tissues (Hajdu, 2005). Soon after World War I, two pharmacists (Louis Goodman and Alfred Gilman) were recruited by the United States Department of Defense to investigate potential therapeutic applications of nitrogen mustard, a chemical warfare agent. Autopsy observations of people exposed to mustard gas during World War I had revealed profound lymphoid and myeloid suppression. Goodman and Gilman reasoned that this

agent could be used to treat lymphoma, since lymphoma is a tumor of lymphoid cells (Goodman *et al*., 1946). These findings launched a new area in medicine in the 1950s to discover chemical molecules that can reach and destroy the cancer cells in the body. In response to early successes in cancer chemotherapy research, the United States Congress created a National Cancer Chemotherapy Service Center (NCCSC) at the National Cancer Institute (NCI) in 1955 to promote drug discovery for cancer. Perhaps the most important breakthrough in cancer therapy occurred in 1965, when James Holland, Emil Freireich and Emil Frei hypothesized that cancer chemotherapy should follow the strategy of antibiotic therapy for tuberculosis with combinations of drugs administered concurrently, each with a different site of action, which would make it extremely difficult for the tumor to develop resistance to the drugs. Since then, the search continued, with the pharmaceutical industry screening for new compounds and scientists performing elaborate clinical trials with ever more complex combinations and higher doses.

2.1.2 Tumor growth and its stages of development.

The growth of tumors can be divided into three stages. Acoording to Alarcon *et al*. (2005), tumor stages are described as avascular growth, angiogenesis, and vascular growth. During the avascular stage, there is no blood supply to the tumor, and the tumor reaches a maximum size limited by the amount of oxygen and nutrients the tumor can receive through its surface, then, some of the tumor cells produce substances known as tumor angiogenic factors (TAFs). When TAFs diffuse to the surrounding vasculature of noncancerous tissue, angiogenesis occurs. In this stage, the vasculature grows toward and into the tumor. Once blood vessels have reached the tumor, the third stage begins: the tumor receives a vast amount of nutrients and can grow larger than was possible during the avascular growth stage.

Furthermore, the vasculature now serves as an avenue for metastasis. Once a tumor enters the vascular growth stage, its potential lethality greatly increases (Alarcon *et al*., 2005).

2.1.3 Phases of Cell Cycle

All cells must traverse these cell cycle phases before and during division as depicted in Figure 2.2.

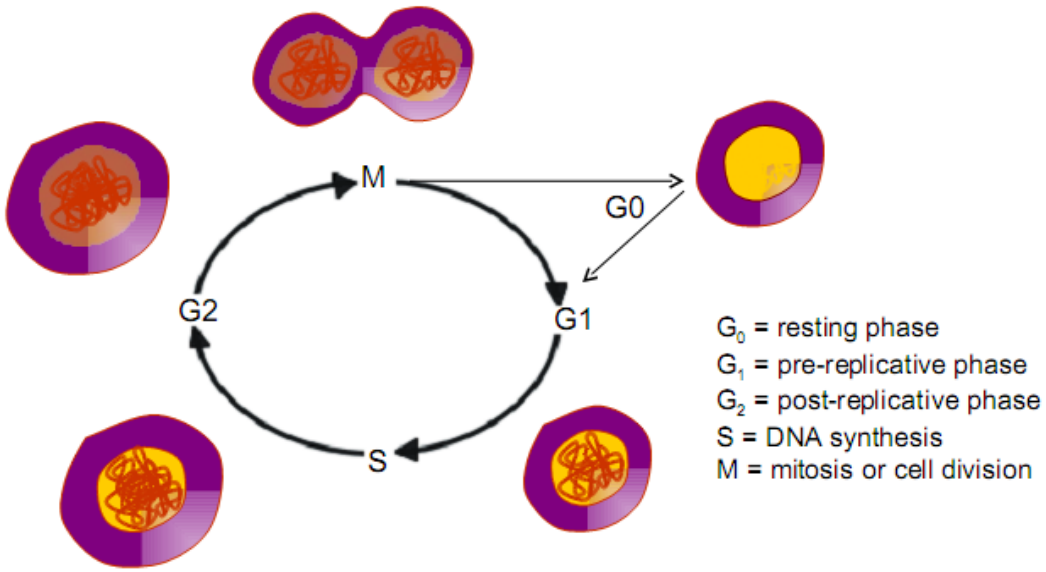

Figure2.2: Phases of Cell cycle

a) S-phase (synthesis) DNA synthesis, chromosomes are duplicated.

b) G2 –phase: preparation for mitosis, specialized protein and RNA synthesis and manufacture of mitotic spindle apparatus.

c) M-phase: mitosis (prophase, metaphase and telophase), (chromosomal condensation, spindle formation).

d) G1 –phase: Prereplicative phase, cell increaes in size & prepares to copy DNA, enzymes necessary for DNA synthesis are synthesized, several hours to days, can re-enter S phase or G_o phase.

e) G_o –phase: quiescent, non-dividing (resting) state, cell stops dividing temporarily or permanently, inactive metabolically

2.1.4 Signaling Pathway and tumor development

Tumour /cancer development is a result of very complex mechanism. There are many signalling pathways playing important role in development of tumour as shown in Figure 2.3.

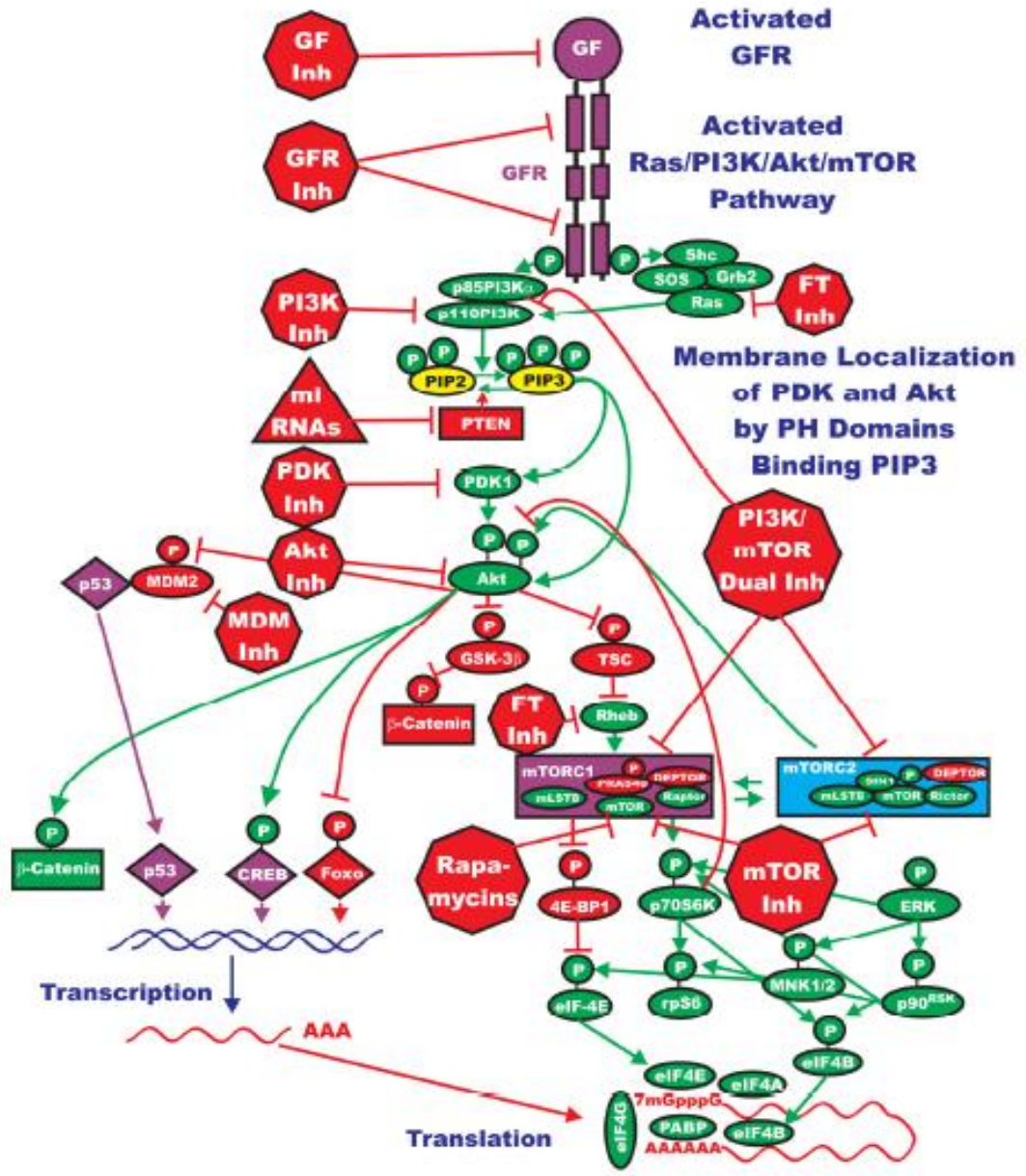

Figure-2.3: Complex signalling pathway in development of tumour

Unrestricted cellular proliferation and decreased sensitivity to apoptotic-inducing agents are typically associated with activation of these

pro-survival pathways. Dysregulated signaling through the Ras/Raf/MEK/ERK and PI3K/PTEN/Akt/ mTOR pathways is often the result of genetic alterations in critical components in these pathways or upstream activators. These pathways have important role in normal and neoplastic tissue growth and controlling the expression of these pathways could help to design the better therapy for cancer (Steelman *et al*., 2011). Significant advances in cancer biology may lead to customised therapy based on targeting specific genes and pathways. The main signaling pathways that could provide roadmaps for therapy include the following: growth promoting pathways (Epidermal Growth Factor Receptor/Ras/PhosphatidylInositol 3-Kinase), growth inhibitory pathways (p53/Rb/P14ARF, STK11), apoptotic pathways (Bcl-2/Bax/Fas/FasL), DNA repair and immortalisation genes (Brambilla and Gazdar, 2009).

Cyclooxygenase (COX)-2 suppresses phosphatidylinositol 3-kinase (PI3-K) activity, which represses antioxidant response element (ARE)_NF-E2 related factor 2 (Nrf2)-mediated transcriptional response in human chondrocytes. The resultant decrease in antioxidant capacity of sheared chondrocytes contributes to their apoptosis (Healy *et al.*, 2005).

The activation of specific receptors triggers intracellular signals (i.e., NFκB, p38, or MAPKs mediated), which regulate pro-inflammatory cytokine expression, such as interleukin 1 beta (IL1β), tumor necrosis factor alpha (TNFα), interleukin 6 (Il6), together with chemokines and cell adhesion proteins (Kulinsky, 2007), in turn, leading to the recruitment and the activation of immune cells. This hypothesis is supported by the findings that the tumor microenvironment is characterized by the infiltration with different types of immune cells (i.e., dendritic cells, lymphocytes, and macrophages) responsible for the release of cytokines (Kulinsky, 2007).

The Ras/PI3K/PTEN/Akt/mTOR pathway plays a key role in regulating p53 activity. This pathway plays a key role in regulating critical proteins involved in protein translation especially those necessary for the translation of "weak" mRNAs (mTORC1,). This pathway also indicates that Akt can result in the activation of downstream mTOR which can subsequently serve as either a negative feed back to inactivate Akt by p70S6K or activate Akt by mTORC2 (Steelman *et al.*, 2011). These pathways overview provide potential sites of therapeutic intervention.

2.2 Classification of Anti-cancer (antineoplastic) agents

2.2.1 Classification by World Health Organization's collaborating centre

World Health Organization's collaborating centre for drug statistics methodology has suggested the following classification (Anonymous, 2009).

A) Alkylating agents

a. Nitrogen mustard analogues: cyclophosphamide, chlorambucil, melphalan.

b. Alkyl sulfonates: busulfan, treosulfan and mannosulfan

c. Ethylenimines: thiotepa, altretamine, carboquone and triaziquone

d. Nitrosoureas: streptozocin, carmustine, lomustine, fotemustine and ranimustine

e. Epoxides: etoglucid

f. Other alkylating agents: dacarbazine, temozolomide, mitobronitol, mitobronit ol , pipobroman

B) Antimetabolites

a. Folic acid analogues: methotrexate, raltitrexed and pemetrexed

b. Purine analogues: mercaptopurine, tioguanine, cladribine, fludarabine, clofarabine and nelarabine

c. Pyrimidine analogues: cytarabine, fluorouracil, tegafur, carmofur, gemcitabine, capecitabine, azacitidine and decitabine

C) Plant alkaloids and other natural products

a. Vinca alkaloids and analogues: vinblastine, vinorelbine, vincristine and vindesine.

b. Podophyllotoxin derivatives: etoposide and teniposide

c. Colchicine derivatives: demecolcine

d. Taxanes: paclitaxel, docetaxel, poliglumex and cabazitaxel

e. Other plant alkaloids and natural products: trabectedin

D) Cytotoxic antibiotics and related substances

a. Actinomycines: dactinomycin

b. Anthracyclines and related substances: doxorubicin, daunorubicin, epirubicin, aclarubicin, zorubicin, idarubicin, mitoxantrone, pirarubicin, valrubicin, amrubicin and pixantrone

c. Other cytotoxic antibiotics: bleomycin, plicamycin, mitomycin, ixabepilone

E) Other antineoplastic agents

a. Platinum compounds: cisplatin, carboplatin, oxaliplatin and satraplatin.

b. Methylhydrazines: procarbazine

c. Monoclonal antibodies: edrecolomab, cetuximab, panitumumab, trastuzumab, catumaxomab, rituximab, gemtuzumab, alemtuzumab and bevacizumab

d. Sensitizers used in photodynamic/radiation therapy: porfimer sodium, methyl aminolevulinate, aminolevulinic acid, temoporfin and efaproxiral

e. Protein kinase inhibitors: imatinib, gefitinib, erlotinib, sunitinib, sorafenib, dasatinib, lapatinib, nilotinib, and temsirolimus.

f. Other antineoplastic agents: hydroxycarbamide (hydroxyurea), tretinoin, celecoxib and meloxicam.

2.2.2 Classification based on targeted therapy

Targeted therapy based antineoplastics have been classified in different groups as follows.

A) CI Monoclonal antibodies "mab"

a. Receptor tyrosine kinase (i) ErbB:HER1/EGFR. e.g: Cetuximab, Panitumumab
   (ii) HER2/neu e.g Trastuzumab
b. Others for solid tumors (i) EpCAM .e.g Catumaxomab, Edrecolomab
   (ii) VEGF-A e.g. Bevacizumab
c. Leukemia/lymphoma (i) Lymphoid CD20 e.g Ibritumomab, Ofatumumab, Rituximab, Tositumomab
   (ii) CD30 .e.g Brentuximab
   (iii) CD52 .e.g Alemtuzumab
   (iv) Myeloid CD33.e.g Gemtuzumab

B) Tyrosine-kinase inhibitors ("-nib")

a. Receptor tyrosine kinase:
   i. ErbB: HER1/EGFR. e.g: Erlotinib, Gefitinib, Vandetanib
   ii. HER1/EGFR and HER2/neu e.g Afatinib, Lapatinib, Neratinib
   iii. RTK class III: C-kit and PDGFR e.g Axitinib, Pazopanib, Sunitinib, Sorafenib, Toceranib.
   iv. FLT3 e.g Lestaurtinib
   v. VEGFR e.g Axitinib, Cediranib, Pazopanib, Regorafenib, Semaxanib, Sorafenib, Sunitinib, Toceranib, Vandetanib.
b. Non receptor:
   (i) bcr-abl e.g Dasatinib, Imatinib, Nilotinib

(ii) Src e.g Bosutinib

(iii) Janus kinase e.g Lestaurtinib, Ruxolitinib

(iv) EML4-ALK e.g Crizotinib

c. Others

(i) fusion protein against VEGF e.g Aflibercept

(ii) Proapoptotic peptide against ANXA2 and prohibitin e.g Adipotide

(iii) exotoxin against IL-2 e.g Denileukin diftitox

2.3 Pharmacology of erlotinib

2.3.1 Physico-chemical properties of erlotinib

Erlotinib is a quinazolinamine with the chemical name N-(3-ethynylphenyl)–6,7-bis (2-methooxyethoxy)-4-quinazolinamine. Erlotinib hydrochloride has the following molecular formula: $C_{22}H_{23}N_3O_4$.HCL and a molecular weight of 429.90. Erlotinib hydrochloride is very slightly soluble in water, slightly soluble in methanol and practically insoluble in acetonitrile, acetone, ethyl acetate and hexane. Aqueous solubility of erlotinib hydrochloride is dependent on pH with increased solubility at a pH of less than 5 due to protonation of the secondary amine (Pollack *et al.*, 1999).

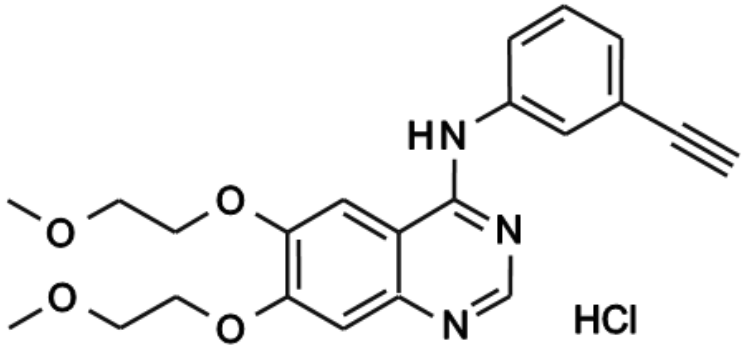

Figure 2.4: The chemical structure of erlotinib.

2.3.2 Pharmacodynamic of erlotinib

Epidermal growth factor receptor (EGFR) belongs to a family of four receptors: ErbB-1 (EGFR), ErbB-2 (HER2/neu), ErbB-3 (HER3), and ErbB-4 (HER4) responsible for cell survival (Ciardiello and Tortora 2001). EGFR is a transmembrane receptor with an internal tyrosine kinase (TK) domain which is phosphorylated after the binding of the ligand to the receptor. The activation of this domain will then stimulate several internal signaling pathways (Ras/MAPK/PI3K/Akt/etc) which in turn affects cell proliferation, differentiation and survival (Herbst, 2004). There is evidence to suggest that this process can promote cancer development and metastasis (Chan *et al.*, 1999; Engebraaten *et al.*, 1993). There are several methods of inhibiting the EGFR pathway including monoclonal EGFR antibodies and small molecule inhibitors of TK.

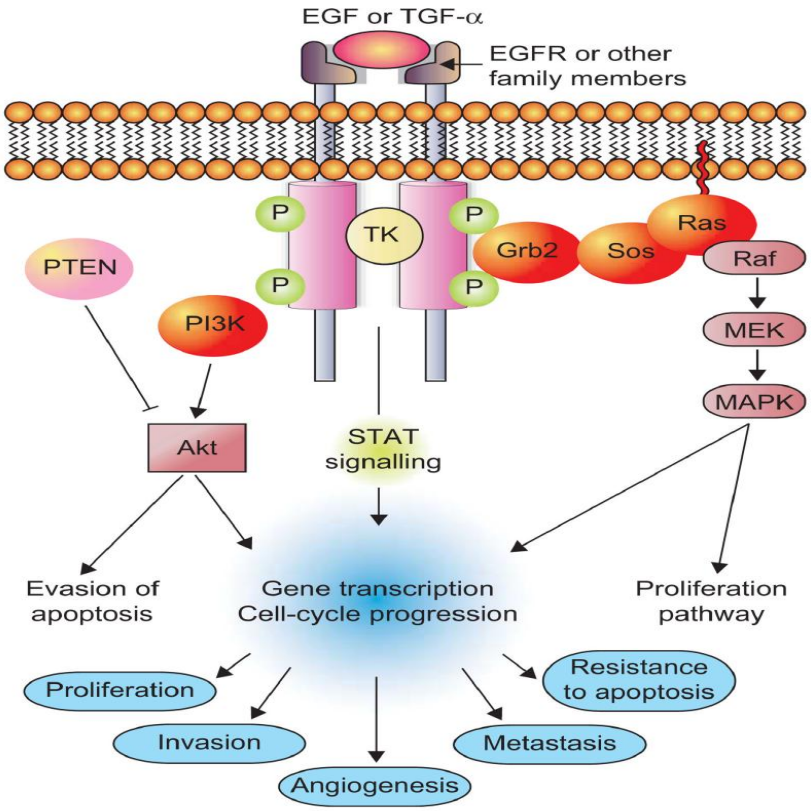

Figure-2.5: Mechanism of action of erlotinib

A different method of blocking EGFR is by inhibiting the cytoplasmic TK domain. Gefitinib and erlotinib are both available small molecule act as EGFR TK inhibitors. Gefitinib was initially approved in the United States based on encouraging response rate and survival in phase II studies (Fukuoka *et al.,* 2003), but was subsequently pulled from the North American market when a randomized phase III trial (ISEL) failed to show a survival benefit versus placebo (Thatcher *et al.,* 2005). Erlotinib, however is currently approved for use as second-line or third-line therapy in patients with non-small cell lung cancer (NSCLC) based on the landmark BR.21 trial which showed a statistically significant survival advantage for the drug versus placebo (Shepherd *et al*., 2005), as well as in combination with gemcitabine in locally advanced or metastatic pancreatic cancer (Moore *et al.,* 2007).

Erlotinib is an EGFR-TK inhibitor. It inhibits the tyrosin kinase activity of EGFR by competing with ATP for the ATP-binding site within the intracellular tyrosin kinase domain of the EGFR. The EGFR and other ErbB family cell membrane receptors (ErbB-2/Neu/HER2, ErbB-3/Neu/HER3, ErbB-1/HER1 and ErbB-4/Neu/HER4) are important mediators for cell growth, differentiation and survival. EGF-like growth factors bind to and activate one or more receptor of the ErbB family. Erlotinib reduces HER1/EGFR autophosphorylation in intact tumor cells with a median inhibitory concentration of 20 nmol/L (7.9 ng/mL), inhibits epidermal growth factor-dependent cell proliferation at nanomolar concentrations, and blocks cell-cycle progression at the G1 phase (Pollack *et al.,* 1999).

2.3.3 Pharmacokinetics and metabolism of erlotinib

Erlotinib is extensively metabolised into multiple products (Ling *et al.,* 2006), including an active O-desmethyl metabolite, OSI-420. Cytochrome P450 3A4 (CYP3A4) plays a prominent role in the metabolism of this agent (Li *et al.,* 2007[a]).

Erlotinib is subject to extensive first-pass metabolism following oral administration and also influenced by intestinal and/or hepatic CYP3A4 activity. The CYP3A4 is again inhibited by BAS 100, a novel spiro-ortho-ester mechanism-based inhibitor present in grapefruit juice (Li *et al.,* 2006, 2007[b]), which alter the pharmacokinetics of erlotinib in mice. Since the grapefruit effect was first reported in the early 1990s (Bailey *et al.,* 1991), the ingestion of grapefruit juice has been shown to enhance the systemic exposure of a number of orally administered drugs (Bailey *et al.,* 2004).

Smoking also dramatically increases activity of CYP1A2, another enzyme involved in erlotinib metabolism and this is hypothesized to be one reason for lack of efficacy of the drug in smokers, due to increased clearance (Li *et al.,* 2007[a]). Ongoing trials are exploring dose escalation of erlotinib in smokers to see if this can be overcome.

2.3.3.1 Pharmacokinetics in human

Clinical pharmacokinetics of erlotinib in patients with solid tumors has been investigated by Lu *et al.* (2006). Patients treated with erlotinib as a single agent, the oral clearance was 3.95 L/h, the oral volume of distribution was 233 L, and the absorption rate was 0.95 h^{-1}. The median erlotinib half-life based on patient population was 36.2 hours. Total bilirubin, alpha1-acid glycoprotein, and smoking status were the most important factors affecting clearance.

Geoerger *et al.* (2009) carried out the multicenter phase I study to determine the recommended dose (RD) of erlotinib, a small-molecule EGFR TKI, in patients ≤ 21 years as monotherapy or in combination with radiation therapy (RT). Four dose levels were planned: 75, 100, 125 and 150 mg/m²/day. Mean (± SD) apparent CL and volume of distribution for erlotinib were 143.7 mL/h/kg (± 66.3) and 3.5 L/kg (± 3.0), respectively. Mean half-life for erlotinib was 20.4 hours.

Hanauske *et al.* (2007) reported the pharmacokinetics of erlotinib at dose of 100 mg/day orally in patients with advanced solid tumors. Cmax and Tmax were 1,519.50 ± 257.06 ng/mL and 3.65 ± 0.60 h respectively. AUC (0-24 hr) was found to be 22,809.48 ± 4,256.52 ng˙hr/mL. The drug was detected in the body for 24.65 ± 0.43 h.

Clinical pharmacokinetics of erlotinib in patients with advanced pancreas cancer has been investigated by Ma *et al.* (2010). Patients treated with erlotinib as a single agent at the dose rate of 150 mg/kg orally, the maximum concentration of the drug in plasma was 1033.4 ± 382.7 ng/ml which was observed at 3.95 h after oral administration. The area under curve (AUC) was 28,385 ± 12,854 ng˙hr/mL. The elimination-half life was found to be 15.91 ± 2.16 h.

L*in*g *et al.* (2006) evaluated pharmacokinetic parameters of erlotinib after a single 100 mg oral dose of erlotinib to male volunteers. The maximum concentration of the drug in plasma was 1.39 ± 0.39 ug/ml which was observed at 1.4 ± 1.0 h after oral administration. The area under curve (AUC) was 14.8 ± 5.3 ug˙hr/mL. The elimination-half life was found to be 8.10 ± 3.5 h.

2.3.3.2 Pharmacokinetics in animals

Smith *et al.*, (2008) evaluated pharmacokinetics of erlotinib administered orally at the dose rate of 10 mg/kg in mice. The maximum concentration of the drug in plasma was 2323 ng/ml which was observed at 0.5 h after oral administration in mice. The area under curve (AUC) was 17957 ng˙hr/mL. The elimination-half life was found to be 3.1h.

Marchetti *et al.*, (2008) studied the pharmacokinetics of erlotinib after p.o. and i.p. administration in Bcrp1/Mdr1a/1b-/- (triple-knockout) and WT mice. In vivo, systemic exposure as well as bioavailability of erlotinib after oral administration (5

mg/kg) showed statistically significant increase in Bcrp1/Mdr1a/1b-/- knockout mice (60.4%) compared with WT mice (40.0%; P = 0.02). Erlotinib is transported efficiently by P-gp and BCRP/Bcrp1in vitro. In vivo, absence of P-gp and Bcrp1 significantly affected the oral bioavailability of erlotinib. Possible clinical consequences for drug-drug and drug-herb interactions in patients in the gut between P-gp/BCRP-inhibiting substrates and oral erlotinib need to be addressed.

2.3.4 Pharmacological effect of erlotinib

Zerbe *et al.* (2006) reported that erlotinib (EGFR inhibitor) reduced tumor burden in male mice by two fold compared to vehicle control (12.7 ± 1.2 vs 26.2 ± 2.5 mg, respectively). Further, erlotinib decreased the contents of pEGFR in uninvolved lungs and lung tumors, particularly in males. They concluded that adenomas from male mice in this early lung cancer model were responsive to erlotinib treatment, possibly because of a greater dependence of male tumor growth on the EGFR pathway compared to females.

Harsha *et al.* (2008) reported that targeted therapeutic approaches have the potential to transform cancer therapy as exemplified by the success of several tyrosine kinase inhibitors. The comprehensive profiling of tyrosine kinases and their substrates was carried out using a panel of low passage pancreatic cancer cell lines, which exhibited the pancreatic cancer cell lines, P196 showed dramatic up regulation of tyrosine kinase activity as compared to non-neoplastic cells. A careful analysis of activated tyrosine kinase pathways revealed aberrant activation of epidermal growth factor receptor pathway in this cell line. Mouse xenograft based studies using EGFR inhibitor erlotinib confirmed EGFR pathway to be responsible for proliferation in these tumors. By a systematic study across low passage pancreatic cancer cell lines and mice carrying cancer xenografts, they have demonstrated activated epidermal

growth factor receptor as an attractive candidate for targeted therapy in a subset of cancers.

Lu *et al.* (2008) investigated the effect and mechanism of action of erlotinib, an epidermal growth factor receptor (EGFR) small molecule tyrosine kinase inhibitor (TKI), in the human pancreatic cancer cell line BxPC-3 both in vitro and in vivo. Erlotinib, as a single agent, repressed BxPC-3 cell growth in a dose-dependent manner, triggered G_1 arrest and induced cell apoptosis, and suppressed capillary formation of endothelium in vitro. Expressions of VEGF were significantly down-regulated at a high concentration of 200 μmol/L; however, the expressions of bcl-2 and bcl-xl were decreased at 50 μmol/L. In vivo, erlotinib-treated mice demonstrated a reduced tumor volume, weight and microvessel density as compared to the control. Immunohistochemical staining showed decreased expression of EGFR and RT-PCR had lower VEGF expression in treated mice. They concluded that inhibition of EGFR may be a promising adjuvant chemotherapy strategy in pancreatic (and possibly other) cancer treatment.

The human epidermal growth factor receptors HER1/EGFR and HER2 offer potential targets for treating non-small cell lung cancer (NSCLC). The antitumor efficacy of erlotinib, a HER1/EGFR tyrosine-kinase inhibitor, was investigated in relation to HER1/EGFR and HER2 expression in five NSCLC xenograft models by Friess *et al.* (2006). Tumor-bearing mice were randomized to daily oral erlotinib 50 mg/kg, or vehicle controls for 20-50 days. The antitumor efficacy of erlotinib was measured through tumor volume, serum tumor markers and tumor biomarkers. Tumor HER1/EGFR and HER2 expression were analyzed immunohistochemically. Erlotinib reduced tumor volume in three NSCLC models. It also reduced serum tumor marker levels and the extent of inhibition correlated with tumor growth inhibition.

HER1/EGFR and HER2 expression differed between the five tumor models, suggesting that expression level does not predict response to treatment. Erlotinib showed differing antitumor activity in five NSCLC models, suggesting that its antitumor effect is independent of HER1/EGFR and HER2 over-expression.

2.3.5 Interaction of erlotinib with other drugs

There are many novel/targeted agents currently in clinical trials in combination with erlotinib. Bevacizumab was combined with erlotinib in a phase I/II study in patients with relapsed non-squamous NCSLC (Herbst *et al.,* 2005; Sandler *et al.*, 2006). The addition of erlotinib blocks the EGFR and the synthesis of angiogenic factors including vascular endothelial growth factor (VEGF) which in turn prevents endothelial response to VEGF. Furthermore, there is some evidence to suggest that bevacizumab inhibits the EGFR autocrine function (Petit *et al*., 1997; Hirata *et al*., 2002). There was no drug limiting toxicity reported in the phase I portion and 34 patients were treated at the phase II doses with erlotinib 150 mg daily and bevacizumab 15 mg/kg every 21 days. The most common toxicities were diarrhea, rash, hematuria and proteinuria with no treatment related deaths. There was a 20% partial response (PR) and 65% stable disease(SD) with a median overall survival(OS) of 12.6 months and a PFS of 6.2 months. Nine tumors were tested for EGFR mutations in exons 19–21 and 23 and only 2 had the mutation (1 partial response and 1 stable disease). Confirmation of these exciting preliminary results is being sought in two international phase III trials, ATLAS, and Beta. The ATLAS trial (N = 1150) is a randomized double-blind, placebo-controlled phase IIIb trial that compares bevacizumab with or without erlotinib after completion of first-line chemotherapy with bevacizumab for advanced NSCLC (non-squamous). The Beta trial (N = 650) randomizes patients requiring second-line therapy to erlotinib with or without

bevacizumab. These promising results with dual EGFR/VEGFR inhibition have also been seen with single drugs that target both receptors. The one furthest in development is ZD6474 (vandetanib). This compound has been directly compared to gefitinib in phase II testing with favorable results (Natale *et al*., 2006). This has led to an ongoing trial of the compound versus erlotinib.

Higgins *et al.* (2003) reported that two human non-small cell lung cancer (NSCLC) cell lines H460a and A549 express similar numbers of epidermal growth factor receptors (HER1/EGFR). When grown as subcutaneous tumors in athymic mice, these cells display different tumor growth kinetics, having a doubling time of approximately 5 and 10 days, respectively. Their goal was to explore the antitumor activity of combinations of erlotinib with gemcitabine or cisplatin in mice bearing H460a and A549 tumors. Antitumor activity was observed with each agent in both models at their respective MTD (erlotinib 100 mg/kg: 71% tumor growth inhibition (TGI), 93% TGI; Gemcitabine 120 mg/kg: 93% TGI, 75% TGI; Cisplatin 6 mg/kg: 81% TGI, 88% TGI). When each compound was administered at 1/4 MTD, suboptimal antitumor activity was seen. Combinations of gemcitabine or cisplatin with erlotinib were assessed at 1/4 MTD to determine whether erlotinib potentiated activity of gemcitabine or cisplatin. In both NSCLC models, suboptimal doses of gemcitabine (30 mg/kg q3d) or cisplatin (1.5 mg/kg q6d) with erlotinib (25 mg/kg qd) were well tolerated. For the slow growing A549 tumor, significant TGI was seen in the gemcitabine/erlotinib and cisplatin/erlotinib combinations (103% and 90%, respectively), with partial regressions. The enhanced tumor growth inhibition obtained when erlotinib was combined with either gemcitabine or cisplatin was significant compared to that obtained with monotherapy ($p<0.05$). For the faster growing H460a tumor, significant tumor growth inhibition was seen in the

gemcitabine/erlotinib combination (86%) and in the cisplatin/erlotinib combination (53%). However, tumor growth inhibition with these combinations was not significantly better than that achieved with monotherapy (p=0.05). These results show that erlotinib effectively inhibits fast and slow growing tumors alone and in combination with chemotherapy, but slow growing tumors appear to be more responsive.

Reckamp *et al.* (2008) evaluated the effect of TG01, a new potent COX-2 inhibitor in combination with erlotinib in metastatic or recurrent non-small cell lung cancer (NSCLC) patients. Female patients with recurrent or metastatic NSCLC were treated with erlotinib (150 mg PO daily) and escalating doses of TG01 (100 - 1200) PO daily). Six patients have been enrolled (3 at TG01 100mg; 3 at TG01 200mg). There have been no dose limiting toxicity; most common adverse events include G1 and G2 nausea, diarrhea and rash (2 pts each). TG01 and erlotinib have been safely administered in doses evaluated to date.

2.3.6 Adverse effects of erlotinib

Perez-Soler *et al.* (2004) reported the most common drug-related adverse events in patients treated with erlotinib 150 mg/day. Fifty-six patients (98%) had at least one drug related adverse event, 38 patients (67%) had drug-related adverse events with a maximum severity of grade 1 or 2, and 17 patients (30%) had at least one grade 3 drug-related adverse event. Less than 10% of patients showed signs of ocular toxicity attributable to erlotinib therapy; no incidence exceeded grade 2. Dysphagia, pruritus, fatigue, dyspnea, decreased appetite, and anxiety were the only drug-related grade 3 events reported in two patients, and none was reported in more than two patients (4%). Interstitial pneumonia and grade 4 events were not reported.

The patients receiving erlotinib for treatment of NSCLC, pancreatic cancer or other advanced solid tumors leads serious Interstitial Lung Disease (ILD)-like events, including fatalities. The patients suspected of having ILD-like events included pneumonitis, radiation pneumonitis, hypersensitivity pneumonitis, interstitial pneumonia, interstitial lung disease, obliterative bronchiolitis, pulmonary fibrosis, Acute Respiratory Distress Syndrome and lung infiltration (Anonymous, 2011).

Small-molecule tyrosine kinase inhibitors (TKIs) targeting the epidermal growth factor receptor (EGFR) pathways are used clinically for patients with non-small cell lung cancer (NSCLC). It is well established that somatic mutations in the kinase domain of the EGFR are strongly associated with the tumor response and clinical outcomes in patients with NSCLC receiving EGFR-TKIs. Although the most common adverse events are skin rash and diarrhea, the most serious adverse effect reported is drug-related interstitial lung disease (ILD) (Inoue *et al.,* 2003; Lynch *et al*., 2004; Paez *et al.,* 2004; Ando *et al*., 2006; Mitsudomi and Yatabe, 2007)

Cases of hepatorenal syndrome, acute renal failure (including fatalities) and renal insufficiency have been reported. Some were secondary to baseline hepatic impairment while others were associated with severe dehydration due to diarrhea, vomiting, and/or anorexia or concurrent chemotherapy use. In the event of dehydration, particularly in patients with contributing risk factors for renal failure (e.g. pre-existing renal disease, medical conditions or medications that may lead to renal disease, or other predisposing conditions including advanced age), erlotinib therapy should be interrupted and appropriate measures should be taken to intensively rehydrate the patient. Periodic monitoring of renal function and serum electrolytes is recommended in patients at risk of dehydration (Anonymous, 2011).

Cases of hepatic failure and hepatorenal syndrome (including fatalities) have been reported during use of erlotinib, particularly in patients with baseline hepatic impairment. Therefore, periodic liver function testing (transaminases, bilirubin, and alkaline phosphatase) is recommended. Gastrointestinal perforation (including fatalities) has been reported in patients receiving erlotinib. Patients receiving concomitant anti-angiogenic agents, corticosteroids, NSAIDs, and/or taxane based chemotherapy, or who have prior history of peptic ulceration or diverticular diseases are at increased risk. Corneal perforation or ulceration has been reported during use of erlotinib. Other ocular disorders including abnormal eyelash growth, keratoconjunctivitis sicca or keratitis have been observed with erlotinib treatment and are known risk factors for corneal ulceration/perforation (Anonymous, 2011).

Cutaneous toxicity is common in patients treated with erlotinib. Dose reductions and discontinuation of therapy are needed in some patients. There are several theories on the etiology of these skin reactions. EGFR is expressed on the keratinocytes, sebaceous gland cells, and the outer sheath of hair follicles (Lee *et al*., 2004). EGFR inhibition can result in follicle occlusion and acneiform eruption with inflammation (Journagan and Obadiah, 2006). Individuals normally present with a papulopustular rash affecting the face and the upper trunk occurring in the first weeks of therapy (Luu *et al*., 2007).

Hanauske *et al.* (2007) reported that erlotinib has been associated with elevated alanine aminotransferase and bilirubin. In this study, there was no apparent relationship between the level of erlotinib exposure and the degree of elevation of alanine aminotransferase or bilirubin.

2.3.7 Therapeutic uses of erlotinib

Erlotinib has shown a survival benefit in the treatment of lung cancer in phase III trials. The SATURN (Sequential Tarceva in Unresectable NSCLC) study found that erlotinib added to chemotherapy improved progression free survival (PFS) significantly with erlotinib vs placebo, the response rate (12%) and disease control rate (40.8%) was also significantly ($p < 0.01$) increased with erlotinib vs placebo. (Cappuzzo *et al.*, 2009). The U.S. Food and Drug Administration (FDA) has approved for the treatment of locally advanced or metastatic non-small cell lung cancer that has failed at least one prior chemotherapy regimen. In November 2005, the FDA approved erlotinib in combination with gemcitabine for treatment of locally advanced, unresectable, or metastatic pancreatic cancer.

In lung cancer, erlotinib has been found effective in patients with or without EGFR mutations, but appears to be more effective in the group of patients with EGFR mutations. Erlotinib may be used for treatment of polycythemia vera and other myeloproliferative disorders (Li *et al.*, 2007[a]).

Erlotinib, tyrosine kinase inhibitor (TKI) targeting the epidermal growth factor receptor (EGFR), have shown remarkable activity in a portion of patients with non-small-cell lung cancer (NSCLC). Currently, randomized phase III trials of erlotinib as initial treatment of patients with EGFR mutant lung cancer are ongoing. In the setting of these recent developments, a review of the data regarding the use of erlotinib or gefitinib as initial therapy in the treatment of NSCLC is warranted.

2.4 Pharmacology of meloxicam

2.4.1 Physico-chemical properties

It is chemically designated as 4-hydroxy-2-methyl-*N*-(5-methyl-2-thiazolyl)-2*H*-1, 2-benzothiazine-3-carboxamide 1, 1-dioxide. The molecular weight is 351.4.

Its empirical formula is $C_{14}H_{13}N_3O_4S_2$ and it has the following structural formula (Anonymous, 2004). Meloxicam is a yellow solid, practically insoluble in water, with higher solubility observed in strong acids and bases and very slightly soluble in methanol (Abd Elbary, 2001).

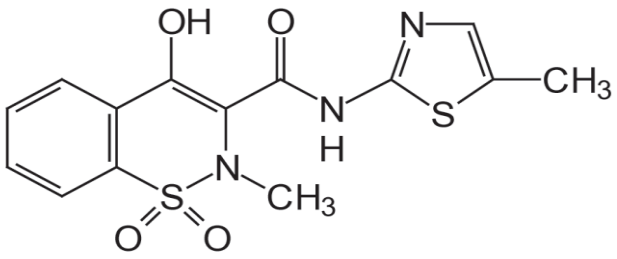

Fig. 2.6 Chemical structure of meloxicam

2.4.2 Mechanism of action of meloxicam

Meloxicam is an NSAID of the oxicam class, which acts by inhibiting prostaglandin synthesis and inducible COX-2, thereby exerting anti inflammatory, anti-exudative, analgesic and antipyretic effects (Friton *et al.,* 2003; Hirsch *et al.,* 2003; Papatsas *et al.,* 2004). Non-steroidal anti-inflammatory drugs (NSAIDs) have been used by humans in various forms for more than 3,500 years. Their popularity is still enormous; it is estimated that humans around the world consume around 40,000 metric tons of aspirin each year, equating to about 120 billion aspirin tablets of 300 mg. Despite this long history and large volume, the mechanisms of how NSAID's achieve their actions are still not completely unravelled (Vane, 2000).

Some 30 years ago, it was first revealed that all these drugs reduced the formation of prostaglandins and that ability was associated with inhibition of the enzyme cyclo-oxygenase (COX), which converts arachidonic acid to the prostaglandin precursor prostaglandin H2 (PGH2). In the early 1990's the existence of two isoforms of COX, COX-1 and COX-2, was demonstrated, leading to the classification of all NSAIDs according to their specificity to each of these isoforms

(Warner and Mitchell, 2002). Recently, meloxicam showed marked anti-endotoxic activity. It has been found to inhibit the production of thromboxane B2, induced by intravenous *E. coli* endotoxin to animals and the intramammary administration of *E. coli* endotoxin to lactating cows (Friton *et al.,* 2003; Hirsch *et al.,* 2003; Papatsas *et al.,* 2004).

During the last decade, numerous studies have shown that NSAIDs are chemopreventive for cancer and, to a lesser extent, breast and lung cancer in humans. The use of NSAIDs to abrogate tumor growth also has been demonstrated extensively in murine models of colorectal cancer, as well as in human clinical cancer cases (Giardiello, 1996; Thun, 1996; Husain *et al.,* 2002). They also exhibit anticancer activity by several mechanisms including induction of apoptosis, inhibition of angiogenesis, and decreased invasiveness and metastatic potential. These effects have been documented in several preclinical studies. Similar effects are also seen in dogs administered piroxicam for transitional cell carcinoma (Mohammed *et al.,* 2002).

The beneficial effects of NSAIDs in colorectal carcinogenesis have been established by numerous population-based studies and confirmed in 2 large, randomized clinical trials with the prodrug sulindac and the selective COX-2 inhibitor celecoxib (Giardiello *et al.,* 1993; Steinbach *et al.,* 2000). The results showed that these drugs inhibited the growth of intestinal polyps and caused regression of existing tumors in patients with familial adenomatous polyposis.

2.4.3 Pharmacokinetics of meloxicam.

Favourable kinetic properties of meloxicam like good absorption, longer elimination half-life and optimum bioavailability make it an ideal and suitable NSAID for use in animals (Busch *et al*., 1998). The pharmacokinetic behaviour of meloxicam has been investigated in horses (Lees *et al*., 1991; Toutain *et al*., 2004),

dogs (Gohel, 2006; Montoya *et al*., 2004), piglets (Fosse *et al.*, 2008), donkey (Sinclair *et al.*, 2006), rats (Aguilar-Mariscal *et al.*, 2007; Habashi and Jamali, 2008), chickens (Baert and De Backer, 2002), ostriches (Baert *et al*., 2002), vulture (Naidoo *et al.*, 2008), rabbits (Turner *et al.*, 2006), sheep and goats (Shukla *et al*., 2007) and humans (Busch *et al*., 1998).

Meloxicam is well absorbed from the gastrointestinal tract, which is reflected by a high absolute bioavailability of 89% following oral administration. Meloxicam has almost complete bioavailability when administered orally with food. Maximum plasma concentrations of meloxicam, at steady state, are achieved within seven to eight hours for the tablet, capsule and the oral suspension. Meloxicam is highly plasma protein bound drug (95-99%). It has a long plasma half-life, enabling less frequent dosage schemes. Turck *et al.* (1996) revealed that meloxicam has prolonged and almost complete absorption and the drug is more than 99.5% bound to plasma proteins. Busch *et al.* (1998) has found meloxicam to be highly bound to plasma proteins in rats (99.5-99.7%), mice (96.8%), dogs (97%) and minipigs (96%). meloxicam penetrate into synovial fluid to give concentration approximately half of those in plasma and low volume of distribution.

Meloxicam undergoes extensive hepatic biotransformation. Meloxicam is extensively metabolized, with only traces of the drug appearing unchanged in urine and feces. The main metabolites are formed by hydroxylation and further oxidation of the methyl group of the thiazolyl moiety (Schmid *et al*., 1995). Meloxicam is excreted predominantly in the form of metabolites and excretion occurs to equal extents in urine and faeces. Less than 5% of the daily dose is excreted unchanged in faeces, while only traces of the parent compound are excreted in urine. Turck *et al.* (1996) have studied meloxicam metabolism and noted four biologically inactive metabolites,

which are excreted in both urine and faeces. *In-vitro* studies indicated that cytochome CYP 2C9 plays an important role in this metabolic pathway with a minor contribution of the CYP 3A4 isozyme.

2.4.3.1 Pharmacokinetics in human

Rani *et al.* (2004) studied pharmacokinetics of meloxicam following oral administration of 15 mg tablets in human after an overnight fast and reported that pharmacokinetic parameters *viz.* maximum plasma concentration (Cmax), time to reach maximum plasma concentration (Tmax), area under the plasma concentration-time curve (AUC0-∞), elimination half life (t1/2), volume of distribution (Vd/F) and clearance (Cl/F) were 1.3763 (0.25) mg/l, 2.91 (2.02) h, 35.79 (8.53) mg.h/l, 30.50 (9.60) h, 19.04 (7.16) l and 0.44 (0.10) l/h respectively.

Burgos-Vargas *et al.* (2004) evaluated pharmacokinetics of meloxicam in patients with juvenile rheumatoid arthritis (n=18). Following administration of meloxicam at dose of 0.25-mg/kg body weight up to a maximum of 15 mg in children, geometric mean value of Cmax, AUC0-∞, apparent clearance, apparent volume of distribution, and elimination half-life values were 1.24 µg/mL, 25.6 µg•h/mL, 0.17 mL/min/kg, 0.19 L/kg, and 13.4 hours in the younger group and 1.89 µg/mL, 35.8 µg•h/mL, 0.12 mL/min/kg, 0.13 L/kg, and 12.7 hours for the older group, respectively.

2.4.3.2 Pharmacokinetics in animals

Toutain *et al.,* (2004), reported pharmacokinetics of meloxicam in horses. Values of clearance, volume of distribution and terminal half-life were 34 ± 0.5 mL/kg/h, 0.12 ± 0.018 L/kg and 8.54 ± 3.02 h respectively. During once-daily administration for 14 days, drug accumulation was not detected.

Gohel (2006) studied the pharmacokinetic of meloxicam following intramuscular and oral routes in dogs. The elimination half-lives for I.M. and oral route of administration were 23.41 ± 0.93 h and 21.18 ± 1.22 h, respectively. The values of area under curve were 33.40 ± 2.02 μg.h/mL and 32.62 ± 2.14 μg.h/mL for I.M. and oral route of administration, respectively. The value of area under first moment of curve were found to be 1259 ± 35.1 $\mu g.h^2$/mL and 1607.29 ± 185 $\mu g.h^2$/mL. The values of MRT were 38.38 ± 2.46 and 48.72 ± 3.63 h, respectively. Values of F for I.M. and oral study were 0.96 ± 0.06 and 0.95 ± 0.07 % respectively.

Sinclair *et al*. (2006) investigated comparative pharmacokinetics of meloxicam in clinically normal horses and donkeys after IV bolus administration of meloxicam (0.6 mg/kg). Mean values of area under the curve, total body clearance and volume of distribution (Vd) were 18.8 ± 7.31 and 4.6 ± 2.55 μg/mL/h, 34.7 ± 9.21 and 187.9 ± 147.26 mL/kg/h and 270 ± 160 and 93.2 ± 33.74 mL/kg in horses and donkeys, respectively. They found significant difference in values of Vd between donkeys and horses and also reported that horses and donkey had greater Cl for meloxicam, indicating a rapid elimination of the drug from plasma.

Aguilar-Mariscal *et al*. (2007) studied the pharmacokinetics of meloxicam in rats following oral doses of 3.2, 5.6 or 10 mg/kg body weight and reported increased blood concentrations reaching a dose-dependent maximal concentration in about 2 h. They also observed an increase in C_{max} and AUC as a function of the dose. But no statistically significant difference was observed in AUC/dose or C_{max}/dose between doses.

Carpenter *et al.* (2009) evaluated the single and multiple-dose pharmacokinetics of meloxicam after oral administration to the rabbit (*Oryctolagus cuniculus*). They determined the pharmacokinetics of meloxicam following a single

dose and 10-day period of dosing at 0.2 mg/kg body weight orally daily in eight clinically normal rabbits (*Oryctolagus cuniculus*). After oral administration, mean ± standard deviation values for area under the curve were 1.8 ± 0.50 and 2.1 ± 0.55 µg × h/ml and maximum plasma concentrations were 0.17 ± 0.06 and 0.24 ± 0.07 µg/ml for day 1 and day 10, respectively. They reported half-life of 8 hr. and suggested administration of meloxicam at a dosage of 0.2 to 0.3 mg/kg orally every 24 hr. Meloxicam administered at 0.2 mg/kg oral daily for 10 day was well tolerated by the rabbits.

Ingvast-Larsson *et al.* (2011) studied pharmacokinetics of meloxicam (0.5 mg/kg body weight) in adult goats ($n = 8$) after single intravenous and oral administration. In the adult goats after intravenous administration the terminal half-life, steady-state volume of distribution was and total body clearance were 10.9 ± 1.7 h, 0.245 ± 0.06 L/kg, 17.9 ± 4.3 mL/h/kg respectively. Whereas following oral administration, bioavailability, C_{max} *and* T_{max} were 79 ± 19%, 736 ± 184 ng/mL, 15 ±5 h respectively. Although the terminal half-life was similar to the intravenous value, 11.8 ± 1.7 h.

Wasfi *et al.* (2012) studied the pharmacokinetics and metabolism of meloxicam (0.6 mg/kg) in camels (*Camelus dromedarus*) after intravenous administration ($n = 6$). They detected meloxicam in plasma following 10 days i.v. administration in camels using a sensitive liquid chromatography mass spectrometry (LC/MS/MS) method and reported elimination half-life ($t_{1/2\beta}$), total body clearance (Cl_t) and volume of distribution at steady state (V_{SS}) 40.2 ± 16.8 h, 1.94 ± 0.66 ml·kg/h. 92.8 ± 13.7 ml/kg respectively and also reported detection of unconjugated meloxicam in urine.

2.4.4 Pharmacological effects of meloxicam

Meloxicam has anti-inflammatory, analgesic and antipyretic activity as it is inhibitor of prostaglandin synthesis. It is used worldwide as a NSAID and is categorized as a selective COX-2 inhibitor. It has been reported that meloxicam has an inhibitory effect on colorectal cancer cells and non small-cell lung cancer cells (Tsubouchi *et al*., 2000). NSAIDs have been used for a long time in the treatment of inflammatory diseases and were first recognized for their potential anticancer activity after comparing cancer incidence in individuals that did and did not routinely use these agents.

More effective and less toxic therapy is needed to prevent tumour progression and recurrence. Prostanoids produced by the arachidonic acid pathway play an important role in multiple stages of carcinogenesis and progression of cancer. Cyclooxygenase (COX) enzymes are known to be involved in carcinogenesis at several organ sites. Cyclooxygenase (COX) exists in 2 isoforms out of which Cyclooxygenase-1 is constitutively expressed in normal tissues and is essential for several important physiologic functions while Cyclooxygenase-2 is selectively overexpressed in neoplastic and inflammatory tissues (Ramalingam and Belani, 2004). Emerging genetic evidence suggests that COX-2 is important in carcinogenesis, and expression of COX-2 is up-regulated in many types of human carcinoma (Abou-Issa *et al..*, 2001). Many studies have also shown that COX-2 is over-expressed both in human cancers and in chemically induced rodent and murine cancers (Ristimaki *et al.*, 2001; Suzuki *et al.*, 2009). Non–small-cell lung cancer (NSCLC), especially adenocarcinomas, overexpresses COX-2, which contributes to the progression of malignancy by several mechanisms. In carcinoma in situ, COX-2 expression was significantly associated with disease recurrence. COX-2 inhibitors

have chemopreventive effects in both experimental and clinical settings in cancers (Abou-Issa *et al.,* 2001). This represents the basis of therapy with COX-2 inhibitors (Ramalingam and Belani, 2004).

Although the chemopreventive and antitumorigenic activity of NSAIDs against human and rodent cancers has been characterized extensively, the molecular responses and efficacy in canine cancer have not been elucidated clearly; however, because of the conserved nature of prostaglandin biosynthesis across species, it is entirely logical to expect mechanisms and efficacy in dogs to be similar to those in humans and rodents. Carprofen, piroxicam, and meloxicam are the most common NSAIDs prescribed in animals. Both piroxicam and meloxicam inhibit COX-2 activity and induce apoptosis in cell culture, and meloxicam has a greater selectivity for COX-2 in healthy dogs (Sugimoto and Narumiya, 2007). Piroxicam was tested in dogs with naturally occuring, invasive transitional cell carcinoma of the urinary bladder, resulting in remission in 12 of 18 cases (Mohammed *et al.,* 2002). These results were strongly associated with induction of apoptosis and a reduction in urine basic fibroblast growth factor concentration. Administration of piroxicam to human cancer patients with pulmonary metastases induced minor remission in 5 of 31 individuals (Breau *et al.,* 1989). Clinical efficacy of COX-2 inhibitors in the treatment of NSCLC has also been evaluated (Ramalingam and Belani, 2004).

Celecoxib is a highly selective COX-2 inhibitor and has been shown to have antitumor effect in human tumor xenograft models of colon (Masferrer *et al.,* 2000), breast (Blumenthal *et al.,* 2001), prostate (Liu *et al.,* 2000) and Lewis lung carcinoma (Masferrer *et al.,* 2000). The *in-vitro* antiproliferative effect of celecoxib has been attributed to the induction of apoptosis. Singh *et al*. (2006) reported that celecoxib enhances the antitumor effect of docetaxel in lung tumors (xenograft mice model by

A549 lung cancer cell line) in mice. Meloxicam is a selective COX-2 inhibitor, which has been tested for its chemopreventive effects in bladder cancer but not in other types of cancer (Hattori *et al.*, 2006).

A wide range of molecular mechanisms responsible for the antitumorigenic effects of NSAIDs have been implicated in laboratory studies, with the most obvious being, the inhibition of COX-2 and reduction of prostaglandin synthesis. Vigorous investigation of this aspect of NSAID activity has made it clear that prostaglandin-independent mechanisms are also involved, particularly with respect to the induction of apoptosis.

Much of the focus on development of NSAIDs as chemopreventive drugs has been on their use as selective COX-2 inhibitors. COX-2 expression is low in most tissues with some important exceptions and expression is increased by cytokines and growth factors up-regulated during inflammation and within tumors (Spugnini *et al.*, 2005). The importance of COX- 2 in tumorigenesis has been demonstrated clearly in studies with COX-2 knockout mice, which are significantly less susceptible to chemically or genetically induced tumors of the skin, intestine, and mammary gland (Tiano *et al.*, 2002).

Fact of antitumorigenic activity of NSAIDS in humans stimulates cancer research in other species, including dogs in which, COX-1 has not been studied as a chemopreventive target gene, but it has been reported that treatment of a canine mammary carcinoma cell line with NS-398 (a specific COX-2 inhibitor) significantly blocked prostaglandin E2 (PGE2) synthesis and reduced cell proliferation (Brunelle *et al.*, 2006). In study of Brunelle *et al.* (2006) neoplastic canine mammary cell lines constitutively overexpressed COX-2 and inhibition of COX-2 decreased PGE2 production and cell proliferation, supporting a role for COX-2 and prostaglandins in

canine mammary tumorigenesis. Number of such studies also has investigated the expression of COX-1 and COX-2 in canine tumor tissues, primarily by immunostaining in mammary, prostate, ovarian, colorectal, nasal, and bladder carcinomas and in osteosarcoma. Although most of these studies demonstrate upregulation of COX-2 protein expression in tumors, this should not be interpreted to directly implicate functional activity in generating any particular bioactive product. The human colorectal cancer cell line HT-29 highly expresses COX-2 protein as determined by immunohistochemistry and Western blot analysis; however, there is no detectable PGE2 in these cells as assessed by high performance liquid chromatography (Hsi *et al.,* 2000). Similarly, the clinical response in dogs with transitional cell carcinoma treated with piroxicam therapy was not predicted by intratumoral COX-2 expression before treatment (Mutsaers *et al.,* 2005).

2.4.5 Safety and adverse effects of meloxicam

Distel *et al*. (1996) investigated a global safety analysis of data from meloxicam clinical studies, focusing on gastrointestinal (GI) adverse events. With respect to all GI adverse events, meloxicam at 7.5 and 15 mg were significantly better than all comparators in a pooled analysis of double-blind studies in rheumatoid arthritis (RA) and osteoarthritis (OA). Meloxicam improved GI safety profile is likely to be due to its preferential inhibition of inducible COX-2 relative to constitutive COX-1.

Schoenfeld (1999) studied gastrointestinal safety profile of meloxicam and with two additional trials using endoscopy to assess the affect of meloxicam on gastric mucosa of healthy volunteers suggested that piroxicam caused no greater mucosal damage than meloxicam.

Safety and efficacy of meloxicam in the treatment of osteoarthritis were evaluated by Yocum *et al.* (2000) and they reported that the incidence of all adverse events was low at each dosage of meloxicam than for diclofenac, but greater than for placebo. However, the incidence of gastrointestinal adverse events and dropout rates of such events was the same for meloxicam as for placebo and lower than for diclofenac.

Major clinical sign of NSAID toxicity related to liver damage is increase in levels of AST, ALT or AKP and presence or absence of hyperbilirubinemia and hypoalbuminemia. Alkaline phosphatase and alanine aminotransferase concentrations were significantly increased compared with baseline with meloxicam (0.2 mg kg^{-1}) and ketoprofen (2 mg/kg) in dogs (Deneuche *et al.*, 2004).

Swarup *et al.* (2007) evaluated safety of meloxicam to critically endangered Gyps vultures and other scavenging birds in India and recommended introduction of meloxicam as rapidly as possible across the Indian sub-continent as an alternative to diclofenac.

Luna *et al.* (2007) reported adverse effects of long-term oral administration of carprofen, etodolac, flunixin meglumine, ketoprofen and meloxicam in adult dogs. Assessement of dogs with Gastroscopy before and after the end of treatment, they detected gastric lesions in all dogs treated with etodolac, ketoprofen and flunixin, and 1 of 6 treated with carprofen. They concluded that carprofen induced the lowest frequency of gastrointestinal adverse effects, followed by meloxicam. On day 7, bleeding time was significantly longer in dogs treated with meloxicam, ketoprofen and flunixin, compared with control dogs. Clotting time increased significantly in all groups except those treated with etodolac. At day 90, clotting time was significantly shorter in flunixin treated dogs, compared with lactose-treated dogs.

In addition to sharing many therapeutic activities, NSAIDs induce certain unwanted toxic effects. The most common is a propensity to induce gastric or intestinal ulceration that can sometimes be accompanied by anemia from the resultant blood loss (Gabriel *et al*., 1991; Figueras *et al*., 1994).

The safety profile of meloxicam has been evaluated in well controlled target animal safety studies in the dog. Dogs treated with placebo, 1X, 3X and 5X label dosages were closely monitored over a 180 day (26 weeks) period. The study determined that there were no drug related adverse effects on clinical observations, normal body weight gain, and food consumption, physical and behavioral examinations (Anonymous, 2004).

Vijayakumar and Dinnath (2006) studied the ulcerogenic effect of meloxicam, in rats by the method reported by Nagarsenker *et al*. (2000). They concluded that meloxicam, physical mixture and stable disease showed significant ulcerogenic potential compared to the control in rats treated chronically for seven consecutive days (4 mg/kg meloxicam, 4 mg/kg of meloxicam in physical mixture or stable disease, p.o). The physical mixture and stable disease showed less ulcerogenic potential with the ulcer score of 1.3 ± 0.30 and 0.7 ± 0.12, respectively as compared to meloxicam (1.75±0.14). Further, stable disease possessed significantly less ulcerogenic potential as compared with pure meloxicam and physical mixture. The results indicated that stable disease and physical mixture protect the gastric mucosa from injury, which is in accordance with analgesic effects of poorly water soluble NSAID in physical mixture and stable disease (Sahin and Librowski, 2003).

MacDonald *et al*. (2003) estimated that meloxicam and the coxibs, have been channeled towards high risk patients, and to estimate the risk of gastrointestinal haemorrhage associated with the use of drugs. For that they were used the UK

General Practice Research Database, this study included 7.1 thousand patient years exposure to meloxicam, 1.6 thousand patient years exposure to coxibs, and 628 thousand patient years exposure to older nonspecific NSAIDs. They found that most risk factors for gastrointestinal hemorrhage were more prevalent among patients prescribed the newer NSAIDs. Adjusting for these risk factors reduced the relative risks of gastrointestinal haemorrhage on meloxicam and coxibs versus older non-specific NSAIDs to 0.84 (95% confidence interval 0.60, 1.17) and 0.36 (0.14, 0.97), respectively.

Magarwadiya *et al*. (2002) studied the safety profile of meloxicam in four buffalo calves at dose of rate of 5 mg/30 kg body weight for five days. Blood samples were collected at 0 (treated as normal animal) 3, 6, 9, 12, 48, 72, 96, 120 and 144 hours, and compared various hematological, and biochemical parameters with o hour value. They concluded that none of hematological parameter was found to be significantly affected and in the biochemical parameter they found significantly higher plasma total protein values at 48, 120, and 144 hours. Plasma albumin had significantly higher value at 48, 96, and 144 hours of drug administration.

Marilac *et al*. (2003) studied the margin of safety of meloxicam in dogs (n=5). There was a significant ($p \leq 0.05$) leukocytosis, neutrophilia and lymphopenia in 60% of the dogs. The higher dosage group dogs had significant anemia with hematocrit and hemoglobin reduction. The higher dosage group also showed mild to severe gastro enteritis.

2.4.6 Interaction of meloxicam with other drugs

Drug-drug interactions can be categorized into those originating from pharmacokinetic mechanisms and pharmacodynamic mechanisms. Pharmacokinetic interactions are those that result in alterations of drug absorption, distribution,

metabolism and elimination. Pharmacodynamic interactions occur when one drug affects the actions of another drug. The potential for drug-drug interactions is an important aspect of overall drug safety. Meloxicam has been widely used in the clinical field because of their high clinical efficacy and safety, and are often used concomitantly with other drugs. Reports on the interactions of meloxicam with other drugs in animals are limited.

Drugs of the NSAID class should not be used concurrently as the potential for the aforementioned side effects increases. For similar reasons, NSAIDS should not be used in conjunction with corticosteroid hormones such as prednisone, dexamethasone, etc. Pfizer recommends a 5 to 7 day rest period when changing from one NSAID to another. Allow at least one week between prednisone and meloxicam.

Taking meloxicam with other non-steroidal anti-inflammatory drugs (NSAIDs) or pain relievers, such as ibuprofen, naproxen, aspirin or acetaminophen, may cause an overdose. Drinking more than three alcoholic beverages a day, while on meloxicam treatment may increase risk of gastrointestinal problems such as stomach bleeding, ulcers, or holes in stomach or intestines. Other drugs that may adversely interact with meloxicam include: cidofovir, diuretics, lithium, pemetrexed, warfarin, cholestyramine, methotrexate and angiotensin-converting enzyme (ACE) inhibitors, such as benazepril, captopril, enalapril, fosinopril, lisinopril and quinapril.

If meloxicam is used concurrently with phenobarbital, these two drugs interact and neither may work well if they are used together. Angiotensin converting enzyme (ACE) inhibitors such as enalapril, benazepril, or captopril may not be effective in the presence of meloxicam. This is because ACE inhibitors depend on the dilation of blood vessels in the kidneys and such dilation can be interfered by NSAIDs.

There is a possible interaction of EGFR and COX-2; EGFR activation results in the induction of COX-2 expression through nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) activation (Richardson *et al.*, 2003). Therefore, a combination of COX-2 and EGFR inhibitors would be a reasonable and promising strategy for the chemoprevention of cancer. Hattori *et al.* (1998) reported that both meloxicam and gefitinib, when given as single agents, significantly inhibited rat bladder carcinogenesis with no adverse effects. They concluded that a combination of these drugs would be worth studying to test their synergistic effects.

Singh *et al.* (2004) evaluated the antitumor effect of celecoxib alone and in combination with docetaxel in mice bearing s.c human lung adenocarcinoma, A549 tumors and reported 94.2% inhibition of tumor growth by the combined treatment of celecoxib with docetaxel as compared to 70.2 and 76.6% tumor growth inhibition observed with docetaxel alone and celecoxib alone treatments, respectively. All the treatments showed a statistically significant inhibition of tumor growth, as compared to the tumor volume of vehicle treated control mice at 28 days suggesting possibility of obtaining superior antitumor effect by the combination of celecoxib with docetaxel.

Dumka *et al.* (2008) reported effect of meloxicam (0.5 mg/kg S.C.) on pharmacokinetic of Levofloxacin (4 mg/kg I.V.) in cross breed calves. Mean values for half life ($t_{1/2}$), volume of distribution (Vz), clearance (Cl) and area under curve (AUC) of Levofloxacin were 1.61 ± 0.07 and 2.16 ± 0.08 h, 0.74 ± 0.03 and 1.38 ± 0.05 L/kg and 0.317 ± 0.004 and 0.45 ± 0.02 L/kg.h, and 12.7 ± 0.20 and 9.02 ± 0.34 µg.h/mL, after administration of Levofloxacin as single drug and in combination with meloxicam, respectively. They concluded that concurrent administration of meloxicam significantly alters the pharmacokinetic of Levofloxacin in calves.

Suzuki *et al.* (2009) reported that COX-2 overexpression has been observed in many malignancies including lung cancer. Recent pre-clinical studies reveled that selective COX-2 inhibitors have demonstrated promising results when used with chemotherapy. Meloxicam in combination with carboplatin and weekly paclitaxel chemotherapy showed promising activity with encouraging survival. A combination of sulindac, a COX-2 inhibitor and EKI-569, an EGFR tyrosine kinase inhibitor resulted in a synergistic effect in preventing mice intestinal neoplasia (Torrance *et al.*, 2000).

2.4.7 Therapeutic uses of meloxicam

Meloxicam acts by inhibition of prostaglandin synthesis, thereby exerting anti–inflammatory, analgesic and antipyretic properties. Meloxicam is indicated for the short term symptomatic therapy of acute exacerbations of osteoarthrosis, long term symptomatic treatment of rheumatoid arthritis or ankylosing spondylitis.

Meloxicam was found superior in this regard as compared to other traditional anti–inflammatory drugs used in veterinary medicine. The drug has been found to be highly effective in chonic arthritis/synovitis in canines (Henderson *et al.*, 1994; VanBree *et al.,* 1994; Cardini *et al.,* 1995). Samad and Gaikwad (2000) have carried out the preliminary field trials to evaluate the efficacy of meloxicam in various inflammatory conditions of bovine, caprine and canine species of animals. They have found meloxicam at (5 mg/30 kg) to be a potent anti–inflammatory agent in the treatment of different inflammatory conditions such as lameness, myositis, synovitis, mammilitis, mastitis, arthritis, otitis and pneumonia.

Engelhardt *et al.* (1996) revealed that in pleurisy of rat, meloxicam was twice as potent as tenoxicam, 3 times as potent as Flurbiprofen, 8 times as potent as Diclofenac and 20 times as potent as Tenidap in regards to inhibition of prostaglandin

synthesis. Okkinga and Salmon (1998) suggested the suitability of meloxicam with long acting antibiotics in the treatment of calf pneumonia also reported maintainence of therapeutic effect over thee days after injection of meloxicam.

Goldman *et al.* (1998) reported that meloxicam inhibits the growth of colorectal cancer cells. Cyclooxygenase-2 has been reported to play an important role in colorectal carcinogenesis. They evaluated effects of meloxicam (COX-2 inhibitor) on the growth of two colon cancer cell lines that express COX-2 (HCA-7 and Moser-S) and a COX-2 negative cell line (HCT-116). The growth rate of these cells was measured following treatment with meloxicam. HCA-7 and Moser-S colony size were significantly reduced following treatment with meloxicam; however, there was no significant change in HCT-116 colony size with treatment. They have also performed *in-vivo* studies to evaluate the effect of meloxicam on the growth of HCA-7 cells when xenografted into nude mice and observed a 51% reduction in tumor size after 4 weeks of treatment. Analysis of COX-1 and COX-2 protein levels in HCA-7 tumor lysates revealed a slight decrease in COX-2 expression levels in tumors taken from mice treated with meloxicam and no detectable COX-1 expression. Thus, meloxicam significantly inhibited HCA-7 colony and tumor growth but had no effect on the growth of the COX-2 negative HCT-116 cells.

Francesca *et al.* (2009) studied efficacy of meloxicam in a patient with juvenile polyposis syndrome characterized by the occurrence of multiple hamartomatous polyps affecting the gastrointestinal (GI) tract (Zbuk and Eng, 2007). As with the other hamartomatous syndromes described, there is an increased risk of colon cancer that arises from adenomaous components present in the juvenile polyps (Schreibman *et al.,* 2005). Recent studies reported up-regulation of cyclooxygenase-2 (COX-2) in colorectal polyps (Kurland *et al.,* 2007). Experimental studies using cell

lines and animal models demonstrated an ability of COX-2 inhibitors to prevent tumour proliferation (Fujimura *et al.,* 2006). Therefore, chemoprevention with a new class of nonsteroidal anti-inflammatory drugs, COX-2 inhibitors, has been suggested as an adjunct therapy to endoscopic polypectomy (Brazowski et al., 2005). Recently Francesca *et al.* (2009) reported a case of 11-year-old female with JPS, who was treated successfully with selective COX-2 inhibitor, meloxicam. The efficacy of meloxicam was found in chemoprevention of recurrent hamartomatous polyp in JPS. This treatment may be a useful adjunct therapy to repeated endoscopic polypectomies in this rare condition.

Tsuchida *et al.* (2005) reported inhibitory effect of meloxicam, a COX-2 inhibitor, on N-nitrosobis (2-oxopropyl) amine induced biliary carcinogenesis in Syrian hamsters.

American Society of Clinical Oncology reported a pilot study of successful treatment with meloxicam, of patients with extra-abdominal desmoid tumors. Later on Tumor Research centre in 2012 has also reported the inhibitory effects of Meloxicam on the growth of human liver cancer cell line HepG2 and agiogenesis.

Nishida *et al.* (2012) reported transition of treatment for patients with extra-abdominal desmoid tumors and suggested conservative treatment with meloxicam as a promising novel modality for patients with extra-abdominal desmoid tumors.

Meloxicam has been found to inhibit the growth of colon cancer growth with HCA-7 cell line in xenograft nude mice (Goldman *et al.,* 1998). There was 51% reduction of tumor size along with decreased level of COX-2 expression level in tumors within 4 weeks of treatment with meloxicam at dose rate of 20 mg/kg, i.p. in mice.

Kundu and Fulton (2002) reported that oral administration of either a selective COX-2 inhibitor (celecoxib) or a selective COX-1 inhibitor (SC560) to mice with established tumors by murine mammary tumor cell line 410 results in significant inhibition of tumor growth. Administration of the dual inhibitor, indomethacin leads to even better growth control. Metastatic capacity was also reduced by treatment of tumor-bearing mice with either COX-1 or COX-2 selective inhibitors. Pretreatment of tumor cells with COX inhibitors also reduced metastatic success, indicating that tumor cells may be a direct target of action by COX inhibitors.

Tanaka *et al.* (2005) investigated the uses and limitations of cyclooxygenase-(COX) 2 inhibition using clinically relevant doses of oral rofecoxib in the treatment of murine models of non–small-cell lung cancer (NSCLC) and observed that clinically relevant doses of the COX-2 inhibitor rofecoxib given orally were effective in inhibiting the growth of small (but not large) tumors in 3 murine NSCLC cell lines tested and in preventing recurrences after surgical debulking. They suggested COX-2 inhibition as an adjuvant therapy for surgically resectable NSCLC.

Pancreaticobiliary maljunction (PBM) is a high risk factor in biliary tract carcinoma. The chemopreventive action of a COX-2 inhibitor (meloxicam) on N-nitrosobis (2-oxopropyl) amine (BOP)-induced gallbladder cancer in hamster PBM models was investigated by Tsuchida *et al.* (2005). They have concluded that meloxicam suppresses carcinogenesis in hamster PBM models and its mechanism may be based on the suppression of cell growth.

Mun and Hwang (2009) assessed the anti-tumor effect of meloxicam on the human oral cavity squamous cell carcinoma xenografted in nude mice, depending on the timing of treatment. Their results suggested the possibility of novel applications of

selective COX-2 inhibitors for chemoadjuvant and chemo-preventive treatment of oral cavity cancer.

2.5 Pharmacology of Metformin

Metformin is now established as a first-line antidiabetic therapy for the management of type 2 diabetes.

2.5.1 Physico-chemical properties of metformin

Metformin hydrochloride (N, N-dimethyl imido dicarbo nimidic diamide hydrochloride) is a white to off-white crystalline compound with a molecular formula of $C_4H_{11}N_5$ (figure 2.7) and a molecular weight of 165.63. It is freely soluble in water and is practically insoluble in acetone, ether and chloroform. The pKa is 12.4 and the pH of a 1% aqueous solution is 6.68.

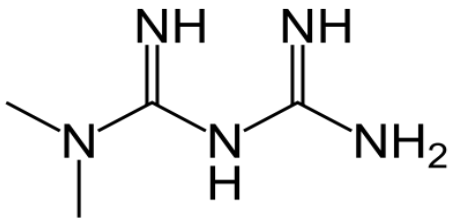

Figure 2.7: Chemical structure of metformin

2.5.2 Mechanism of action of Metformin

Metformin improves glucose tolerance in patients with type 2 diabetes mellitus by lowering both basal and postprandial plasma glucose (Knowler *et al*., 2002). The glucose lowering effect of metformin is mainly a consequence of reducing hepatic 5-glucose production (Hundal et al., 2000). Primary site of action of metformin appears to be in hepatocyte mitochondria, where it disrupts respiratory chain oxidation of complex-I substrates (glutamate). Inhibition of cellular respiration decreases gluconeogenesis (Dominguez *et al.,* 1996). Metformin also facilitates insulin induced suppression of gluconeogenesis from several substances including

lactate, pyruvate, glycerol and amino acids (Wiernsperger and Bailey, 1999) and opposes the gluconeogenic actions of glucagons (Dominguez *et al*., 1996). Metformin activates insulin and tyrosine kinase activity in insulin-like growth factor-1 receptor of vascular smooth muscle cells independently of insulin action (Khurana and Malik, 2010). Thus, metformin has metabolic effects on insulin-sensitive tissues that may contribute to its glucose-lowering effect.

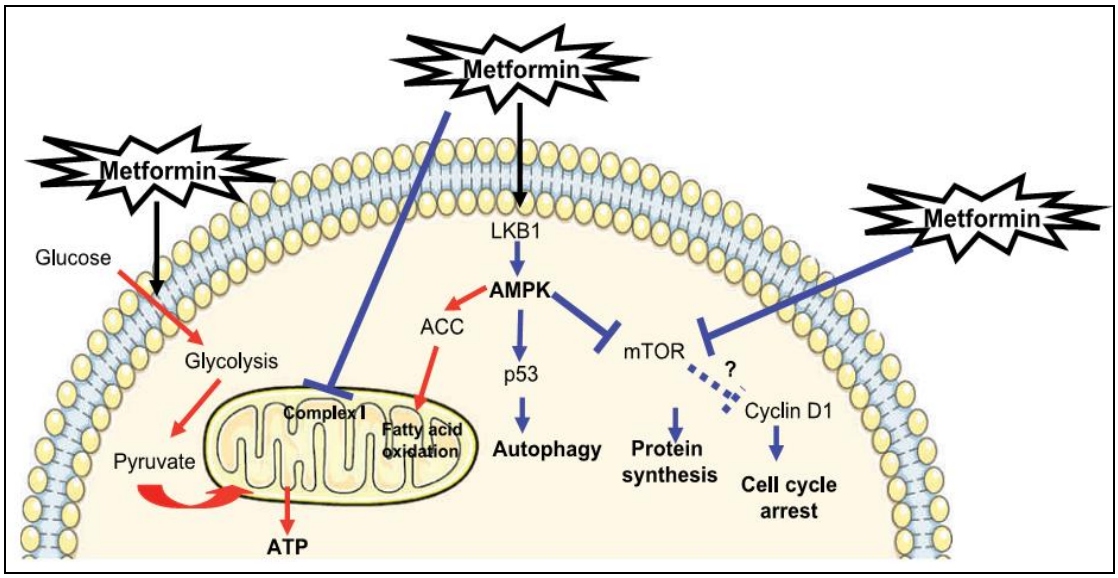

Figure-2.8: Mechanism of action on cellular metabolism and cell proliferation.

Metformin has been shown to reduce free fatty acid oxidation by 10% to 30% (Perriello *et al.*, 1994; Bailey *et al.*, 1996). Elevated levels of free fatty acids contribute to increased hepatic glucose production and development of insulin resistance. Increased fatty acid oxidation inhibits key enzymes of the glycolytic pathway by accumulation of acetyl coenzyme A and citrate, by-products of free fatty acid oxidation (Kelley and Mandarino, 2000). Increased glucose 6-phosphate concentration, in turn inhibits the hexokinase enzyme, resulting in reduced glucose uptake and oxidation (Kelley and Mandarino, 2000). By decreasing free fatty acid levels, metformin not only improves insulin sensitivity but also helps to correct impaired insulin secretion by beta-cell. Metformin also improves hyperglycemia by

attaining high concentration in the small intestine and decreasing intestinal absorption of glucose (Ikeda *et al.,* 2000), an action that may contribute to a decrease in postprandial blood glucose levels. Metformin decreases hepatic glucose production, improves peripheral insulin sensitivity, decreases gastrointestinal glucose absorption and indirectly improves pancreatic beta-cell response to glucose by reducing glucose toxicity and free fatty acid levels (Patane *et al.,* 2000). The cellular metabolism including glycolysis and protein synthesis as well as cell proliferation including autophagy and cell cycle arrest is depicted in figure 2.8.

The anticancer effects of metformin are associated with both direct (insulin-independent) and indirect (insulin-dependent) actions of the drug as depicted in Figure 2.9. The indirect, insulin-dependent effects of metformin are mediated by the ability of AMPK (adenosine monophosphate kinase) to inhibit the transcription of key gluconeogenesis genes in the liver and stimulate glucose uptake in muscle, thus reducing fasting blood glucose and insulin (Cusi *et al*., 1996). The insulin-lowering effects of metformin play a major role in its anticancer activity since insulin has mitogenic and prosurvival effects and tumor cells often express high levels of the insulin receptor, indicating a potential sensitivity to the growth promoting effects of the hormone (Frasca *et al*., 2008). Further, obesity and high insulin levels are adverse prognostic factors for a number of cancers particularly those of the breast, prostate and colon (Frasca *et al*., 2008). Indeed, metformin suppresses the stimulatory effects of obesity and hyperinsulinemia on lung tumor growth in mice by improving insulin sensitivity, lowering circulating insulin and activating AMPK signaling (Algire *et al*., 2008). In addition, metformin reduced circulating insulin levels by 22% and improved insulin sensitivity by 25% in non-diabetic women with breast cancer, highlighting the insulin-lowering effects of metformin as a potential mechanism of action in the

treatment of breast cancer (Goodwin *et al*., 2008). The direct, insulin-independent effects of metformin originate from LKB1-mediated activation of AMPK and a reduction in mTOR signaling and protein synthesis in cancer cells (Dowling *et al*., 2007). AMPK impacts mTOR via phosphorylation and activation of the tumor suppressor tuberous sclerosis complex 2 (TSC2, tuberin), which negatively regulates mTOR activity (Inoki *et al*., 2003). mTOR is a key integrator of growth factor and nutrient signals and is a critical mediator of the phosphatidylinositol-3-kinase/ protein kinase B/Akt (PI3K/PKB/Akt) signaling pathway, which is one of the most frequently deregulated molecular networks in human cancer (Steelman *et al*., 2011). Metformin mediated AMPK activation leads to an inhibition of mTOR signaling, a reduction in phosphorylation of its major downstream effectors, the eukaryotic initiation factor 4E-binding proteins (4E-BPs) and ribosomal protein S6 kinases (S6Ks), and an inhibition of global protein synthesis and proliferation in a number of different cancer cell lines (Dowling *et al*., 2007; Alimova *et al*., 2009)

Some recent reports raise the possibility that metformin may mediate additional anticancer effects independently of AMPK, LKB1, and TSC2 (Kalender *et al*., 2010; Foretz *et al.,* 2010). Indeed, metformin reduced mTOR signaling independently of AMPK and TSC2 by inhibiting Rag GTPase-mediated activation of mTOR (Kalender *et al*., 2010). Paradoxically, at least in one cell model system, loss of function of LKB1 sensitized cells to the inhibitory effects of metformin under conditions of low glucose (Algire *et al*., 2011). Moreover, metformin reduced hepatic gluconeogenesis by lowering hepatic energy levels in the absence of AMPK and LKB1 (Foretz *et al.,* 2010). While these additional effects are intriguing, LKB1-dependent suppression of mTOR signaling remains the key mechanism of antitumor action of metformin.

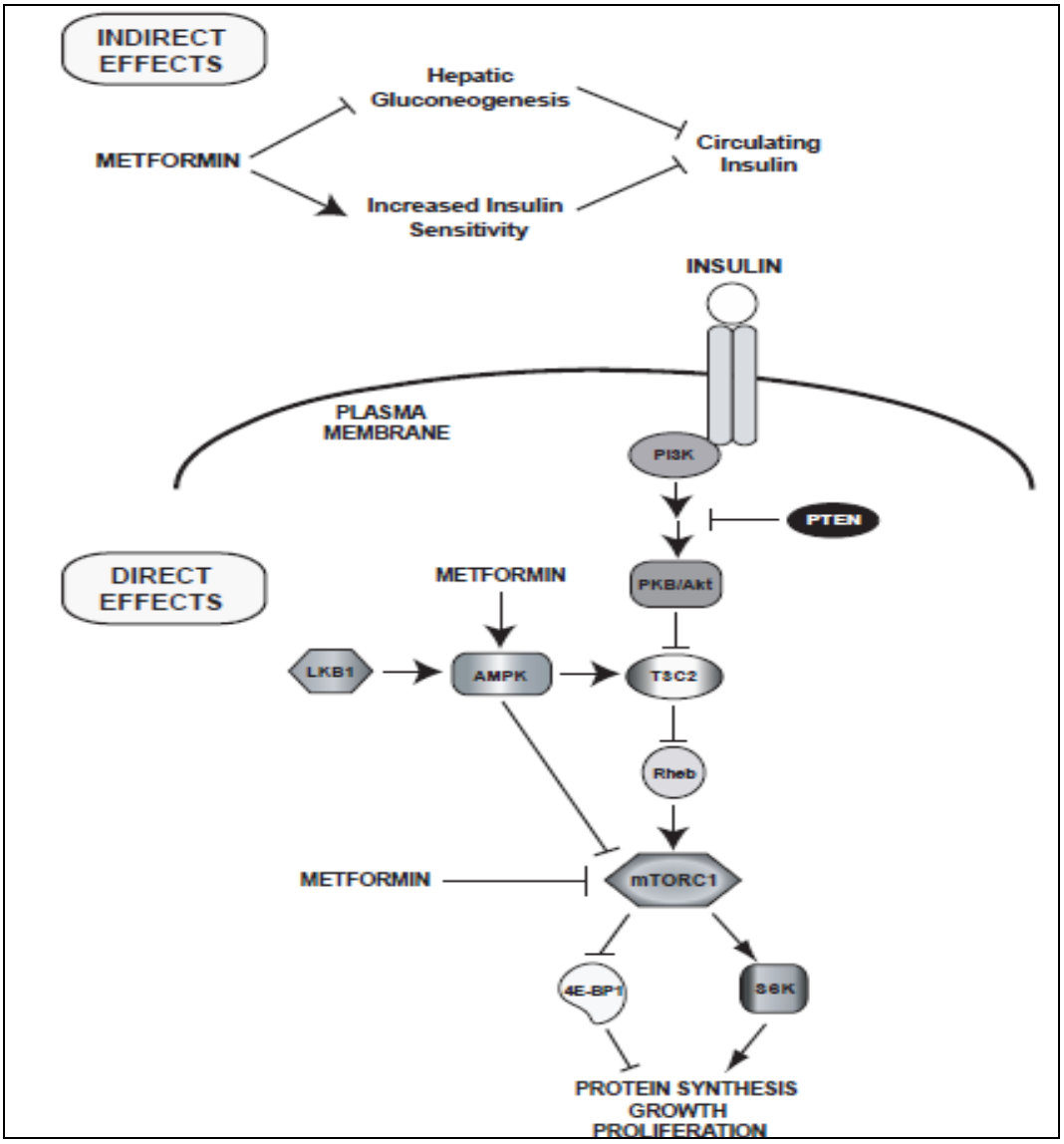

Figure 2.9: Direct and indirect effects of metformin on cancer.

2.5.3 Pharmacokinetics of metformin

2.5.3.1 Pharmacokinetics in human

The absolute bioavailability following oral administration of metformin given under fasting conditions is approximately 50% to 60% (Scheen, 1996). C_{max} and T_{max} after a single oral dose of 850mg metformin in healthy adults are 1.60 ± 0.38 µg/mL and 2.75 ± 0.81 h, respectively. The extent of absorption of metformin is influenced by food and gastrointestinal transit time. Food decreases the extent of and slightly delays the absorption of metformin, as 40% lower mean C_{max}, 25% lower AUC and

35-minute prolongation of T_{max} following administration of a single 850-mg tablet of metformin with food, compared to the same tablet strength administered fasting. At the usual doses and dosing schedules, steady state plasma concentrations are reached within 24 to 48 h. The drug concentrations are generally less than 1µg/mL and C_{max} does not exceed 5µg/mL even at maximum doses.

The average apparent volume of distribution (Vd) of 850mg metformin following a single oral dose is 654 ± 358 L. The drug is negligibly bound to plasma proteins (Davidson and Peters, 1997). However, partitions into erythrocytes as a function of time most likely represents a secondary compartment of distribution. Intravenous single-dose studies in normal subjects demonstrate that metformin is excreted unchanged in the urine and does not undergo hepatic metabolism (no metabolites have been identified in humans) nor biliary excretion (Davidson and Peters, 1997). Renal clearance is approximately 3.5 times greater than creatinine clearance, which indicates that tubular secretion is the major route of metformin elimination. Following oral administration, approximately 90% of the absorbed drug is eliminated via the renal route within the first 24 h, with a plasma elimination t1/2 of approximately 4.0 to 8.7 h. Renal function impairment leads to increased levels of metformin in plasma (Lalau *et al.*, 1989). In blood, the elimination t1/2 is approximately 17.6 h; suggesting that the erythrocyte mass may be a compartment of distribution.

Charles *et al*. (2006) studied population pharmacokinetics of metformin in late pregnancy. Blood samples were obtained in the third trimester of pregnancy from women with gestational diabetes or type 2 diabetes. A cord blood sample also was obtained at the delivery time from these women. Plasma metformin concentrations were assayed by a new, validated, reverse-phase HPLC method. Mean (range)

metformin concentrations in cord plasma and in maternal plasma were 0.81 (range, 0.1-2.6) mg/L and 1.2 (range, 0.1-2.9) mg/L, respectively. Typical population values (interindividual variability, CV%) for allometrically scaled maternal clearance and volume of distribution were 28 L/h/70 kg (17.1%) and 190 L/70 kg (46.3%), giving a derived population-wide half-life of 5.1 hours. Neither maternal age nor weight significantly influenced the pharmacokinetics. The pharmacokinetics was similar to those in non pregnant patients.

Hughes *et al.* (2006) evaluated the Effect of pregnancy on the pharmacokinetics of metformin. Women with Type 2 diabetes mellitus taking metformin throughout pregnancy were studied on two occasions, once at 28–36 weeks gestation and once at least 8 weeks postpartum. Metformin concentrations were lower in pregnancy in six subjects, with a mean (95% CI) AUC_{0-4} that was 69% (53.6, 84.8) of the postpartum value. The AUC_{0-4} of one subject was higher in pregnancy at 142% of the postpartum value. Overall, the mean (95% CI) AUC_{0-4} during pregnancy for all seven subjects was 80% (51.3, 107.8) of the postpartum value. These results suggest that the clearance of metformin increases in pregnancy as a result of enhanced renal elimination.

Scheen, (1996) studied Clinical pharmacokinetics of metformin. Metformin has an absolute oral bioavailability of 40 to 60%, and gastrointestinal absorption is apparently complete within 6 hours of ingestion. Metformin is rapidly distributed following absorption and does not bind to plasma proteins. No metabolites or conjugates of metformin have been identified. The absence of liver metabolism clearly differentiates the pharmacokinetics of metformin from that of other biguanides, such as phenformin. Metformin undergoes renal excretion and has a mean plasma elimination half-life after oral administration of between 4.0 and 8.7 hours.

Therapeutic levels may be 0.5 to 1.0 mg/L in the fasting state and 1 to 2 mg/L after a meal.

Ping *et al.* (2002) reported relative bioavailability and pharmacokinetics of metformin hydrochloride enteric dissolving tablets by HPLC. The pharmacokinetics and bioavailability of metformin hydrochloride enteric dissolve tablets were compared with stomach dissolving tablets. The parameters of the two formulations for metformin, Cmax of enteric and stomach dissolving tablets were 2.9 ± 0.7mg/L and 2.6 ± 0.6 mg/L , T max 2.6 ± 0.4h and 2.3 ± 0.5 h; T 1/2Ka 0.87 ± 0.26 h and 0.81 ± 0.23 h; T 1/2Ke 1.6 ± 0.4 and 1.9 ± 0.6 h, AUC 12.2 ± 1.1 and 11.9 ± 1.2 mg/L/h respectively. The relative bioavailability of enteric dissolving tablets was 103.0 ± 12.0 %. There was no significant difference in pharmacokinetic parameters.

Baozhong *et al.* (2006) investigated pharmacokinetics and bioequivalence of compound Metformin hydrochloride capsule in Chinese healthy male volunteers. Each subject was randomized to take either two compound capsule (one meformin hydrochloride/glibenclamide:250 mg/2.5 mg) or co-administered capsule (one meformin hydrochloride 500 mg and one glibenclamide 2.5 mg). The samples were analyzed by HPLC for the concentrations determination for metformin hydrochloride and glibenclamide. After administration of compound or co-administered capsules, the main pharmcokinetic parameters were C_{max} (1112 ± 340) and (1040 ± 340) (ng/ml,) $t_{1/2\beta}$ (2.5±0.7) and (2.5±0.9) h; AUC (6018±1123.8) and (6070±1 626.8) ng·h/ml for metformin hydrochloride; (65.7±23.4) and (69.1±20.8) ng/ml, (3.1±0.9) and (3.2±0.9) h, (303.2±81.8) and (318.2±92.3) ng·h/ml for glibenclamide, respectively. The relative bioavailability of metformin hydrochloride and glibenclamide was (102±17.5) % and (97.0±15.9) %.

2.5.3.2 Pharmacokinetics in animals

Choi *et al.* (2006) reported dose-independent pharmacokinetics of metformin in rats. Pharmacokinetic parameters of metformin were evaluated after intravenous and oral administration (50, 100, and 200 mg/kg) in rats. The total AUC $_{(0-\infty)}$ values were dose-proportional after both intravenous and oral dose ranges studied. After oral administration (100 mg/kg), 4.39% of oral dose was not absorbed and extent of absolute oral bioavailability (*F*) value was 29.9%. The gastrointestinal first-pass effect of metformin was 53.8% of oral dose in rats (the gastric and intestinal first-pass effects were 23.1 and 30.7%, respectively), and the hepatic first-pass effect was 27.1% after absorption into the portal vein. Since 41.8% of oral metformin was absorbed into the portal vein, the value of 27.1% is equivalent to 11.3% of oral dose. The first-pass effects of metformin in the lung and heart were almost negligible in rats.

Gui-chun *et al.* (2008) studied pharmacokinetics and tissues distribution of metformin in rats. After a single intraperitoneal administration of metformin with a dose of 100 mg/kg, the plasma samples were taken at 0, 10, 20, 30, 60, 120, 240, 480 and 720 min for determination of the pharmacokinetic parameters of metformin in rat blood. The various organ samples were also taken from another group of animals for the distribution study of the drug. The main pharmacokinetic parameters were as follow: AUC 4676 ± 171 mg.min/L, CL 21.4±0.8 mL.min/kg, $t_{1/2}$ 50±5 min, t_{max} 20.0±1.0 min, C_{max} 59±4mg/L. Metformin were detected in all tissue samples 10 min after administration.

2.5.4 Pharmacological effects of metformin

Gagnon *et al.* (2009) reported that the use of metformin may be associated with better survival of lung cancer patients. Mazzone *et al.* (2010) studied the effect

of metformin and thiazolidinedione on lung cancer and reported that diabetic patients with lung cancer who were previously exposed to metformin and/or thiazolidinedione are less likely to present with metastatic disease, more likely to present with an adenocarcinoma and may survive longer. A potential mechanism of this effect is through the activation of AMP-activated protein kinase (AMPK). When cells are faced with energy stresses, AMPK functions to restore energy balance by inhibiting synthetic pathways and stimulating catabolic pathways. This function may serve to protect against lung cancer development or progression.

Liu *et al.* (2011) evaluated the effects of metformin on renal cell carcinoma (RCC) and its underlying mechanisms. A xenograft model was used to study the effects of metformin on RCC tumor growth. They demonstrated that metformin effectively inhibits cell proliferation in 786-O and OS-RC-2 RCC cell lines. Moreover, metformin down-regulated cyclin D1 expression and induced G0/G1 cell cycle arrest in these cells. Further study revealed metformin induced the activation of AMP-activated protein kinase (AMPK) and inhibited mammalian target of rapamycin (mTOR), which is a central regulator of protein synthesis and cell growth, and negatively regulated by AMPK. Most importantly, daily treatment of mice with metformin prevented RCC tumor growth in a xenograft model. Metformin was able to induce G0/G1 cell cycle arrest and inhibit RCC growth in vitro and in vivo. These results suggest that metformin may be a potential therapeutic agent for the treatment of RCC.

Rattan *et al.* (2011) investigated the efficacy of metformin alone and in combination with cisplatin in vivo against ovarian cancer. A2780 ovarian cancer cells were injected intraperitoneally in nude mice. A2780-induced tumors in nude mice were used. Upon administration of metformin in drinking water, significant reduction

of tumor growth accompanied by inhibition of tumor cell proliferation as well as decreased live tumor size and mitotic cell count was observed. Metformin-induced activation of AMPK/mTOR pathway was accompanied by decreased microvessel density and vascular endothelial growth factor expression. Metformin treatment inhibited the growth of metastatic nodules in the lung and significantly potentiated cisplatin-induced cytotoxicity resulting in approximately 90% reduction in tumor growth compared with treatment by either of the drugs alone. Collectively, their data showed that in addition to inhibiting tumor cell proliferation, metformin treatment inhibits both angiogenesis and metastatic spread of ovarian cancer. Overall, this study provides a strong rationale for use of metformin in ovarian cancer treatment.

Metformin was found to inhibit proliferation of most cultured breast cancer cell lines (Zhuang and Miskimins, 2008). Inhibition of cell proliferation was associated with arrest within G0/G1 phase of the cell cycle. In sensitive breast cancer lines, the reduction in cyclin D1 led to release of sequestered CDK inhibitors. Cell cycle arrest in response to metformin requires CDK inhibitors in addition to AMPK activation and cyclin D1 down regulation. Many cancers are associated with loss or downregulation of CDK inhibitors and the results may be relevant to the development of anti-tumor reagents that target the AMPK pathway.

2.5.5 Safety and adverse effects of metformin

Metformin is contraindicated in patients who are hypersensitive to the drug and in the patients with renal disease or renal dysfunction (e.g., as suggested by serum creatinine levels ≥ 1.5 mg/dL in males, ≥ 1.4 mg/dL in females or abnormal creatinine clearance) and acute or chronic metabolic acidosis, including diabetic ketoacidosis, with or without coma.

Metformin is inexpensive drug widely used by type 2 diabetics who overproduce insulin. But new researches suggest that it could be useful in breast cancer prevention and treatment. It was found that metformin can also act on lung cancer tumour growth in mice that have been exposed to a common carcinogen in cigarettes. Moreover, new studies suggest that it could be tested for colon cancer too. It's thought the drug works by targeting a cancer tumor's stem cells which, if not killed off, can allow various cancer cell advances in cancer management types to regenerate. Each tablet costs 21 cents and must be taken twice daily. Despite the low price, the cost to run such a clinical trial, which involves collecting blood samples, is expected to run at least $15-million. The trial is expected to include 3,582 patients in Canada and the United States who are undergoing standard cancer treatment plus metformin or placebo for up to five years. Until the results are in, patients should not use it unless it is prescribed for diabetes or they are on the clinical trial, where they can be properly monitored (Dowling, 2011).

A small study showed a link between the use of metformin during pregnancy and preeclampsia (high blood pressure requiring immediate medical attention). However, this could not be proven by other studies. Other studies compared the use of metformin with insulin during pregnancy and found that metformin did not increase the risk of complications for mothers and babies. There have been some reports of jaundice (high levels of bilirubin in the system) in babies exposed to metformin during pregnancy, but these reports do not prove that metformin was the cause of the jaundice. One study looked at the outcome of pregnancy after treatment of polycystic ovarian syndrome with metformin throughout pregnancy. Infants were found to have normal birth weight and height. At 6 months of age, these

infants had normal weight, height, and social and motor development (Glueck *et al.*, 2001)

Metformin appears to be safe during breastfeeding. Three studies have shown that metformin is transferred into breast milk in very small amounts. Another study found that infants of mothers who received metformin throughout pregnancy and while breastfeeding achieved the same growth at six months of age as infants who were formula-fed. (Glueck *et al*., 2006).

The main side effects associated with metformin treatment are the gastrointestinal symptoms of nausea, diarrhoea, flatulence, bloating, anorexia, metallic taste and abdominal pain. These symptoms occur with variable degrees in patients and in most cases resolve spontaneously. The severity of side effects can be reduced by gradual administration of metformin. A start dose of 500 mg daily during the main meal of the day for 1-2 weeks can lessen the side effects and allow tolerance to develop (Nestler, 2008). Slow release metformin can be associated with fewer side effects. Metformin can also lead to vitamin B12 malabsorption in the distal ileum in approximately 10-30% of patients which is an effect dependent on age, dose and duration of treatment (Ting *et al.,* 2006). Rarely, lactic acidosis can occur, mainly in diabetic patients, which is a serious condition that can potentially be fatal. However, unless there is a contraindication to taking metformin such as renal disease the risk of lactic acidosis is negligible (Salpeter *et al.,* 2003). Additional, adverse reactions are reported in $\geq$ 1.0- $\leq$ 5.0% of patients: hypoglycemia, myalgia, lightheaded, dyspnea, nail disorder, rash, sweating, taste disorder, chest discomfort, chills, flu-like syndrome, flushing and palpitation. Lactic acidosis, a serious and potentially lethal metabolic condition, has occurred with all biguanides, but rarely with metformin

(Tomayko and Reynolds, 1989). Strict observation of contraindications and prescribing precautions substantially reduces this risk.

2.5.6 Interaction of metformin with other drugs

Glyburide

In a single-dose interaction study in type 2 DM patients, co-administration of metformin and glyburide does not result in any changes in either pharmacokinetics or pharmacodynamics of metformin. Decreases in glyburide AUC and Cmax are observed, but are highly variable. The single-dose nature of this study and the lack of correlation between glyburide blood levels and pharmacodynamic effects make the clinical significance of this interaction uncertain.

Furosemide

A single-dose, metformin-furosemide drug interaction study in healthy subjects demonstrates that pharmacokinetic parameters of both compounds are affected by co-administration. When administered with metformin, the C_{max} and AUC of furosemide are 31% and 12% smaller, respectively, than when administered alone, and the terminal $t_{1/2}$ is decreased by 32%, without any significant change in furosemide renal clearance. No information is available about the interaction of metformin and furosemide when co-administered chronically.

Nifedipine

A single-dose, metformin-nifedipine drug interaction study in normal healthy volunteers demonstrates that co-administration of nifedipine increases plasma metformin C_{max} and AUC by 20% and 9%, respectively, and increases the amount of metformin excreted in the urine. Tmax and t1/2 are unaffected. Nifedipine appears to enhance the absorption of metformin. Metformin has minimal effects on nifedipine.

Cationic drugs

Cationic drugs (e.g., amiloride, digoxin, morphine, procainamide, quinidine, quinine, ranitidine, triamterene, trimethoprim, or vancomycin) that are eliminated by renal tubular secretion theoretically have the potential for interaction with metformin by competing for common renal tubular transport systems. Such interaction between metformin and oral cimetidine has been observed in normal healthy volunteers in both single-and multiple-dose, metformin-cimetidine drug interaction studies, with a 60% increase in peak metformin plasma and whole blood concentrations and a 40% increase in plasma and whole blood metformin AUC. There is no change in elimination t1/2 in the single-dose study. Metformin has no effect on cimetidine pharmacokinetics.

Other Drugs

Certain drugs tend to produce hyperglycemia and may lead to loss of glycemic control. These drugs include the thiazides and other diuretics, corticosteroids, phenothiazines, thyroid products, estrogens, oral contraceptives, phenytoin, nicotinic acid, sympathomimetics, calcium channel blocking drugs, and isoniazid. When such drugs are administered to a patient receiving metformin, the patient should be closely observed for loss of blood glucose control. When such drugs are withdrawn from a patient receiving metformin, the patient should be observed closely for hypoglycemia.

2.5.7 Therapeutic uses of metformin

Gotlieb *et al.* (2008) studied i*n vitro* metformin anti-neoplastic activity in epithelial ovarian cancer. Metformin may reduce cancer risk and improve cancer prognosis. They evaluated its effect on epithelial ovarian cancer cell lines. The OVCAR-3 and OVCAR-4 cell lines were exposed to metformin with and without cisplatin. Levels of total and phosphorylated AMPK, p70S6K and S6K were

evaluated by Western blotting following exposure to metformin. Metformin induces dose and time dependent growth inhibition of OVCAR-3 and OVCAR-4 cell lines. Metformin growth inhibition was partly abolished by the AMPK inhibitor. Western blotting demonstrated that metformin at cytotoxic concentrations, induced AMPK phosphorylation and decreased p70S6K and S6K phosphorylation, suggesting the mechanism for its anti-proliferative action. Metformin significantly inhibits the growth of ovarian cancer cell lines and potentiates cisplatin. Further pre-clinical studies are being conducted to determine the applicability of metformin in the treatment of ovarian cancer.

Sahra *et al.* (2010) reported that metformin exerts an antitumoral effect *in vitro* and *in vivo* through a decreased cyclin D1 level. They investigated the effect of metformin on human prostate cancer cell proliferation *in vitro* and *in vivo*. Metformin inhibited the proliferation of DU145, PC-3 and LNCaP cancer cells with a 50% decreased cell viability and had a modest effect on normal prostate epithelial cell line P69. Metformin activated the AMP kinase pathway, a fuel sensor signaling pathway. However, inhibition of the AMPK pathway using siRNA against the two catalytic subunits of AMPK did not prevent the antiproliferative effect of metformin in prostate cancer cells. Oral and intraperitoneal treatment with metformin led to a 50 and 35% reduction of tumor growth, respectively, in mice bearing xenografts of LNCaP. Metformin also caused strong reduction of cyclin D1 protein level in tumors providing evidence for a mechanism that contribute to the antineoplastic effects as suggested by recent epidemiological studies.

Gagnon *et al.* (2009) evaluated protective effect of metformin in lung cancer patients (n=850). The factors that were included in the model were age, gender, stage, histology and metformin use. 850 patients (F: M=375:475; mean age of 66) were

diagnosed since 2000 and followed in pulmonary oncology outpatient clinic for NSCLC. 79 (9%) patients were receiving treatment with metformin for their co-morbid type 2 diabetes. The use of metformin is associated with a 37% increase in survival.

Mazzone *et al.* (2010) evaluated effect of metformin in lung cancer with diabetic patients. The medical records of 157 diabetic patients who had a history of lung cancer were reviewed. Lung cancer characteristics were compared between the group that had been exposed to metformin and/or Thiazolidinedione (TZDs) prior to their lung cancer diagnosis and those who had not received either of these medications prior to their lung cancer diagnosis. They reported that diabetic patients with lung cancer who are previously exposed to metformin and/or TZDs are less likely to present with metastatic disease and may survive longer.

Issam *et al.* (2010) reported that there is increasing evidence of a potential efficacy of metformin as an anticancer drug. First, epidemiological studies show a decrease in cancer incidence in metformin-treated patients. Second, metformin decreases insulin resistance and indirectly reduces insulin level, a beneficial effect because insulin promotes cancer cell growth. Third, several reports outline a direct inhibitory effect of metformin on cancer cell growth and an antitumoral action. Finally, metformin activates the AMP activated protein kinase (AMPK) pathway, a major sensor of the energetic status of the cell, which has been proposed as a promising therapeutic target in cancer.

Algire *et al.* (2010) reported that Metformin blocks the stimulative effect of a high-energy diet on colon carcinoma growth *in vivo* and is associated with reduced expression of fatty acid synthase. They investigated the effects of a high-energy diet on growth of an *in vivo* colon cancer model and suggested a potential role of

metformin in the management of a metabolically defined subset of colon cancers.

Bhalla *et al.* (2012) reported that metformin prevents liver tumorigenesis by inhibiting pathways driving hepatic lipogenesis. Recently it has become appreciated that type II diabetes increases the risk of developing HCC. This represents a patient population that can be identified and targeted for cancer prevention. A role of metformin in HCC is suggested by studies linking metformin intake for control of diabetes with a reduced risk of HCC. Although a number of preclinical studies show the anticancer properties of metformin in a number of tissues, no studies have directly examined the effect of metformin on preventing carcinogenesis in the liver, one of its main sites of action. In addition, restoring lipogenic gene expression by ectopic expression of the lipogenic transcription factor SREBP1c rescues metformin-mediated growth inhibition.

CHAPTER- III

MATERIALS AND METHODS

3.1 Experimental animals

The present study was conducted in adult healthy severe combined immunodeficient (SCID) male mice. Healthy males were selected after physical and behavioral examination. Total of 222 SCID male mice of 5-8 weeks of age were used for this study, which were procured from Animal Research Facility, Zydus Research Centre, Cadila Healthcare Ltd, Moraiya, Ahmedabad-382213, Gujarat, India.

The experimental protocol for general procedures and use of animals for conducting this study was approved by the Institutional Animal Ethics Committee (IAEC). All necessary husbandry procedures were adopted to keep the mice free from stress.

3.1.1 Husbandry

3.1.1.1 Animal housing

All mice were housed in groups of 3 mice in sterilized solid floor conventional polypropylene cages in aseptic environment in animal isolator throughout the study period. Autoclaved corncob was used as bedding material and cages were changed once a week. Corncob was analysed periodically for pesticide and microbial contaminants and the levels were found to be within the acceptable limits.

3.1.1.2 Environmental conditions

The temperature in the experimental room was maintained between 22 ± 3 ºC and the relative humidity between 30 – 70%. The photoperiod in the experimental room followed a light: dark cycle of 12:12 hours throughout the study period. Animals were housed under aseptic environment in animal isolator throughout the study

period

3.1.1.3 Diet and drinking water

The experimental animals were provided with gamma irradiated standard laboratory animal diet (Teklad 18, Rodent Pellet feed, Harlan laboratories, USA) and purified autoclaved drinking water *ad libitum*. Proximate and microbial contaminant analyses of feed were carried out periodically as per the schedule, and the results were found to be within acceptable limits.

3.1.1.4 Sanitation

Cages and bedding material were changed twice in a week, whereas the water bottles were changed daily. Floor of experimental room and all worktops were swept and mopped with disinfectant solution every day.

3.1.2 Animal and cage identification

In each cage, animals were identified with individual tail marking. Animal number one and two were marked on base and middle of tail, respectively, whereas animal number three was kept unmarked. The cage label was specific to treatment group, indicating cage No., species, strain, sex, experiment start date, experiment end date, test item, group/dose, feed type, study director and IAEC protocol No.

3.1.3 Acclimatization

All animals were acclimatized to experimental conditions for seven days before grouping and dosing. A thorough veterinary examination was performed before randomization and animals found free of obvious health abnormalities were used for the study.

3.1.4 Randomization and grouping of animals

For pharmacokinetic study, a composite study designed was planned for blood collection from mice. Total 168 SCID male mice were randomly assigned to seven

treatment groups. Each group consisting of total 24 mice, which were further randomly divided into 4 sub-groups (A, B, C and D) of 6 animals each. Total 12 blood sampling time points were distributed among the 4 sub groups of 6 animals each (each animal/sub group was not bled more than three time points) for each group. The single time point concentration data set was made by pooling sample time points of four sub group for each group. The same procedure of blood collection was repeated for other sub group of each treatment groups. Group I, II and III were used for individual drug pharmacokinetic, and group IV, V, VI and VII were used for combinational drug pharmacokinetic studies as shown in Table 3.1.

Another group of 54 SCID mice were used for evaluation of pharmacological and toxicological effects of individual drug and in combination. These animals were divided in nine groups; each having 6 animals. Group I and II were used as Negative and positive control respectively whereas, group III to IX were used as different treatment group as shown in Table 3.2.

Table 3.1: Pharmacokinetic study: experimental outline showing animal No., cage No. and data set No. for various treatment groups.

Group No.	Treatment Group	Cage No.	Animal No.	Data Set No.
I	Erlotinib	1-8	1-24	1-6
II	Meloxicam	9-16	25-48	7-12
III	Metformin	17-24	49-72	13-18
IV	Erlotinin + Meloxicam	25-32	73-96	19-24
V	Erlotinin + Metformin	33-40	97-120	25-30
VI	Meloxicam + Metformin	41-48	121-144	31-36
VII	Erlotinin + Meloxicam + Metformin	49-56	145-168	37-42

Table 3.2: Pharmaco–toxicology study: experimental outline showing animal No., cage No. and data set No. for various treatment groups.

Group No.	Treatment Group	Cage No.	Animal No.	Data Set No.
I	Negative control (without tumor)	1-2	1-6	1-6
II	Positive (tumour) control	3-4	7-12	7-12
III	Erlotinib	5-6	13-18	13-18
IV	Meloxicam	7-8	19-24	19-24
V	Metformin	9-10	25-30	25-30
VI	Erlotinib + Meloxicam	11-12	31-36	31-36
VII	Erlotinib + Metformin	13-14	37-42	37-42
VIII	Meloxicam + Metformin	15-16	43-48	43-48
IX	Erlotinib + Meloxicam + Metformin	17-18	49-54	49-54

3.2 Drugs and chemicals

Erlotinib, meloxicam, metformin, bisoprolol and tenoxicam pure base powder were obtained from Ms. Zydus Research Centre, Ahmedabad. Acetonitrile of HPLC grade was purchased from Merck India Ltd., Mumbai. Methanol, Paraffin wax, Hematoxylin and Xylene were procured from SD Fine Chemical Ltd., Mumbai. Twin-80 (polysorbate) was procured from Merk, Germany and hydroxylpropyl methylcellulose (HPMC) was procured from Colorcon Asia pvt ltd .Eosin was procured from Qualigens fine chemicals, Mumbai. Reagents for Hematological analysis were purchased from Abbott (USA) and reagents for serum biochemical analysis were purchased from Roche (Germany). Chloroform, Isopropanol, Di ethyl Pirocarbonate (DEPC), TRI and RNAse free water were purchased from sigma, Hi Capacity cDNA synthesis kit, Optical Reaction plates and Optical adhesive covers were procured from AB Biosytem and SYBR Green PCR Kit was procured from quantifast, Qualigen.

3.3 Experimental protocol and design

Test items	: Erlotinib, meloxicam and metformin
Strain & species	: Severe Combined Immuno Deficient (SCID) mice.
Sex	: Male
Source of animal	: Animal Research Facility (ARF), Zydus Research Centre, Ahmedabad, India
Age at initiation	: 5-8 Weeks
Route of administration	: Oral and intra peritoneal

3.3.1 Dose preparation and dosing of formulation

Erlotinib, meloxicam and metformin were formulated in Tween 80 and in 2% HPMC (prepared in purified water). The concentration strength of drug was adjusted with 2 % HPMC by keeping Tween 80 concentration at 0.25 % in final formulation. Doses were calculated according to body weight of animals and administrated as per concentration strength of formulation. Erlotinib (30 mg/kg) and Metformin (100 mg/kg) were administered by oral route using oral gavage needle, where as meloxicam (20 mg/kg) was administered by intra peritoneal route with sterile 1ml tuberculin syringe and needle of 26G (0.45mm x 13mm) at abdomen region of animal.

3.3.2 Plan of work

The study was undertaken in two phases as described in Table 3.3 and 3.4.

Table 3.3: Pharmacokinetic study design for mono and combinational treatment of erlotinib (ERT), meloxicam (MEX) and metformin (MET).

Groups	No. of Animals	Treatment	No. of Data Set*
I	sub group –A (6 animals)	ERT (30 mg/kg)	1-6
	sub group –B (6 animals)	ERT (30 mg/kg)	
	sub group –C (6 animals)	ERT (30 mg/kg)	
	sub group –D (6 animals)	ERT (30 mg/kg)	
II	sub group –A (6 animals)	MEX (20 mg/kg)	7-12
	sub group –B (6 animals)	MEX (20 mg/kg)	
	sub group –C (6 animals)	MEX (20 mg/kg)	
	sub group –D (6 animals)	MEX (20 mg/kg)	
III	sub group –A (6 animals)	MET (100 mg/kg)	13-18
	sub group –B (6 animals)	MET (100 mg/kg)	
	sub group –C (6 animals)	MET (100 mg/kg)	
	sub group –D (6 animals)	MET (100 mg/kg)	
IV	sub group –A (6 animals)	ERT(30 mg/kg) + MEX(20 mg/kg)	19-24
	sub group –B (6 animals)	ERT(30 mg/kg) + MEX(20 mg/kg)	
	sub group –C (6 animals)	ERT(30 mg/kg) + MEX(20 mg/kg)	
	sub group –D (6 animals)	ERT(30 mg/kg) + MEX(20 mg/kg)	
V	sub group –A (6 animals)	ERT(30 mg/kg) + MET(100 mg/kg)	25-30
	sub group –B (6 animals)	ERT(30 mg/kg) + MET(100 mg/kg)	
	sub group –C (6 animals)	ERT(30 mg/kg) + MET(100 mg/kg)	
	sub group –D (6 animals)	ERT(30 mg/kg) + MET(100 mg/kg)	
VI	sub group –A (6 animals)	MEX(20 mg/kg) + MET(100 mg/kg)	31-36
	sub group –B (6 animals)	MEX(20 mg/kg) + MET(100 mg/kg)	
	sub group –C (6 animals)	MEX(20 mg/kg) + MET(100 mg/kg)	
	sub group –D (6 animals)	MEX(20 mg/kg) + MET(100 mg/kg)	
VII	sub group –A (6 animals)	ERT(30 mg/kg) + MEX(20 mg/kg)+ MET(100 mg/kg)	37-42
	sub group –B (6 animals)	ERT(30 mg/kg) + MEX(20 mg/kg)+ MET(100 mg/kg)	
	sub group –C (6 animals)	ERT(30 mg/kg) + MEX(20 mg/kg)+ MET(100 mg/kg)	
	sub group –D (6 animals)	ERT(30 mg/kg) + MEX(20 mg/kg)+ MET(100 mg/kg)	

*****Final data set was prepared for six animals by pooling data of sub groups A, B, C and D for each treatment group.

Table 3.4: Pharmaco-toxicological study design for mono and combinational treatments of erlotinib (ERT), meloxicam (MEX) and metformin (MET).

Groups	No. of animals	Treatment	Animal No.
I	6	Negative Control	1-6
II	6	Positive(tumor)Control	7-12
III	6	ERT (30 mg/kg)	13-18
IV	6	MEX(20 mg/kg)	19-24
V	6	MET(100 mg/kg)	25-30
VI	6	ERT (30 mg/kg) + MEX(20 mg/kg)	31-36
VII	6	ERT (30 mg/kg) + MET(100 mg/kg)	37-42
VIII	6	MEX(20 mg/kg) + MET(100 mg/kg)	43-48
IX	6	ERT (30 mg/kg) + MEX(20 mg/kg) + MET(100mg/kg)	49-54

3.4 Pharmacokinetic study of mono and combinational treatments of erlotinib, meloxicam and metformin

Randomizations for pharmacokinetic study, grouping of animals (168) were followed as described in 3.1.4.

3.4.1 Dosage and administration of drugs

Seven groups were used for pharmacokinetic interaction study. Each group consisting 24 male SCID mice, which was further divided in 4 sub group. The drugs given to all groups of animals are described in Table 3.3. The erlotinib and metformin were administered orally whereas, meloxicam was administered intra peritoneally.

3.4.2 Collection of blood and plasma samples

Blood was collected from retro-orbital plexus. Blood sample (0.25 ml) was collected in 0.5 ml capacity centrifuge tube at 5, 10, 20, 40 minutes, 1, 2, 4, 6, 8, 12,

24 and 48 hours after administration of drug and/or combination of drugs. The 5 minute, 1 hr, 8 hr blood samples were collected from sub group A of each group. The 10 minute, 2 hr, 12 hr blood samples were collected from sub group B of each group. The 20 minute, 4 hr, 24 hr blood samples were collected from sub group C of each group. The 40 minute, 6 hr, 48 hr blood samples were collected from sub group D of each group. The single time point concentration data set was made by pooling sample time points of four sub group for each group. The control plasma of same strain animals was used as 0 minute (before drug administration) sample. Blood was collected from all animals in heparinized tubes and centrifuged at 3000 rpm for 10 minutes at ambient temperature to obtain plasma. Separated plasma was transferred to labeled cryovials. Plasma samples were stored at - 70 ^{0}C in deep freezer until analysis of drug.

3.5 Estimation of erlotinib and metformin concentration in plasma and tissue by liquid chromatography-mass spectrometry (LC-MS/MS)

3.5.1 Bio-analytical method

LC-MS/MS System: Integrated system of model Co-sense Bio-analysis system, Liquid Chromatograph, Shimadzu Corporation, Koyoto, Japan (This include HPLC pump, Autosampler, UV-Visible Detector).

HPLC pump : LC-20-AD, Prominance, Pump, Shimadzu, Japan

Degasser : DGU20A$_{5,}$ Prominance, Vacuum Degasser, Shimadzu, Japan

Autosampler : SIL-HTc Autosampler, Shimadzu, Japan

Sample tray : 1.0 ml sample cooler, Shimadzu, Japan

Column oven : CTO-20 A column Oven, Shimadzu, Japan

Detector : API 3200 LC-MS/Ms system, PE SCIEX, Canada

Data system : Analyst software version 1.4.2

3.5.2 Chromatographic conditions (erlotinib and metformin)

Mobile phase : (A:B), 5mM Ammonium Formate + 0.13% Formic acid in water (Sol A), Acetonitrile (Sol B).

Gradient : Ratio of Solution A and B of mobile phase at time 0.01 (45:55), 3 (20:80), 4 (20:80), 4.5 (45:55), were programmed.

Auto sampler rinsing solvent : Methanol: Acetonitrile: Water (40:40:20 %v/v)

Auto sampler temperature : 10 °C

Column description : BAL/COL/07/75[YMC CN 150*4.6mm, 5u

Column temperature : Ambient (25 ± 5^0C)

Flow rate : 1.0 ml / min with splitter (80:20), 80 % in waste

Injection volume : 2 µL

Run time : 6.2 minutes.

Retention time : Erlotinib – 3.11 (± 0.5 min) (Analyte-1)

Metformin- 2.35 (± 0.5 min) (Analyte-2)

Bisoprolol - 2.75 (± 0.5 min) (Internal standard)

3.5.3 Detector settings

Ionization Mode : Electro spray – positive mode

Scan type : MRM

Curtain Gas (CUR) : 30

Collision Gas (CAD) : Medium

Ion Spray Voltage (IS) : 4500 V

Temperature (TEM) : 550 °C

GS 1 : 70

GS 1	:	80
Interface heater	:	ON
Dwell time	:	300 Sec.

Erlotinib

Declustering Potential (DP)	:	70.00 V
Entrance Potential	:	12.00 V
Collision cell entrance Potential	:	20.77 V
Collision cell energy	:	32.00 V
Collision cell exit potential	:	1.00 V

Metformin

Declustering Potential (DP)	:	25.00 V
Entrance Potential	:	11.00 V
Collision cell entrance Potential	:	12.06 V
Collision cell energy	:	38.00 V
Collision cell exit potential	:	1.00 V

Bisoprolol (internal standard)

Declustering Potential (DP)	:	36.00 V
Entrance Potential	:	12.00 V
Collision cell entrance Potential	:	18.54 V
Collision cell energy	:	33.00 V
Collision cell exit potential	:	1.00 V

3.5.4 Preparation of stock solution of erlotinib, metformin and internal standard.

3.5.4.1 Diluent: It was prepared by mixing of methanol: water in the ratio of 80:20.

3.5.4.2 Stock solution A of erlotinib and metformin

Accurately weighed 10.0 mg of Erlotinib and Metformin API grade powder were dissolved separately in diluent and final volume was made up to 10 ml in volumetric flasks to get final concentration of erlotinib and metformin to 1000 µg/ml.

3.5.4.3 Stock Solution B of internal standard (Bisoprolol)

Accurately weighed 1.0 mg of Bisoprolol was dissolved in diluent and final volume was made up to 100 ml in volumetric flask to get final concentration of bisoprolol to10µg/ml.

3.5.5 Working solutions

Working solutions for calibration standards and quality control samples of erlotinib and metformin were prepared as described in the Table 3.5. Working Solution of Internal Standard was prepared by diluting 0.5 ml of stock solution B up to 10 ml with diluent in volumetric flask to get final concentration of 500 ng/ml.

Table 3.5: Preparation of working solution of erlotinib and metformin

Sr. No.	Identification	Preparation procedure	Concentration of erlotinib and metformin (ng/ml)
1	CAL-1/LLOQ-QC	100 µL of CAL-4 to 1 ml	1000
2	CAL-2	500 µL of CAL-3 to 1 ml	2500
3	CAL-3	50 µL of CAL-8 to 1 ml	5000
4	CAL-4	100 µL of CAL-8 to 1 ml	10000
5	CAL-5	20 µL of stock solution-A to 1 ml	20000
6	CAL-6	40 µL of stock solution-A to 1 ml	40000
7	CAL-7	60 µL of stock solution-A to 1 ml	60000
8	CAL-8	100 µL of stock solution-A to 1 ml	100000
9	Low-QC(LQC)	150 µL of CAL-4 to 1 ml	1500
10	Medium-QC(MQC)	100 µL of HQC to 1 ml	8000
11	High-QC(HQC)	80 µL of Stock solution-A to 1 ml	80000

[CAL=calibration point, LLOQ=lower limit of quantitation, LQC= low quality control, MQC=medium quality control, HQC= high quality control]

3.5.6 Sample preparation

3.5.6.1 Spiked calibration standards and quality control of sample

A 45 µL of the drug free plasma/tissue or organ homogenate samples of SCID mice were transferred in 2 ml centrifuge tube. 5 µL of each working solutions of erlotinib or metformin and 5 µL of working solution of Bisoprolol (IS) was added to each tube. Contents were mixed by vortexing for about 30 sec. Standard and quality control samples were extracted by plasma protein precipitation method as per section 3.5.6.3.

3.5.6.2 Study samples

A 50 µL of the plasma/tissue or organ homogenate samples were transferred in 2 ml cryovials. 5 µL of working solution of internal standard was added to each sample. The contents were mixed by vortexing for 30 sec. The samples were extracted by plasma protein precipitation method as per section 3.5.6.3.

3.5.6.3 Method of sample extraction (plasma protein precipitation method)

Acetonitrile (800 µL) was added to each prepared plasma/tissue or organ homogenate sample as well as standards. The contents were vortexed for 1 minute. Samples were centrifuged at 10000 rpm for 5 min. Supernatant was transferred in HPLC vials for LC-MS/MS analysis.

3.5.7 Validation of LC-MS/MS method

The final drug concentration in plasma/tissue or organ homogenate samples for standard were 100, 250, 500, 1000, 2000, 4000, 6000 and 10000 ng/ml. The sensitivity of erlotinib and metformin assay was 100 ng/ml. The assay was sensitive, reproducible and linearity was observed from 100 to 10000 ng/ml. The mean correlation coefficient (R^2) of Erlotininb and Metformin was 0.9998 and 0.9995, respectively. The lower limit of quantification (LLOQ) was 100 ng/ml. The absolute

recovery of drug was measured by comparison of the areas of drug after injection of the extracted sample with those obtained after injection of the standard containing equivalent concentration of the drug. The mean recovery of erlotinib and metformin from plasma was 80.00 % and 95 % at 100 ng/ml, respectively. Intraday and interday precision (±15%) and accuracy (±10%) were within standard limits. Validation parameters indicated that the method was reliable, reproducible and accurate.

3.6 Estimation of meloxicam concentration in plasma and tissue by liquid chromatography-mass spectrometry (LC-MS/MS).

3.6.1 Bio-analytical method

LC-MS/MS System : Integrated system of model Co-sense Bio-analysis system, Liquid Chromatograph, Shimadzu Corporation, Koyoto, Japan (This include HPLC pump, Autosampler, UV-Visible Detector).

HPLC pump : LC-20-AD, Prominance, Pump, Shimadzu, Japan

Degasser : DGU20A_5, Prominance, Vacuum Degasser, Shimadzu, Japan

Autosampler : SIL-HTc Autosampler, Shimadzu, Japan

Sample tray : 1.0 ml sample cooler, Shimadzu, Japan

Column oven : CTO-20 A column Oven, Shimadzu, Japan

Detector : API 3200 LC-MS/Ms system, PE SCIEX, Canada

Data system : Analyst software version 1.4.2

3.6.2 Chromatographic conditions (meloxicam)

Mobile phase : (A:B), 0.1% Ammonia in water (Solution A), Acetonitrile (Solution B)

Gradient : Isocratic

Auto sampler rinsing solvent	:	Methanol: Water (80:20 %v/v)
Auto sampler temperature	:	10 °C
Column description	:	BAL/COL/11/03[ACE C18 50*4.6mm, 5u
Column temperature	:	Ambient (25 ± 5 °C)
Flow rate	:	1.0 ml / min with splitter (80:20), 80 % in waste
Injection volume	:	2 µL
Run time	:	2.0 minutes.
Retention time	:	Meloxicam - 0.42 (± 0.5 min) (Analyte-1)
		Tenoxicam- 0.41 (± 0.5 min) (Analyte-2)

3.6.3 Detector settings

Ionization Mode	:	Electro spray – positive mode
Scan type	:	MRM
Curtain Gas (CUR)	:	20
Collision Gas (CAD)	:	Medium
Ion Spray Voltage (IS)	:	4500 V
Temperature (TEM)	:	550 ˚C
GS 1	:	60
GS 1	:	60
Interface heater	:	ON
Dwell time	:	300 Sec.

Meloxicam

Declustering Potential (DP)	:	22.0 V
Collision cell entrance Potential	:	25.13 V

Tenoxicam (internal standard)

Declustering Potential (DP) : 25.00 V

Entrance Potential : 24.61 V

3.6.4 Preparation of stock solution of meloxicam and internal standard

3.6.4.1 Diluent: It was prepared by mixing of methanol: water in the ratio of 80:20.

3.6.4.2 Stock solution A of meloxicam

Accurately weighed 10.0 mg of meloxicam API grade powder was dissolved in diluent and volume was made upto 10 ml in volumetric flask to get concentration of meloxicam to 1000 µg/ml.

3.6.4.3 Stock solution B of internal standard (tenoxicam)

Accurately weighed 1.0 mg of tenoxicam was dissolved in diluent and final volume of 10 ml was made in volumetric flask to get concentration of tenoxicam to 100µg/ml.

3.6.5 Preparation of working solutions

Working solutions for calibration standards and quality control samples of meloxicam were prepared as described in the Table 3.6. Working solution of internal standard was prepared by diluting 0.8 ml of stock solution B up to 10 ml with diluent in volumetric flask to get final concentration of 8µg/ml.

Table 3.6: Preparation of working solution of meloxicam

Sr. No.	Identification	Preparation procedure	Concentration of meloxicam (ng/ml)
1	CAL-1/LLOQ-QC	100 µL of CAL-4 to 1 ml	1000
2	CAL-2	500 µL of CAL-3 to 1 ml	2500
3	CAL-3	50 µL of CAL-8 to 1 ml	5000
4	CAL-4	100 µL of CAL-8 to 1 ml	10000
5	CAL-5	20 µL of stock solution-A to 1 ml	20000
6	CAL-6	40 µL of stock solution-A to 1 ml	40000
7	CAL-7	60 µL of stock solution-A to 1 ml	60000
8	CAL-8	100 µL of stock solution-A to 1 ml	100000
9	Low-QC(LQC)	150 µL of CAL-4 to 1 ml	1500
10	Medium-QC(MQC)	100 µL of HQC to 1 ml	8000
11	High-QC(HQC)	80 µL of Stock solution-A to 1 ml	80000

[CAL=calibration point, LLOQ= lower limit of quantitation, LQC= low quality control, MQC=medium quality control, HQC= high quality control]

3.6.6 Sample preparation

3.6.6.1 Spiked calibration standards and QC sample

The volume of 45 µL of the drug free plasma/tissue or organ homogenate of SCID mice was transferred in 2 ml centrifuge tube. A 5 µL of each working solution of meloxicam and 5 µL of working solution of Tenoxicam (IS) was added to each tube. Contents were mixed by vortexing for about 30 sec. Standard and quality control samples were extracted by plasma protein precipitation method as per section 3.6.6.3.

3.6.6.2 Study samples

A 50 µL of the plasma/tissue or organ homogenate samples were transferred in 2 ml micro centrifuge tube. 5 µL of working solution of internal standard was added to each sample. The contents were mixed by vortexing for 30 sec. The samples were extracted by solid phase extraction method as per section 3.6.6.3.

3.6.6.3 Method of extraction (solid phase extraction method)

A 45 µL of the plasma/tissue or organ homogenate sample was transferred in 2 ml micro centrifuge tube. A 5 µL of working solution of internal standard and 50µL of 0.01% v/v formic acid in purified water were added to each sample. The contents were mixed by vortexing for 30 sec.

Conditioning of the cartridge (Strio-E, 30mg 1cc) was done by adding 1 mL methanol followed by 1 mL water. The samples were loaded in sequence of blank, zero samples, calibration standards, quality control samples and test samples. The cartridge was washed twice by adding 1 mL water. Samples were eluted in 1 mL of extraction solvent {0.01% ammonium in water: Acetonitrile (20:80% v/v)}, which were vortexed and transferred into HPLC vials for analysis.

3.6.7 Validation of LC-MS/MS method:

The final drug concentration in plasma/ tissue or organ homogenate for standardization were 100, 250, 500, 1000, 2000, 4000, 6000 and 10000 ng/mL. The sensitivity of meloxicam assay was 100 ng/ml. The assay was sensitive, reproducible and linearity was observed from 100 to 10000 ng/ml. The mean correlation coefficient (R^2) of meloxicam was 0.9985. The lower limit of quantification (LLOQ) was 100 ng/ml. The absolute recovery of drug was measured by comparison of the areas of drug after injection of the extracted sample with those obtained after injection of the standard solution containing equivalent concentrations of the drug. The mean recovery of meloxicam from plasma was 85 % at 100 ng/ml. Intraday and inter day precision (±15%) and accuracy (±10%) were within standard limits. Validation parameters indicated that the method was reliable, reproducible and accurate.

3.7 Pharmacokinetic analysis

A calibration plot of known concentration of erlotinib, meloxicam and metformin was plotted against instrument response. The linear regression was derived by applying $1/X^2$ weighing factor. Erlotinib, meloxicam and metformin concentration from unknown plasma samples were determined from linear regression equation (y=ax+b), where x=concentration of erlotinib, meloxicam and metformin in unknown plasma samples, y=Peak area ratio of erlotinib, meloxicam and metformin to internal standard, a= slope of calibration curve and b=intercept of calibration curve.

Pharmacokinetic parameters (T_{max}, C_0, C_{max}, $AUC_{(0-t)}$, $AUC_{(0-\infty)}$, $T_{1/2}$, Vd, CL and K_{el}, MRT) were derived by using non-compartmental analysis, WinNonlin software version 5.2.1 (Pharsight Corporation, USA).

3.8 Pharmaco-toxicological study of erlotinib, meloxicam and metformin alone and in combination with each other in SCID mice following repeated administration

Fifty four healthy male SCID mice were employed to assess pharmacological and toxicological effect of erlotinib (30 mg/kg, p/o), meloxicam (20 mg/kg, i/p), and metformin (100 mg/kg, p/o) alone and in combination with each other and all three drugs simultaneously following repeated administration at 24 hour interval for 28 days. Pharmaco-toxicological effects of these drugs alone and in combination with each other were assessed by studying the following parameters.

1. Tumor size reduction
2. Expression of *akt, pten, raf1, AMPK* and *p70S6k genes* in tumor tissue
3. Hematology
4. Serum biochemistry
5. Histopathological examination

3.8.1 Development of xenograft model

A resemblance of lung adeno carcinoma was made by developing a mice xenograft model using A549 cell line. A 549 (lung adenocarcinoma) cell line was cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum and 1% penicillin-streptomycin in 5% CO2 at 37°C. Fifty four SCID male mice of 5-8 weeks age were selected randomly and injection site was prepared by removing hair at right side flank region. Each animal except normal control was injected with 0.2 ml cells (5.0X 10^6 cells) subcutaneously at injection site with 23 gauge needle. Normal control was injected subcutaneously with 0.2 ml DMEM. Animals were observed daily for any abnormal manifestation. Tumor development was checked twice a week. On development of measurable tumor growth (100 mm^2) animals were grouped according to size of tumor in eight groups (group II to IX) with 6 animals in each group.

Fifty four animals were allotted in nine groups each consisting of 6 male SCID mice for evaluation of Pharmaco-toxicological effect of individual drug as well as in combination. Group I served as negative (without tumor) control and received only vehicle. Group II served as positive (tumor) control and received only vehicle as a treatment. The group III, IV and V received erlotinib (30 mg/kg, p.o), meloxicam (20 mg/kg, i/p) and metformin (100 mg/kg, p.o), respectively. The group VI and VII received erlotinib (30 mg/kg, p.o) along with meloxicam (20 mg/kg, i/p) and metformin (100 mg/kg, p.o), respectively. The group VIII received Metformin (100 mg/kg, p.o) along with meloxicam (20 mg/kg, i/p), whereas group IX received all three drugs. These animals were administered with defined doses of drugs and administration was repeated at 24 hours interval for 28 days.

3.8.2 Observations

All animals were observed daily for any clinical manifestation throughout the period of study.

3.8.2.1 Physical examination and behavioral observations

A careful physical examination was done at initiation of study. Animals were examined daily through out the study for changes in skin, fur, eyes, mucous membrane, occurrence of secretions and excretions and autonomic activity (e.g. lacrimation, piloerection, pupil size, unusual respiratory pattern) and palpable mass. Changes in gait, posture and response to handling were also observed.

3.8.2.2 Tumor size and mortality

Tumor volume was measured once a week in all animals of treatment groups and positive (tumor) control group by digital vernier calipers. Mortality, if any was recorded. The tumor volume was calculated from two dimensional measurements as per following formula.

$$\text{Tumor Volume} = (\text{Length X Width}^2)/2$$

The relative tumor volume (RTV) was calculated by evaluating the mean tumor volume of treatment group on specific experimental day "T" Vs the mean tumor volume of the same group on day 0 of treatment "T_0". It is represented as per following formula.

$$RTV = T / T_0$$

The relative tumor growth inhibition ratio was calculated by mean relative tumor volume growth of treatment group mice divided by the mean relative tumor volume growth of control group mice on specific day of treatment.The difference in efficacy between treatment group were expressed as percentage of tumor growth inhibition (TGI%) and calculated as per following formula.

$$[1- (T-T_0) / (C-C_0)] \text{ X } 100$$

where "T" represent the mean tumor volume of treatment group on specific experimental day, "T_0" represent the mean tumor volume of the same group on day 0 of treatment , "C" represents the mean tumor volume of control group on specific experimental day and "C_0" represent the mean tumor volume of the control group on day 0 of treatment.

3.8.2.3 Body weight and feed consumption

Body weight and feed consumption were recorded weekly throughout the study period.

3.8.3 Blood sample collection for hematological and biochemical analysis

Blood samples were collected from retro-orbital plexus into clean sterilized plain and EDTA added centrifuge tube on 0 day (before drug administration) and 28th day for hematological and serum biochemical analysis.

3.8.3.1 Hematological estimations

Hematological estimation was carried out on the day of collection of blood sample. The samples were analyzed by using automatic hematology analyzer (CELL-DYN®3700, Abbott Lab., USA) for assessment of the following parameters.

1. Hemoglobin
2. Hematocrit value
3. Total leukocyte count
4. Different leukocyte count

3.8.3.2 Serum biochemical estimations

Blood samples for serum biochemical estimation were allowed to clot and then centrifuged at 4000 rpm for 10 minutes at ambient temperature and serum was collected. Serum biochemical parameters mentioned in Table 3.7 were analyzed using automatic biochemical analyzer (Daytona IR200, Randox Ltd., India & COBAS c311, ROCHE, Germany).

Table 3.7: Serum biochemical parameters and their detection method

Parameters	Methods	Unit
Glucose	GODPOD/ Hexokinase	mg/dL
Aspartate Amino Transferase (AST)	UV kinetic/ Modified IFCC	IU/L
Alanine Amino Transferase (ALT)	UV kinetic/ Modified IFCC	IU/L
Alkaline Phophatase (ALP)	UV kinetic/ Modified IFCC	IU/L
Total Bilirubin (TB)	Sulphuric acid DMSO/ Diazonium Salt/Ion, W/o Blank	mg/dL
Creatinine	Alkaline Picrate method	mg/dL
Creatinine Kinase (CK)	IFCC	U/L
Urea	Urease Kinetic method	mg/dL
Blood Urea Nitrogen (BUN)	Calculated	mg/dL
Lactate Dehydrogenase (LDH)	UV assay Lactate to Pyruvate (IFCC)	U/L
Acid Phosphatase (ACP)	Colorimetric method	U/L
Uric acid	Uricase Perox Colorimetric method/ Uricase Enzymatic colorimetric test	mg/dL

3.8.4 Necropsy and organ weight

All treatment and control group (I to IX) animals were sacrificed on day 28. Postmortem examination was performed in the confined disinfected laboratory to determine the presence or absence of gross pathological lesions.

Post mortem examination was made by systemic approach (i.e. gross changes in organ size, shape and any visible lesions). Detailed post mortem lesions from all the animals were recorded. For gross lesions liver, lungs, kidneys, heart, G.I tract and tumor site were observed.

The organs like liver, lungs, kidneys, heart and tumor were collected, cleaned using blotting paper and then weighed on analytical balance. Weights of all the organs were recorded as absolute values.

3.8.4.1 Collection of tissue samples

For gene expression, tissue distribution of drug and histopathological changes liver, lungs, kidneys, heart and tumor mass tissue were collected, weighed and divided in one, two or three pieces as per requirement, one for gene expression, second for drug tissue distribution in target organ and third for histopathological examination. The tumor mass and lung tissue were collected for all three parameters, whereas heart, liver and kidney tissue were collected for only histopathological examination. The tumor tissue collected for gene expression were kept in pre-labeled vial and immediately transferred to liquid nitrogen. The tissues collected for drug distribution in target organs were kept in pre-labeled vial and immediately transferred to -70 °C deep fridge. The tissue collected for histopathological examinations were transferred in 10% neutral buffered formalin and preserved for processing. Tissues were processed, embedded in paraffin and stain with H & E and were observed under light microscope.

3.9 Drug distribution study

3.9.1 Collection of tissue

On day of termination the target organ tissue were collected for drug distribution study as described in section no.3.8.4.1. Lung and tumor tissues were collected for drug distribution study from all animals. Both tissues were thoroughly washed with purified water to remove blood clots and connective tissues. Finally, both tissues were washed with saline and stored in deep freeze at –75 ± 5° C till homogenate preparation and further analysis.

3.9.2 Preparation of tissue homogenates

Tissues were weighed and homogenized using tissue homogenizer in cold Tris-Sucrose buffer, pH 7.4 to get 0.33 g of tissue/ml of homogenate. Tissue homogenates were stored at -70 ± 5 ℃, till analysis.

3.9.3 Extraction procedure (tissue extraction)

Erlotinib and metformin were extracted from tissue by protein precipitation method as mentioned in section no 3.5.6.3. Meloxicam from tissue was extracted by solid phase extraction method as mentioned at section no. 3.6.6.3. All samples were mixed by vortexing for 1.0min. Samples were centrifuged at 10000 rpm for 5 minutes and clear supernatant was transferred to HPLC vials for LC-MS/MS analysis.

3.9.4 Estimation of drug concentration

The concentration of erlotinib, meloxicam and metformin in tissue extracted samples were determined using a validated LC-MS/MS bioanalytical method.

The analytical method, chromatographic conditions, LC-MS/MS configuration, detector parameters etc. were same as mentioned in section No 3.5 for erlotinib and metformin and section No. 3.6 for meloxicam. The stock and working solutions for erlotinib, metformin and internal standard (bisoprolol) were prepared as mentioned in section No 3.5.4 and 3.5.5 for tissue homogenate analysis. Same way the stock and working solutions for meloxicam and internal standard (tenoxicam) were prepared as mentioned in section No 3.6.4 and 3.6.5.

The sample preparation for system suitability, blank tissue homogenate (control group animals) samples and study samples were carried out as per procedure given in section No 3.9.2 and 3.9.3.

3.10 Gene expression study

3.10.1 RNA extraction

Total RNA from tissue sample was extracted following TRI reagent based protocol. Frozen tissue sample weighing 100 mg was taken in 2 ml centrifuge tube and 1 mL TRI reagent (100mg : 1mL) was added. Sample was homogenized immediately using a conventional homogenizer at maximum speed. 0.2 ml chloroform was added and kept at room temperature for 5 minutes followed by centrifugation of mixture at 12000 rcf for 20 min. Upper transparent layer was collected in another 1.5 mL tube than equal volume of 2-iso propanol is added, mixed and centrifuged at 12000 rcf for 20 min. Pellet of RNA formed at the bottom of the tube was washed twice with 70% alcohol and dried. The pellet was then resuspended in 50 µL nuclease free water.

3.10.2 Quantitation of RNA

RNA was quantified by Spectrophotometer (Eppendorf) by measuring absorbance at 260 nm. 2 µL sample was mixed with 198 µL milli-Q water and absorbance was taken at 260 nm. The quantified RNA again diluted to make the concentration @ 100 ng/ µL and loaded on the agarose gel to check the quality. The gel picture was normalized with help of IMAGE-J software to get equal amount of RNA in all samples.

3.10.3 cDNA synthesis

The high capacity cDNA reverse transcription kit from Applied Biosystem was used for cDNA synthesis. To synthesize the cDNA, 1 µg of total RNA was used from the diluted sample and all the components including total RNA (template), primer solutions, 10x RT buffer, dNTP mix and RNAse free water were thawed on ice and used for reaction. RNAse inhibitor was diluted to a final concentration of 10 U/µL

in ice-cold 1x RT buffer and mixed by vortexing for 5 seconds. Master mix was prepared according to the table given below. Template RNA (1 µg) was added to individual tubes containing the master mix and mixed thoroughly by pipetting.

Component used for cDNA synthesis

Component	Vol./reaction	Final conc.
10 X Buffer RT	2.0 µl	1X
dNTP (5mM each dNTP)	2.0 µl	0.5 mM each
Random primer (10X)	2.0 µl	1 µM
RNAse inhibitor (10 U/µl)	1.0 µl	10 U/20 µl
Reverse Transcriptase enzyme	1.0 µl	4 U/20 µl
Template RNA	10.0 µl	1 µg
RNAse free water	Up to 20 µl	-

* Tubes were kept on ice throughout the preparation.

After the formulation (master mix) each sample was run in thermal cycling condition for cDNA synthesis as given bellow,

Process	Temp. (°C)	Time(min.)
Annealing	25	10
Incubation	37	120
Inactivation	85	5 Sec.
Store	4	∞

Synthesized cDNA was stored at -15 to -25 °C until use.

3.10.4 Quality check of cDNA

The quality of cDNA was checked by agarose gel electrophoresis. The gel picture was normalized with help of IMAGE-J software to get equal amount of cDNA in all samples.

3.10.5 Real Time Polymerase Chain Reaction (RT-PCR)

Expression of differential mRNA was quantified by Real Time PCR and analyzed using Applied Biosystems 7300 SDS software.

3.10.6 RT primers

The primers were designed by using online NCBI software (http://www.ncbi.nlm.nih.gov) and commercially obatained from Sigma. The primers used are given in Table 3.8.

Table 3.8: Primers sequence design for RT-PCR

Genes	Accession No.	Primer sequences (5'-3')	Annealing (°C)
RAF1	NM002880.3	F- TTGCACGCTGACCACGTCCC	60 °C
		R- GGAGAAGCCAGCAGGCACCAC	
AMPK	NM006253.4	F- CGCTGAGGGGTGGTGAAGCG	60 °C
		R- CAACCCTGCCGACTCAGCCG	
PTEN	NM000314.4	F- TATGCGCTGCGGCAGGATACG	60 °C
		R- GTTGAGCCGCTGTGAGGCGA	
P70S6K	NM003161.2	F- ACGGCTTTTACCCAGCCCCG	60 °C
		R- GCGGGCTCTGAGGATGAGCTG	
AKT	NM001014431.1	F- AGCCCACCCTTCAAGCCCCA	60 °C
		R- TGTGTGGACAGCGAGCGCAG	

3.10.6.1 Quick RT

All the primers were checked by one step RT PCR Qiagen Kit to check its annealing temperature and amplified product.

Reaction mixture was setup as given below:

Components	Vol/Reaction (µl)
5X Buffer	2.0
dNTPs (10mM)	0.8
Quick RT enzyme (4U)	0.4
Farward Primer (10µm)	0.3
Reverse Primer (10µm)	0.3
RNA	2.0
Milli Q	4.2

These samples were run in PCR Thermal cycler (AB system 2730) with condition as given bellow.

Step	Temperature and Time
Incubation	50 ^{o}C 30 min
Initial denaturation	95 ^{o}C 15 min
Denaturation	95 ^{o}C 30 Sec
Annealing	60 ^{o}C 45 Sec
Extension	72 ^{o}C 1 min
Repeat for 30cycles	
Final Extension	72 ^{o}C 7 min
Hold	4 ^{o}C

Amplified products were run on 1% agarose gel to check amplification.

3.10.7 Preparation of SYBR Green Master mix (for a single 20 µL)

Ingredients	Volume (µl)	Final Concentration
2X SYBR PCR Master Mix(Quantifast)	10	1X
Forward primer (10 µM stock)	2	1 µM
Reverse primer (10 µM stock)	2	1 µM
cDNA	2 (consider the volume, which has been already added to the well)	
RNase free Water	up to make final volume 20	

3.10.8 Preparation of the plate for running the PCR

A 18 µl of the reaction mix was added to the necessary well containing a cDNA and air bubble was removed by spinning.

These samples were run in ABI 7300 RT PCR as per following condition. Dissociation/melting curve was done after completion of the SYBR green reaction.

Thermal cycling parameters for SYBR green

Step	Temperature and Time	Remarks
Incubate	95°C 5 minutes	-
Repeat for 40 cycles	-	-
Denaturation	95°C 10 seconds	-
Annealing and Extension	60°C 32 seconds	Florescence was detected at this stage.

3.10.9 Relative quantitation

Gene quantitation was achieved using the C_T (Cycle Threshold) comparative method and is expressed as 'n-fold up or down regulation of transcription' in relation to a calibrator which is represented by the smallest signal detectable for that specific gene. For relative quantification by the comparative C_T method, values are expressed relative to a reference sample, called the calibrator. The expression of selected genes was calibrated by that of the reference gene, mice β actin, at each time point and converted to the relative expression ratio (fold of expression), where,

Fold of Expression=$2^{-\Delta\Delta C_T}$

$\Delta\Delta C_T$ = Average ΔC_T of target– Average ΔC_T of calibrator

ΔC_T = Average C_T of target- Average C_T of endogenous control

3.11 Histopathology

All the organs collected were processed in automatic vacuumed tissue processor (Leica ASP 300, Leica microsystem, Germany) and then paraffin tissue blocks of all the tissues were prepared in automatic tissue embedding station (Microm EC 350-1, Microm international, New york). Sections of all organs were taken at 4 microns by automatic microtome machine (Leica RM 2155, Leica microsystem, Germany) on the glass slide. Tissue sections were stained with Haematoxylin and

Eosin (H & E) in automatic staining machine (Leica auto stainer XL, Leica microsystem, Germany). Mounting of stained slide was done with automatic cover slipper (Leica CV 5030, Leica microsystem, Germany) The stained slides were observed under microscope and histological lesions were recorded. The criteria for scoring the lesions was performed by severity grade such as minimal (< 20 %), mild (21-50 %), moderate (51-75 %) and severe (76-100%) depending on the area of distribution of lesions.

3.12 Statistical analysis

The data obtained for pharmacokinetic parameters, body weight, hematological parameters, serum biochemical parameters and organ weights were analyzed statistically. The statistical procedure used for analysis of above data was unpaired two tail `t` test and one way ANOVA. Where $p \leq 0.05$ was considered as statistically "significant" and $p \leq 0.01$ was considered as statistically "highly significant". Graphs and figures were prepared using software Graph Pad Prism (Version 5.00). The data were described using the statistical parameters viz. arithmetic mean, standard error of mean (SEM), Relative change and % change.

CHAPTER – IV

RESULTS

The present study was conducted in male SCID mice to investigate pharmacokinetics of erlotinib (30 mg/kg, p.o), meloxicam (20 mg/kg, i.p) and metformin (100 mg/kg, p.o) alone and in combination with each other as well as all three drugs together at same dose rate by single administration. Additionally, pharmaco-toxicological effects of erlotinib (30 mg/kg), meloxicam (20 mg/kg) andf metformin (100 mg/kg) were evaluated by repeated administration for 28 days in xenograft tumor model developed in SCID male mice. The plasma samples were assayed for erlotinib, meloxicam and metformin concentration using Liquid chromatography with mass spectrometry (LC-MS/MS) procedure. Calibration curves were prepared for standard known concentration of drugs in normal SCID mice plasma. The actual drug concentration for erlotinib, meloxicam and metformin in plasma samples obtained from SCID mice were calculated by comparing with standard calibration curve of kinetic study. For pharmaco-toxicological effect tumor volume measurement, gene expression study, hematological and serum biochemical analysis, tissue distribution study and microscopic examination were carried out.

4.1 Plasma levels of erlotinib following oral administration alone and in combination with meloxicam (i.p) and/or metformin (p.o) in male SCID mice

The plasma levels of erlotinib as a function of time after its single dose administration (30 mg/kg, p.o) alone and in combination with meloxicam (20 mg/kg, i.p.) and/or metformin (100 mg/kg, p.o) in male SCID mice are depicted in Table 4.1 to 4.4. The plasma concentration of erlotinib in male SCID mice is represented as mean ± SEM at different time intervals.

Following oral administration of Erlotinib alone, the mean peak plasma level of drug was observed 16372.63 ± 846.59 ng/ml at 0.167 hrs, which slowly declined to 6969.17 ± 309.70 ng/ml at 4 hrs and thereafter the drug concentration in plasma diminished slowly and was detectable (709.25 ± 78.61 ng/ml) up to 24 hrs. In case of oral administration of erlotinib in combination with intra peritoneal administration of meloxicam, the mean peak plasma level of erlotinib was observed 17741.60 ± 2918.54 ng/ml at 0.167 hrs, which gradually declined to 6988.27 ± 381.15 ng/ml at 4 hrs and finally reached to 414.05 ± 84.34 ng/ml at 24.00 hrs. Same way oral administration of erlotinib in combination with metformin, the mean peak plasma level of erlotinib was observed 13400.85 ± 882.54 ng/ml at 0.167 hrs, which gradually declined to 4567.18 ± 456.98 ng/ml at 4 hrs and finally reached to 385.70 ± 114.87 ng/ml at 24.00 hrs. When erlotinib was administered along with meloxicam and metformin, the mean peak plasma level of erlotinib was observed 13600.97 ± 1176.92 ng/ml at 0.167 hrs, which gradually declined to 3558.74 ± 250.68 ng/ml at 4 hrs and finally reached to 463.43 ± 42.72 ng/ml at 24.00 hrs. The plasma drug concentration was not detected in samples collected after 24 hrs post oral administration of erlotinib alone, erlotinib with meloxicam, intraperitoneally, erlotinib with metformin, orally and erlotinib along with both meloxicam and metformin.

Table 4.1: Plasma concentrations (ng/ml) of erlotinib following single dose oral administration (30 mg/kg) in SCID male mice

Time (h)	Plasma concentration (ng/ml)						
	Animal No.						Mean ± SEM
	M1	M2	M3	M4	M5	M6	
0.083	10110.12	14060.77	11661.16	16088.43	9381.12	10391.09	11948.78 ± 1065.83
0.167	19918.76	16038.41	17518.72	14310.57	15778.51	14670.80	16372.63 ± 846.59
0.333	14243.16	16336.69	18167.51	15955.19	14574.66	15409.52	15781.12 ± 577.14
0.667	11066.70	12629.19	11678.03	11796.19	11974.24	17345.65	12748.33 ± 942.14
1	14510.25	11308.42	12862.37	9682.63	9999.71	13061.99	11904.23 ± 773.90
2	11600.16	10297.59	9473.23	9143.84	10997.15	11354.35	10477.72 ± 413.07
4	6235.25	7760.37	6907.78	8035.71	6378.13	6497.76	6969.17 ± 309.70
6	2974.00	4490.48	2684.53	2072.96	3582.39	5174.07	3496.41 ± 474.99
8	2695.98	3809.92	3129.29	2831.40	3483.12	3545.96	3249.28 ± 178.16
12	1107.25	1623.09	1118.85	725.29	1610.84	962.11	1191.24 ± 146.57
24	ND	486.93	621.46	960.41	623.95	853.50	709.25 ± 78.61
48	ND	ND	ND	ND	ND	ND	ND

Table 4.2: Plasma concentrations (ng/ml) of erlotinib following single dose administration (30mg/kg, p.o) along with meloxicam (20 mg/kg, i.p.) in SCID male mice

Time (h)	Plasma concentration (ng/ml)						
	Animal No.						Mean ± SEM
	M19	M20	M21	M22	M23	M24	
0.083	10406.15	17620.15	13552.94	12670.21	11275.91	10340.80	12644.36 ± 1122.10
0.167	26540.75	12108.24	14970.65	26719.33	15717.74	10392.90	17741.60 ± 2918.54
0.333	15756.19	15243.24	16141.42	12422.05	11640.74	16452.46	14609.35 ± 837.82
0.667	14024.06	15995.47	13985.86	13727.06	15130.71	8980.13	13640.55 ± 995.99
1	13683.89	16998.75	14891.47	13326.09	13310.57	9673.80	13647.43 ± 979.57
2	12307.13	9347.65	10456.50	18240.30	12377.87	8022.37	11791.97 ± 1462.39
4	8383.43	7064.02	6692.91	5483.55	7137.12	7168.60	6988.27 ± 381.15
6	3798.64	4081.08	2727.83	4715.17	4618.89	5260.54	4200.36 ± 360.93
8	3166.47	2248.60	2135.65	3080.78	4976.02	3464.53	3178.67 ± 419.32
12	1583.08	445.80	1085.95	1708.98	1198.43	1172.53	1199.13 ± 181.51
24	246.74	347.74	540.43	590.53	123.01	635.88	414.05 ± 84.34
48	ND	ND	ND	ND	ND	ND	ND

Table 4.3: Plasma concentrations (ng/ml) of erlotinib following single dose administration (30 mg/kg, p.o) along with metformin (100 mg/kg, p.o) in SCID male mice

Time (h)	Plasma concentration (ng/ml)						
	Animal No.						Mean ± SEM
	M25	M26	M27	M28	M29	M30	
0.083	8125.17	13877.26	10423.78	14325.09	12121.23	10892.05	11627.43 ± 945.84
0.167	11571.01	15510.87	16324.61	13324.47	12866.82	10807.29	13400.85 ± 882.54
0.333	10212.88	13221.53	11514.37	14051.10	13634.62	10718.13	12225.44 ± 661.79
0.667	11086.25	11730.54	10107.66	13075.09	11420.28	11674.46	11515.72 ± 394.93
1	10574.23	11775.39	10852.06	12118.05	10707.07	9951.45	10996.37 ± 328.61
2	6417.60	9917.75	9907.85	7949.71	6666.94	7621.07	8080.15 ± 624.67
4	3455.38	3573.36	4157.54	6359.72	5375.10	4481.97	4567.18 ± 456.98
6	3874.32	3521.31	4195.84	2711.57	2636.43	3747.94	3447.90 ± 260.60
8	3236.44	2163.12	3071.61	2315.49	2245.87	2341.48	2562.34 ± 190.00
12	1189.53	892.91	999.51	1516.42	1180.20	2187.38	1327.66 ± 192.55
24	144.06	ND	ND	318.45	ND	694.60	385.70 ± 114.87
48	ND	ND	ND	ND	ND	ND	ND

Table 4.4: Plasma concentrations (ng/ml) of erlotinib following single dose administration (30 mg/kg, p.o) along with meloxicam (20 mg/kg, i.p) and metformin (100 mg/kg, p.o) in SCID male mice

Time (h)	Plasma concentration (ng/ml)						
	Animal No.						Mean ± SEM
	M37	M38	M39	M40	M41	M42	
0.083	10844.02	11959.39	16022.83	9373.03	14734.74	9417.19	12058.53 ± 1133.60
0.167	10006.69	12230.83	12090.56	15378.17	13682.12	18217.45	13600.97 ± 1176.92
0.333	13824.83	10392.49	12744.20	13386.06	14807.61	12964.57	13019.96 ± 604.43
0.667	10427.67	9793.26	9072.59	11264.90	11671.57	11834.40	10677.40 ± 450.62
1	11678.60	9403.50	9772.73	9756.52	11066.83	11297.19	10495.90 ± 392.84
2	7668.23	5956.30	6637.74	7499.58	8782.63	8632.04	7529.42 ± 450.05
4	2500.54	3769.25	3227.61	3993.97	3660.70	4200.38	3558.74 ± 250.68
6	2474.62	2600.53	3131.96	2925.23	2015.65	1524.90	2445.48 ± 242.11
8	1615.79	2246.46	3021.45	2686.17	1806.22	3138.48	2419.09 ± 258.35
12	913.71	1159.39	1417.42	718.38	1502.30	810.91	1087.02 ± 132.79
24	449.99	427.36	582.33	ND	542.25	315.24	463.43 ± 42.72
48	ND	ND	ND	ND	ND	ND	ND

Table 4.5: Comparison of mean plasma concentration (ng/ml) of erlotinib following single dose administration of erlotinib (30 mg/kg, p.o) alone and in combination with meloxicam (20 mg/kg, i.p) and/or metformin (100 mg/kg, p.o) in SCID male mice (n=6)

Time (hr)	Erlotinib concentration (ng/ml)			
	Erlotinib	Erlotinib + Meloxicam	Erlotinib + Metformin	Erlotinib + Meloxicam + Metformin
0.083	11948.78 ± 1065.83	12644.36 ± 1122.10	11627.43 ± 945.84	12058.53 ± 1133.60
0.167	16372.63 ± 846.59	17741.60 ± 2918.54	13400.85 ± 882.54	13600.97 ± 1176.92
0.333	15781.12 ± 577.14	14609.35 ± 837.82	12225.44 ± 661.79**	13019.96 ± 604.43*
0.667	12748.33 ± 942.14	13640.55 ± 995.99	11515.72 ± 394.93	10677.40 ± 450.62
1	11904.23 ± 773.90	13647.43 ± 979.57	10996.37 ± 328.61	10495.90 ± 392.84
2	10477.72 ± 413.07	11791.97 ± 1462.39	8080.15 ± 624.67	7529.42 ± 450.05
4	6969.17 ± 309.70	6988.27 ± 381.15	4567.18 ± 456.98**	3558.74 ± 250.68**
6	3496.41 ± 474.99	4200.36 ± 360.93	3447.90 ± 260.60	2445.48 ± 242.11
8	3249.28 ± 178.16	3178.67 ± 419.32	2562.34 ± 190.00	2419.09 ± 258.35
12	1191.24 ± 146.57	1199.13 ± 181.51	1327.66 ± 192.55	1087.02 ± 132.79
24	709.25 ± 78.61	414.05 ± 84.34	385.70 ± 114.87	463.43 ± 42.72
48	ND	ND	ND	ND

**significant at $p<0.01$, *significant at $p<0.05$

Comparison of mean plasma concentration (ng/ml) of erlotinib alone and in combination with meloxicam and/or metformin is depicted in Table 4.5. The results showed that the mean peak concentration time remained same in erlotinib alone and in combination with meloxicam and/or metformin. The mean peak plasma concentration value of erlotinib was observed little higher in combination with meloxicam and lower in combination with metformin and both in comparison to alone. The concentration of erlotinib in plasma was observed till 24 hrs in erlotinib alone and in combination with meloxicam, metformin and both. The plasma concentrations of erlotinib at 8 hrs post administration in erlotinib alone and in combination with meloxicam remained considerably higher in comparison to other two combinations. The plasma concentration of erlotinib in combination with metformin and all three drugs together was significantly low at 0.33h and 4h post administration of drug. The comparative plasma concentration of erlotinib in all four conditions is presented as semi logarithmic graph in Figure 4.1.

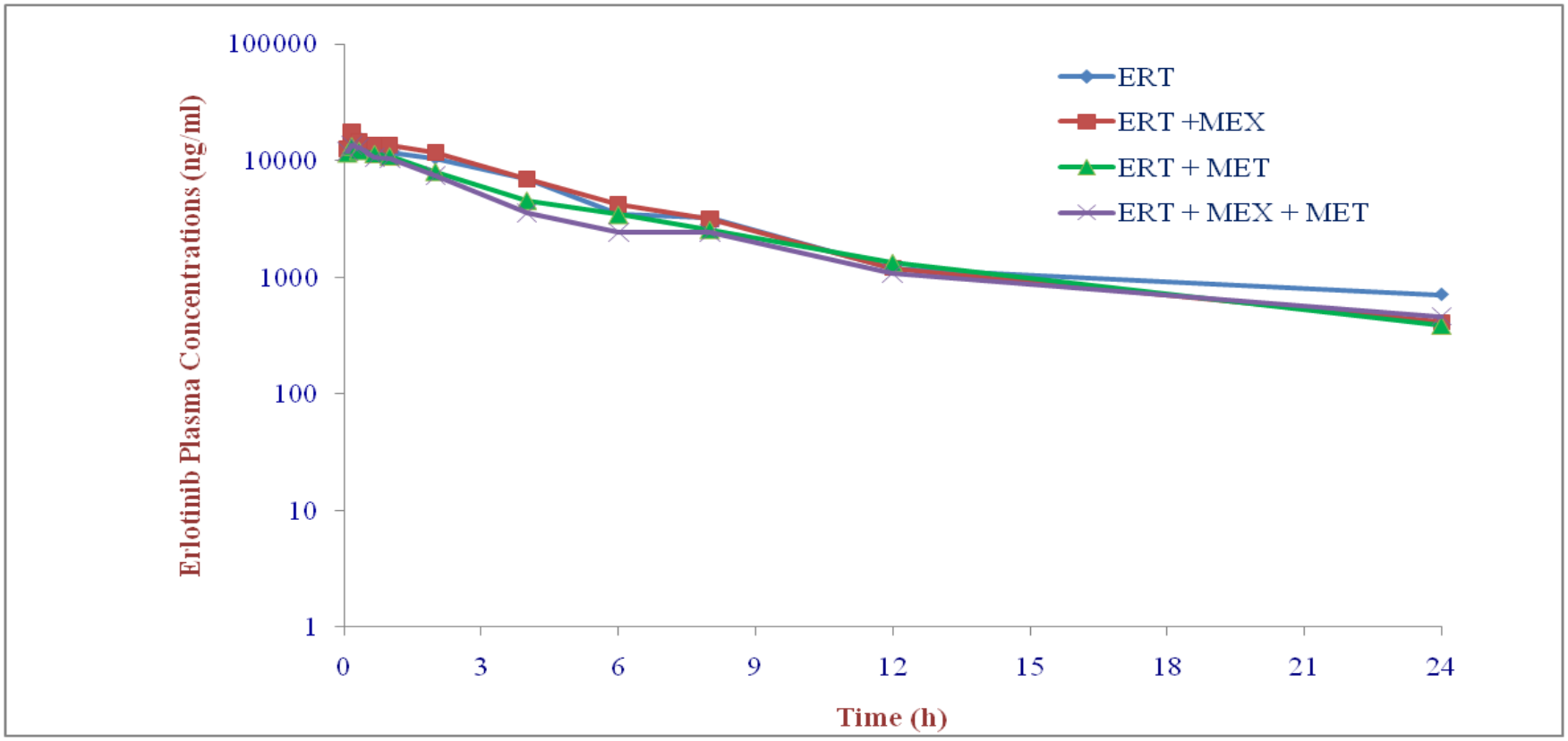

Figure 4.1: Semi logarithmic plot of erlotinib plasma concentration following single dose administration of erlotinib alone (30 mg/kg, p.o), in combination with meloxicam (20 mg/kg, i.p), in combination with metformin (100 mg/kg, p.o) and in combination with meloxicam (20 mg/kg, i.p) and metformin (100 mg/kg, p.o) in SCID male mice (n=6).

4.2 Plasma levels of meloxicam following intraperitoneal administration as alone and in combination with erlotinib and/or metformin in male SCID mice

The plasma levels of meloxicam as a function of time after its single dose administration (20 mg/kg, i.p) in male SCID mice alone and in combination with erlotinib (30 mg/kg, p.o) or/and metformin (100 mg/kg, p.o) are depicted in Table 4.6 to 4.9. The plasma concentration of meloxicam in male SCID mice is represented as mean ± SEM at different time intervals.

Following intraperitoneal administration of meloxicam alone, the mean peak plasma level of drug was observed 44858.46 ± 3486.54 ng/ml at 0.33 hrs, which slowly declined to 10660.24 ± 381.76 ng/ml at 4 hrs and thereafter the drug concentration in plasma diminished slowly and was detectable (160.24 ± 9.75 ng/ml) up to 24 hrs. In case of intraperitoneal administration of meloxicam in combination with oral administration of erlotinib, the mean peak plasma level of drug was observed 49994.17 ± 2928.14 ng/ml at 0.33 hrs, which gradually declined to 11869.03 ± 1141.80 ng/ml at 6 hrs and finally reached to 382.34 ± 106.23 ng/ml at 24.00 hrs. Same way intraperitoneal administration of meloxicam in combination with metformin, the mean peak plasma level of meloxicam was observed 46540.83 ± 3879.53 ng/ml at 0.33 hrs, which gradually declined to 10268.29 ± 652.65 ng/ml at 4 hrs and finally reached to 245.65 ± 31.90 ng/ml at 24.00 hrs. When the meloxicam was administered along with erlotinib and metformin, the mean peak plasma level of meloxicam was observed 39648.94 ± 2190.42 ng/ml at 0.33 hrs, which gradually declined to 9266.74 ± 749.79 ng/ml at 4 hrs and finally reached to 163.29 ± 12.29 ng/ml at 24.00 hrs. The plasma drug concentration was not detected in samples collected after 24 hrs post intraperitoneal administration of meloxicam alone, meloxicam with erlotinib orally, meloxicam with metformin orally and meloxicam along with both erlotinib and metformin.

Table 4.6: Plasma concentrations (ng/ml) of meloxicam following single dose intraperitoneal administration (30 mg/kg) in SCID male mice

Time (hr)	Plasma concentration (ng/ml)						
	Animal No.						Mean ± SEM
	M7	M8	M9	M10	M11	M12	
0.083	25542.42	37075.02	21816.85	22289.47	23359.59	34350.58	27405.65 ± 2701.73
0.167	39879.74	34687.67	37568.56	23236.96	42559.76	35830.77	35627.24 ± 2734.69
0.333	45600.94	54552.91	38382.21	55270.40	40545.17	34799.11	44858.46 ± 3486.54
0.667	31035.76	32866.01	32982.71	55862.04	44062.42	34018.94	38471.31 ± 3959.60
1	30563.23	30604.72	24775.93	21607.56	21556.07	25191.91	25716.57 ± 1660.70
2	21253.17	21568.60	14545.07	16656.78	14715.65	15890.22	17438.25 ± 1296.19
4	15056.00	10951.97	9311.37	8545.09	13974.59	6122.43	10660.24 ± 1381.76
6	3291.94	3777.97	5866.75	3653.17	3629.94	4204.22	4070.66 ± 378.79
8	1273.25	1263.00	2791.22	2837.18	3248.53	2904.78	2386.33 ± 359.64
12	1050.76	1433.79	808.61	1373.59	1435.97	1651.38	1292.35 ± 124.97
24	151.56	175.25	ND	145.35	193.88	135.15	160.24 ± 9.75
48	ND	ND	ND	ND	ND	ND	ND

Table 4.7: Plasma concentrations (ng/ml) of meloxicam following single dose administration (20 mg/kg, i.p) along with erlotinib (30 mg/kg, p.o) in SCID male mice

Time (hr)	Plasma concentration (ng/ml)						
	Animal No.						Mean ± SEM
	M19	M20	M21	M22	M23	M24	
0.083	34073.37	11446.28	24408.69	22472.22	32271.47	32059.92	26121.99 ± 3502.06
0.167	48275.49	49304.70	34088.83	33535.69	34563.84	35802.34	39261.82 ± 3031.46
0.333	48556.39	51259.32	53417.40	38804.60	60487.19	47440.12	49994.17 ± 2928.14
0.667	34320.06	39754.95	32853.68	32872.19	34020.14	28891.39	33785.40 ± 1433.29
1	26910.03	23920.75	31329.88	24686.76	28196.93	23505.68	26425.00 ± 1228.12
2	24703.88	21317.01	30959.91	22408.61	26543.02	21228.54	24526.83 ± 1540.02
4	21732.26	19423.09	17378.48	18813.86	20695.15	18188.92	19371.96 ± 658.52
6	10746.54	14029.20	10226.45	16254.29	11412.56	8545.18	11869.03 ± 1141.80
8	8731.16	8794.45	4776.72	7638.79	8600.34	3643.03	7030.75 ± 920.01
12	3126.63	3060.87	3812.66	2680.23	3522.25	2317.54	3086.70 ± 221.80
24	ND	ND	146.25	635.41	169.88	577.84	382.34 ± 106.23
48	ND	ND	ND	ND	ND	ND	ND

Table 4.8: Plasma concentrations (ng/ml) of meloxicam following single dose administration (20 mg/kg, i.p) along with metformin (100 mg/kg, p.o) in SCID male mice

Time (hr)	Plasma concentration (ng/ml)						
	Animal No.						Mean ± SEM
	M31	M32	M33	M34	M35	M36	
0.083	13914.73	22070.48	25325.16	20058.76	20558.91	21209.23	20522.88 ± 1525.48
0.167	29184.54	27239.46	25893.56	28596.85	26611.53	24800.82	27054.46 ± 672.64
0.333	40797.50	53461.82	42782.05	37617.37	42029.04	62557.18	46540.83 ± 3879.53
0.667	36977.89	28189.24	28833.00	38725.14	30381.46	34098.13	32867.47 ± 1798.75
1	17585.31	21880.19	21887.60	14347.58	20948.32	20113.53	19460.42 ± 1211.67
2	13492.58	13337.72	11103.62	16842.91	15919.97	13377.56	14012.39 ± 841.43
4	9612.41	11927.93	10649.58	11978.68	7789.90	9651.24	10268.29 ± 652.65
6	4283.27	4203.08	3807.07	3693.89	2727.01	2740.85	3575.86 ± 281.60
8	2640.64	2760.05	3201.84	2851.36	2813.54	2619.55	2814.50 ± 86.14
12	1480.63	1392.76	1470.05	1526.33	1117.56	1201.42	1364.79 ± 68.11
24	225.25	285.15	213.15	178.01	385.27	187.08	245.65 ± 31.90
48	ND	ND	ND	ND	ND	ND	ND

Table 4.9: Plasma concentrations (ng/ml) of meloxicam following single dose administration (20 mg/kg, i.p) along with erlotinib (30 mg/kg, p.o) and metformin (100 mg/kg, p.o) in SCID male mice

Time (hr)	Plasma concentration (ng/ml)						
	Animal No.						Mean ± SEM
	M37	M38	M39	M40	M41	M42	
0.083	26234.31	23053.87	15910.69	26467.51	27610.95	33177.00	25409.06 ± 2329.21
0.167	26725.07	34319.56	26368.31	41760.14	39007.23	37793.88	34329.03 ± 2647.67
0.333	35715.40	38853.56	31881.46	46128.37	44582.76	40732.13	39648.94 ± 2190.42
0.667	28914.51	28287.88	27396.52	39542.51	32763.00	25903.09	30467.92 ± 2041.67
1	29894.80	25920.31	26941.55	30733.62	24855.05	19305.42	26275.12 ± 1674.75
2	21794.24	19257.06	20755.42	21258.02	18482.70	14866.46	19402.32 ± 1039.82
4	6919.96	9048.39	9066.50	9362.49	8639.73	12563.39	9266.74 ± 749.79
6	5118.10	5600.09	6536.76	4986.15	5304.28	7220.98	5794.39 ± 364.00
8	3753.20	3215.08	3073.75	3778.18	3216.04	3103.80	3356.67 ± 131.49
12	2436.88	2623.30	2814.69	2081.64	3288.97	2345.10	2598.43 ± 171.42
24	142.00	ND	184.58	ND	ND	ND	163.29 ± 12.29
48	ND	ND	ND	ND	ND	ND	ND

Table 4.10: Comparison of mean plasma concentrations (ng/ml) of meloxicam following single dose administration of meloxicam(20 mg/kg, i.p) alone and in combination with erlotinib and/or metformin in SCID male mice (n=6)

Time (hr)	Meloxicam concentration (ng/ml)			
	Meloxicam	Meloxicam + Erlotinib	Meloxicam + Metformin	Meloxicam + Erlotinib + Metformin
0.083	27405.65 ± 2701.73	26121.99 ± 3502.06	20522.88 ± 1525.48	25409.06 ± 2329.21
0.167	35627.24 ± 2734.69	39261.82 ± 3031.46	27054.46 ± 672.64	34329.03 ± 2647.67
0.333	44858.46 ± 3486.54	49994.17 ± 2928.14	46540.83 ± 3879.53	39648.94 ± 2190.42
0.667	38471.31 ± 3959.60	33785.40 ± 1433.29	32867.47 ± 1798.75	30467.92 ± 2041.67
1	25716.57 ± 1660.70	26425.00 ± 1228.12	19460.42 ± 1211.67*	26275.12 ± 1674.75
2	17438.25 ± 1296.19	24526.83 ± 1540.02**	14012.39 ± 841.43	19402.32 ± 1039.82
4	10660.24 ± 1381.76	19371.96 ± 658.52**	10268.29 ± 652.65	9266.74 ± 749.79
6	4070.66 ± 378.79	11869.03 ± 1141.80**	3575.86 ± 281.60	5794.39 ± 364.00
8	2386.33 ± 359.64	7030.75 ± 920.01**	2814.50 ± 86.14	3356.67 ± 131.49
12	1292.35 ± 124.97	3086.70 ± 221.80**	1364.79 ± 68.11	2598.43 ± 171.42**
24	160.24 ± 9.75	382.34 ± 106.23	245.65 ± 31.90	163.29 ± 12.29
48	ND	ND	ND	ND

**significant at p<0.01, *significant at p<0.05

Comparison of mean plasma concentration (ng/ml) of meloxicam alone and in combination with erlotinib and/or metformin is depicted in Table 4.10. The results showed that the mean peak concentration time 0.333h remained same in meloxicam alone and in combination with erlotinib and/or metformin. The mean peak plasma concentration of meloxicam was observed little higher in combination with erlotinib and metformin whereas lower in combination with both in comparison to alone. The concentration of meloxicam in plasma was observed till 24 hrs in meloxicam alone and combination with erlotinib and/or metformin. The plasma concentrations of meloxicam at 6 hrs post administration in combination with erlotinib remained considerably high in comparison to other two combinations and meloxicam alone treatment groups. The mean plasma concentration of meloxicam is significantly high in combination with erlotinib at 2h, 4h, 6h, 8h, 12h and in combination with all three drugs at 12h post administration. The comparative plasma concentration of meloxicam

in all four conditions (i.e meloxicam alone, meloxicam with erlotinib, meloxicam with metformin and meloxicam with erlotinib and metformin) is presented as semi logarithmic graph in Figure 4.2.

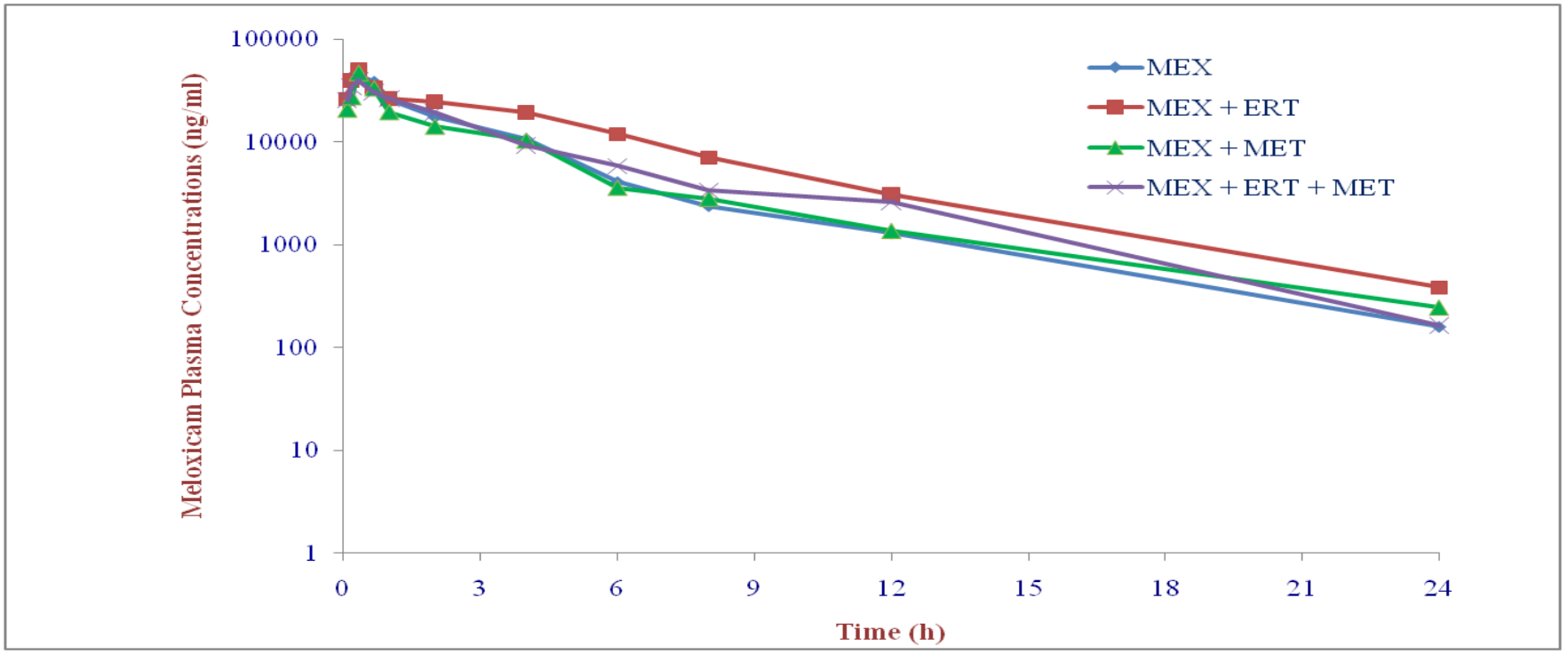

Figure 4.2: Semi logarithmic plot of meloxicam plasma concentration following single dose administration of meloxicam alone (20 mg/kg, i.p), in combination with erlotinib (30 mg/kg, p.o), in combination with metformin (100 mg/kg, p.o) and in combination with erlotinib (30 mg/kg, p.o) and metformin (100 mg/kg, p.o) in SCID male mice (n=6).

4.3 Plasma levels of metformin following oral administration as alone and in combination with erlotinib and/or meloxicam in male SCID mice

The plasma levels of metformin as a function of time after its single dose administration (100 mg/kg, p.o) alone and in combination with erlotinib (30 mg/kg, p.o) and/or meloxicam (20 mg/kg, i.p) in male SCID mice are depicted in Table 4.11 to 4.14.

Following oral administration of metformin alone, the mean peak plasma level of drug was observed 5782.29 ± 441.77 ng/ml at 0.33h, which slowly declined to 1142.31 ± 121.83 ng/ml at 4h and thereafter the drug concentration in plasma diminished fast and was detectable (202.32 ± 27.47 ng/ml) up to 12h only. In case of oral administration of metformin in combination with oral administration of erlotinib, the mean peak plasma level of drug was observed 6307.71 ± 577.57 ng/ml at 0.33h, which gradually declined to 1463.02 ± 208.69 ng/ml at 4h and finally reached to 166.02 ± 18.99 ng/ml at 12h. Same way oral administration of metformin in combination with intraperitoneal administration of meloxicam, the mean peak plasma level of metformin was observed 7724.44 ± 384.36 ng/ml at 0.67 hrs, which gradually declined to 2216.40 ± 292.12 ng/ml at 4 hrs and finally reached to 126.25 ± 11.12 ng/ml at 12h. The drug was not detected in plasma samples collected after 12h post oral administration of metformin along with meloxicam. When the metformin was administered along with erlotinib and meloxicam, the mean peak plasma level of metformin was observed 9490.58 ± 963.64 ng/ml at 0.33 hrs, which gradually declined to 2017.30 ± 186.74 ng/ml at 4 hrs and finally reached to 218.14 ± 36.09 ng/ml at 12h. The plasma drug concentration was not detected in the samples collected after 12 hrs post oral administration of metformin alone, metformin with erlotinib orally, metformin with meloxicam intraperitoneally, and metformin along with both erlotinib and meloxicam.

Table 4.11: Plasma concentrations (ng/ml) of metformin following single dose administration (100 mg/kg, p.o) in SCID male mice

Time	Plasma concentration (ng/ml)						
	Animal No.						Mean ± SEM
	M13	M14	M15	M16	M17	M18	
0.083	2883.39	1524.43	2909.47	2331.97	2494.00	1746.81	2315.01 ± 234.99
0.167	4123.92	2715.03	2567.95	3434.56	2006.38	3200.32	3008.03 ± 302.60
0.333	5705.46	5461.62	6748.68	5894.62	6952.91	3930.44	5782.29 ± 441.77
0.667	4036.74	7104.58	6377.26	6217.10	5464.42	4397.15	5599.54 ± 488.46
1	6246.05	2464.87	6317.26	5624.13	2464.00	4155.54	4545.31 ± 730.48
2	2688.22	1268.44	2110.41	3019.65	4311.67	1491.92	2481.72 ± 457.18
4	1011.47	866.35	1247.12	1512.06	790.65	1426.21	1142.31 ± 121.83
6	351.05	522.05	519.11	671.89	637.97	857.74	593.30 ± 70.09
8	272.52	251.68	395.93	479.79	253.41	423.41	346.12 ± 40.52
12	216.79	220.75	190.91	150.25	120.71	314.51	202.32 ± 27.47
24	ND	ND	ND	ND	ND	ND	ND
48	ND	ND	ND	ND	ND	ND	ND

Table 4.12: Plasma concentrations (ng/ml) of metformin following single dose administration (100 mg/kg, p.o) along with erlotinib (30 mg/kg, p.o) in SCID male mice

Time	Plasma concentration (ng/ml)						
	Animal No.						Mean ± SEM
	M25	M26	M27	M28	M29	M30	
0.083	2462.57	4379.76	3337.60	4887.97	5498.00	3630.77	4032.78 ± 451.26
0.167	7492.84	4644.90	5057.84	8389.01	6910.35	3962.74	6076.28 ± 721.21
0.333	6152.10	5697.80	7344.14	5258.89	8585.38	4807.96	6307.71 ± 577.57
0.667	5394.36	5714.92	6891.78	5189.61	5707.32	5977.62	5812.60 ± 243.28
1	4435.53	4933.93	3856.80	4654.59	4093.86	3356.25	4221.83 ± 233.73
2	2804.97	3925.58	2568.48	2538.54	2378.57	2494.86	2785.16 ± 235.11
4	1026.25	1453.38	1421.93	1046.70	1399.06	2430.79	1463.02 ± 208.69
6	500.63	542.11	670.00	560.44	488.56	358.05	519.97 ± 41.72
8	160.87	279.79	561.32	519.29	517.39	269.92	384.76 ± 68.60
12	ND	ND	ND	190.58	112.36	195.13	166.02 ± 18.99
24	ND	ND	ND	ND	ND	ND	ND
48	ND	ND	ND	ND	ND	ND	ND

Table 4.13: Plasma concentrations (ng/ml) of metformin following single dose administration (100 mg/kg, p.o) along with meloxicam (20 mg/kg, i.p) in SCID male mice

Time	Plasma concentration (ng/ml)						
	Animal No.						Mean ± SEM
	M31	M32	M33	M34	M35	M36	
0.083	2893.27	2738.32	2316.44	3942.67	5275.06	6252.91	3903.11 ± 640.30
0.167	3711.17	4620.22	4052.08	4441.71	6677.49	9672.05	5529.12 ± 930.55
0.333	4992.35	4927.93	9538.50	6711.73	7660.19	10011.27	7306.99 ± 930.55
0.667	7053.47	7578.34	7008.32	8797.30	9001.17	6908.06	7724.44 ± 384.36
1	5686.53	5669.03	7046.41	7218.44	6847.76	8268.54	6789.45 ± 404.68
2	3287.17	4186.83	3171.23	4765.43	5382.46	4868.75	4276.98 ± 366.12
4	1956.51	1334.68	2896.56	2309.89	3167.16	1633.57	2216.40 ± 292.12
6	519.28	508.22	578.08	551.08	695.85	493.81	557.72 ± 30.30
8	255.11	318.43	285.68	217.78	222.42	315.38	269.13 ± 18.13
12	ND	124.15	129.36	ND	ND	125.24	126.25 ± 1.12
24	ND	ND	ND	ND	ND	ND	ND
48	ND	ND	ND	ND	ND	ND	ND

Table 4.14: Plasma concentrations (ng/ml) of metformin following single dose administration (100 mg/kg, p.o) along with erlotinib (30 mg/kg, p.o) and meloxicam (20 mg/kg, i.p) in SCID male mice

Time	Plasma concentration (ng/ml)						
	Mice number						Mean ± SEM
	M37	M38	M39	M40	M41	M42	
0.083	4099.33	4155.18	4346.89	5415.61	6210.81	4933.97	4860.30 ± 340.47
0.167	5725.81	5117.24	5184.99	4210.81	9133.21	5210.64	5763.78 ± 702.97
0.333	9211.39	8421.16	9414.78	12191.16	11895.77	5809.19	9490.58 ± 963.64
0.667	6265.81	6079.48	6516.69	7110.78	5896.28	3591.86	5910.15 ± 494.61
1	6773.04	5375.85	5855.49	5932.82	5371.66	3419.80	5454.78 ± 457.57
2	3000.97	5130.50	5497.16	6064.92	4181.40	3724.96	4599.99 ± 473.64
4	2719.45	2032.36	2397.10	1587.15	1709.10	1658.66	2017.30 ± 186.74
6	1055.98	556.62	474.17	808.34	885.67	536.19	719.49 ± 94.70
8	426.41	492.87	396.01	701.10	846.10	461.66	554.02 ± 73.14
12	136.17	338.37	ND	154.59	284.29	177.29	218.14 ± 36.09
24	ND	ND	ND	ND	ND	ND	ND
48	ND	ND	ND	ND	ND	ND	ND

Table 4.15: Comparison of mean plasma concentrations (ng/ml) of metformin (100 mg/kg) following single dose administration of metformin (100 mg/kg, p.o) alone and in combination with erlotinib and/or meloxicam in SCID male mice (n=6)

Time (hr)	Metformin concentration (ng/ml)			
	Metformin	Metformin + Erlotinib	Metformin + Meloxicam	Metformin + Erlotinib + Meloxicam
0.083	2315.01 ± 234.99	4032.78 ± 451.26	3903.11 ± 640.30	4860.30 ± 340.47**
0.167	3008.03 ± 302.60	6076.28 ± 721.21*	5529.12 ± 930.55	5763.78 ± 702.97
0.333	5782.29 ± 441.77	6307.71 ± 577.57	7306.99 ± 930.55	9490.58 ± 963.64*
0.667	5559.27 ± 509.49	5812.60 ± 243.28	7724.44 ± 384.36**	5910.15 ± 494.61
1	4101.37 ± 894.37	4221.83 ± 233.73	6789.45 ± 404.68*	5454.78 ± 457.57
2	2965.92 ± 501.76	2785.16 ± 235.11	4276.98 ± 366.12	4599.99 ± 473.64*
4	1142.31 ± 121.83	1463.02 ± 208.69	2216.40 ± 292.12**	2017.30± 186.74*
6	593.30 ± 70.09	519.97 ± 41.72	557.72 ± 30.30	719.49 ± 94.70
8	346.12 ± 40.52	384.76 ± 68.60	269.13 ± 18.13	554.02 ± 73.14
12	202.32 ± 27.47	166.02 ± 18.99	126.25 ± 1.12	218.14 ± 36.09
24	ND	ND	ND	ND
48	ND	ND	ND	ND

**significant at $p<0.01$, *significant at $p<0.05$

Comparison of mean plasma concentration (ng/ml) of metformin alone and in combination with erlotinib and/or meloxicam is depicted in Table 4.15. The results showed that the mean peak concentration time of metformin in combination with meloxicam was observed at 0.667h, whereas the mean peak concentration time in metformin alone and in combination with erlotinib and all three drugs together remained at 0.33h. The mean peak plasma concentration value of metformin was observed significantly higher in combination with meloxicam and all three drug together, whereas little higher in combination with erlotinib in comparison to alone. The concentration of metformin in plasma was observed till 12h in metformin alone and combination with erlotinib and/or metformin. The plasma concentrations of metformin at 4h and 6h post administration in combination with meloxicam and all three drugs together remained considerably high in comparison to metformin alone and metformin with erlotinib treatment groups. The mean plasma concentration of metformin was significantly high in combination

with erlotinib at 0.167h, in combination with meloxicam at 0.667h, 1h, 4h and in combination with all three drug together at 0.0833h, 0.33h, 2h and 4h post administration of drug. The comparative plasma concentration of metformin in all four conditions (i.e metformin alone, metformin with erlotinib, metformin with meloxicam and metformin with erlotinib and meloxicam) is presented as semi logarithmic graph in Figure 4.3.

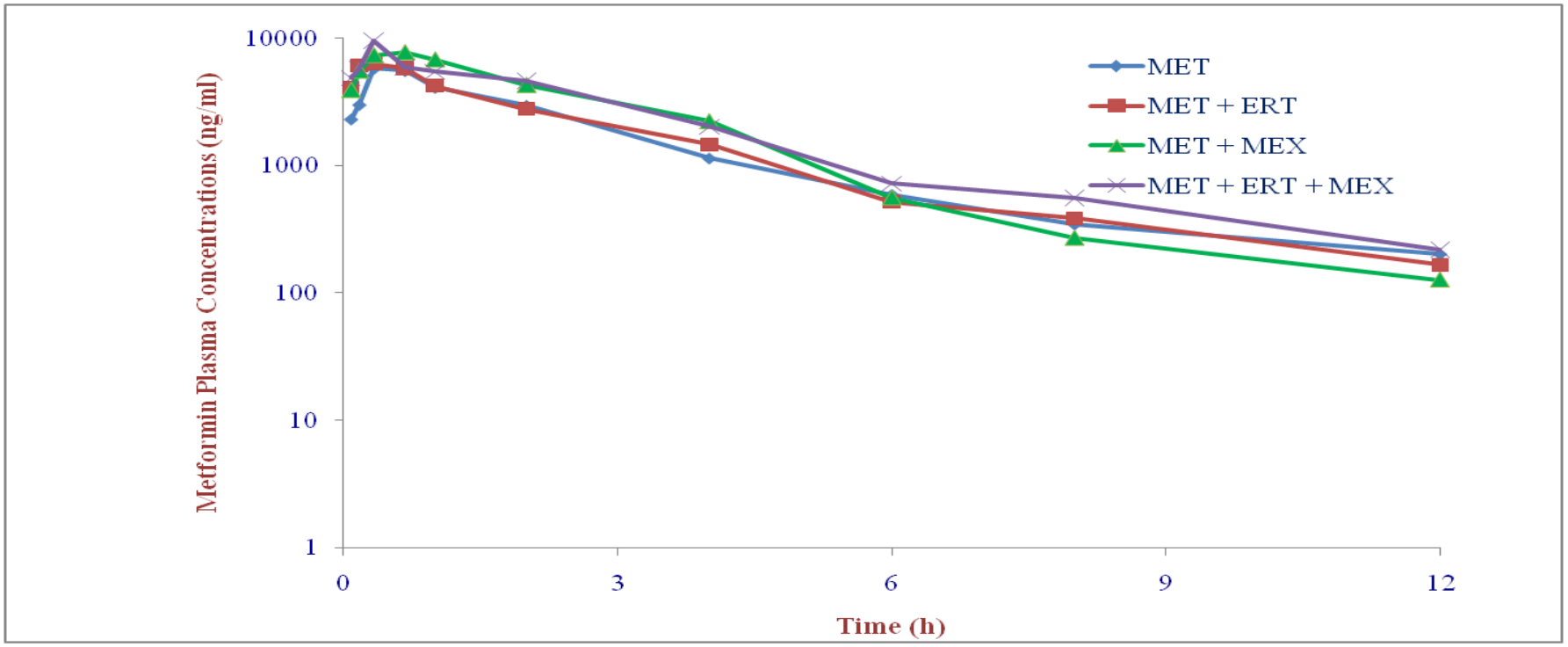

Figure 4.3: Semi logarithmic plot of metformin plasma concentration following single dose administration of metformin alone (100 mg/kg, p.o), in combination with erlotinib (30 mg/kg, p.o), in combination with meloxicam (20 mg/kg, i.p) and in combination with erlotinib (30 mg/kg, p.o) and meloxicam (20 mg/kg, i.p) in SCID male mice (n=6).

4.4 Pharmacokinetic parameters of erlotinib following oral administration as alone and in combination with meloxicam and/or metformin in SCID male mice

Various pharmacokinetic parameters calculated from plasma concentration – time profile after single dose administration of erlotinib (30 mg/kg, p.o) alone and in combination with meloxicam (20 mg/kg, i.p.) and/or metformin (100 mg/kg, p.o) in male SCID mice are depicted in Table 4.16 to 4.19. The comparison of the calculated parameter for erlotinib alone and in combination with meloxicam and/or metformin is depicted in Table 4.20.

The results showed that the calculated T_{max} remained lowest in erlotinib with meloxicam and all three drugs together treatment groups in comparison to erlotinib with metformin and erlotinib alone. The calculated C_{max} of erlotinib was observed highest in combination with meloxicam treatment group when compared to other treatment groups. The $AUC_{(0-\infty)}$ was significantly low in treatment group of erlotinib with metformin ($p<0.05$) and erlotinib with meloxicam and metformin ($p<0.01$). The calculated clearance rate was also observed significantly higher in treatment group of erlotinib with metformin ($p<0.01$) and all three drugs together ($p<0.01$) in comparison to erlotinib alone treatment group.

4.5 Pharmacokinetic parameter of meloxicam following intraperitoneal administration as alone and in combination with erlotinib and/or metformin in SCID male mice

Various pharmacokinetic parameters calculated from plasma concentration – time profile after single dose administration of meloxicam (20 mg/kg, i.p) alone and in combination with administration of erlotinib (30 mg/kg, p.o) or metformin (100 mg/kg, p.o) in male SCID mice are depicted in Table 4.21to 4.24.

The comparison of the calculated parameter for meloxicam alone and in combination with erlotinib and/or metformin is depicted in Table 4.25.

The results showed that the calculated Tmax remained lower in meloxicam with erlotinib and all three drugs together treatment group in comparison to meloxicam with metformin and meloxicam alone treatment group. The calculated C_{max} of meloxicam was observed highest in combination with erlotinib treatment group in comparison to other treatment groups. The $AUC_{(0-\infty)}$ was significantly high in treatment group of meloxicam with erlotinib ($p<0.01$) in comparison to the meloxicam alone treatment group. The calculated clearance rate was also observed significantly lower in treatment group of meloxicam with erlotinib ($p<0.05$) and all three drugs together ($p<0.05$) in comparison to meloxicam alone treatment group. The volume of distribution was also significantly low in meloxicam with erlotinib treatment group whereas significantly high in meloxicam with metformin group in comparison to meloxicam alone treatment group. The MRT was also significantly higher in meloxicam with erlotinib treatment group in comparison to meloxicam alone treatment group.

4.6 Pharmacokinetic parameter of metformin following oral administration as alone and in combination with erlotinib and/or meloxicam in male SCID mice

Various pharmacokinetic parameters calculated from plasma concentration – time profile after single dose administration of metformin (100 mg/kg, p.o) alone and in combination with administration of erlotinib (30 mg/kg, p.o) and/or meloxicam (20 mg/kg, i.p) in male SCID mice are depicted in Table 4.26 to 4.29. The comparison of the calculated parameter for metformin alone and in combination with erlotinib and/or meloxicam is depicted in Table 4.30.

The result showed that the calculated T_{max} remained lower in metformin with erlotinib and all three drugs together treatment group in comparison to metformin with meloxicam and metformin alone treatment group. The calculated C_{max} of metformin was observed higher in metformin with meloxicam and all three drugs together group in comparison to other treatment groups. The AUC $_{(0-\infty)}$ was significantly high in treatment group of metformin with meloxicam ($p<0.01$) and all three drugs together ($p<0.01$) in comparison to the meloxicam alone treatment group. The calculated clearance rate was also observed significantly lower in treatment group of metformin with meloxicam ($p<0.05$) and all three drugs together ($p<0.05$) in comparison to meloxicam alone treatment group. The volume of distribution was also significantly low in all treatment groups in comparison to metformin alone treatment group. The half life of metformin with meloxicam was significantly lower in comparison to metformin alone treatment group.

Table 4.16: Pharmacokinetic parameters of erlotinib following single dose administration of erlotinib (30 mg/kg, p.o) in male SCID mice

PK parameters	Animal No.						Mean ± SEM
	M1	M2	M3	M4	M5	M6	
T_{max} (h)	0.17	0.33	0.33	0.33	0.33	0.67	0.29 ± 0.09
C_{max} (ng/ml)	19918.76	16336.69	18167.51	16088.43	15778.51	17345.65	17272.59 ± 640.80
$T_{1/2}$ (h)	3.42	5.56	6.44	6.75	6.91	7.43	6.08 ± 0.59
$AUC_{(0-\infty)}$ (h.ng/ml)	72540.63	90730.01	82152.74	81225.58	87415.62	94643.56	84784.69 ± 3209.24
Vz (ml)	2042.63	2653.38	3391.40	3594.13	3419.15	3399.50	3083.37 ± 247.42
Cl (ml/hr)	413.56	330.65	365.17	369.34	343.19	316.98	356.48 ± 14.03
MRT (h)	3.36	5.49	5.25	5.61	5.74	5.41	5.14 ± 0.36

Table 4.17: Pharmacokinetic parameters of erlotinib following single dose administration of erlotinib (30 mg/kg, p.o) along with meloxicam (20 mg/kg, i.p) in male SCID mice

PK parameters	Animal No.						Mean ± SEM
	M19	M20	M21	M22	M23	M24	
T_{max} (h)	0.17	1.0	0.33	0.17	0.17	0.33	0.21 ± 0.04
C_{max} (ng/ml)	26540.75	17620.15	16141.42	26719.33	15717.74	16452.46	19865.31 ± 2154.82
$T_{1/2}$ (h)	9.80	10.64	6.28	13.23	3.27	6.24	8.24 ± 1.48
$AUC_{(0-\infty)}$ (h.ng/ml)	97846.01	81045.30	79898.96	117005.14	88075.44	82470.64	91056.92 ± 5850.21
Vz (ml)	4336.51	5680.97	3403.03	4892.56	1606.08	3273.50	3865.44 ± 584.29
Cl (ml/hr)	306.60	370.16	375.47	256.40	340.62	363.77	335.50 ± 18.90
MRT (h)	6.55	6.26	4.88	8.51	4.75	5.76	6.12 ± 0.56

Table 4.18: Pharmacokinetic parameters of erlotinib following single dose administration of erlotinib (30 mg/kg, p.o) along with metformin (100 mg/kg, p.o) in male SCID mice

PK parameters	Animal No.						Mean ± SEM
	M25	M26	M27	M28	M29	M30	
T_{max} (h)	0.17	0.17	0.17	0.33	0.33	0.083	0.27 ± 0.09
C_{max} (ng/ml)	11571.01	15510.87	16324.61	14325.09	13634.62	11674.46	13840.11 ± 797.51
$T_{1/2}$ (h)	3.71	3.75	3.69	5.72	3.92	7.95	4.79 ± 0.71
$AUC_{(0-\infty)}$ (h.ng/ml)	60965.48	60714.26	64964.42	72839.03	59177.49	80297.94	66493.10 ± 3418.82
Vz (ml)	2632.04	2673.20	2456.53	3398.86	2866.33	4285.88	3052.14 ± 279.98
Cl (ml/hr)	492.08	494.12	461.79	411.87	506.95	373.61	456.74 ± 21.69
MRT (h)	5.26	3.33	3.72	5.22	3.58	6.64	4.63 ± 0.53

Table 4.19: Pharmacokinetic parameters of erlotinib following single dose administration of erlotinib (30 mg/kg, p.o) along with meloxicam (20 mg/kg, i.p) and metformin (30 mg/kg, p.o) in male SCID mice

PK parameters	Animal No.						Mean ± SEM
	M37	M38	M39	M40	M41	M42	
T_{max} (h)	0.33	0.17	0.33	0.17	0.33	0.17	0.21 ± 0.04
C_{max} (ng/ml)	13824.83	12230.83	16022.83	15378.17	14807.61	18217.45	15080.29 ± 828.43
$T_{1/2}$ (h)	7.86	6.86	7.19	3.26	8.18	6.42	6.63 ± 0.72
$AUC_{(0-\infty)}$ (h.ng/ml)	58687.85	59394.73	68369.23	54849.82	69891.94	63522.62	62452.70 ± 2400.15
Vz (ml)	5799.81	4998.49	4553.92	2569.16	5062.44	4372.81	4559.44 ± 446.48
Cl (ml/hr)	511.18	505.10	438.79	546.95	429.23	472.27	483.92 ± 18.55
MRT (h)	5.14	5.72	6.19	3.53	5.62	4.75	5.16 ± 0.38

Table 4.20: Comparison of Pharmacokinetic parameter of erlotinib following single dose administration of erlotinib (30 mg/kg, p.o) and in combination with meloxicam (20 mg/kg, i.p) and/or metformin (100 mg/kg, p.o) in male SCID mice

PK parameters	Erlotinib	Erlotinib + Meloxicam	Erlotinib + Metformin	Erlotinib + Meloxicam + Metformin
T_{max} (h)	0.29 ± 0.09	0.21 ± 0.04	0.27 ± 0.09	0.21 ± 0.04
C_{max} (ng/ml)	17272.59 ± 640.80	19865.31 ± 2154.82	13840.11 ± 797.51	15080.29 ± 828.43
$T_{1/2}$ (h)	6.08 ± 0.59	8.24 ± 1.48	4.79 ± 0.71	6.63 ± 0.72
$AUC_{(0-\infty)}$ (h.ng/ml)	84784.69 ± 3209.24	91056.92 ± 5850.21	66493.10 ± 3418.82*	62452.70 ± 2400.15**
Vz (ml)	3083.37 ± 247.42	3865.44 ± 584.29	3052.14 ± 279.98	4559.44 ± 446.48
Cl (ml/hr)	356.48 ± 14.03	335.50 ± 18.90	456.74 ± 21.69**	483.92 ± 18.55**
MRT (h)	5.14 ± 0.36	6.12 ± 0.56	4.63 ± 0.53	5.16 ± 0.38

**significant at $p<0.01$, *significant at $p<0.05$

Table 4.21: Pharmacokinetic parameters of meloxicam following single dose administration (20 mg/kg, i.p) in male SCID mice

PK parameters	Animal No.						Mean ± SEM
	M7	M8	M9	M10	M11	M12	
T_{max} (h)	0.33	0.33	0.33	0.67	0.67	0.33	0.42 ± 0.09
C_{max} (ng/ml)	45600.94	54552.91	38382.21	55862.04	44062.42	35830.77	45715.22 ± 3343.07
$T_{1/2}$ (h)	4.42	4.52	2.22	3.81	4.13	3.61	3.79 ± 0.34
$AUC_{(0-\infty)}$ (h.ng/ml)	133855.55	133408.11	110115.66	124318.24	129209.19	113092.92	123999.94 ± 4181.79
Vz (ml)	953.58	977.77	582.89	884.90	922.24	922.06	873.90 ± 59.61
Cl (ml/hr)	149.41	149.92	181.63	160.88	154.79	176.85	162.24 ± 5.67
MRT (h)	3.08	3.21	2.82	3.37	3.69	3.76	3.32 ± 0.15

Table 4.22: Pharmacokinetic parameters of meloxicam following single dose administration (20 mg/kg, i.p.) along with administration of erlotinib (30 mg/kg, p.o) in male SCID mice

PK parameters	Animal No.						Mean ± SEM
	M19	M20	M21	M22	M23	M24	
T_{max} (h)	0.33	0.33	0.33	0.33	0.33	0.33	0.33 ± 0.00
C_{max} (ng/ml)	48556.39	51259.32	53417.40	38804.60	60487.19	47440.12	49994.17 ± 2928.14
$T_{1/2}$ (h)	2.99	2.95	3.01	4.10	2.91	5.10	3.51 ± 0.37
$AUC_{(0-\infty)}$ (h.ng/ml)	200250.47	196613.40	201681.61	200482.12	214207.14	168458.56	196948.88 ± 6206.02
Vz (ml)	430.26	432.96	430.42	589.81	391.34	873.95	524.79 ± 75.33
Cl (ml/hr)	99.87	101.72	99.17	99.76	93.37	118.72	102.10 ± 3.52
MRT (h)	3.71	3.83	4.43	4.95	4.61	4.46	4.33 ± 0.19

Table 4.23: Pharmacokinetic parameters of meloxicam following single dose administration (20 mg/kg, i.p.) along with administration of metformin (100 mg/kg, p.o) in male SCID mice

PK parameters	Animal No.						Mean ± SEM
	M31	M32	M33	M34	M35	M36	
T_{max} (h)	0.33	0.33	0.33	0.67	0.33	0.33	0.39 ± 0.06
C_{max} (ng/ml)	40797.50	53461.82	42782.05	38725.14	42029.04	62557.18	46725.46 ± 3798.13
$T_{1/2}$ (h)	4.34	4.74	4.24	4.06	6.09	4.47	4.66 ± 0.30
$AUC_{(0-\infty)}$ (h.ng/ml)	111609.54	120086.69	111248.75	118188.75	109311.66	112469.99	113819.23 ± 1751.21
Vz (ml)	1121.31	1139.66	1099.12	991.89	1607.09	1146.10	1184.20 ± 87.65
Cl (ml/hr)	179.20	166.55	179.78	169.22	182.96	177.83	175.92 ± 2.66
MRT (h)	3.91	3.84	3.99	3.87	3.76	3.44	3.80 ± 0.08

Table 4.24: Pharmacokinetic parameters of meloxicam following single dose administration (20 mg/kg, i.p.) along with erlotinib (30 mg/kg, p.o) and metformin (100 mg/kg, p.o) in male SCID mice

PK parameters	Animal No.						Mean ± SEM
	M37	M38	M39	M40	M41	M42	
T_{max} (h)	0.33	0.33	0.33	0.33	0.33	0.33	0.33 ± 0.00
C_{max} (ng/ml)	35715.40	38853.56	31881.46	46128.37	44582.76	40732.13	39648.95 ± 2190.41
$T_{1/2}$ (h)	3.43	4.52	3.63	3.87	5.91	3.27	4.10 ± 0.40
$AUC_{(0-\infty)}$ (h.ng/ml)	134236.47	134419.64	137243.99	142308.68	147468.88	127457.44	137189.18 ± 2845.93
Vz (ml)	736.32	969.87	763.78	783.93	1155.38	740.02	858.22 ± 69.29
Cl (ml/hr)	148.99	148.79	145.73	140.54	135.62	156.92	146.10 ± 3.02
MRT (h)	4.17	3.11	4.49	2.86	3.12	3.31	3.51 ± 0.27

Table 4.25: Comparison of Pharmacokinetic parameter of meloxicam following single dose administration of meloxicam (20 mg/kg, i.p) and in combination with erlotinib (30 mg/kg, p.o) and/or metformin (100 mg/kg, p.o) in male SCID mice

PK parameters	Meloxicam	Meloxicam + Erlotinib	Meloxicam + Metformin	Meloxicam + Erlotinib + Metformin
T_{max} (h)	0.40 ± 0.09	0.33 ± 0.00	0.39 ± 0.06	0.33 ± 0.00
C_{max} (ng/ml)	45715.22 ± 3343.07	49994.17 ± 2928.14	46725.46 ± 3798.13	39648.95 ± 2190.41
$T_{1/2}$ (h)	3.79 ± 0.34	3.51 ± 0.37	4.66 ± 0.30	4.10 ± 0.40
$AUC_{(0-\infty)}$ (h.ng/ml)	123999.94 ± 4181.79	196948.88 ± 6206.02**	113819.23 ± 1751.21	137189.18 ± 2845.93
Vz (ml)	873.90 ± 59.61	524.79 ± 75.33*	1184.20 ± 87.65*	858.22 ± 69.29
Cl (ml/hr)	162.24 ± 5.67	102.10 ± 3.52**	175.92 ± 2.66	146.10± 3.02*
MRT (h)	3.32 ± 0.15	4.33 ± 0.19**	3.80 ± 0.08	3.51 ± 0.27

**significant at $p<0.01$, *significant at $p<0.05$

Table 4.26: Pharmacokinetic parameters of metformin following single dose administration (100 mg/kg, p.o) in male SCID mice

PK parameters	Animal No.						Mean ± SEM
	M13	M14	M15	M16	M17	M18	
T_{max} (h)	1.00	0.67	0.33	0.67	0.33	0.67	0.61 ± 0.10
C_{max} (ng/ml)	6246.05	7104.58	6748.68	6217.10	6952.91	4397.15	6277.75 ± 404.25
$T_{1/2}$ (h)	4.04	4.04	3.15	2.49	2.79	3.65	3.36 ± 0.27
$AUC_{(0-\infty)}$ (h.ng/ml)	17196.21	13259.95	17964.74	19346.90	16732.72	18812.46	17218.83 ± 885.71
Vz (ml)	33925.51	43912.07	25287.25	18559.54	24029.76	27987.81	28950.33 ± 3629.19
Cl (ml/hr)	5815.24	7541.51	5566.46	5168.79	5976.32	5315.62	5897.32 ± 350.95
MRT (h)	2.37	2.68	2.51	2.66	2.43	3.20	2.64 ± 0.12

Table 4.27: Pharmacokinetic parameters of metformin following single dose administration (100 mg/kg, p.o) along with erlotinib (30 mg/kg, p.o) in male SCID mice

PK parameters	Animal No.						Mean ± SEM
	M25	M26	M27	M28	M29	M30	
T_{max} (h)	0.17	0.67	0.33	0.17	0.33	0.67	0.39 ± 0.09
C_{max} (ng/ml)	7492.84	5714.92	7344.14	8389.01	8585.38	5977.62	7250.65 ± 487.30
$T_{1/2}$ (h)	1.49	1.56	2.61	3.43	2.36	2.53	2.33 ± 0.30
$AUC_{(0-\infty)}$ (h.ng/ml)	15562.32	18535.52	18642.40	17742.53	17926.53	17625.56	17672.48 ± 454.97
Vz (ml)	13828.61	12110.27	20185.48	27868.67	19015.37	20736.01	18957.40 ± 2289.64
Cl (ml/hr)	6425.78	5395.05	5364.12	5636.18	5578.33	5673.58	5678.84 ± 158.06
MRT (h)	1.89	2.08	2.21	2.66	2.52	2.75	2.35 ± 0.14

Table 4.28: Pharmacokinetic parameters of metformin following single dose administration (100 mg/kg, p.o) along with meloxicam (20 mg/kg, i.p) in male SCID mice

PK parameters	Animal No.						Mean ± SEM
	M31	M32	M33	M34	M35	M36	
T_{max} (h)	0.67	0.67	0.33	0.67	0.67	0.33	0.56 ± 0.07
C_{max} (ng/ml)	7053.47	7578.34	9538.50	8797.30	9001.17	10011.27	8663.34 ± 464.89
$T_{1/2}$ (h)	1.54	2.44	1.91	1.30	1.25	2.29	1.79 ± 0.21
$AUC_{(0-\infty)}$ (h.ng/ml)	18994.29	20171.06	23725.53	24046.59	27465.35	25635.64	23339.74 ± 1314.84
Vz (ml)	11706.27	17429.11	11588.49	7780.31	6575.41	12898.16	11329.62 ± 1581.91
Cl (ml/hr)	5264.74	4957.60	4214.87	4158.59	3640.95	3900.82	4356.26 ± 255.94
MRT (h)	2.15	2.33	2.43	2.06	2.18	2.07	2.20 ± 0.06

Table 4.29: Pharmacokinetic parameters of metformin following single dose administration (100 mg/kg, p.o) along with erlotinib (30 mg/kg, p.o) and meloxicam (20 mg/kg, i.p) in male SCID mice

PK parameters	Animal No.						Mean ± SEM
	M37	M38	M39	M40	M41	M42	
T_{max} (h)	0.33	0.33	0.33	0.33	0.33	0.33	0.33 ± 0.00
C_{max} (ng/ml)	9211.39	8421.16	9414.78	12191.16	11895.77	5809.19	9490.58 ± 963.64
$T_{1/2}$ (h)	2.08	3.55	1.46	2.46	3.26	2.69	2.58 ± 0.31
$AUC_{(0-\infty)}$ (h.ng/ml)	24355.65	25791.76	25022.91	27620.74	26581.47	18575.70	24658.04 ± 1303.61
Vz (ml)	12315.56	19858.65	8402.65	12829.97	17704.22	20918.03	15338.18 ± 2005.80
Cl (ml/hr)	4105.82	3877.21	3996.34	3620.47	3762.02	5383.38	4124.21 ± 261.29
MRT (h)	2.66	2.66	2.10	2.51	2.78	2.79	2.59 ± 0.11

Table 4.30: Comparison of Pharmacokinetic parameter of metformin following single dose administration of metformin (100 mg/kg, p.o) and in combination with erlotinib (30 mg/kg, p.o) and/or meloxicam (20 mg/kg, i.p) in male SCID mice

PK parameters	Metformin	Metformin + Erlotinib	Metformin + Meloxicam	Metformin + Erlotinib + Meloxicam
T_{max} (h)	0.61 ± 0.10	0.39 ± 0.09	0.56 ± 0.07	0.33 ± 0.00
C_{max} (ng/ml)	6277.75 ± 404.25	7250.65 ± 487.30	8663.34 ± 464.89	9490.58 ± 963.64**
$T_{1/2}$ (h)	3.36 ± 0.27	2.33 ± 0.30	1.79 ± 0.21**	2.58 ± 0.31
$AUC_{(0-\infty)}$ (h.ng/ml)	17218.83 ± 885.71	17672.48 ± 454.97	23339.74 ± 1314.84**	24658.04 ± 1303.61**
Vz (ml)	28950.33 ± 3629.19	18957.40 ± 2289.64*	11329.62 ± 1581.91**	15338.18 ± 2005.80**
Cl (ml/hr)	5897.32 ± 350.95	5678.84 ± 158.06	4356.26 ± 255.94 **	4124.21 ± 261.29**
MRT (h)	2.64 ± 0.12	2.35 ± 0.14	2.20 ± 0.06	2.59 ± 0.11

**significant at $p<0.01$, *significant at $p<0.05$

4.2 Studies on assessment of pharmaco-toxicological effect of erlotinib (30 mg/kg), meloxicam (20 mg/kg) and metformin (100 mg/kg) alone and in combination with each other and all three drugs together

4.2.1 Effect on Clinical symptoms and mortality

Animals of all the groups were observed daily for physical and behavioral changes up to 28 days of treatment period. Few animals of group VI (ERT+ MEX) and IX (ERT+ MEX + MET) revealed clinical signs such as piloerection, staring eyes, hunched posture, uncoordinated gait attributable to administration of erlotinib in combination with meloxicam. Two animals from group VI (ERT+ MEX) were found dead on day 21st and 22nd; one animal died on day 23rd in group VII (ERT + MET) and two animals from group IX (ERT+ MEX + MET) found dead on day 20th and 22nd during treatment period. Animals of other treatment groups were found clinically normal throughout the study period.

4.2.2 Effect on Tumor volume

The tumor volume of each animal in tumor control and all treatment groups were measured once a week. The dimension of tumor was measured by digital vernier caliper and the volume was calculated by [(width^{2}X length)/2] for development of tumor. The grouping was done based on mean tumor volume on day 0.

The effect of erlotinib, meloxicam and metformin administration as alone and in combination with each other for 28 days on mean tumor volume (mm^3) in xenograft model of SCID male mice is depicted in Table 4.31. The tumor volume was measured on day 0, 7, 14, 21, and 28th day during the treatment period. A considerable reduction was observed in tumor volume of treatment group compared to the tumor control. The tumor size have shown the reducing trend in tumor volume growth on 1st, 2nd ,3rd and 4th week in treatment group compared to the tumor control group except group

V. It was observed that growing pattern of tumor was high during 3rd and 4th week. On 28th day, the tumor size of control group was 1718.66 ± 561.01 mm^3 which is more than 10 times the size on day 0. On 28th day, the approx. tumor size of groups III, IV and V was 7 to 8 times higher whereas in groups VI, VII, VIII and IX was 5 to 6 times higher than it's size on day 0. The linear development of tumor volume and its comparison within group II to IX is presented as graphical form in Figure 4.4

Table 4.31: Effect of erlotinib, meloxicam and metformin administration as alone and in combination with each other for 28 days on mean tumor volume (mm^3) in xenograft model of SCID male mice (n=6)

Days	0	7	14	21	28
Group-I (Normal control)	NA	NA	NA	NA	NA
Group-II (Tumor control)	149.05 ± 14.30	545.39 ± 257.14	677.51 ± 202.93	1333.28 ± 502.75	1718.66 ± 561.01
Group-III (ERT)	142.28 ± 32.54	227.14 ± 51.70	368.05 ± 102.38	650.02 ± 94.43	939.99 ± 10.70
Group-IV (MEX)	177.70 ± 12.55	447.94 ± 54.58	596.12 ± 93.00	868.29 ± 130.38	1262.03 ± 5.80
Group-V (MET)	186.82 ± 21.63	495.49 ± 114.48	638.17 ± 117.15	1181.84 ± 281.60	1535.54 ± 251.80
Group-VI (ERT+MEX)	151.32 ± 56.51	193.58 ± 35.52	354.49 ± 75.30	433.39 ± 144.08	869.72 ± 54.51
Group-VII (ERT+MET)	145.25 ± 25.24	315.06 ± 42.50	391.46 ± 39.81	661.69 ± 102.78	912.13 ± 99.06
Group-VIII (MET +MEX)	169.50 ± 49.33	231.06 ± 62.00	391.98 ± 81.78	648.95 ± 99.23	1010.22 ± 69.47
Group-IX (ERT+MEX+MET)	151.13 ± 14.58	182.17 ± 19.46	285.84 ± 98.18	412.86 ± 43.93	805.98 ± 36.93

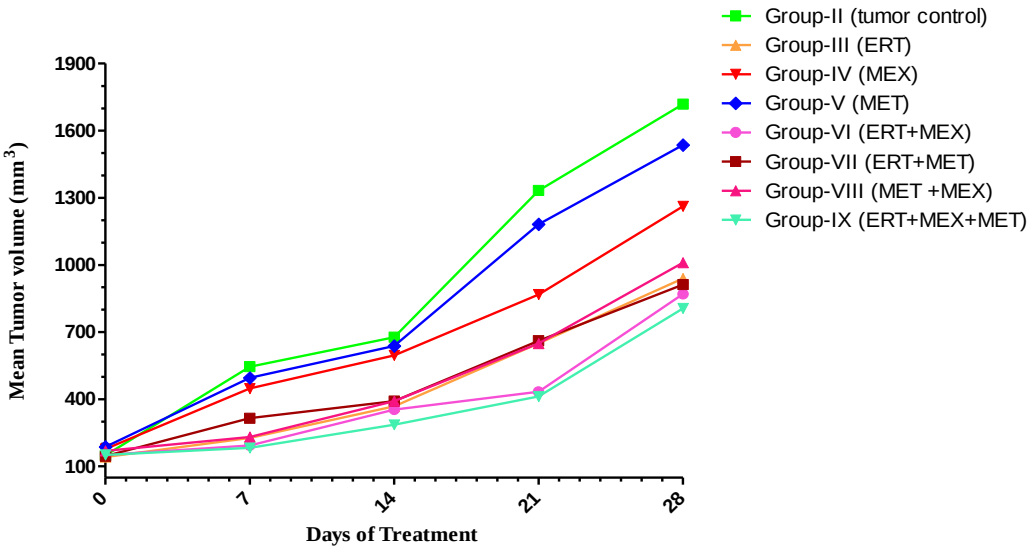

Figure 4.4: Effect of erlotinib, meloxicam and metformin administration as alone and in combination with each other for 28 days on mean tumor volume (mm3) in xenograft model of SCID male mice (n=6).

The effect of erlotinib, meloxicam and metformin administration as alone and in combination with each other for 28 days in xenograft model of SCID male mice was also evaluated by calculating percent reduction in tumor volume (%), which is depicted in Table No. 4.32. The mean percentage tumor volume reduction was calculated in comparison to tumor volume of control group for corresponding week. Results showed that, the tumor growth was considerably inhibited in groups III, VI, VII, VIII and IX on 1st and 2nd week of the treatment. The reduction in tumor volume was observed more than 50 % in groups III,VI, VII,VIII and IX on 3rd week of the treatment whereas on 4th week only treatment group IX showed more than 50 % tumor volume reduction in comparison to tumor control group. The treatment group IX shown marked percentage reduction in tumor volume on 1st, 2nd, 3rd and 4th week of the treatment as compared with tumor control. Overall, the groups III, VI, VII and VIII showed considerable reduction in tumor volume in comparison to tumor control group. The percent reduction of tumor volume in all treatment groups in comparison to control group for respective week is presented as graphical form in Figure 4.5 and 4.6.

Table 4.32 : Effect of erlotinib, meloxicam and metformin administration as alone and in combination with each other for 28 days on percent reduction of tumor volume (%) in xenograft model of SCID male mice (n=6)

Treatment Groups	Study Day				
	0	7	14	21	28
Group-II (Tumor control)	100.00	100.00	100.00	100.00	100.00
Group-III (ERT)	NA	58.35	45.68	51.25	45.31
Group-IV (MEX)	NA	17.87	12.01	34.88	26.57
Group-V (MET)	NA	9.15	5.81	11.36	10.65
Group-VI (ERT+MEX)	NA	64.51	47.68	67.49	49.40
Group-VII (ERT+MET)	NA	42.23	42.22	50.37	46.93
Group-VIII (MET +MEX)	NA	57.63	42.14	51.33	41.22
Group-IX (ERT+MEX+ MET)	NA	66.60	57.81	69.03	53.10

NA = Not Applicable

The effect of erlotinib, meloxicam and metformin administration as alone and in combination with each other for 28 days in xenograft model of SCID male mice was also evaluated by mean relative tumor volume, which is depicted in Table 4.33. The mean relative tumor volume (RTV) was calculated by dividing the mean tumor volume on any day by the mean tumor volume at the start of the treatment. The treatment group IX showed considerable reduction in mean relative tumor volume as compared to tumor control group. The other treatment groups also showed substantial reduction in mean relative tumor volume as compared to tumor control group. The mean RTV of control group on 28^{th} day was observed 11.53 times higher than its 0 day value. The RTV reduction was observed in descending pattern in treatment group V >IV >III >VII >VIII >VI >IX. The lowest RTV was observed in treatment group IX. The graphical presentation of relative tumor volume is depicted in Figure 4.7.The effect of erlotinib, meloxicam and metformin treatment as alone and in combination with each other is also presented by relative tumor volume at 4, which is depicted in Figure 4.8.

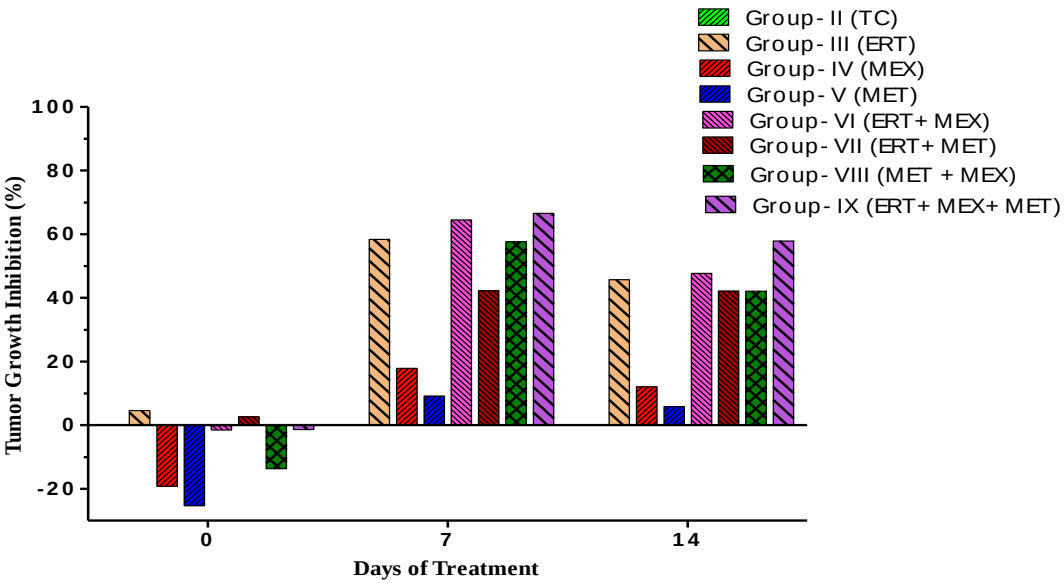

Figure 4.5: Effect of erlotinib, meloxicam and metformin administration as alone and in combination with each other for 7 and 14 days on Tumor growth Inhibition (%) in xenograft model of SCID male mice (n=6)

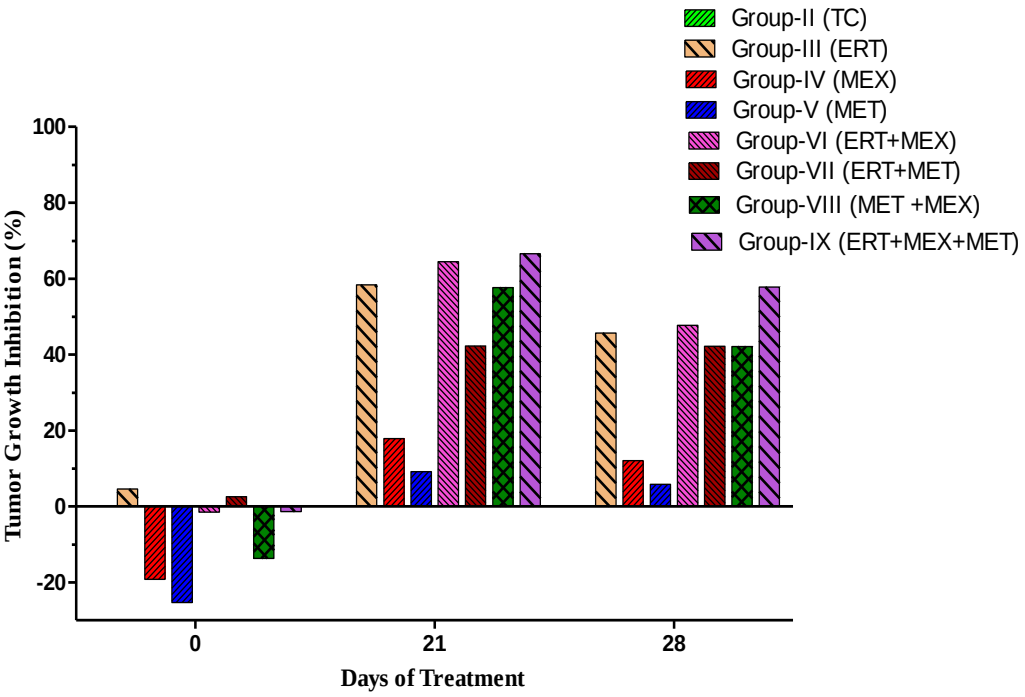

Figure 4.6: Effect of erlotinib, meloxicam and metformin administration as alone and in combination with each other for 21 and 28 days on Tumor growth Inhibition(%) in xenograft model of SCID male mice (n=6)

Table 4.33 :Effect of erlotinib, meloxicam and metformin administration as alone and in combination with each other for 28 days on mean relative tumor volume in xenograft model of SCID male mice (n=6)

Treatment Groups	Study Day				
	0	7	14	21	28
Group-II (Tumor Control)	1.00	3.66	4.55	8.94	11.53
Group-III (ERT)	1.00	1.60	2.59	4.57	6.61
Group-IV (MEX)	1.00	2.53	3.35	4.89	7.10
Group-V (MET)	1.00	2.65	3.42	6.33	8.22
Group-VI (ERT+MEX)	1.00	1.28	2.34	2.86	5.75
Group-VII (ERT+MET)	1.00	2.17	2.70	4.56	6.28
Group-VIII (MET +MEX)	1.00	1.36	2.31	3.83	5.96
Group-IX (ERT+MEX+MET)	1.00	1.21	1.89	2.73	5.33

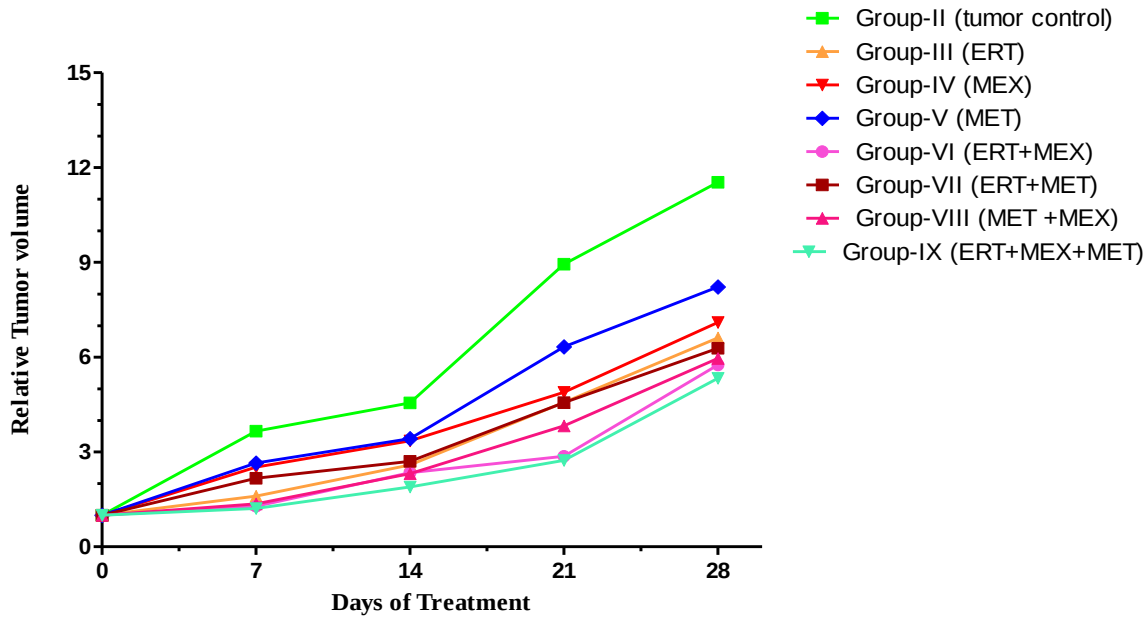

Figure 4.7: Effect of erlotinib, meloxicam and metformin administration as alone and in combination with each other for 28 days on mean relative tumor volume (%) in xenograft model of SCID male mice (n=6)

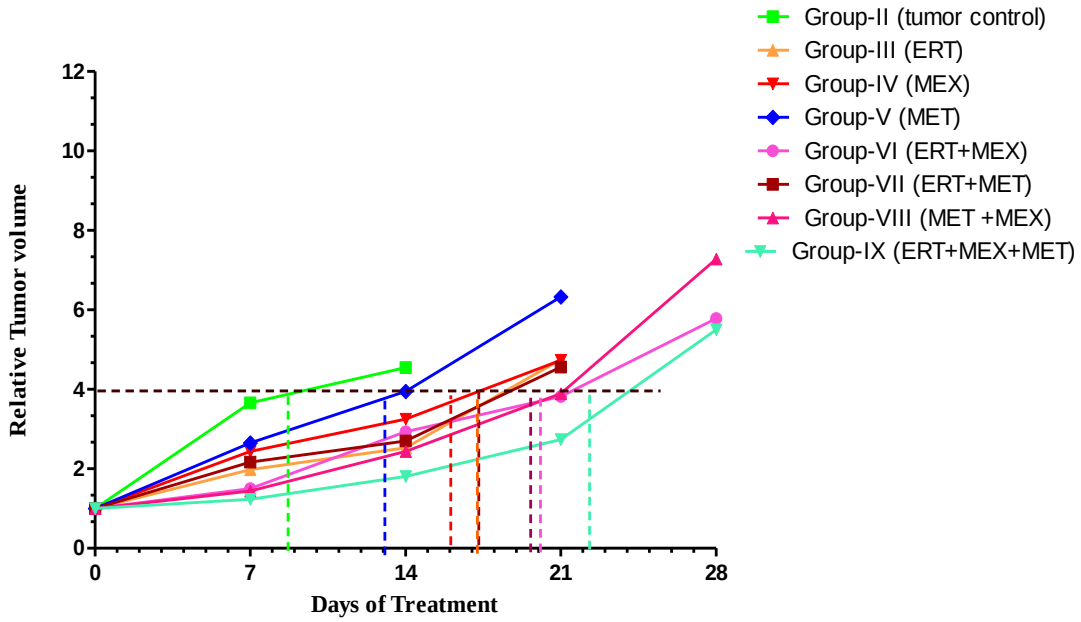

Figure 4.8: Effect of erlotinib, meloxicam and metformin administration as alone and in combination with each other on time to reach mean relative tumor volume 4 in xenograft model of SCID male mice (n=6)

4.2.3 Effect on Body weight

Weekly body weight of all animals including both control and treatment groups of erlotinib, meloxicam and metformin alone and in combination with each other and all three drugs together were recorded till day 28 of experiment and is presented in Table 4.34. The effect of erlotinib, meloxicam and metformin alone and in combination with each other is graphically depicted in Figure 4.9.

The mean body weight was significantly reduced ($p < 0.05$) in treatment group VII in comparison to group II in 1st, 2nd, 3rd and 4th week of the study. The mean body weights of groups III, VI, VII and IX was significantly reduced ($p < 0.05$) in comparison to group II on 3rd week of the study. On 4th week of study, the mean body weight of groups VI and VII was significantly reduced ($p < 0.05$) in comparison to group II. The body weights of animals in tumor control and metformin treated groups were nearly similar to the normal control.

Table 4.34: Effect of daily administration erlotinib, meloxicam and metformin as alone and in combination with each other for 28 days on body weight in xenograft model of SCID male mice (n=6)

Treatment group	Body Weight (g)				
	D0	W1	W2	W3	W4
Group-I (Normal Control)	24.4 ± 0.63	25.10 ±0.56	25.56 ±0.48	26.08 ±0.44	26.52 ±0.44
Group-II (Tumor Control)	26.28 ±0.76	28.30 ±0.67	28.02 ±0.65	29.16 ±0.67	28.34 ± 0.74
Group-III (ERT)	25.75 ±0.71	25.83 ±0.31	25.67 ±0.30	26.15 ±0.34*	26.00 ±0.46
Group-IV (MEX)	26.65 ±0.76	26.52 ±0.87	27.04 ±0.77	28.22 ±0.32	27.73 ±0.68
Group-V (MET)	26.42 ±0.95	26.72 ±0.86	25.84 ±1.19	28.23 ±0.44	28.60 ±0.49
Group-VI (ERT+MEX)	24.38 ±0.52	24.30 ±0.96	24.33 ±0.89	24.25 ±0.70*	23.25 ±0.32*
Group-VII (ERT+MET)	24.22 ±0.82	24.54 ±0.86*	21.25 ±1.07*	22.50 ±0.05*	22.50 ±0.10*
Group-VIII (MET +MEX)	25.40 ±0.51	24.80 ±0.66	26.93 ±0.89	26.90 ±1.39	26.70 ±1.42
Group-IX (ERT+MEX+MET)	26.40 ±0.82	25.22 ±0.73	24.03 ±0.27	25.13±0.66*	25.43 ±0.69

D=Day, W=Week, *Significant at p<0.05

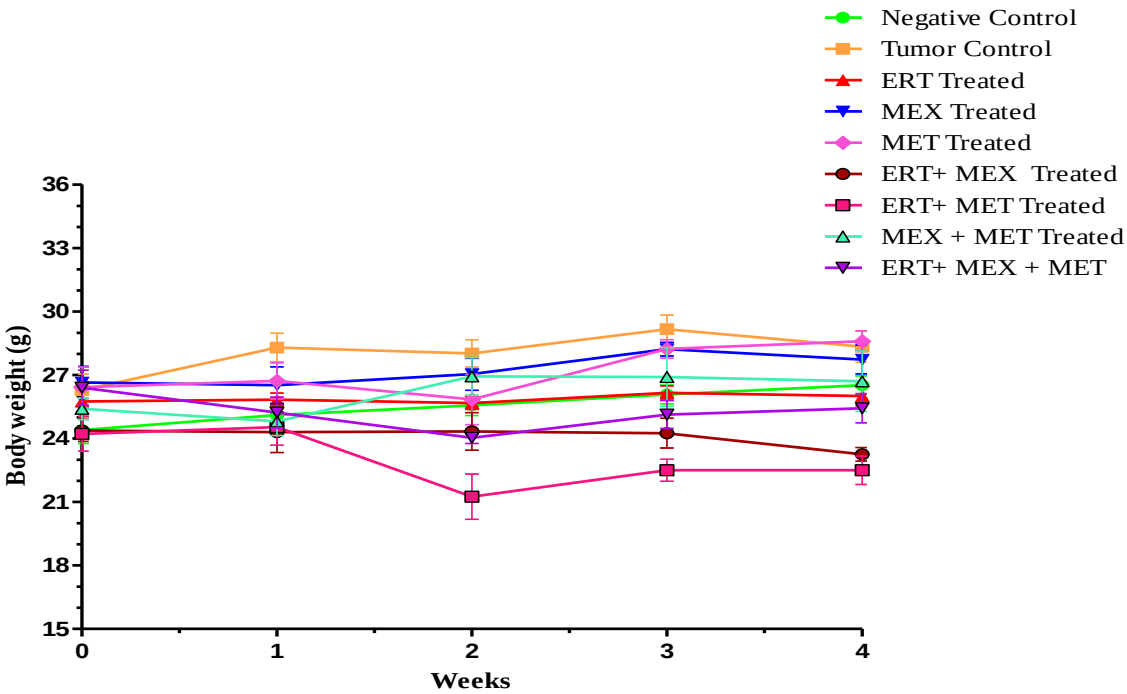

Figure 4.9: Effect of daily administration of erlotinib, meloxicam and metformin as alone and in combination with each other for 28 days on body weight of xenograft model of SCID male mice (n=6)

4.2.4 Effect on Feed consumption

Feed consumption was measured weekly for all animals of control and treatment groups of erlotinib, meloxicam and metformin alone and in combination with each other and all three drug together on day 7, 14, 21 and 28 of the experiment and is presented in Table 4.35. The feed consumption comparison as mean value is depicted in Figure 4.10. Feed consumption in SCID male mice of treatment groups was not changed significantly as compared to that of control animals at the end of four weeks.

Table 4.35: Effect of daily administration of erlotinib, meloxicam and metformin as alone and in combination with each other for 28 days on feed consumption of xenograft model of SCID male mice (n=6)

Treatment group	Feed consumption (g)			
	W1	W2	W3	W4
Group-I (Normal Control)	3.15	3.69	3.37	3.39
Group-II (Tumor Control)	3.91	3.73	3.10	2.70
Group-III (ERT)	3.08	2.15	2.61	3.09
Group-IV (MEX)	3.51	3.93	2.96	3.29
Group-V (MET)	3.73	2.83	2.36	2.67
Group-VI (ERT+MEX)	3.49	2.08	2.74	3.28
Group-VII (ERT+MET)	3.19	2.30	2.73	2.61
Group-VIII (MET +MEX)	3.06	2.26	2.89	3.01
Group-IX (ERT+MEX+MET)	2.94	1.90	2.21	3.09

W=Week

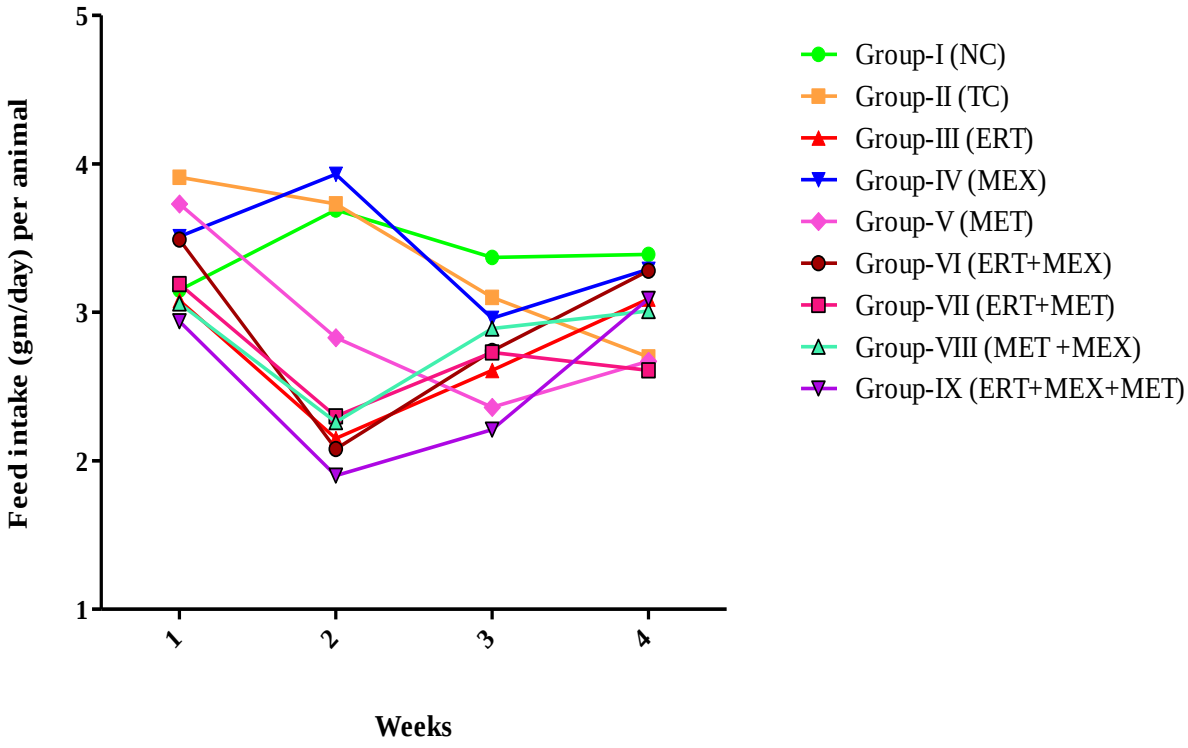

Figure 4.10 : Effect of daily administration of erlotinib, meloxicam and metformin as alone and in combination with each other for 28 days on feed consumption of xenograft model of SCID male mice

4.2.5 Effect on Hematological parameters of SCID male mice treated with erlotinib, meloxicam, metformin alone and in combination with each other and all three drugs together.

The mean values of total WBC count, total RBC count, haemoglobin, hematocrit, and differential leukocyte count (neutrophil, lymphocyte, basophil, eosinophil and monocyte) of erlotinib, meloxicam, metformin alone and in combination with each other and all drug together treated SCID male mice are presented in Tables 4.36 and graphically depicted in Figures 4.11 to 4.23.

The mean value of WBC and % neutrophil of group VII was significantly high when compared to group II. The mean value of all other hematological parameter of male SCID mice treated with erlotinib, meloxicam, metformin alone and in combination with each other and all three drugs together for 28 days did not differ significantly from the corresponding values observed in tumor control animals. The WBC count of group VI was also considerably decreased in comparison to group II. The mean WBC value of groups II and all other treatment groups (III to IX) is slightly higher in comparison to negative control (group-I). The neutrophil % of Tumor control and all treatment groups was considerably high in comparison to negative or normal control, whereas the lymphocytes % and eosinophil % were decreased in tumor control and treatment group in comparison to negative control. The monocyte % in group V is significantly higher than group II.

Table 4.36: Effect of daily administration of erlotinib, meloxicam and metformin as alone and in combination with each other for 28 days on hematology parameter of xenograft model of SCID male mice (n=6)

Treatment Group	WBC $10^3/\mu L$	RBC $10^6/\mu L$	HGB g/dL	HCT %	MCV fL	MCH pg	MCHC g/dL	PLT $10^3/\mu L$	N% %	L% %	M% %	E% %	B% %
Group-I (Negative control)	1.17 ± 0.15	7.10 ± 0.13	11.25 ± 0.15	41.07 ± 0.67	57.85 ± 0.48	15.85 ± 0.12	27.40 ± 0.10	1212.83 ± 71.00	58.82 ± 1.96	31.25 ± 1.94	4.02 ± 1.01	5.35 ± 0.52	0.54 ± 0.25
Group-II (Tumor control)	2.30 ± 0.71	6.68 ± 0.23	10.58 ± 0.33	38.23 ± 1.29	57.28 ± 1.16	15.83 ± 0.28	27.68 ± 0.14	1096.33 ±134.53	72.73 ± 5.66	20.57 ± 5.63	4.11 ± 0.63	2.27 ± 0.89	0.33 ± 0.14
Group-III (ERTB)	2.52 ± 0.77	7.09 ± 0.21	10.90 ± 0.36	39.66 ± 1.30	56.38 ± 0.46	15.57 ± 0.08	27.57 ± 0.13	1250.50 ± 67.05	73.76 ± 3.80	17.08 ± 3.52	5.27 ± 1.90	3.65 ± 0.68	0.42 ± 0.13
Group-IV (MEX)	2.23 ± 0.48	6.45 ± 0.22	10.32 ± 0.34	38.03 ± 1.40	59.02 ± 1.44	15.98 ± 0.30	27.12 ± 0.19	1158.83 ± 62.20	75.92 ± 4.02	15.97 ± 2.73	5.74 ± 1.95	2.13 ± 0.99	0.25 ± 0.07
Group-V (MET)	2.53 ± 0.38	6.76 ± 0.15	10.63 ± 0.27	38.45 ± 0.96	56.85 ± 0.49	15.75 ± 0.11	27.70 ± 0.12	1179.33 ± 93.51	68.90 ± 4.73	16.96 ± 3.27	9.71** ± 2.00	3.65 ± 0.91	0.78 ± 0.17
Group-VI (ERTB+MEX)	1.58 ± 0.47	6.87 ± 0.15	10.92 ± 0.18	40.20 ± 0.85	58.60 ± 1.28	15.93 ± 0.26	27.18 ± 0.23	1208.50 ± 60.91	74.02 ± 4.34	17.70 ± 3.99	5.31 ± 2.07	2.76 ± 0.70	0.26 ± 0.14
Group-VII (ERTB+MET)	5.48** ± 1.16	6.50 ± 0.10	10.09 ± 0.21	36.00 ± 0.82	54.78 ± 0.51	15.43 ± 0.10	28.17 ± 0.15	1138.17 ± 51.82	80.82 ± 5.99	13.96 ± 6.02	2.81 ± 0.97	1.98 ± 1.04	0.43 ± 0.21
Group-VIII (MET +MEX)	2.31 ± 0.42	6.68 ± 0.34	10.29 ± 0.44	37.60 ± 1.71	56.35 ± 0.67	15.47 ± 0.18	27.42 ± 0.21	1231.50 ± 55.76	73.57 ± 5.35	16.26 ± 3.03	6.04 ± 1.80	3.41 ± 1.16	0.71 ± 0.35
Group-IX (ERTB+MEX+MET)	3.47 ± 0.52	6.80 ± 0.18	10.67 ± 0.22	38.55 ± 0.67	56.78 ± 0.78	15.72 ± 0.16	27.65 ± 0.20	1359.83 ± 64.57	79.30 ± 3.65	12.60 ± 2.95	4.91 ± 1.40	2.69 ± 0.59	0.49 ± 0.30

**significant at $p<0.01$, *significant at $p<0.05$

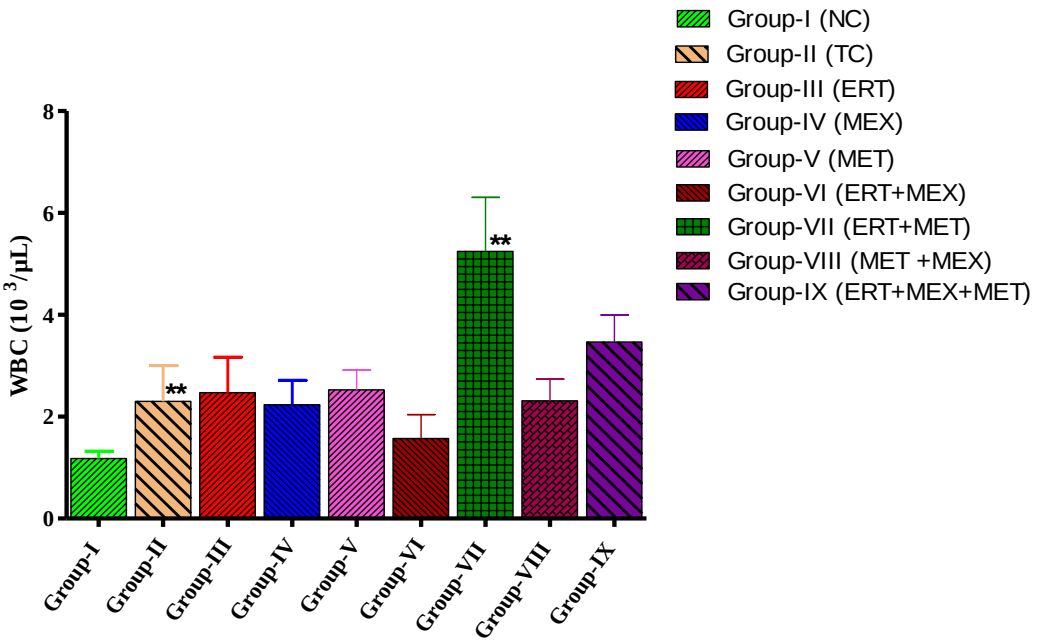

Figure 4.11: Effect of daily administration of erlotinib, meloxicam and metformin as alone and in combination with each other for 28 days on total leukocyte count (10^3/ µL) of xenograft model of SCID male mice

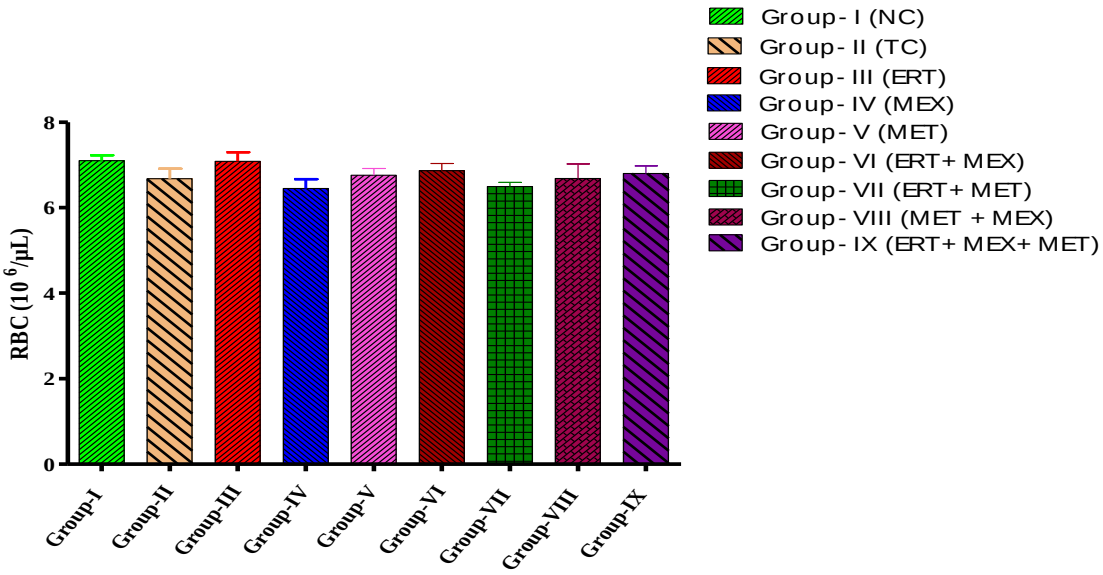

Figure 4.12: Effect of daily administration of erlotinib, meloxicam and metformin as alone and in combination with each other for 28 days on total Red Blood cells (10^6/ µL) of xenograft model of SCID male mice

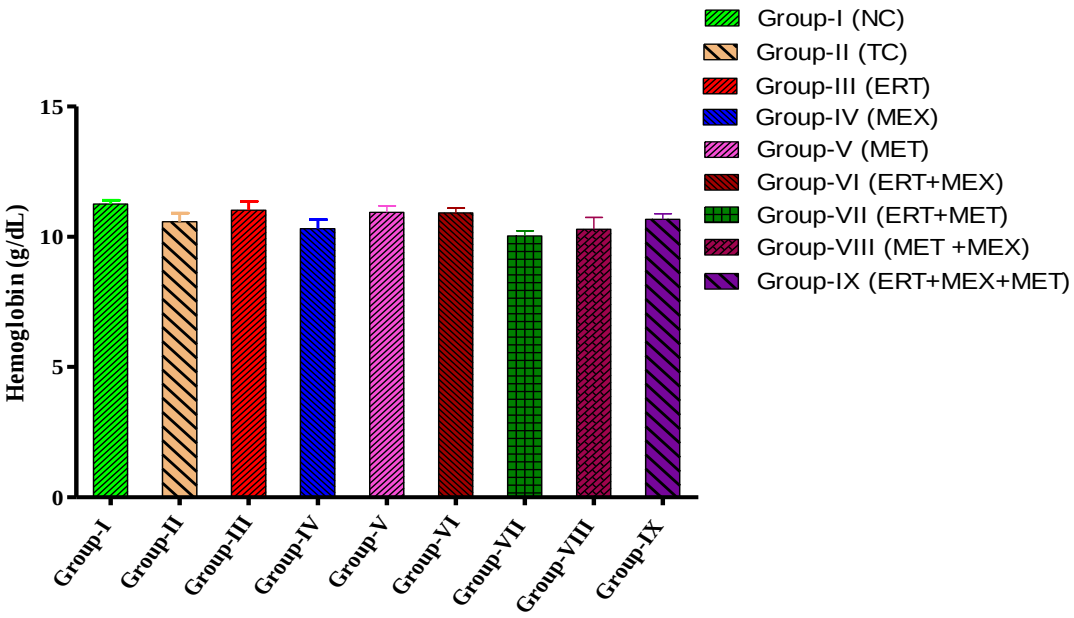

Figure 4.13: Effect of daily administration of erlotinib, meloxicam and metformin as alone and in combination with each other for 28 days on hemoglobin level (g/dL) of xenograft model of SCID male mice

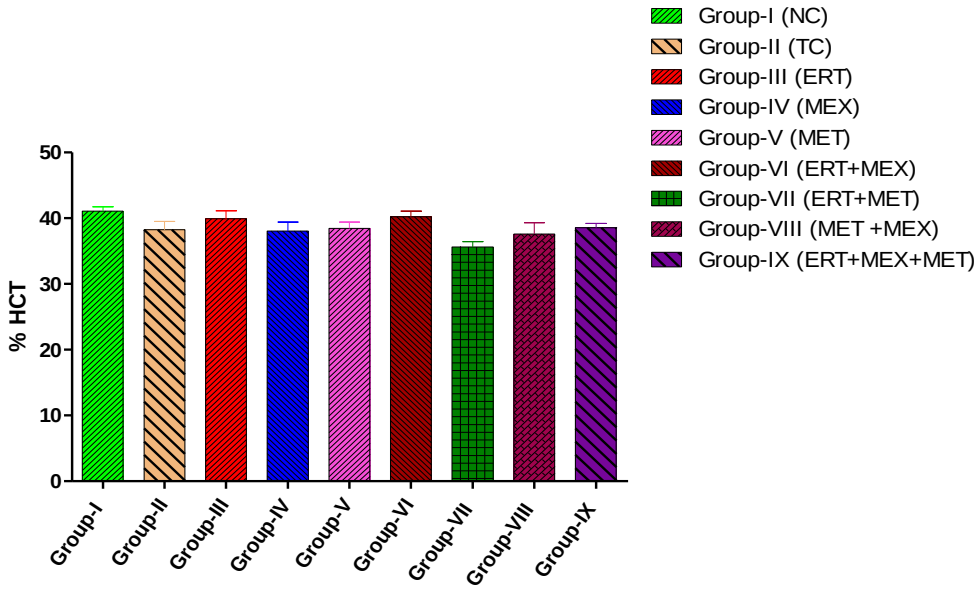

Figure 4.14: Effect of daily administration of erlotinib, meloxicam and metformin as alone and in combination with each other for 28 days on hematocrit value (%) of xenograft model of SCID male mice

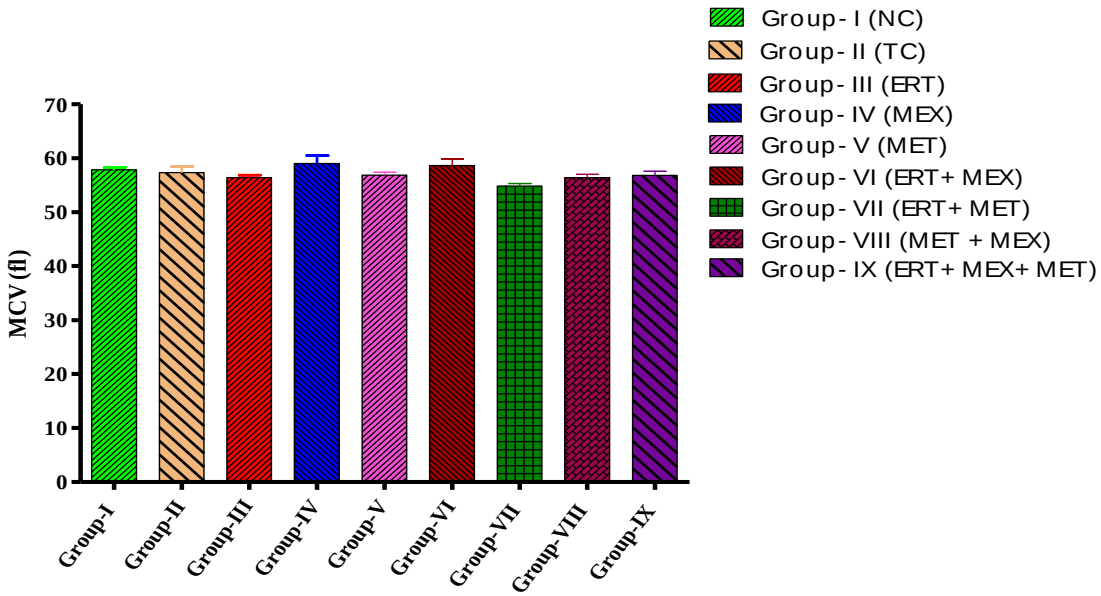

Figure 4.15: Effect of daily administration of erlotinib, meloxicam and metformin as alone and in combination with each other for 28 days on MCV (fL) of xenograft model of SCID male mice

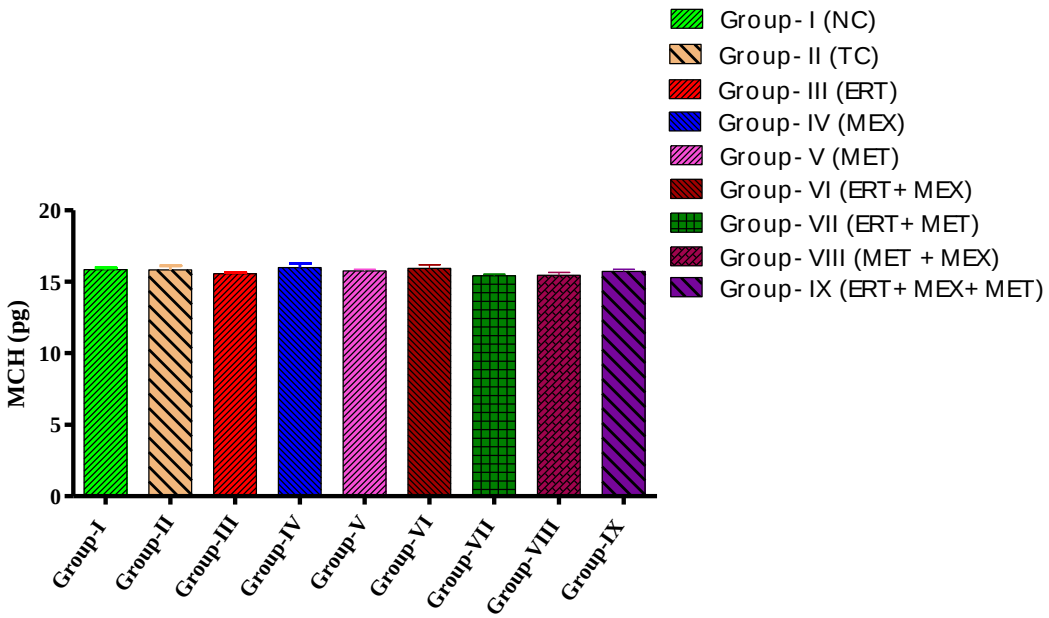

Figure 4.16: Effect of daily administration of erlotinib, meloxicam and metformin as alone and in combination with each other for 28 days on MCH (pg) of xenograft model of SCID male mice

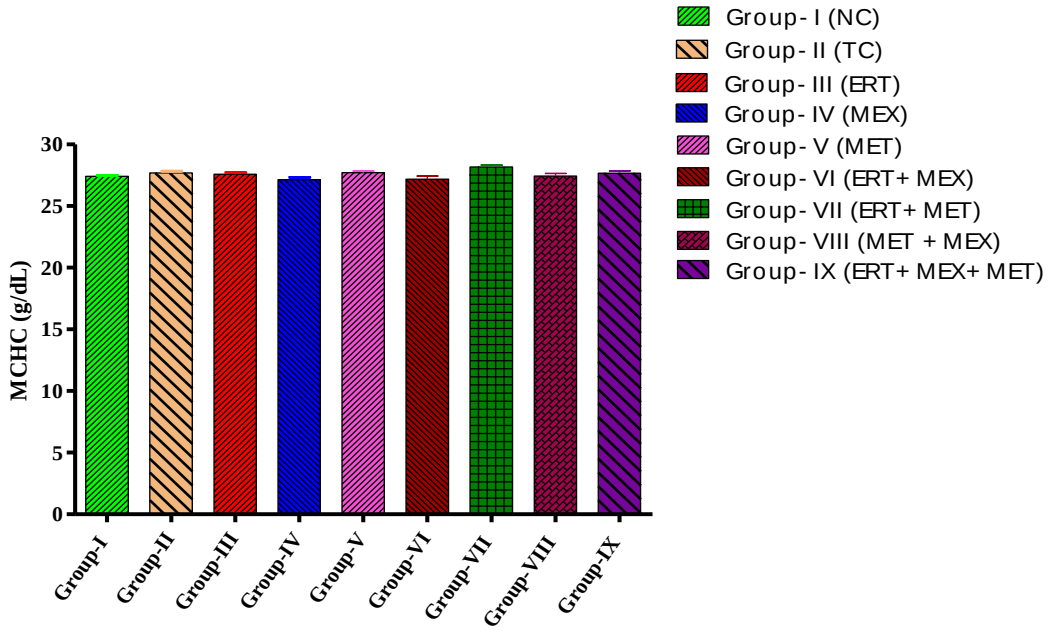

Figure 4.17: Effect of daily administration of erlotinib, meloxicam and metformin as alone and in combination with each other for 28 days on MCHC (g/dL)of xenograft model of SCID male mice

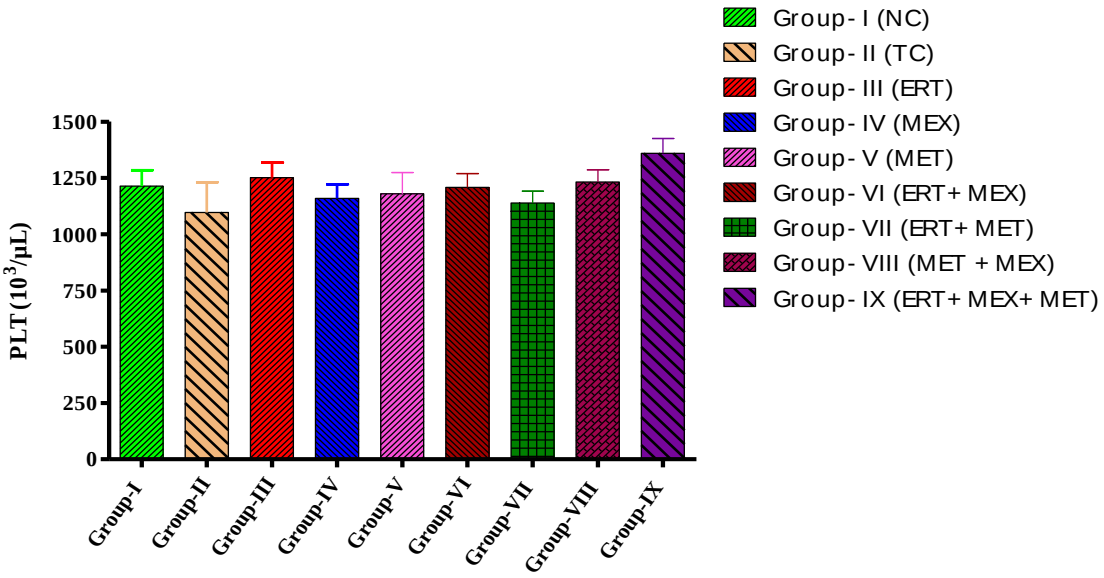

Figure 4.18: Effect of daily administration of erlotinib, meloxicam and metformin as alone and in combination with each other for 28 days on platelet count (10^3/µL) of xenograft model of SCID male mice

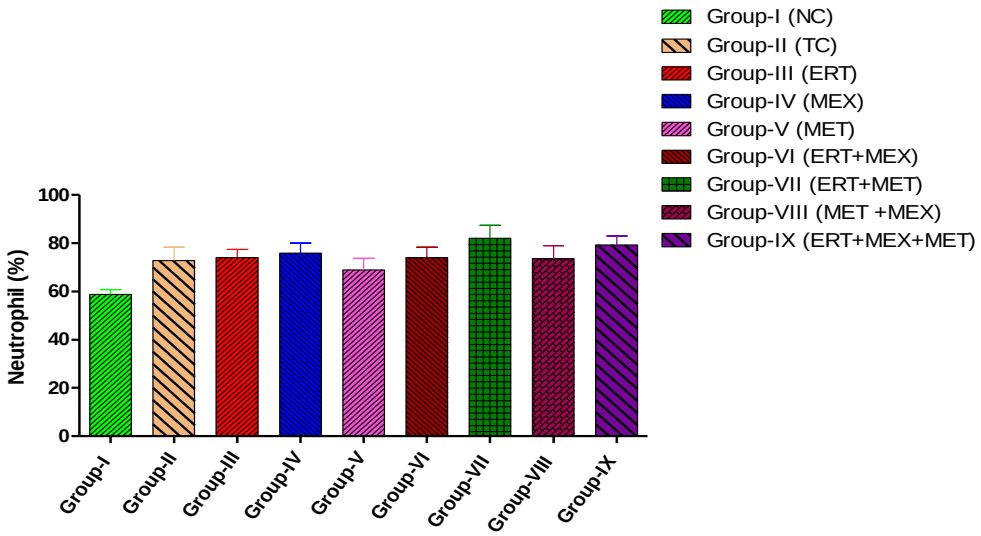

Figure 4.19: Effect of daily administration of erlotinib, meloxicam and metformin as alone and in combination with each other for 28 days on neutrophil count (%) of xenograft model of SCID male mice

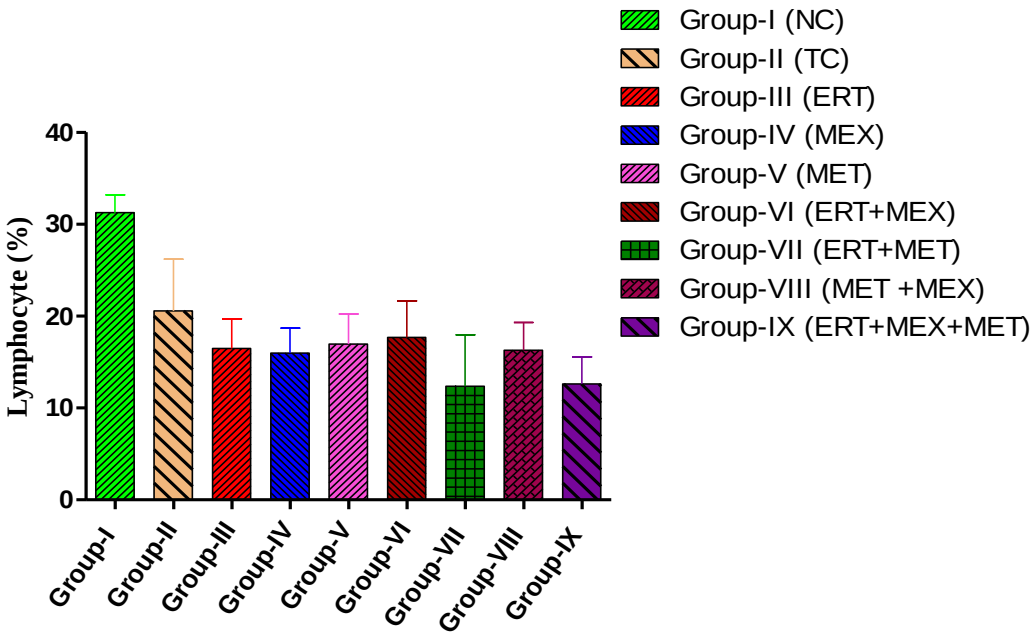

Figure 4.20: Effect of daily administration of erlotinib, meloxicam and metformin as alone and in combination with each other for 28 days on lymphocyte count (%) of xenograft model of SCID male mice

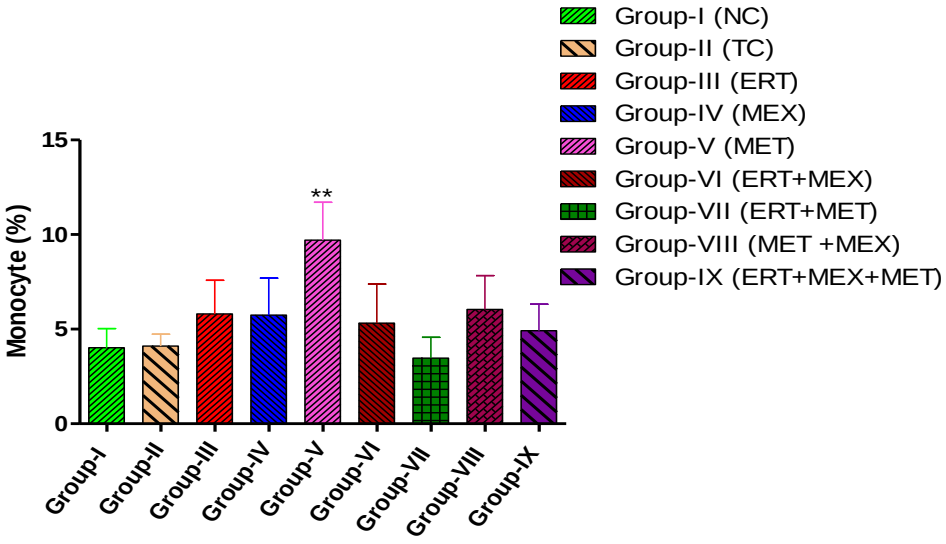

Figure 4.21: Effect of daily administration of erlotinib, meloxicam and metformin as alone and in combination with each other for 28 days on monocyte count (%) of xenograft model of SCID male mice

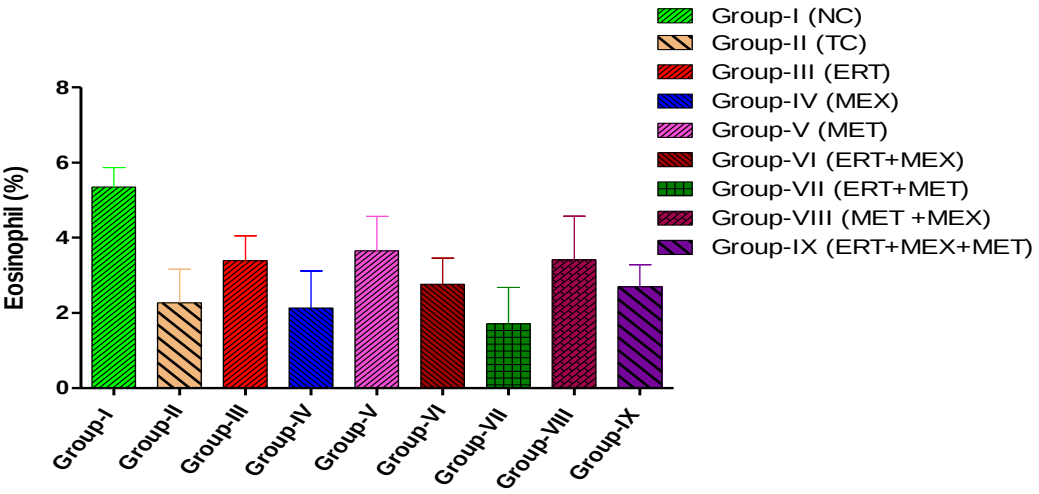

Figure 4.22: Effect of daily administration of erlotinib, meloxicam and metformin as alone and in combination with each other for 28 days on eosinophil count (%) of xenograft model of SCID male mice

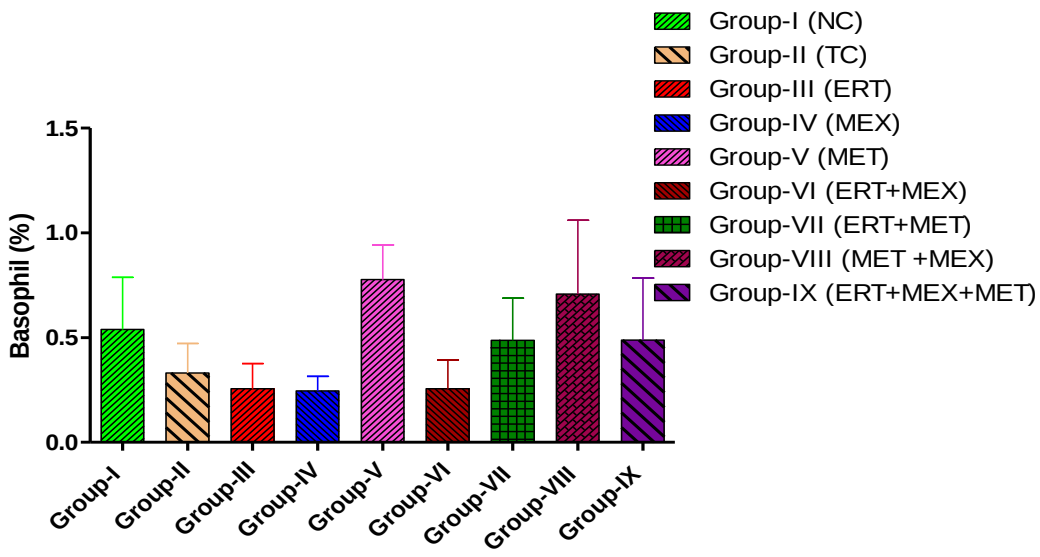

Figure-4.23: Effect of daily administration of erlotinib, meloxicam and metformin as alone and in combination with each other for 28 days on basophil count (%) of xenograft model of SCID male mice

4.2.6 Effect on Biochemical parameters

The mean ± SEM values of various biochemical parameters like glucose, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total bilirubin (TB), creatinine, creatine kinase(CK), blood urea nitrogen(BUN), lactate dehydrogenase (LDH) and acid phosphatase after treatment of erlotinib, meloxicam, metformin alone and in combination with each other and all drug together on SCID male mice and its control are presented in Table-4.37 and graphically depicted in Figures -4.24 to 4.33.

The mean value of creatinin kinase (CK) was significantly decreased in group IV whereas in group IX it was significantly high in comparison to group II. The mean value of blood urea nitrogen (BUN) was significantly increased in group VII when compared to group II.

The mean values of serum glucose, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total bilirubin (TBIL), creatinine,

LDH and acid phosphatase did not differ significantly from those of control animals. The mean value of AST, ALT, TBIL and Acid Phosphatase of group II was considerably high than group I. The glucose level in group V, VII and VIII were considerably low, where as AST of group IV and VI was also found markedly low in comparison to group II. The mean serum ALT level was considerably reduced in treatment group IV, V and VII. The ALP level in group V, VII and IX were decreased considerably in comparison to group II. The creatinine kinase (CK) level in treatment group III, V, VI and VII were considerably reduced in comparison to group II. The LDH level in group IV and VI were considerably reduced in comparison to tumor control group –II.

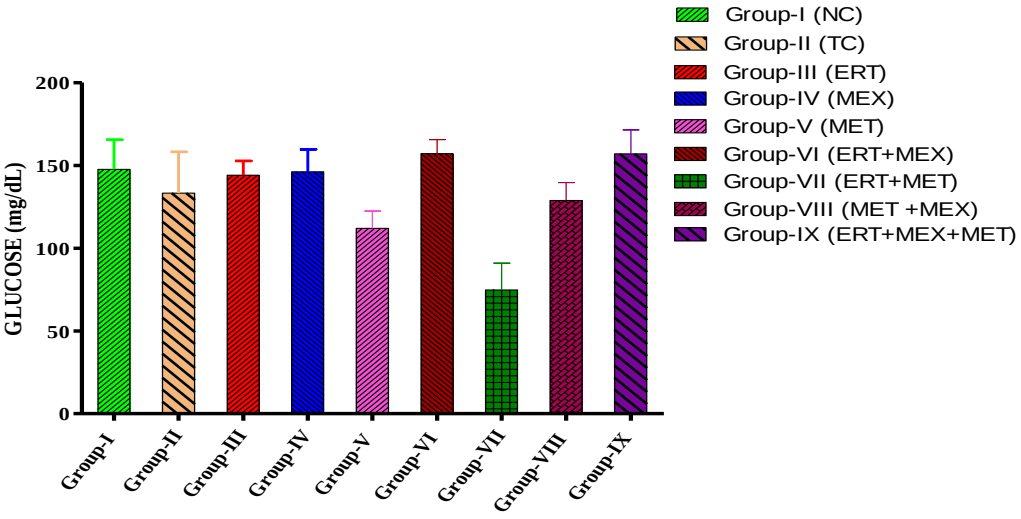

Figure-4.24: Effect of daily administration of erlotinib, meloxicam and metformin as alone and in combination with each other for 28 days on serum glucose level (mg/dL) of xenograft model of SCID male mice

Table 4.37: Effect of daily administration of erlotinib, meloxicam and metformin as alone and in combination with each other for 28 days on biochemical parameters of xenograft model of SCID male mice (n=6)

Treatment Group	Glucose (mg/dL)	AST (U/L)	ALT (U/L)	ALP (U/L)	TBIL (mg/dL)	CREAT (mg/dL)	CK (U/L)	BUN (mg/dL)	LDH (U/L)	Acid Phosphatase (U/L)
Group-I (Normal control)	147.57 ± 17.86	98.98 ± 10.37	26.47 ± 2.24	48.35 ± 1.67	0.12 ± 0.00	0.20 ± 0.02	1016.50 ± 203.20	22.83 ± 0.83	1608.33 ± 119.13	10.68 ± 0.49
Group-II (Tumor control)	133.27 ± 24.89	130.10 ± 29.59	41.70 ± 11.17	44.68 ± 6.61	0.18 ± 0.04	0.17 ± 00	1155.02 ± 143.24	21.17 ± 0.40	1285.50 ± 390.55	14.35 ± 3.53
Group-III (ERT)	144.02 ± 8.68	96.93 ± 14.90	32.20 ± 5.61	47.30 ± 6.56	0.20 ± 0.02	0.17 ± 0.01	485.60 ± 109.28	21.00 ± 0.58	1160.00 ± 182.46	9.93 ± 0.36
Group-IV (MEX)	146.18 ± 13.49	73.13 ± 5.76	21.10 ± 3.20	39.42 ± 2.97	0.12 ± 0.00	0.17 ± 00	210.90 ± 28.10**	24.00 ± 0.52	653.67 ± 61.20	12.62 ± 2.30
Group-V (MET)	111.92 ± 10.44	114.52 ± 15.80	22.70 ± 1.67	35.42 ± 4.23	0.13 ± 0.01	0.19 ± 0.01	494.62 ± 98.04	22.67 ± 0.49	1940.67 ± 154.01	16.20 ± 2.33
Group-VI (ERTB+MEX)	157.12 ± 8.44	82.65 ± 6.92	29.38 ± 3.93	42.22 ± 3.20	0.13 ± 0.01	0.18 ± 0.00	491.92 ± 64.86	21.50 ± 0.67	628.83 ± 63.33	9.48 ± 0.34
Group-VII (ERTB+MET)	74.75 ± 16.19	153.67 ± 29.87	24.50 ± 4.35	24.58 ± 5.63	0.12 ± 0.02	0.15 ± 0.01	683.57 ± 86.46	24.3 ± 0.76*	1429.50 ± 207.56	18.42 ± 1.20
Group-VIII (MET + MEX)	128.78 ± 10.73	163.93 ± 29.63	38.03 ± 7.01	36.07 ± 2.51	0.12 ± 0.01	0.16 ± 0.01	890.90 ± 111.71	22.67 ± 0.80	1348.83 ± 162.47	12.47 ± 1.20
Group-IX (ERTB+MEX+MET)	156.90 ± 14.54	101.83 ± 10.93	28.42 ± 2.56	33.87 ± 3.54	0.23 ± 0.13	0.16 ± 0.01	1916.10 ± 335.93*	21.50 ± 0.72	1141.50 ± 100.59	10.37 ± 1.00

*significant at $p<0.05$, **significant at $p<0.01$

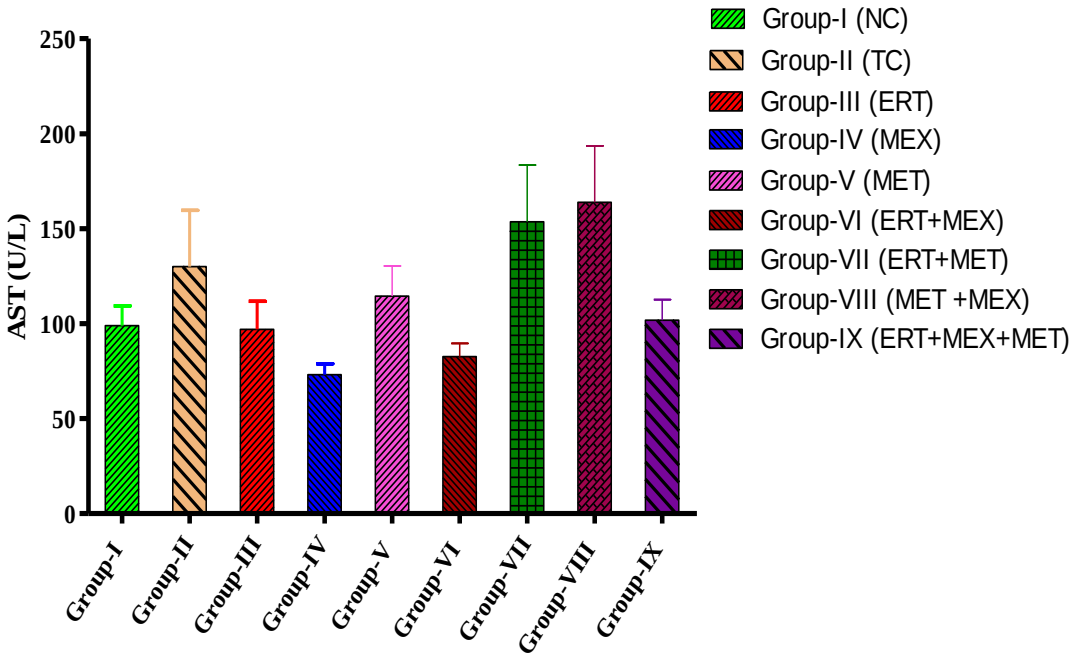

Figure-4.25: Effect of daily administration of erlotinib, meloxicam and metformin as alone and in combination with each other for 28 days on serum aspartate amino transferase (AST) level (U/L) of xenograft model of SCID male mice

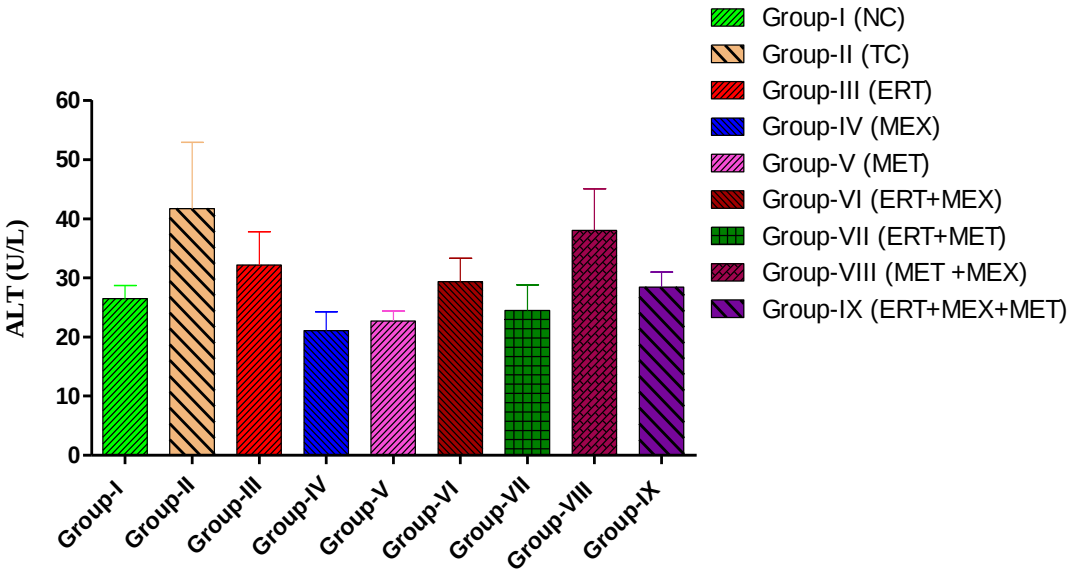

Figure-4.26: Effect of daily administration of erlotinib, meloxicam and metformin as alone and in combination with each other for 28 days on serum alanine aminotransferase (ALT) level (U/L) of xenograft model of SCID male mice

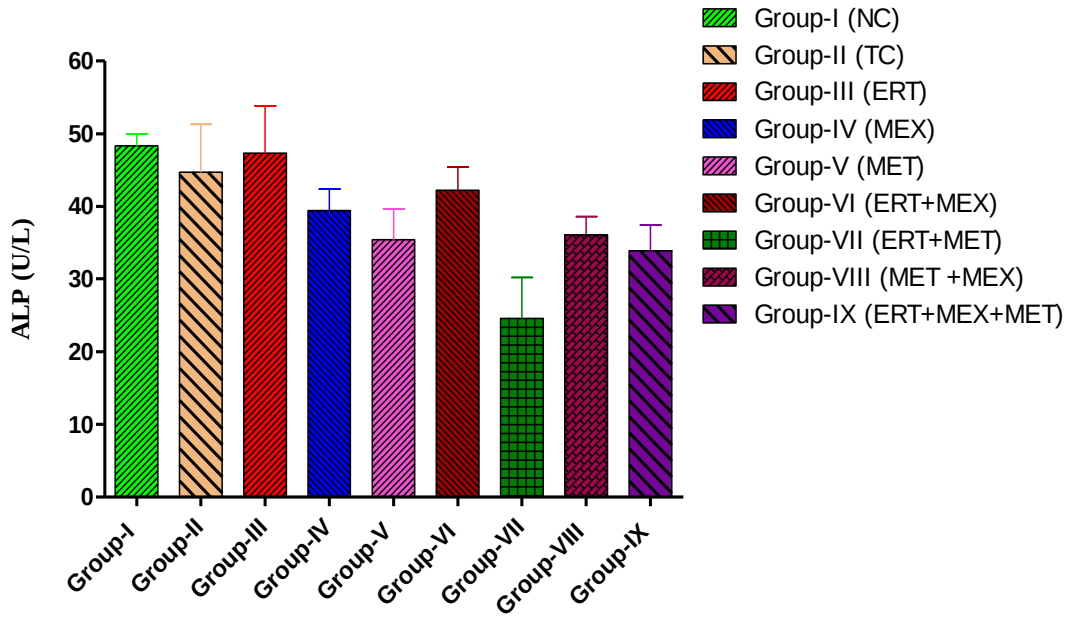

Figure-4.27: Effect of daily administration of erlotinib, meloxicam and metformin as alone and in combination with each other for 28 days on serum alkaline phosphatase (ALP) level (U/L)of xenograft model of SCID male mice

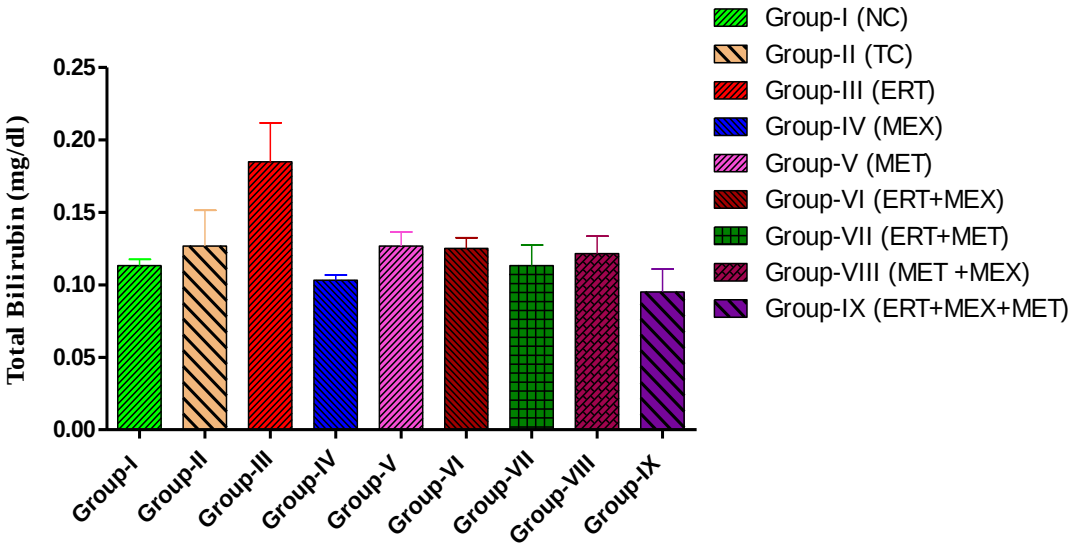

Figure-4.28: Effect of daily administration of erlotinib, meloxicam and metformin as alone and in combination with each other for 28 days on serum total bilirubin (TBIL) level(mg/dL)of xenograft model of SCID male mice

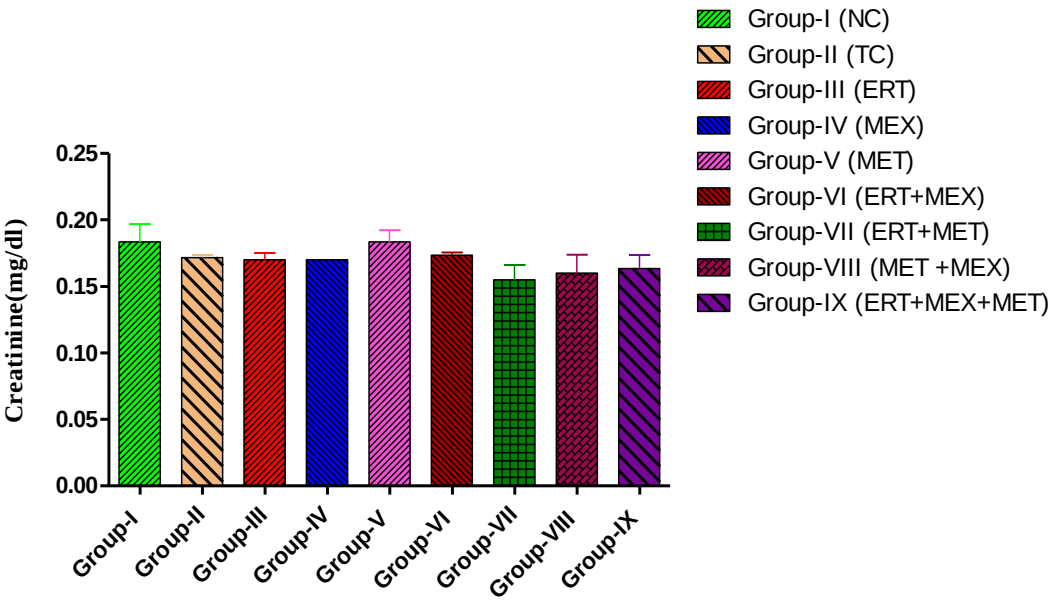

Figure-4.29: Effect of daily administration of erlotinib, meloxicam and metformin as alone and in combination with each other for 28 days on serum creatinine level (mg/dL)of xenograft model of SCID male mice

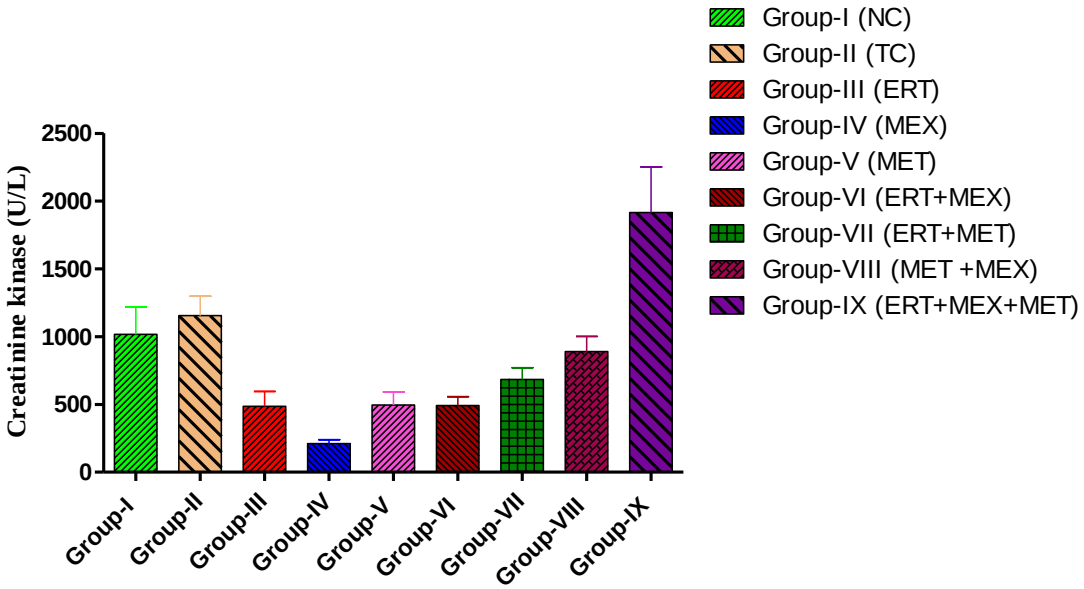

Figure-4.30: Effect of daily administration of erlotinib, meloxicam and metformin as alone and in combination with each other for 28 days on serum creatinine kinase (CK) level (U/L) of xenograft model of SCID male mice

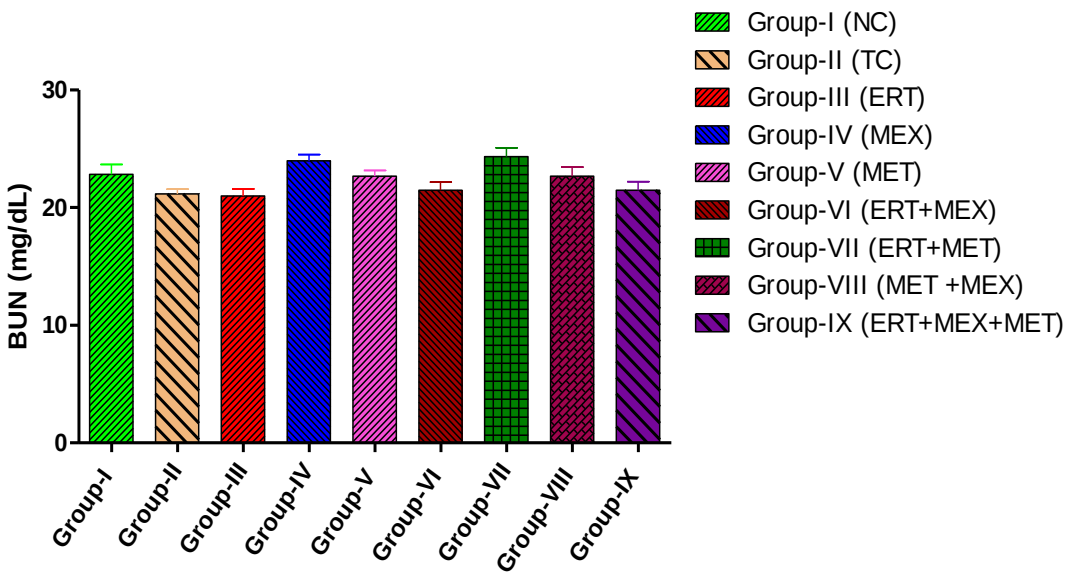

Figure-4.31: Effect of daily administration of erlotinib, meloxicam and metformin as alone and in combination with each other for 28 days on serum blood urea nitrogen (BUN) level (mg/dL) of xenograft model of SCID male mice

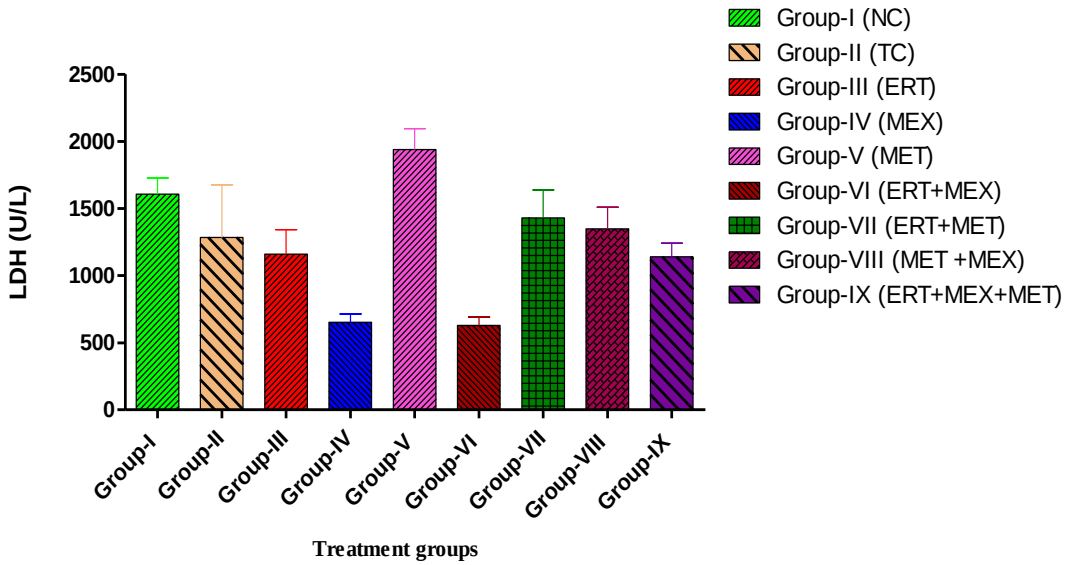

Figure-4.32: Effect of daily administration of erlotinib, meloxicam and metformin as alone and in combination with each other for 28 days on serum lactate dehydrogenase (LDH) level (U/L) of xenograft model of SCID male mice

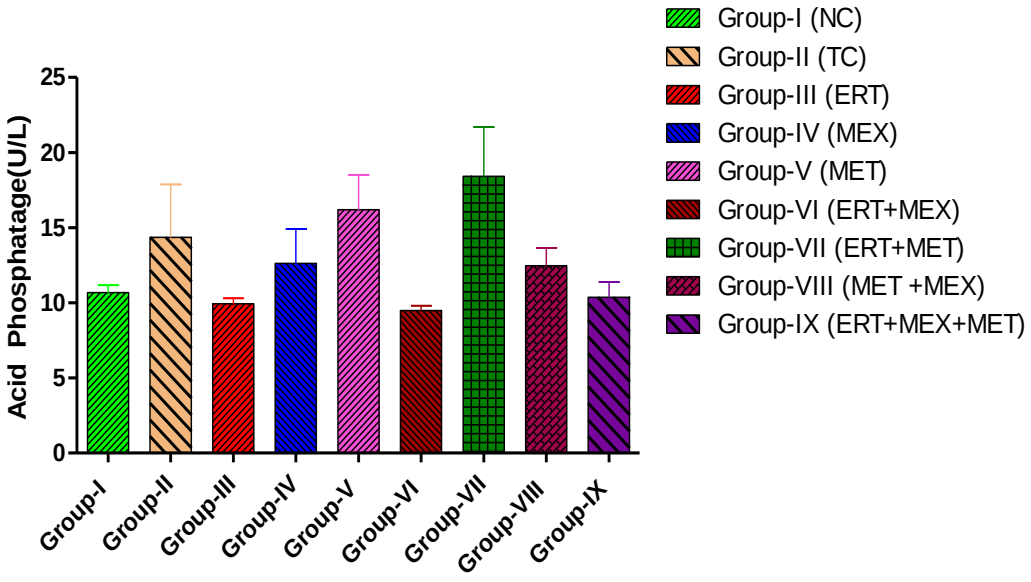

Figure-4.33: Effect of daily administration of erlotinib, meloxicam and metformin as alone and in combination with each other for 28 days on serum acid phosphatase level (U/L) of xenograft model of SCID male mice

2.7 Necropsy

Detailed post mortem examinations of all animals of different groups were performed on 29th day of treatment. The gross pathological lesions observed in individual organs like liver, kidney, lung, stomach, intestine, heart, tumor and its surrounding tissue were recorded. All the male mice animals of group IV, VI, VIII and IX were checked for presence of any ulcerative lesion in stomach and duodenum and no such lesions were found in any of the animals. Gross pathological examination all animals did not reveal any abnormal lesions attributable to the 28 days administration of erlotinib, meloxicam, metformin alone and in combination with each other and all three drugs together.

4.2.8 Effect on Organ weight

The absolute organ weights were obtained for tumor, heart, lung, liver and kidneys of male SCID mice of all groups on 29th day of experiment at the time of necropsy. Results of the absolute organ weight are presented in Table 4.38. Perusal of table indicates

that there was a considerable reduction in mean tumor weight of group III, IV, VI, VII, VIII and IX in comparison to the tumor control group II. The tumor weight reduction was calculated in percentage in comparison to tumor weight of group II. The tumor weight was 46.34 %, 42.73 %, 61.54 %, 37.40 %, 29.24 %, 61.78 % lesser compared to tumor control group II in treatment group III,IV,VI, VII,VIII and IX, respectively. The kidney weight of group VII was significantly reduced in comparison to the group II.

There was no change in mean organ weight of heart, lung and liver after 28 days administration of erlotinib, meloxicam, metformin alone and in combination with each other and all three drugs together. The tumor weight in different treatment group was compared and graphically represented in Figure 4.34. The individual organ weight in different treatment group was compared and graphically represented in Figure 4.35 to 4.38.

Table 4.38: Effect of daily administration of erlotinib, meloxicam and metformin as alone and in combination with each other for 28 days on organ weight of xenograft model of SCID male mice (n=6)

Treatment Group	Tumor	Heart	Lung	Liver	Kidney
Group-I (Negative control)	-	0.13 ± 0.00	0.17 ± 0.01	1.21 ± 0.05	0.43 ± 0.02
Group-II (Tumor control)	1.70 ± 0.49	0.11 ± 0.01	0.18 ± 0.02	1.50 ± 0.18	0.38 ± 0.01
Group-III (ERT)	0.91 ± 0.22	0.10 ± 0.00	0.16 ± 0.00	1.25 ± 0.05	0.33 ± 0.02
Group-IV (MEX)	0.97 ± 0.21	0.12 ± 0.02	0.18 ± 0.02	1.65 ± 0.06	0.34 ± 0.01
Group-V (MET)	1.76 ± 0.05	0.11 ± 0.01	0.16 ± 0.01	1.26 ± 0.09	0.33 ± 0.01
Group-VI (ERT+MEX)	0.65 ± 0.31	0.10 ± 0.00	0.17 ± 0.01	1.33 ± 0.04	0.32 ± 0.01
Group-VII (ERT+MET)	1.06 ± 0.16	0.11 ± 0.00	0.18 ± 0.01	1.16 ± 0.08	0.29 ± 0.02*
Group-VIII (MET +MEX)	1.20 ± 0.41	0.11 ± 0.01	0.17 ± 0.00	1.60 ± 0.02	0.36 ± 0.02
Group-IX (ERT+MEX+MET)	0.65 ± 0.23	0.10 ± 0.00	0.17 ± 0.01	1.35 ± 0.01	0.33 ± 0.00

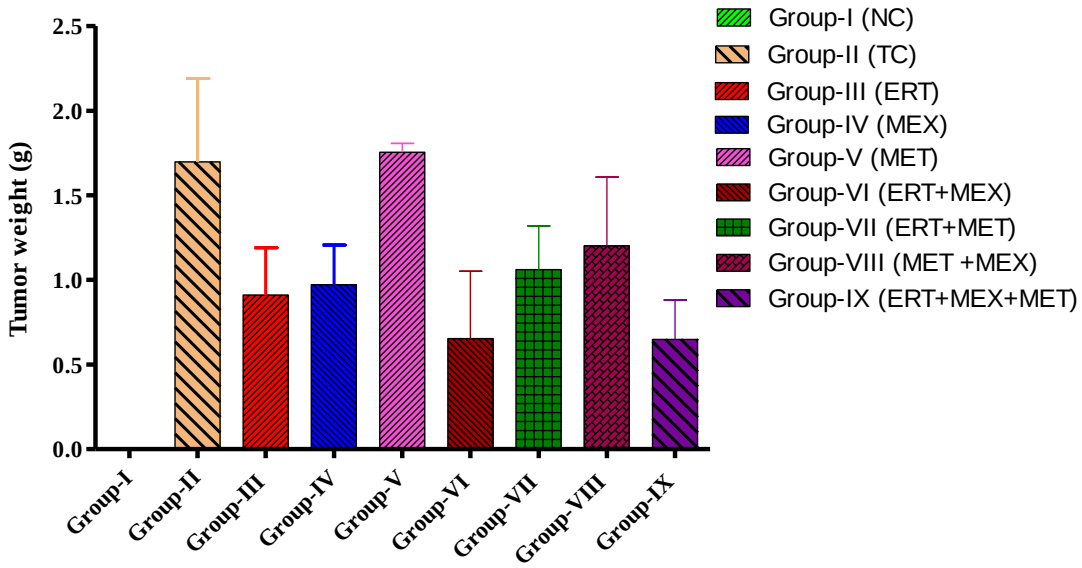

Figure 4.34: Effect of daily administration of erlotinib, meloxicam and metformin as alone and in combination with each other for 28 days on tumor weight (g) of xenograft model of SCID male mice

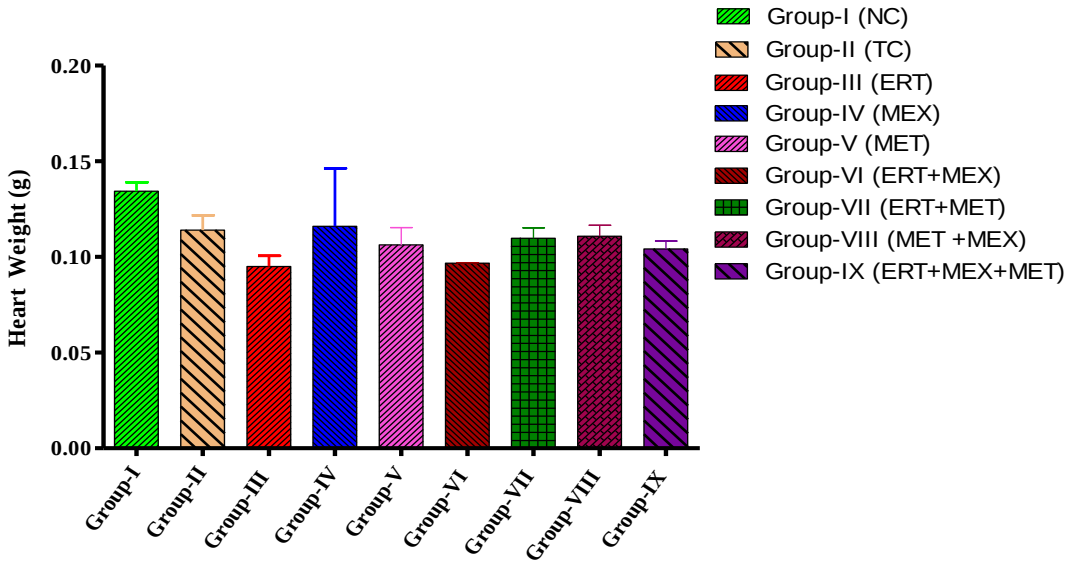

Figure 4.35: Effect of daily administration of erlotinib, meloxicam and metformin as alone and in combination with each other for 28 days on heart weight (g) of xenograft model of SCID male mice

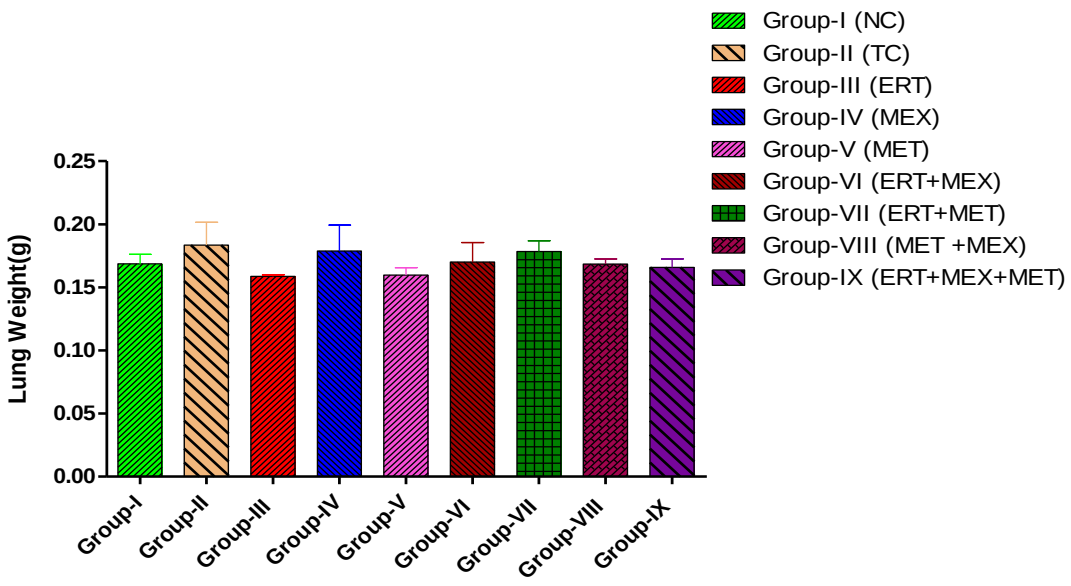

Figure 4.36: Effect of daily administration of erlotinib, meloxicam and metformin as alone and in combination with each other for 28 days on lung weight (g) of xenograft model of SCID male mice

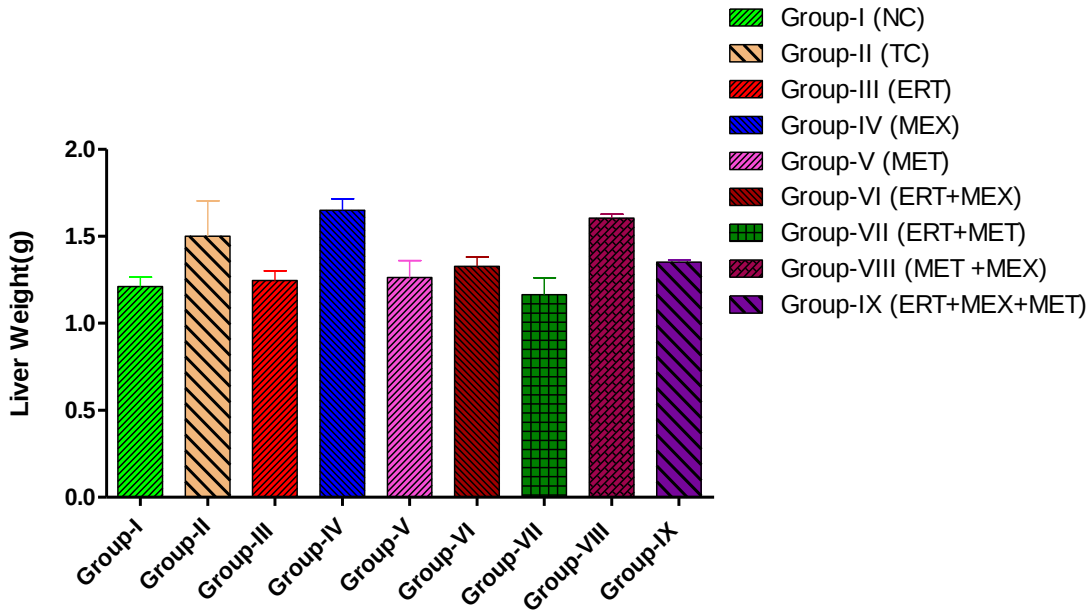

Figure 4.37: Effect of daily administration of erlotinib, meloxicam and metformin as alone and in combination with each other for 28 days on liver weight (g) of xenograft model of SCID male mice

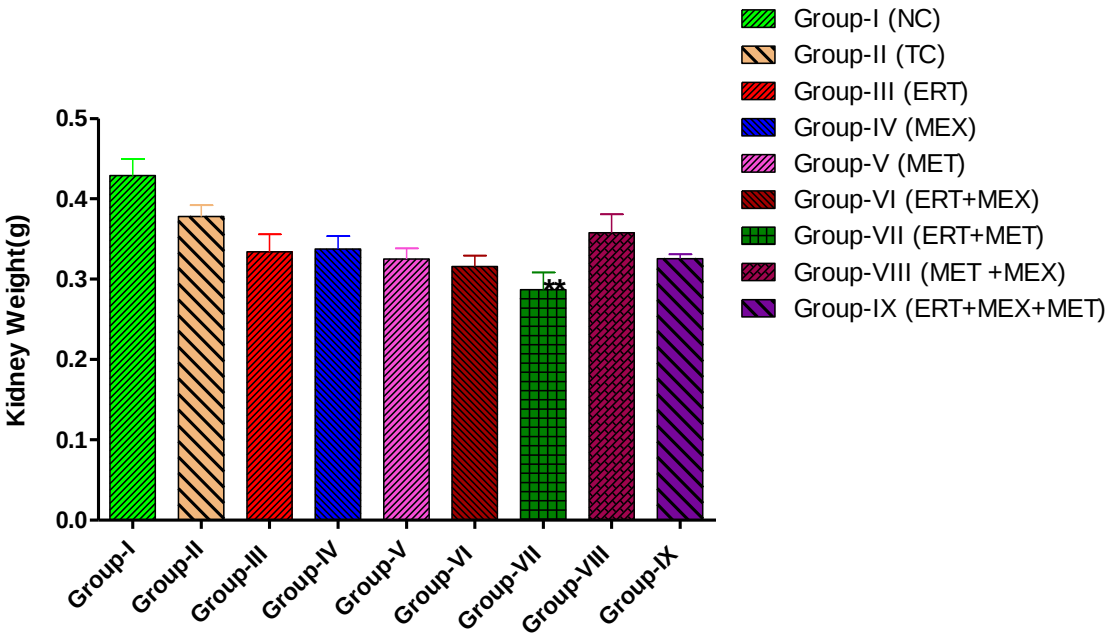

Figure 4.38: Effect of daily administration of erlotinib, meloxicam and metformin as alone and in combination with each other for 28 days on kidney weight (g) of xenograft model of SCID male mice

4.2.9 Drug Distribution in tissue

The tumor and lung tissue from all animals were collected and homogenated for extraction by solid phase extraction method. The extract was measured for the presence of erlotinib, meloxicam and metformin in respective group of treatment but none of the drug was detected in either lung or tumor in any group of the animals.

4.2.10 Effect on Gene expression

The tumor tissue was evaluated for the expression of gene responsible for tumor development and/or apoptosis like AKT, AMPK, P070S6K, PTEN and RAF for change in their expression level and the results obtained are depicted in Table 4.39. There was significant increase in expression of PTEN gene in group no. VI and VII in comparison to the group II. The expression of PTEN gene was increased in all treatment groups in comparison to the tumor control group II. The expression of AKT gene was found to be low in all treatment groups except for significant increase seen in group VIII in

comparison to the group II. The expression of AMPK gene is slightly increased in group III, VI and IX and whereas considerably increased in group V and VII in comparison to the group II. The expression of P070S6K gene was considerably reduced in group VI and VIII in comparison to group II. It is slightly reduced in other group except group IX in comparison to Group II. The RAF expression is considerably reduced in group VIII in comparison to group II. It was slightly decreased in group III, V, VI, VII and IX in comparison to group II. The individual gene expression in different treatment groups was compared and graphically represented in Figure 4.39 to 4.43.

Table 4.39: Effect of daily administration of erlotinib, meloxicam and metformin as alone and in combination with each other for 28 days on tumor gene expression of xenograft model of SCID male mice (n=6)

Treatment group	AKT	AMPK	P070S6K	PTEN	RAF
Group-II (tumor control)	1.04 ± 0.21	1.04 ± 0.22	0.64 ± 0.05	1.34 ± 0.15	1.84 ± 0.29
Group-III (ERTB)	0.84 ± 0.14	1.83 ± 0.43	0.49 ± 0.14	2.77 ± 0.80	1.56 ± 0.39
Group-IV (MEX)	0.79 ± 0.11	1.15 ± 0.15	0.43 ± 0.05	2.04 ± 0.10	1.82 ± 0.53
Group-V (MET)	0.82 ± 0.20	1.93 ± 0.17	0.40 ± 0.11	1.73 ± 0.22	1.04 ± 0.10
Group-VI (ERTB+MEX)	0.56 ± 0.24	1.23 ± 0.33	0.24 ± 0.11	4.21* ± 1.85	1.49 ± 0.40
Group-VII (ERTB+MET)	0.53 ± 0.201	2.01 ± 0.44	0.56 ± 0.31	4.51* ± 0.29	1.41 ± 0.72
Group-VIII (MET +MEX)	2.15* ± 1.84	1.77 ± 0.35	0.39 ± 0.13	2.03 ± 0.54	0.76 ± 0.33
Group-IX (ERTB+MEX+MET)	0.63 ± 0.34	1.63 ± 0.67	0.74 ± 0.25	2.94 ± 0.85	1.45 ± 0.55

*significant at $p<0.05$

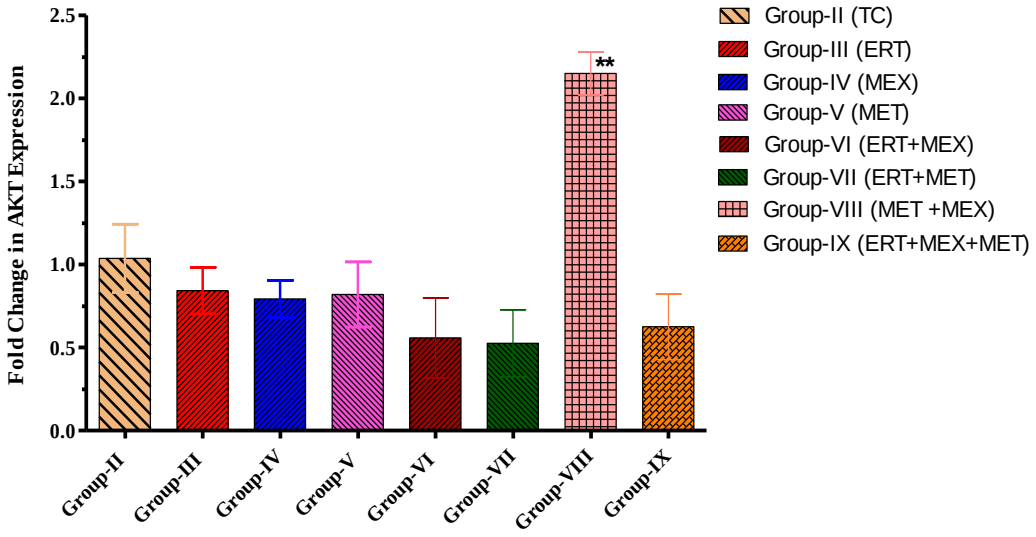

Figure 4.39: Effect of daily administration of erlotinib, meloxicam and metformin as alone and in combination with each other for 28 days on expression of AKT gene in tumor tissue of xenograft model of SCID male mice

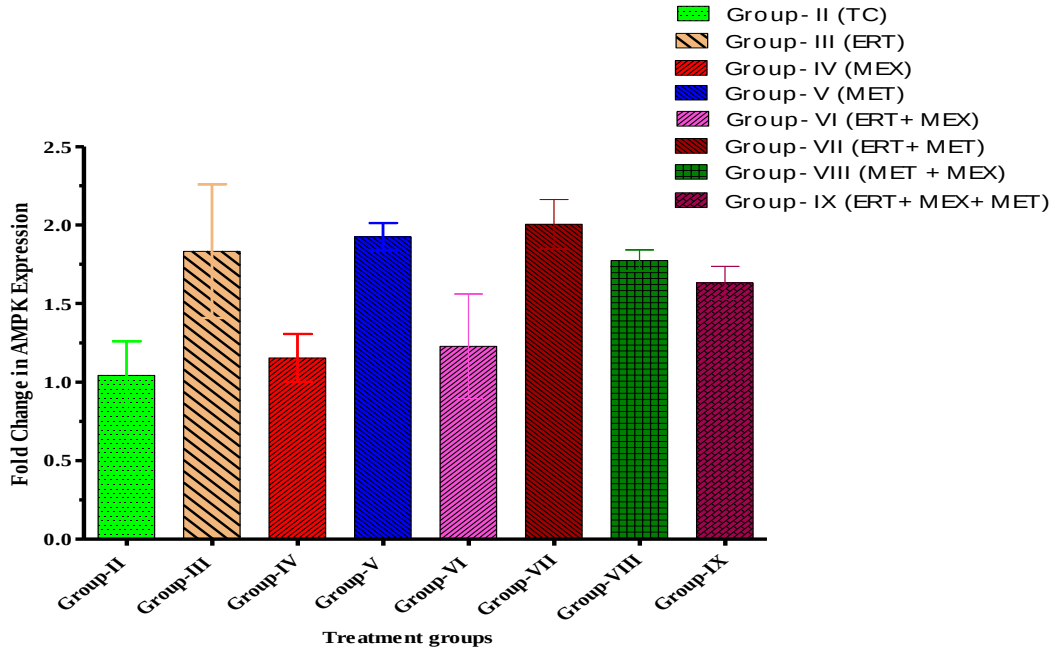

Figure 4.40: Effect of daily administration of erlotinib, meloxicam and metformin as alone and in combination with each other for 28 days on expression of AMPK gene in tumor tissue of xenograft model of SCID male mice

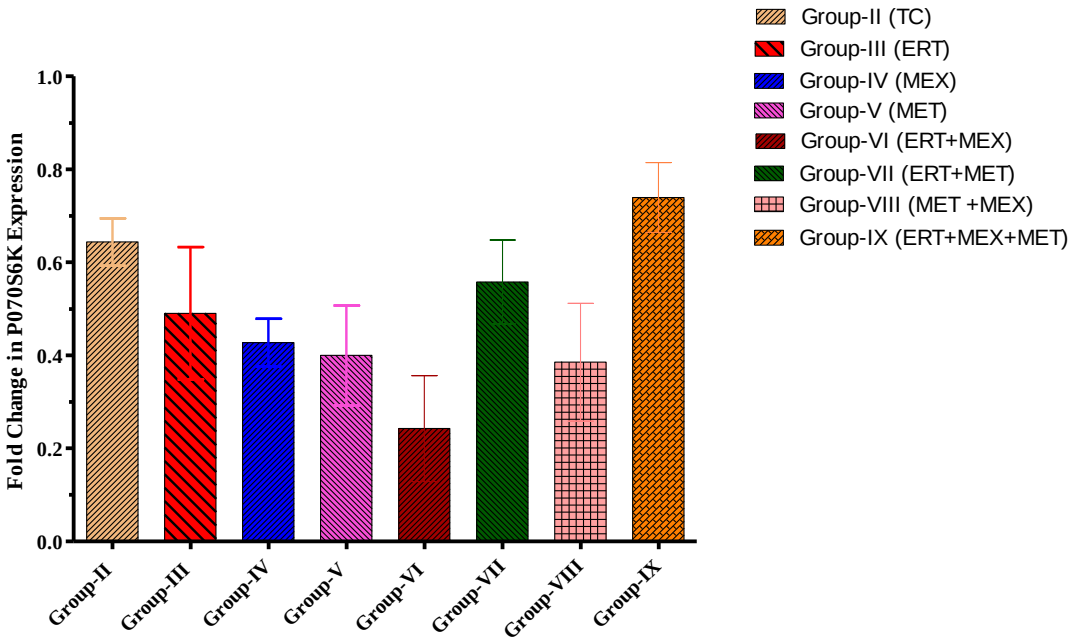

Figure 4.41: Effect of daily administration of erlotinib, meloxicam and metformin as alone and in combination with each other for 28 days on expression of P070S6K gene in tumor tissue of xenograft model of SCID male mice

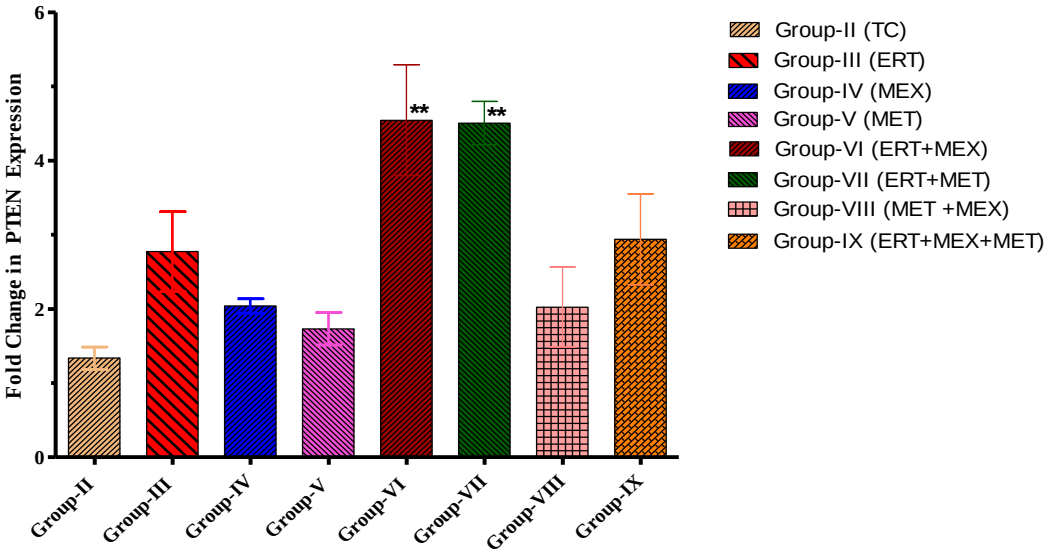

Figure 4.42: Effect of daily administration of erlotinib, meloxicam and metformin as alone and in combination with each other for 28 days on expression of PTEN gene in tumor tissue of xenograft model of SCID male mice

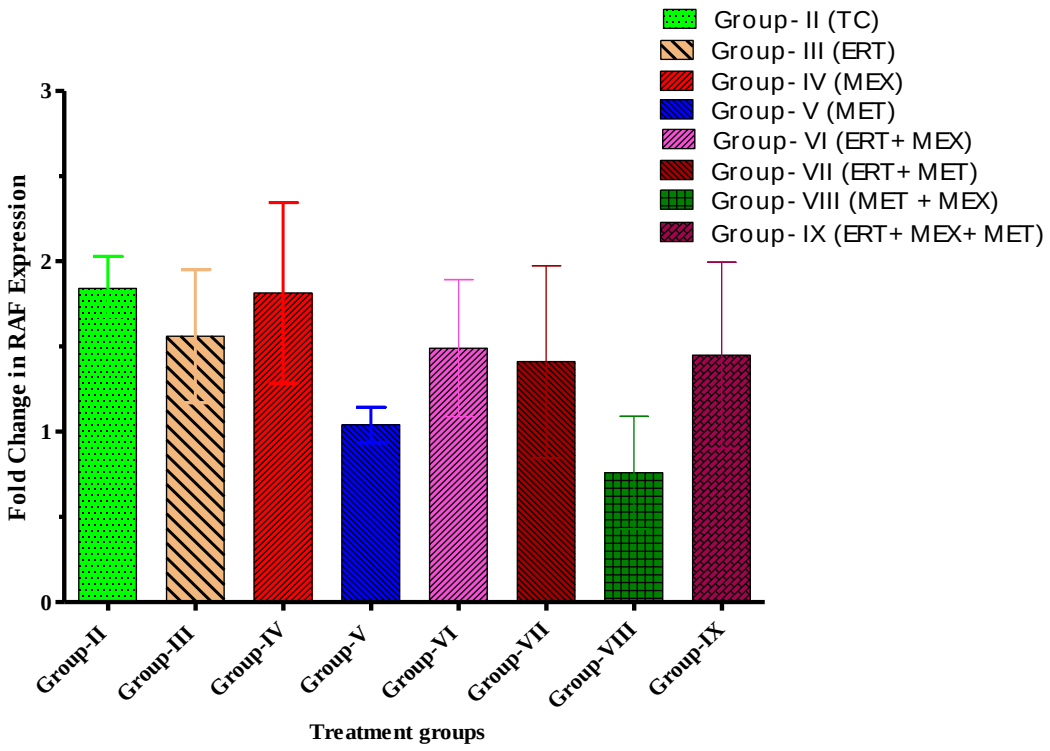

Figure 4.43: Effect of daily administration of erlotinib, meloxicam and metformin as alone and in combination with each other for 28 days on expression of RAF gene in tumor tissue of xenograft model of SCID male mice

4.2.11 Histopathology

Tissues like tumor mass, lungs, liver, heart and kidneys were collected from all the animals of treated and control groups during necropsy. The histopathological slides prepared for the above organs/tissues were subjected for microscopic examination. The changes in tumor mass attributed to be a response to the various treatments as single drug or in combination of drugs is depicted in Table 4.40. Since the major intension was to score the degree of shrinkage of tumor mass due to administration of drugs, the severity of necrosis was main attribute to designate the positive response. Significant differences in histological changes were observed in tumor tissue. The tumor tissue exhibited microscopically necrotic core, mitotic figures, hemorrhage, penetration in surrounding tissue from mild to moderate grade in all treatment groups except treatment group IX where the necrotic core was severe and the mitotic figures observed at minimal to mild

grade in comparison to other groups. The representative photomicrograph is provided in Plate 4.1 to 4.8. The tumor mass of animals in group IX appeared to be reduced to a considerable size as necrosis of tumor mass was severe in these animals. The tumor mass or necrosis of tumor cells was also seen of moderate severity in animals treated in group VI followed by mild to moderate severity in group VIII and mild severity in group VII. The minimal or minimal to mild necrotic changes in tumor mass was seen in animals of groups III, IV and V. The tumor mass was intact and none of the animal revealed necrotic changes in tumor control group. The treated and control group revealed minimal to mild histopathological changes like foci of leucocytic infiltration, alveolar hystiocytosis, necrotic foci, microgranuloma, dilatation of tubules, basophilic tubules and nephropathy are depicted in Table 4.40.

Table 4.40: Effect of daily administration of erlotinib, meloxicam and metformin as alone and in combination with each other for 28 days on histopathological changes on tumor mass and other organs of xenograft model of SCID male mice (n=6)

Histopathological observation	Group-I (NC)	Group-II (TC)	Group-III (ERT)	Group-IV (MEX)	Group-V (MET)	Group-VI (ERT+MEX)	Group-VII (ERT+MET)	Group-VIII (MEX+MET)	Group-IX (ERT+MEX +MET)
Tumor									
Necrotic core	NA	1	1-2	1	1-2	3	2-3	2-3	4
Hemorrhages	NA	3	2-3	3	3	2	1-3	0-3	0-1
Mitotic figures	NA	1-3	1-3	2-3	3	2	2-3	2-3	2
Growth penetrating the surrounding tissue	NA	0-2	0-2	0-1	1	1-2	1-2	1-2	1
Vacuolated cytoplasm	NA	0-2	1-2	1-2	1-2	1	0	0	0
Heart									
Foci of Leucocytic Infiltration	0	0	0	0-1	0	0	0	0	0
Lungs									
Alveolar histiocytosis	0-1	0-2	0-1	0	0-1	0	0	0	0
Foci of Leucocytic Infiltration	0	0	0	0-1	0	0	0	0	0
Liver									
Necrotic foci	0	0-1	0	0-2	0-1	0-1	0	0-2	0
Foci of Leucocytic Infiltration	0	0	0-1	0-2	0	0	0-2	0-2	0-1
Microgranuloma	0	0	0	0	0	0-1	0	0	0
Kidneys									
Dilatation of tubules	0	0	0	0-1	0	0	0-1	0-1	0-1
Basophilic tubules	0	0	0	0-1	0	0	0	0	0
Nephropathy	0	0	0	0	0	0-2	0	0	0

Key: NA = Not applicable, 0 = Normal, 1= Minimal, 2= Mild, 3= Moderate, 4= Severe.

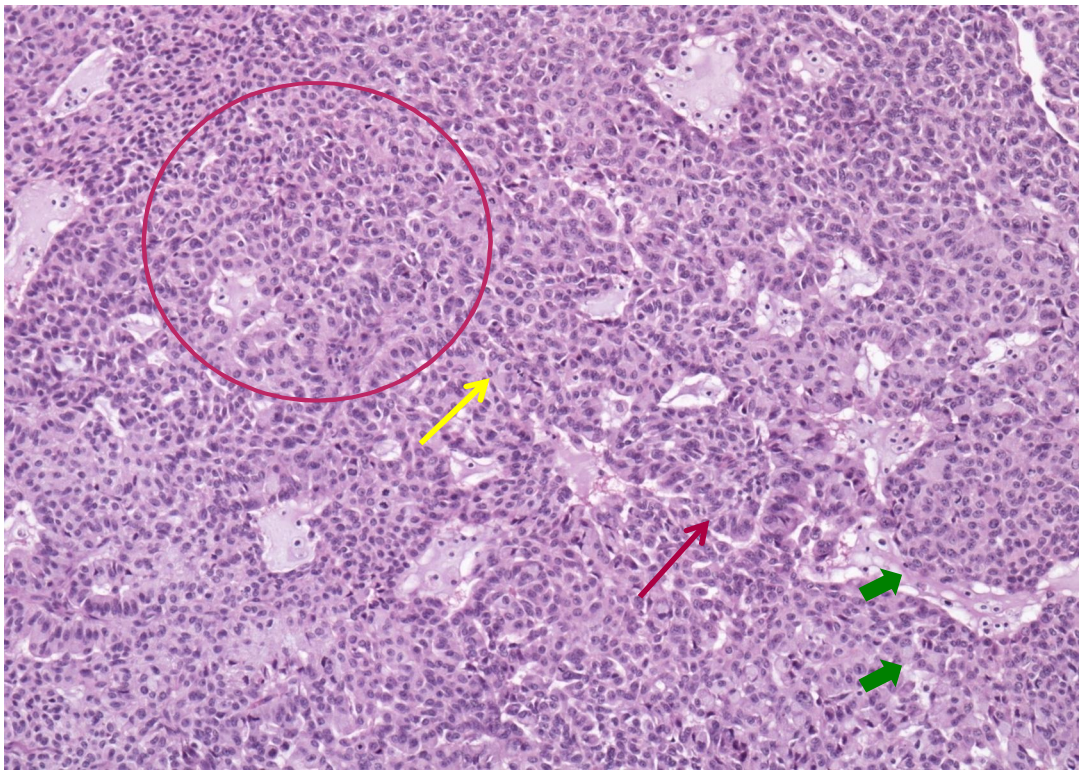

Plate 4.1: Section of tumor mass from tumor control group. Tumor cells arranged to form the glandular structures. Note the abundant cytoplasm (yellow arrow), nuclear pleomorphism (circle), cytoplasmic vacuolation (thick green arrow) and frequent mitotic figures (red arrow) are discernible H & E 20X

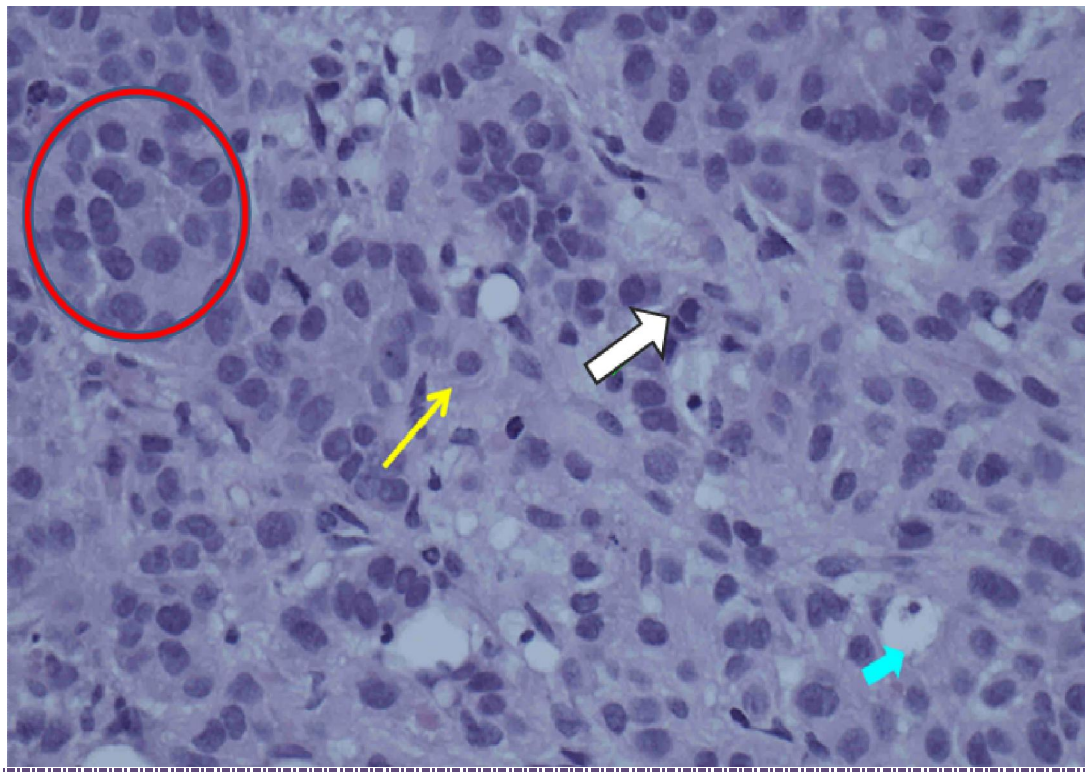

Plate 4.2: Section of tumor mass from tumor control group. Tumor cells arranged to form the glandular structures. Note the abundant cytoplasm, (yellow arrow) nuclear (pleomorphism (circle), cytoplasmic vacuolation (thick small arrow) and mitotic figures (thick white arrow) are discernible H & E 40X

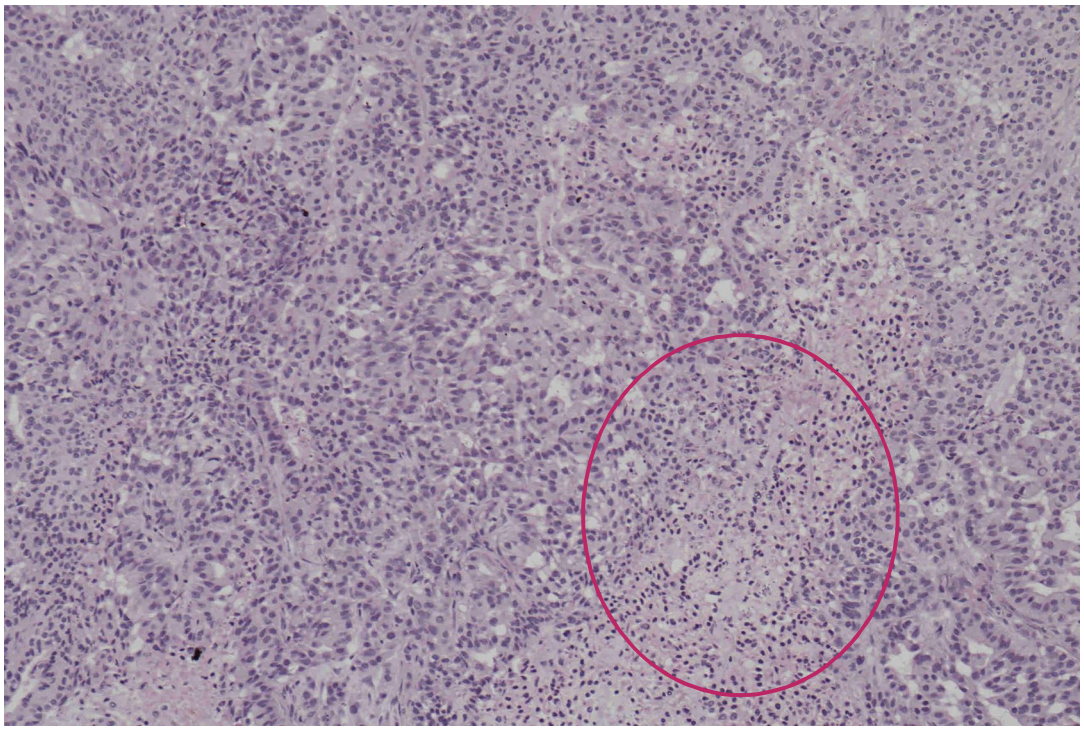

Plate 4.3: Tumor section showing malignant characteristics of epithelial cell with evidence of minimal necrosis (circle) H& E 20X

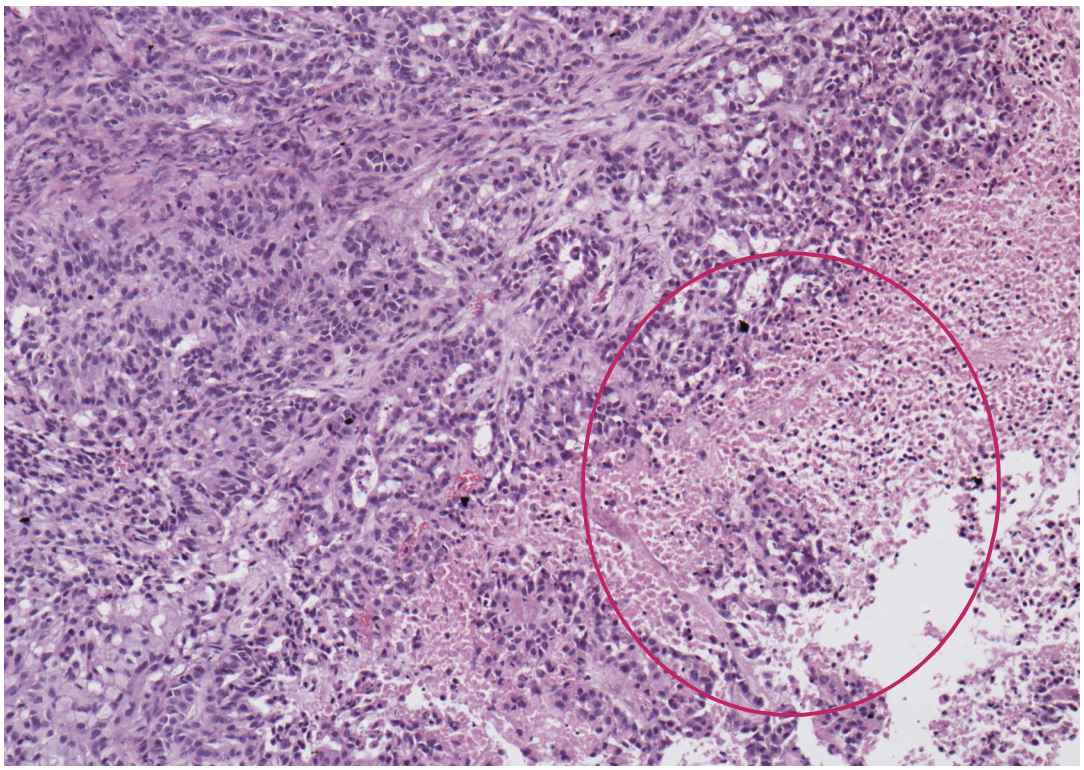

Plate 4.4: Tumor section showing malignant characteristics of epithelial cell with evidence of mild necrotic area in the tumor (circle) H& E 20X

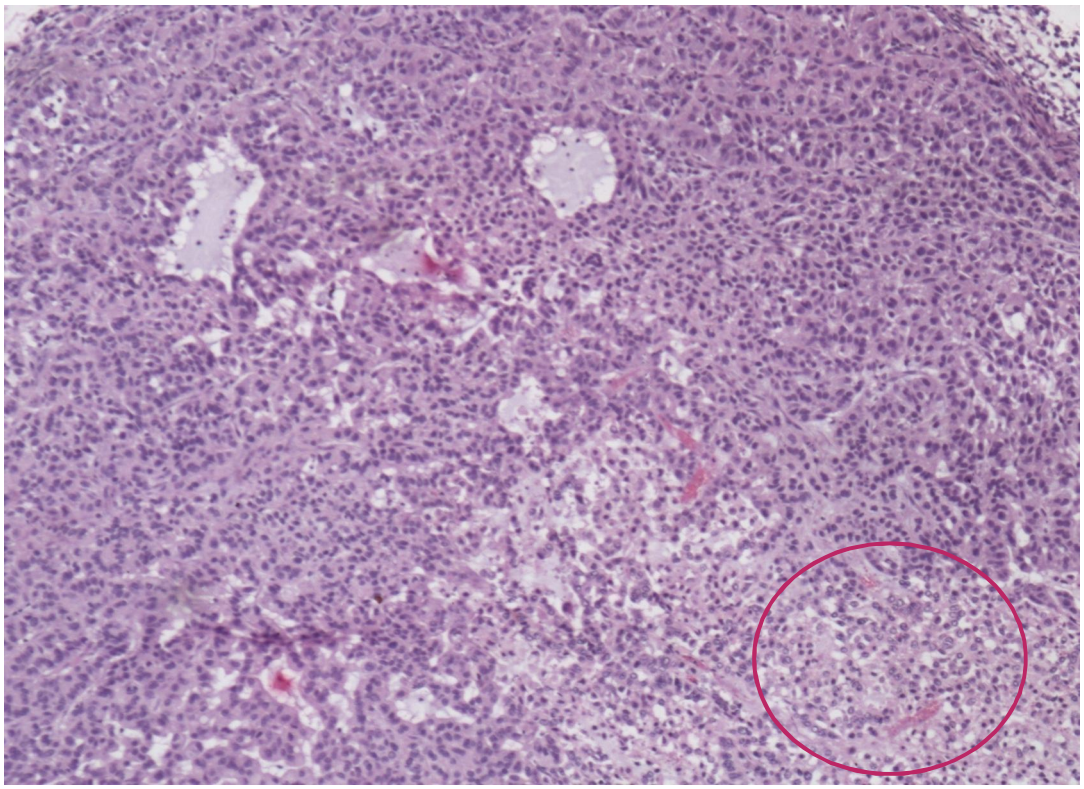

Plate 4.5: Tumor section showing malignant characteristics of epithelial cell with evidence of minimal necrotic area in the tumor (circle) H& E 20X

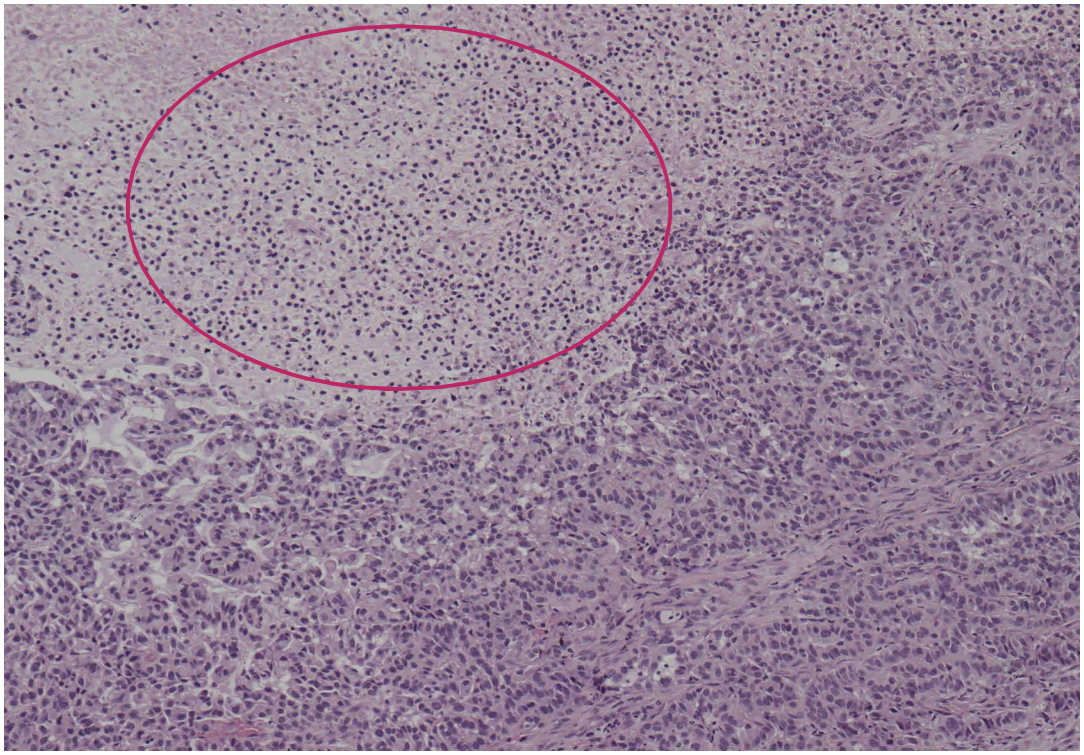

Plate 4.6: Tumor section showing malignant characteristics of epithelial cell with evidence of mild to moderate necrotic area in the tumor (circle) H& E 20X

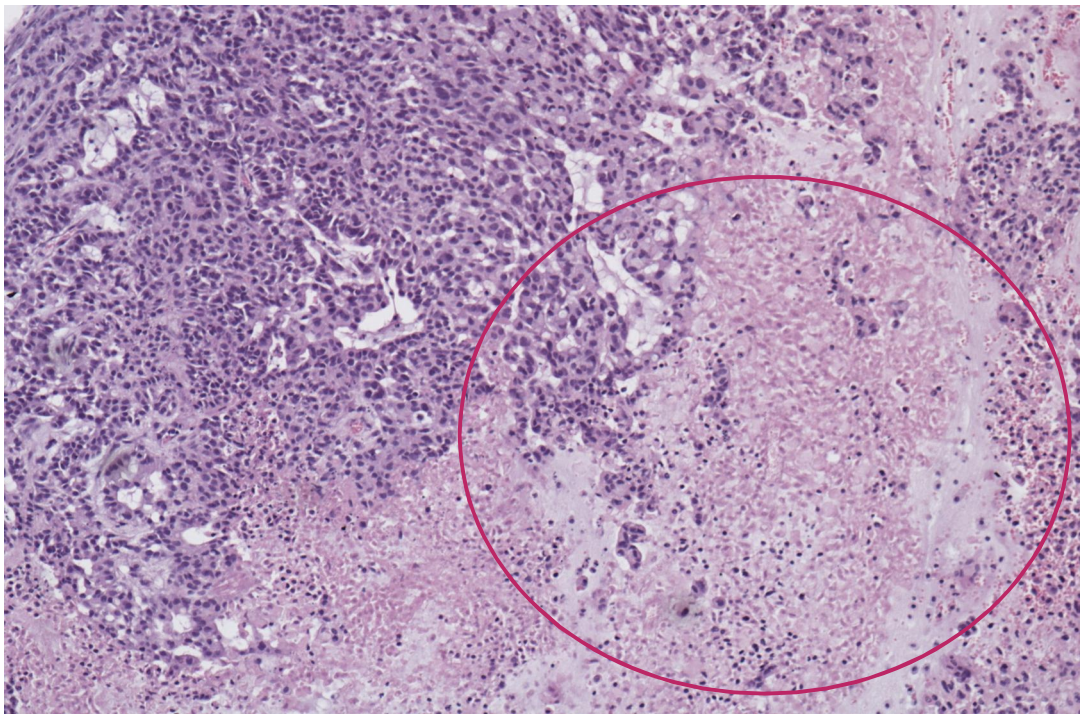

Plate 4.7: Tumor section showing malignant characteristics of epithelial cell with evidence of moderate necrotic area in the tumor (circle) H& E 20X

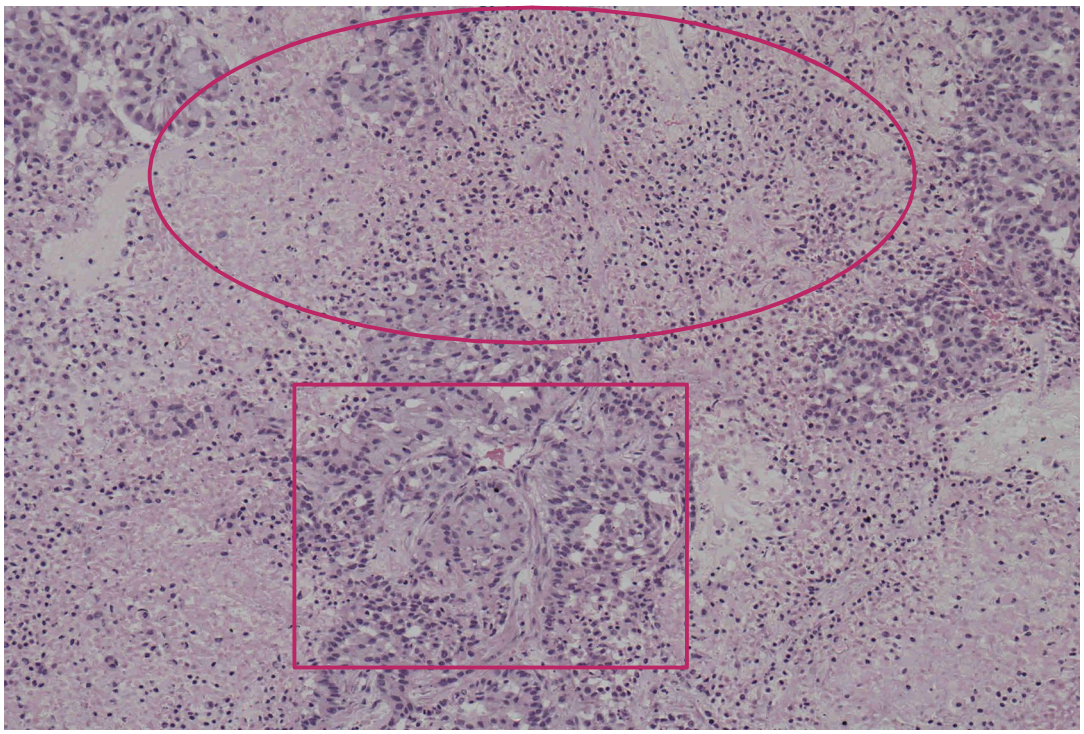

Plate 4.8: Tumor section showing malignant epithelial cells (square) with evidence of severe necrotic area in the tumor (circle) H& E 20X

CHAPTER – V

DISCUSSION

Tyrosine kinase inhibitors are an important class of drugs largely used to treat cancer like non small cell lung carcinoma (NSCLC). With the increasing awareness regarding the mechanism of action, its adverse effect, limitation in its efficacy and development of resistance, numerous new drugs have been synthesized for use in different types of cancer of man and animals. The use of tyrosine kinase inhibitors have been increased for the treatment of cancer especially lung adenocarcinoma. Parallel to development of newer concepts, treatment strategies and understanding of tumor pathology as well as molecular pathways, the concept of combinational drug therapy is emerging.

The tumor pathology is very complex and the progression of tumor depends on many pathways responsible for cell proliferation and apoptosis. The major pathways involved in tumor progression are growth promoting pathways (EGFR/Ras/PI3-Kinase), growth inhibitory pathways (p53/Rb/P14ARF, STK11), apoptotic pathways (Bcl-2/Bax/Fas/FasL), DNA repair and immortalization genes (Brambilla and Gazdar, 2009). In addition to these pathways, Cyclooxygenase (COX)-2 and mTOR pathways play key role in regulating PI3-K activity and P53 activity respectively. The COX-2 suppresses PI_3-K activity, which represses antioxidant response element Nrf_2-mediated transcriptional response in human chondrocytes that results in decreased apoptosis (Healy *et al.*, 2005). The Ras/PI3K/PTEN/Akt/mTOR pathway plays a key role in regulating p53 activity and critical proteins involved in protein translation especially those necessary for the translation of “weak” mRNAs (mTORC1,). The Akt activates downstream mTOR

which can subsequently serve as either a negative feed back to inactivate Akt by p70S6K or activate Akt by mTORC2 (Steelman *et al.*, 2011). These pathways have been studied extensively and still more research is going on and new thoughts are reported every year.

The erlotinib a member of Protein kinase inhibitors is widely used for the treatment of lung adenocarcinoma. It is EGFR inhibitor and very specifically it is tyrosine-kinase inhibitor (TKI). The meloxicam is COX inhibitor and specifically COX-2 inhibitor, whereas the metformin is biguanide, mainly used for diabetes treatment. This drug also has a property as mTOR inhibitor, specifically as AMPK activator and P53 inhibitor (Dowling *et al.*, 2011).

In clinical practice, the analgesic along with antineoplastic drug is necessity to alleviate pain. The carcinogenic conditions are mainly observed at old ages, where there are more chances of having metabolic disorders like diabetes. In such condition, there are always chances of combinational drug therapy like EGFR inhibitors, COX-2 inhibitors as well as mTOR inhibitors for different perspective of treatment. Based on this consideration, a study was carried out to evaluate the effects of erlotinib, meloxicam and metformin alone and in combination with each other and all three drugs together for their pharmaco-toxicological profile. Looking to limitation of the diseased condition and availability of the clinical cases, the study was carried out in xenograft model developed by injecting A549 cell line resembling the lung adenocarcinoma. The pharmacokinetic studies were conducted in normal healthy male SCID mice to evaluate the pharmacokinetic parameters for three drugs individually as well as in combination with each other.

Co-administration of several drugs may result in unpredictable therapeutic outcome (diminished/synergistic therapeutic efficacy or increased/decreased toxicity

of one or more of the administered drugs). Use of non-steroidal anti-inflammatory drugs (NSAIDs) are frequently recommended with antineoplastic agents for the treatment of various carcinogenic condition accompanied by pain and other inflammatory conditions in animals. In veterinary practice, meloxicam is used as anti inflammatory and pain reliever drug in all animals (Lees *et al*., 2004). Pharmacokinetics of an anti cancer drugs may be changed when they are administered with anti-inflammatory drug or with anti diabetic drug in animals/humans. Despite enormous potential for clinical use of these drugs, the data on their pharmacokinetics and safety profile in tumor model of mice are scarce.

Looking to this fact and need of clinical condition, pharmacokinetic parameters and safety evaluation of erlotinib, meloxicam and metformin alone and in combination with each other and all three drugs together were planned. The present study was conducted in two phase; Phase -I was conducted to evaluate the pharmacokinetic parameters after oral administration of erlotinib (30 mg/kg) and metformin (100mg/kg) and intra peritoneal administration of meloxicam (20 mg/kg) in 168 healthy SCID male mice. The phase-II of study was conducted in 54 SCID male mice to evaluate safety and efficacy of erlotinib (30 mg/kg, p.o), metformin (100mg/kg p.o) and meloxicam (20 mg/kg, i.p) administration for 28 days in tumor xenograft model. This study would help in assessment of their therapeutic utility for the treatment of lung adenocarcinoma.

5.1 Plasma levels of erlotinib following oral administration as alone and in combination with meloxicam and/or metformin in SCID male mice

5.1.1 Plasma level of erlotinib

In the present study, erlotinib was administered (30 mg/kg) orally as alone and in combination with meloxicam (20 mg.kg) intraperitoneally and/or metformin (100 mg/kg) orally in normal healthy SCID male mice. Pharmacokinetic studies of erlotinib have previously been conducted in animals like mice (Bailey *et al.*, 1991; Bailey *et al.*, 2004; Li *et al.*, 2006, 2007[a]; Li *et al.*, 2007[b]; Smith *et al.*, 2008; Marchetti *et al*., 2008), beagle dogs (Johnson and Prakash, 1998), SD Rats (Johnson and Prakash, 1997) and in human (Lu *et al.*, 2006; Ling *et al.*, 2006; Hanauske *et al.*, 2007; Geoerger *et al.*, 2009; Ma *et al.*, 2010). The dose of erlotinib 30 mg/kg in present study was selected based on previously reported dose in other experimental studies done in mice (Higgins *et al.*, 2003; Zerbe *et al*, 2006; Smith *et al.*, 2008; Bao *et al.*, 2009; Ueno and Zhang, 2011) and human (Yeo *et al*., 2010). The pharmacokinetic interaction of erlotinib (PO) with BAS-100 (PO) has been evaluated in female balb/c, CYP3A4 transgenic and FVB/NCr wild mice (Smith *et al*, 2008). Similar to that, *in-vivo* and *in-vitro* disposition kinetics of erlotinib with ATP- binding cassette drug transporters ABCB1, ABCG2, and ABCC2 have been evaluated using triple knockout mice and wild type mice (Marchetti *et al*., 2008).

Following oral administration of erlotinib alone, the mean peak plasma level of drug was observed 16372.63 ± 846.59 ng/ml at 0.167 hrs, which slowly declined to 6969.17 ± 309.70 ng/ml at 4 hrs and thereafter the drug concentration in plasma diminished slowly and was detectable (709.25 ± 78.61 ng/ml) up to 24 hrs. The drug was not detected in plasma samples collected after 24 hrs post oral administration of erlotinib in healthy SCID mice.

In case of oral administration of erlotinib in combination with intra peritoneal administration of meloxicam, the mean peak plasma level of erlotinib was observed 17741.60 ± 2918.54 ng/ml at 0.167 hrs, which gradually declined to 6988.27 ± 381.15 ng/ml at 4 hrs and finally reached to 414.05 ± 84.34 ng/ml at 24.00 hrs. Same way oral administration of erlotinib in combination with metformin, the mean peak plasma level of erlotinib was observed 13400.85 ± 882.54 ng/ml at 0.167 hrs, which gradually declined to 4567.18 ± 456.98 ng/ml at 4 hrs and finally reached to 385.70 ± 114.87 ng/ml at 24.00 hrs. The drug was not detected in plasma samples collected after 24 hrs post oral administration of erlotinib along with metformin. When the erlotinib was administered along with meloxicam and metformin, the mean peak plasma level of erlotinib was observed 13600.97 ± 1176.92 ng/ml at 0.167 hrs, which gradually declined to 3558.74 ± 250.68 ng/ml at 4 hrs and finally reached to 463.43 ± 42.72 ng/ml at 24.00 hrs. The drug was not detected in plasma samples collected after 24 hrs post oral administration of erlotinib along with both meloxicam and metformin.

Statistically, there was non-significant difference in mean peak plasma concentrations of erlotinib following its oral administration alone and in combination with meloxicam and/ or metformin in SCID male mice. The mean peak plasma value of erlotinib was observed considerably high in combination with meloxicam and lower in combination with metformin as well as both in comparison to alone. The concentration of erlotinib in plasma was observed till 24 hrs in erlotinib alone and in combination with meloxicam, metformin and both. The plasma concentrations of erlotinib at 8 hrs post administration in erlotinib alone and in combination with meloxicam remained considerably higher in comparison to other two combinations. The plasma concentration of erlotinib with metformin and in combination with meloxicam and metformin both were significantly low ($P < 0.05$) than erlotinib alone

treatment group at 0.33 hrs and 4 hrs post administration of drug. The similar type of study of combination of erlotinib along with pertuzumab was found superior than monotherapy (Friess *et al*., 2005). Prados *et al.* (2006) reported significant reduction in $AUC_{0-\alpha}$ of erlotinib following co-administration of erlotinib with temozolomide (12.00 ± 4.00) as compared to erlotinib alone (22.30 ± 11.00) in human. The lower plasma concentration of drug was probably a result of fast elimination of the drug in metformin combination. This observation is supported by Scarpello and Howlett (2008), who reported that metformin increases the micro vascular blood flow and ultimately increases the micro circulation towards kidney, which may enhances the clearance of the drug.

5.1.2 Plasma levels of meloxicam following intra peritoneal administration alone and in combination with erlotinib or/and metformin in male SCID mice

In the present study, meloxicam was administered (20 mg/kg) intra peritoneally as alone and in combination with erlotinib (30 mg/kg) or/and metformin (100 mg/kg) orally in normal healthy SCID male mice. Pharmacokinetic studies of meloxicam have previously been conducted in animals like horses (Lees *et al*., 1991; Toutain *et al*., 2004), dogs (Montoya *et al*., 2004; Gohel, 2006; Mahmood and Ashraf, 2010), calves (Coetzee *et al.*, 2009), piglets (Fosse *et al.*, 2008), donkey (Sinclair *et al.*, 2006), rats (Aguilar-Mariscal *et al.*, 2007; Habashi and Jamali, 2008), chickens (Baert and De Backer, 2002), ostriches (Baert *et al*., 2002), vulture (Naidoo *et al.*, 2008), rabbits (Turner *et al*., 2006, carpenter *et al*., 2009), sheep and goats (Shukla *et al*., 2007) and humans (Busch *et al*., 1998). The dose of meloxicam 20 mg/kg in present study was selected based on previously reported dose in other experimental studies done in mice (Goldman *et al.*, 1998; Jiang *et al.*, 2009). The pharmacokinetic interaction of meloxicam (PO) with methotrexate has been evaluated in patient with

rheumatoid arthritis (Hubner *et al.*, 1997). Similarly, pharmacokinetic interaction of meloxicam with warferin has been investigated in healthy male volunteers (Turck *et al*., 1997). Sadariya *et al.* (2010) have investigated pharmacokinetic interaction of moxifloxacin and meloxicam following intramuscular administration in rats.

Following oral administration of meloxicam alone, the mean peak plasma level of drug was observed 44858.46 ± 3486.54 ng/ml at 0.33 hrs, which slowly declined to 10660.24 ± 381.76 ng/ml at 4 hrs and thereafter the drug concentration in plasma diminished slowly and was detectable (160.24 ± 9.75 ng/ml) up to 24 hrs. The drug was not detected in plasma samples collected after 24 hrs post intra peritoneal administration of meloxicam in healthy SCID male mice.

In case of intraperitoneal administration of meloxicam in combination with oral administration of erlotinib, the mean peak plasma level of drug was observed 49994.17 ± 2928.14 ng/ml at 0.33 hrs, which gradually declined to 11869.03 ± 1141.80 ng/ml at 6 hrs and finally reached to 382.34 ± 106.23 ng/ml at 24.00 hrs. The drug was not detected in plasma samples collected after 24 hrs post intraperitoneal administration of meloxicam along with erlotinib, orally. Same way intraperitoneal administration of meloxicam in combination with metformin, the mean peak plasma level of meloxicam was observed 46540.83 ± 3879.53 ng/ml at 0.33 hrs, which gradually declined to 10268.29 ± 652.65 ng/ml at 4 hrs and finally reached to 245.65 ± 31.90 ng/ml at 24.00 hrs. The drug was not detected in plasma samples collected after 24 hrs post intraperitoneal administration of meloxicam along with metformin. Whereas, the meloxicam with administration of both erlotinib and metformin, the mean peak plasma level of meloxicam was observed 39648.94 ± 2190.42 ng/ml at 0.33 hrs, which gradually declined to 9266.74 ± 749.79 ng/ml at 4 hrs and finally reached to 163.29 ± 12.29 ng/ml at 24.00 hrs. The drug was not detected in plasma

samples collected after 24 hrs post intraperitoneal administration of meloxicam along with both erlotinib and metformin.

There was non-significant difference in mean peak plasma concentrations of meloxicam following its intraperitoneal administration as alone and in combination with erlotinib or/and metformin in SCID male mice. The mean peak plasma value of meloxicam was observed noticeably higher in combination with erlotinib as well as metformin treatment group whereas considerably lower in combination with both drugs in comparison to meloxicam as alone treatment group. The concentration of meloxicam in plasma was observed till 24 hrs in all treatment groups. The plasma concentrations of meloxicam at 6 hrs post administration remained considerably high in combination with erlotinib in comparison to combination with both drugs as well as combination with metformin treatment groups. The plasma concentration of meloxicam in combination with metformin was significantly low ($P < 0.05$) than meloxicam alone treatment group at 1 hr post administration of drug. The plasma concentration of meloxicam in combination with both erlotinib and metformin was significantly high ($P < 0.01$) than meloxicam alone treatment group at 12 hr post administration of drug. The plasma concentration of meloxicam in combination with erlotinib was significantly high ($P < 0.01$) than meloxicam alone treatment group at 2,4,6,8 and 12 hrs post administration of drug.

Higher plasma concentration of meloxicam may probably due to inhibition of CYP2C9, enzyme responsible for meloxicam metabolism. Similar findings were reported by Haouala *et al.,* (2011) showing increased concentration of ibuprofen, mefenacid, metamizole and diclofenac in human when co administered with tyrosine kinase inhibitors likes imatinib, dasatinib and nilotinib.

5.1.3 Plasma levels of metformin following oral administration alone and in combination with erlotinib or/and meloxicam in male SCID mice

In the present study, metformin was administered (100 mg/kg) orally as alone and in combination with erlotinib (30 mg/kg) and /or meloxicam (20 mg/kg) intra peritoneally in normal healthy SCID male mice. Pharmacokinetic studies of metformin have previously been conducted in animals like ponies (Tinworth *et al*., 2010), horse (Hustace *et al*., 2009), dogs and cats (Heller, 2007), rabbits (Rani *et al*., 2011), rats (Lal and Jain, 2010) and humans (Busch *et al*., 1998**)**. The dose of metformin 100 mg/kg in present study was selected based on previously reported dose in other experimental studies done in mice (Liu *et al*., 2011; Rattan *et al*., 2011). The pharmacokinetic interaction of metformin (PO) with roziglitazone (PO) has been evaluated in mice, rat, dog, monkey and human (EMEA, 2005). Additionally, pharmacokinetic interaction of metformin with centchroman (Lal and Jain, 2010) in rat and metformin with trandolapril (Rani *et al*., 2011) in rabbits have been investigated.

Following oral administration of metformin alone, the mean peak plasma level of drug was observed 5782.29 ± 441.77 ng/ml at 0.33 hrs, which slowly declined to 1142.31 ± 121.83 ng/ml at 4 hrs and thereafter the drug concentration in plasma diminished fast and was detectable (202.32 ± 27.47 ng/ml) up to 12 hrs. The drug was not detected in plasma samples collected after 12 hrs post oral administration of metformin in healthy SCID mice. In case of oral administration of metformin in combination with oral administration of erlotinib, the mean peak plasma level of drug was observed 6307.71 ± 577.57 ng/ml at 0.33 hrs, which gradually declined to

1463.02 ± 208.69 ng/ml at 4 hrs and finally reached to 166.02 ± 18.99 ng/ml at 12.00 hrs. The drug was not detected in plasma samples collected after 12 hrs post oral administration of metformin along with erlotinib, orally. Same way oral administration of metformin in combination with intraperitoneal administration of meloxicam, the mean peak plasma level of metformin observed was 7724.44 ± 384.36 ng/ml at 0.67 hrs, which gradually declined to 2216.40 ± 292.12 ng/ml at 4 hrs and finally reached to 126.25 ± 11.12 ng/ml at 12.00 hrs. The drug was not detected in plasma samples collected after 12 hrs post oral administration of metformin along with meloxicam.

Whereas, in mice treated with metformin with both erlotinib and meloxicam, the mean peak plasma level of metformin was observed 9490.58 ± 963.64 ng/ml at 0.33 hrs, which gradually declined to 2017.30 ± 186.74 ng/ml at 4 hrs and finally reached to 218.14 ± 36.09 ng/ml at 12.00 hrs. The drug was not detected in plasma samples collected after 12 hrs post oral administration of metformin along with both erlotinib and meloxicam.

There was non-significant difference in mean peak plasma concentrations of metformin following its oral administration alone and in combination with erlotinib and/or meloxicam in male SCID mice. The concentration of metformin in plasma was observed till 12 hrs in all treatment groups. The mean T_{max} of metformin in combination with meloxicam was observed at 0.667 hrs, whereas in all other treatment groups it remained at 0.33 hrs. The mean peak plasma concentration of metformin was observed significantly higher in combination with meloxicam ($P<0.05$) and all three drug together ($P<0.01$) in comparison to metformin as alone treatment group. The plasma concentrations of metformin at 4hrs and 6 hrs post administration in combination with meloxicam and all three drugs together remained

considerably high in comparison to metformin alone and metformin with erlotinib treatment groups. The plasma concentration of metformin in combination with both erlotinib and meloxicam was significantly high (P <0.01) than metformin alone treatment group at 0.083 hr post administration of drug. The plasma concentration of metformin in combination with erlotinib was significantly high (P <0.05) than metformin alone treatment group at 0.167 hr post administration of drug. The plasma concentration of metformin in combination with meloxicam was significantly high (P <0.05) than metformin alone treatment group at 0.667,1 and 4 hr post administration of drug. The plasma concentration of metformin in combination with both erlotinib and meloxicam was significantly high (P <0.05) than metformin alone treatment group at 0.33, 2 and 4 hr post administration of drug.

Similarly, significant increase in plasma levels of metformin following combinational treatment with trandolapril has been observed in rabbit (Rani *et al*., 2011). Higher plasma concentration of drug may probably a result of slow elimination of the drug in erlotinib and meloxicam combinational treatment as reported by Rani *et al.* (2011)**.**

5.1.4 Pharmacokinetic parameter of erlotinib following oral administration alone and in combination with meloxicam and/or metformin in male SCID mice

The plasma drug concentrations measured at various time intervals following oral administration of erlotinib alone and in combination with meloxicam and/or metformin in SCID male mice in present study were employed for the calculation of various pharmacokinetic parameters like calculated maximum plasma drug concentration (C_{max}), calculated time of maximum plasma drug concentration (t_{max}), half-life ($t_{1/2}$), area under plasma drug concentration-time curve ($AUC_{0-\alpha}$), volume of

distribution (Vd), clearance (Cl) and mean residence time (MRT).

Following oral administration of erlotinib alone in SCID male mice, calculated t_{max}, calculated C_{max}, $t_{½}$, V_z, $AUC_{0-\infty,}$ Cl, and MRT of erlotinib were 0.29 ± 0.09 h, 17272.59 ± 640.80 ng/ml, 6.08 ± 0.59 h, 3083.37 ± 247.42 ml, 84784.69 ± 3209.24 h.ng/ml, 356.48 ± 14.03 ml/h and 5.14 ± 0.36 h, respectively.

The elimination half life of erlotinib following oral administration in the present study was 6.08 ± 0.59 h, This value is higher than the values of 3.1 h , 2.0 h , 2.1 h observed in mice (Smith *et al*., 2008), rat (Johnson and Prakash, 1997), dog (Johnson and Prakash, 1998), respectively. In contrast, this value is lower than the values of 36.2 h (Lu *et al*., 2006), 20.4 h (Geoerger *et al.*, 2009), 15.91 ± 2.16 h (Ma *et al*., 2010), 8.10 ± 3.5 h (Ling *et al*., 2006) in human.

Following oral administration of erlotinib in combination with meloxicam in SCID male mice, calculated t_{max}, calculated C_{max}, $t_{½}$, V_z, $AUC_{0-\infty,}$ Cl, and MRT of erlotinib were 0.21 ± 0.04 h, 19865.31 ± 2154.82 ng/ml, 8.24 ± 1.48 h, 91056.92 ± 5850.21 h.ng/ml, 3865.44 ± 584.29 ml, 335.50 ± 18.90 ml/h and 6.12 ± 0.56 h, respectively.

Following oral administration of erlotinib in combination with metformin in male SCID mice, calculated t_{max}, calculated C_{max}, $t_{½}$, V_z, $AUC_{0-\infty,}$ Cl, and MRT of erlotinib were 0.27 ± 0.09 h, 13840.11 ± 797.51 ng/ml, 4.79 ± 0.71 h, 66493.10 ± 3418.82 h.ng/ml, 3052.14 ± 279.98 ml, 456.74 ± 21.69 ml/h and 4.63 ± 0.53 h, respectively. Following oral administration of erlotinib in combination with metformin, the mean $AUC_{0-\infty}$ of erlotinib was significantly ($p<0.05$) decreased (66493.10 ± 3418.82 h.ng/ml) as compared to $AUC_{0-\infty}$ (84784.69 ± 3209.24 h.ng/ml) after oral administration of erlotinib alone in SCID male mice. Following oral administration of erlotinib in combination with metformin, the mean Cl of erlotinib

was significant high (456.74 ± 21.69 ml/h) ($p < 0.01$) as compared to Cl (356.48 ± 14.03 ml/h) after oral administration of erlotinib alone in SCID male mice.

Following oral administration of erlotinib in combination with meloxicam and metformin in SCID male mice, calculated t_{max}, calculated C_{max}, $t_{½}$, V_z, $AUC_{0-\infty}$, Cl, and MRT of erlotinib were 0.21 ± 0.04 h, 15080.29 ± 828.43 ng/ml, 6.63 ± 0.72 h, 62452.70 ± 2400.15 h.ng/ml, 4559.44 ± 446.48 ml, 483.92 ± 18.55 ml/h and 5.16 ± 0.38 h, respectively. Following simultaneous administration of erlotinib, meloxicam and metformin in SCID male mice, the mean $AUC_{0-\infty}$ of erlotinib (62452.70 ± 2400.15 h.ng/ml) was observed significantly ($p < 0.01$) less as compared to $AUC_{0-\infty}$ (84784.69 ± 3209.24 h.ng/ml) of erlotinib alone treatment group after oral administration in SCID male mice. Same way the mean Cl of erlotinib (483.92 ± 18.55 ml/h) was found significant high in combination with both meloxicam and metformin ($p < 0.01$) as compared to Cl (356.48 ± 14.03 ml/h) of erlotinib as alone treatment group after oral administration of erlotinib in SCID male mice.

The results showed that the calculated T_{max} of erlotinib remained lowest when administered with meloxicam and in combination of all three drugs in comparison to erlotinib with metformin and erlotinib alone. The calculated C_{max} of erlotinib was observed highest in combination with meloxicam treatment group in comparison to other treatment groups. The $AUC_{(0 - \alpha)}$ was significantly low in treatment group of erlotinib with metformin ($p<0.05$) and erlotinib with both meloxicam and metformin ($p<0.01$). The calculated clearance rate was also observed significantly high in treatment group of erlotinib with metformin ($p<0.01$) and all three drugs together ($p<0.01$) in comparison to erlotinib alone treatment group. These alterations in pharmacokinetic parameters of erlotinib following administration along with meloxicam may be due to decrease in renal blood flow to kidney and also due to

reduction in GFR by meloxicam (Horl, 2010). However, similar alterations have not been observed with animals treated with erlotinib along with meloxicam and metformin which may be due to improved circulation to kidney by metformin (Scarpello and Howlett, 2008).

The lower value of $AUC_{0-\infty}$ in erlotinib with metformin and erlotinib with meloxicam and metformin in present study indicate that less area of body is covered by erlotinib concentration. when given in combination with metformin and all three drugs together. This significant change in $AUC_{0-\infty}$ of erlotinib can be correlate with other phramacokinatic parameter like C_{max}, volume of distribution and clearance. The calculated clearance rate was also observed significantly higher in treatment group of erlotinib with metformin ($p<0.01$) and all three drugs together ($p<0.01$) in comparison to erlotinib alone treatment group. Higher value of clearance of erlotinib in combination with metformin and erlotinib with both meloxicam and metformin in present study suggests that drug is rapidly eliminated from body. This significant increase in clearence of erlotinib when given in combination with metformin might be due to drug like metformin increase the micro circulation in hepatic organ as reported by Scarpello and Howlett (2008), which may be the reason for increased metabolism rate followed by increase clearance. This hypothesis is additionally also supported by Krausova *et al*., (2011), who reported that metformin activate AMPK and the subsequent up-regulation of small heterodimer partner (SHP). SHPo suppresses the functions of several nuclear receptors involved in the regulation of hepatic metabolism, including pregnane X receptor (PXR), which is referred to as a ‘‘master regulator’’ of drug/xenobiotic metabolism.

5.1.5 Pharmacokinetic parameter of meloxicam following intraperitoneal administration alone and in combination with erlotinib or/and metformin in male SCID mice

The plasma drug concentrations measured at various time intervals following intra peritoneal administration of meloxicam alone and in combination with erlotinib and/or metformin in SCID male mice in present study were employed for the calculation of various pharmacokinetic parameters like calculated maximum plasma drug concentration (C_{max}), calculated time of maximum plasma drug concentration (t_{max}), half-life $t_{1/2,}$ area under plasma drug concentration-time curve ($AUC_{0-\infty}$), volume of distribution (Vz), clearance Cl and mean residence time (MRT).

Following intraperitoneal administration of meloxicam alone in male SCID mice, calculated t_{max}, calculated C_{max}, $t_{½}$, V_z, $AUC_{0-\infty,}$ Cl, and MRT of meloxicam were 0.40 ± 0.09 h, 45715.22 ± 3343.07 ng/ml, 3.79 ± 0.34 h, 123999.94 ± 4181.79 h.ng/ml, 873.90 ± 59.61 ml, 162.24 ± 5.67 ml/h and 3.32 ± 0.15 h, respectively.

Following intraperitoneal administration of meloxicam in combination with erlotinib in male SCID mice, calculated t_{max}, calculated C_{max}, $t_{½}$, V_z, $AUC_{0-\infty,}$ Cl, and MRT of meloxicam were 0.33 ± 0.00 h, 49994.17 ± 2928.14 ng/ml, 3.51 ± 0.37 h, 196948.88 ± 6206.02 h.ng/ml, 524.79 ± 75.53 ml, 102.10 ± 3.52 ml/h and 4.33 ± 0.19 h, respectively. The mean $AUC_{0-\infty}$ of meloxicam in combination with erlotinib was very high (196948.88 ± 6206.02 h.ng/ml) and highly significant ($p < 0.01$) as compared to $AUC_{0-\infty}$ of meloxicam (123999.94 ± 4181.79 h.ng/ml) alone treated animals. The mean Vd of meloxicam in combination with erlotinib was (524.79 ± 75.53 ml) significantly low ($p < 0.05$) as compared to Vd of meloxicam (873.90 ± 59.61 ml) in alone treatment group after intraperitoneal administration of meloxicam in SCID male

mice. The mean Cl of meloxicam in combination with erlotinib was very low (102.10 ± 3.52 ml/h) and highly significant ($p < 0.01$) as compared to Cl of meloxicam (162.24 ± 5.67 ml/h) in alone treatment group after intraperitoneal administration of meloxicam in SCID male mice. The mean MRT of meloxicam in combination with erlotinib was increased (4.33 ± 0.19 h) significantly ($p < 0.05$) as compared to MRT of meloxicam (3.32 ± 0.15 h) in alone treatment group after intraperitoneal administration of meloxicam in SCID male mice.

Following intraperitoneal administration of meloxicam in combination with metformin in male SCID mice, calculated t_{max}, calculated C_{max}, $t_{½}$, V_z, $AUC_{0-\infty}$, Cl, and MRT of meloxicam were 0.39 ± 0.06 h, 46725.46 ± 3798.13 ng/ml, 4.66 ± 0.30 h, 113819.23 ± 1751.21 h.ng/ml, 1184.20 ± 87.65 ml, 175.92 ± 2.66 ml/h and 3.80 ± 0.08 h, respectively. The mean V_z of meloxicam in combination with metformin was high (1184.20 ± 87.65 ml) and significant ($p < 0.05$) as compared to Vd of meloxicam (873.90 ± 59.61 ml) given alone after intraperitoneal administration of meloxicam in SCID male mice.

Following intraperitoneal administration of meloxicam in combination with erlotinib and metformin in male SCID mice, calculated t_{max}, calculated C_{max}, $t_{½}$, V_z, $AUC_{0-\infty}$, Cl, and MRT of meloxicam were 0.33 ± 0.00 h, 39648.95 ± 2190.42 ng/ml, 4.10 ± 0.4 h, 137189.18 ± 2845.93 h.ng/ml, 858.22 ± 69.29 ml, 146.10 ± 3.02 ml/h and 3.51 ± 0.27 h, respectively. Following, simultaneous administration of meloxicam, erlotinib and metformin in male SCID mice, significant ($p < 0.05$) decrease in mean Cl of meloxicam (146.10 ± 3.02 ml/h) was observed as compared to Cl (162.24 ± 5.67 ml/h) in alone treatment group after intraperitoneal administration of meloxicam in SCID male mice.

The result showed that the calculated T_{max} remained lower in meloxicam with erlotinib and all three drugs together treatment group in comparison to meloxicam with metformin and meloxicam alone treatment group. The calculated C_{max} of meloxicam was observed highest in combination with erlotinib treatment group in comparison to other treatment groups. The $AUC_{0-\infty}$ was significantly high in treatment group of meloxicam with erlotinib ($p<0.01$) in comparison to the meloxicam alone treatment group. The calculated clearance rate was also observed significantly low in treatment group of meloxicam with erlotinib ($p<0.05$) and all three drugs together ($p<0.05$) in comparison to meloxicam alone treatment group. The volume of distribution (V_z) was also significantly low in meloxicam with erlotinib treatment group whereas significantly high in meloxicam with metformin group in comparison to meloxicam alone treatment group. The MRT is also significantly higher in meloxicam with erlotinib treatment group in comparison to meloxicam alone treatment group.

The higher value of $AUC_{0-\infty}$ in meloxicam with erlotinib in present study indicate that vast area of body is covered by meloxicam concentration. This significant change in $AUC_{0-\infty}$ of meloxicam can be correlated with other phramacokinatic parameter like C_{max}, volume of distribution, clearance and MRT. The calculated clearance rate was also observed significantly lower in treatment group of meloxicam with erlotinib ($p<0.01$) and all three drugs together ($p<0.01$) in comparison to meloxicam alone treatment group. Lower value of clearance of meloxicam in combination with erlotinib and all three drugs together in present study suggests that drug is slowly eliminated from body. This significant decrease in clearence of meloxicam when given in combination with erlotinib and all three drugs together can be correlated with significantly higher value of MRT and lower value of

Vz. The decrease in clearance of meloxicam when given in combination with erlotinib might be due to the extensive metabolism of both the drugs by liver as explained by Busch *et al*., (1998) and Li *et al.*, (2007[a]) as well as the enzyme involved in metabolism of both drugs like CYP3A4, CYP3A5, CYP1A1, CYP1A2 and CYP2C9 as reported by Meineke and Turck (2003) and Li *et al.,* (2007[b]). It is also reported by Busch *et al*., (1998) that 60-65 % metabolite of meloxicam are being excreted through kidney whereas meloxicam may decrease the blood flow to kidney as reported by (Horl, 2010), which may be the reason of lower clearance. The smaller value of volume of distribution in present study is typical of the class NSAIDs which can be correlate with the higher plasma protein biding of the drug meloxicam as reported by Turck *et al.* (1996). The significant increase in volume of distribution of meloxicam given along with metformin in present study is may be due to increase in micro circulation in hepatic organ by metformin as reported by Scarpello and Howlett (2008).

5.1.6 Pharmacokinetic parameter of metformin following oral administration alone and in combination with erlotinib and/or meloxicam in male SCID mice

The plasma drug concentrations measured at various time intervals following oral administration of metformin alone and in combination with erlotinib and/or meloxicam in male SCID mice in present study were employed for the calculation of various pharmacokinetic parameters like calculated maximum plasma drug concentration (C_{max}), calculated time of maximum plasma drug concentration (T_{max}), half-life ($t_{1/2}$), area under plasma drug concentration-time curve ($AUC_{0-\infty}$), volume of distribution (V_z), clearance Cl and mean residence time (MRT).

Following oral administration of metformin alone in male SCID mice, calculated T_{max}, calculated C_{max}, $t_{½}$, V_z, $AUC_{0-\infty,}$ Cl, and MRT of metformin were 0.61 ± 0.10 h, 6277.75 ± 404.25 ng/ml, 3.36 ± 0.27 h, 17218.83 ± 885.71 h.ng/ml, 28950.33 ± 3629.19 ml, 5897.32 ± 350.95 ml/h and 2.64 ± 0.12 h, respectively.

Following oral administration of metformin in combination with erlotinib in SCID male mice, calculated T_{max}, calculated C_{max}, $t_{½}$, V_z, $AUC_{0-\infty,}$ Cl, and MRT of metformin were 0.39 ± 0.09 h, 7250.65 ± 487.30 ng/ml, 2.33 ± 0.30 h, 17672.48 ± 454.97 h.ng/ml, 18957.40 ± 2289.64 ml, 5678.84 ± 158.06 ml/h and 2.35 ± 0.14 h, respectively. The mean V_z of metformin in combination with erlotinib was very low (18957.40 ± 2289.64 ml) and highly significant ($p < 0.05$) as compared to V_z of metformin (28950.33 ± 3629.19 ml) in alone treatment group after oral administration of metformin in SCID male mice.

Following oral administration of metformin in combination with meloxicam in male SCID mice, calculated t_{max}, calculated C_{max}, $t_{½}$, V_z, $AUC_{0-\infty,}$ Cl, and MRT of metformin were 0.56 ± 0.07 h, 8663.34 ± 464.89 ng/ml, 1.79 ± 0.21 h, 23339.74 ± 1314.84 h.ng/ml, 11329.62 ± 1581.91 ml, 4356.26 ± 255.94 ml/h and 2.20 ± 0.06 h, respectively. The mean $t_{1/2}$ of metformin in combination with meloxicam was observed very low (1.79 ± 0.21 h) and highly significant ($p < 0.01$) as compared to $t_{1/2}$ of metformin (3.36 ± 0.27 h) in alone treatment group after oral administration of metformin in SCID male mice. The mean $AUC_{0-\infty}$ of metformin in combination with meloxicam was observed significantly ($p < 0.01$) higher (23339.74 ± 1314.84 h.ng/ml) as compared to $AUC_{0-\infty}$ of metformin (17218.83 ± 885.71 h.ng/ml) in alone treatment group after oral administration of metformin in SCID male mice. The mean V_z of metformin in combination with meloxicam was observed very low (11329.62 ± 1581.91 ml) and highly significant ($p < 0.01$) as compared to V_z of metformin

(28950.33 ± 3629.19 ml) in alone treatment group after oral administration of metformin in SCID male mice. The mean Cl of metformin in combination with meloxicam was observed very low (4356.26 ± 255.94 ml/h) and highly significant ($p < 0.01$) as compared to Cl of metformin (5897.32 ± 350.95 ml/h) in alone treatment group after oral administration of metformin in SCID male mice.

Following oral administration of metformin in combination with erlotinib and meloxicam in male SCID mice, calculated T_{max}, calculated C_{max}, $t_{½}$, V_z, $AUC_{0-\infty}$, Cl, and MRT of metformin were 0.33 ± 0.00 h, 9490.58 ± 963.64 ng/ml, 2.58 ± 0.31 h, 24658.04 ± 1303.61 h.ng/ml, 15338.18 ± 2005.80 ml, 4124.21 ± 261.29 ml/h and 2.59 ± 0.11 h, respectively. Following, simultaneous administration of metformin, meloxicam and erlotinib in SCID male mice, significant ($p < 0.05$) rise was observed in mean calculated C_{max} of metformin (9490.58 ± 963.64 ng/ml) as compared to calculated C_{max} (6277.75 ± 404.25 ng/ml) in alone treatment group after oral administration of metformin in SCID male mice. Following, simultaneous administration of metformin, meloxicam and erlotinib in male SCID mice, significant ($p < 0.05$) rise was observed in mean $AUC_{0-\infty}$ of metformin (24658.04 ± 1303.61 h.ng/ml) as compared to $AUC_{0-\infty}$ (17218.83 ± 885.71 h.ng/ml) in alone treatment group after oral administration of metformin in SCID male mice. Following, simultaneous administration of metformin, meloxicam and erlotinib in male SCID mice, significant ($p < 0.05$) decrease was observed in mean V_z of metformin (15338.18 ± 2005.80 ml) as compared to V_z (28950.33 ± 3629.19 ml) in alone treatment group after oral administration of metformin in SCID male mice. Following, simultaneous administration of metformin, meloxicam and erlotinib in male SCID mice, significant ($p < 0.05$) decrease was observed in mean Cl of

metformin (4124.21 ± 291.29 ml/h) as compared to Cl (5897.32 ± 350.95 ml/h) in alone treatment group after oral administration of metformin in SCID male mice.

The result showed that the calculated T_{max} remained lower in metformin with erlotinib and all three drugs together treatment group in comparison to metformin with meloxicam and metformin alone treatment groups. The calculated C_{max} of metformin was observed higher in metformin with meloxicam and all three drugs together group in comparison to other treatment groups. The $AUC_{(0 - \alpha)}$ was significantly high in treatment group of metformin with meloxicam ($p<0.01$) and all three drugs together ($p<0.01$) in comparison to the meloxicam alone treatment group. The calculated clearance rate was also observed significantly lower in treatment group of metformin with meloxicam ($p<0.05$) and all three drugs together ($p<0.05$) in comparison to meloxicam alone treatment group. The volume of distribution is also significantly low in all treatment groups in comparison to metformin alone treatment group. The half life of metformin with meloxicam is significantly lower in comparison to metformin alone treatment group.

The significant increase of C_{max} and $AUC_{0-\infty}$ observed when metformin administered along with erlotinib and all three drugs together in present study indicate that combinational therapy of metformin along with other drugs increased the C_{max} and AUC value. Similar results have been reported that furosemide, nifedipine and cimetidine have been found to increase the plasma metformin C_{max} and AUC (Lal and Jain, 2010). The higher value of $AUC_{0-\infty}$ in metformin with meloxicam and metformin with erlotinib and meloxicam in present study indicate that vast area of body is covered by metformin concentration. This significant change in $AUC_{0-\infty}$ of metformin can be correlated with other pharmacokinetic parameter like volume of distribution, half life and clearance. The volume of distribution in present study is also found

significantly lower in treatment group of metformin with erlotinib, metformin with meloxicam and all three drugs together. This finding is suggestive of alteration in metformin transport and /or cellular uptake when administered in combination with other drugs. Similar finding have been observed that drugs like tyrosin kinase inhibitior (Imatinib, Dasatinib, Nilotinib, Gefitinib, Erlotinib, Sunitinib, Lapatinib and Soratinib) are competitive inhibitor of metformin uptake through organic cation transporters (OCT) and decrease the volume of distribution at various level (Minematsu and Giacomini, 2011). The calculated clearance rate was also observed significantly lower in treatment group of metformin with meloxicam ($p<0.01$) and all three drugs together ($p<0.01$) in comparison to metformin alone treatment group, which suggests that drug is slowly eliminated from body. This significant decrease in clearance of metformin when given in combination with meloxicam and all three drugs together might be due to alteration in renal clearance of metformin when administered with other drugs. Similar finding have been reported by Rani *et al*. (2011) indicating that metformin clearance is significantly decrease when given in combination with trandolapril. The decrease in clearance of metformin when given in combination with meloxicam might also be due to reduce clearance of drug through kidney as a result of decreased blood flow to kidney in presence of meloxicam as reported by Horl (2010).

5.2 Studies on assessment of pharmaco-toxicological effect of erlotinib (30 mg/kg), meloxicam (20 mg/kg) and metformin (100 mg/kg) alone and in combination with each other and all three togather.

5.2.1 Effect on clinical symptoms and mortality

In the present study, daily observation of animals of all groups was carried out for physical and behavioral changes till day 28 of the experiment. Few SCID male

mice treated with erlotinib along with meloxicam as well as erlotinib with both meloxicam and metformin did reveal symptoms like piloerection with skin rash, staring eyes, hunched posture, itching, pasty skin tone, semisolid faeces, uncordinating gait attributable to the 28 days administration of erlotinib in combination with meloxicam. Saif *et al.* (2008) reported that erlotinib has interference with the follicular and inter follicular epidermal-growth signaling pathway which can play a major role in development of skin rashes and its related side effects. It is also supported by observation of EMEA, 1997 that the ulcerogenic activity in gastro intestinal tract, nephrotoxicity observed due to probably inhibition of constitutive cyclooxygenase. Two animals were found dead after three weeks treatment of erlotinib along with meloxicam, one animal died after 23 days treatment of erlotinib along with metformin and two animals were died after 20 and 22 days treatment of erlotinib along with both meloxicam and metformin. The mortality of animals could be due to toxic effect of erlotinib with meloxicam as the combinational therapy can lead to severe cumulative side effects of both. The similar observations have been reported as side effects of meloxicam (mubic) and reported on drugs information and side effect database (www.drugsdb.com); Li and Perez-Soler (2009) have also reported the EGFR inhibitors class-specific side effects like skin toxicities, papulopustular rash, alter normal development and physiology of the epidermis, rarely life-threatening and may cause significant physical and psycho-social discomfort.

5.2.2 Effect on tumor volume

The tumor volume of each animal in all the groups of animals were measured at weekly interval. The dimension of tumor was measured by digital caliper and the volume was calculated by [(width^{2}X length)/2] for development of tumor. The tumor volume was measured on 0, 7, 14, 21 and 28th day during the treatment. The grouping

was done based on mean tumor volume after attending 100mm^3 and considered as day 0. The tumor volume on day of randomization was in range of 142 to 180 mm^3. The tumor volume of control group was 545.39 ± 257.14 mm^3 on day 7, which rapidly reached to 1333.28 ± 502.75 mm^3 on day 21 and attained the volume of 1718.66 ± 561.01 mm^3 on day of termination. The tumor volume of erlotinib treated group was 227.14 ± 51.70 mm^3 on day 7, which slowly reached to 650.02 ± 94.43 mm^3 on day 21 and finally attained the volume of 939.99 ± 10.70 mm^3 on day of termination. The tumor volume of meloxicam treated group was 447.94 ± 54.58 mm^3 on day 7, which slowly reached to 868.29 ± 130.38 mm^3 on day 21 and finally attained the volume of 1262.03 ± 5.80 mm^3 on day of termination. The tumor volume of metformin treated group was 495.49 ± 114.48 mm^3 on day 7, which rapidly reached to 1181.84 ± 281.60 mm^3 on day 21 and finally attained the volume of 1535.54 ± 251.80 mm^3 on day of termination. The tumor volume of animals treated by erlotinib with meloxicam was 193.58 ± 35.52 mm^3 on day 7, which slowly reached to 433.39 ± 144.08 mm^3 on day 21 and finally attained the volume of 869.72 ± 54.51 mm^3 on day of termination. The tumor volume of animals treated by erlotinib with metformin was 315.06 ± 42.50 mm^3 on day 7, which slowly reached to 661.69 ± 102.78 mm^3 on day 21 and finally attained the volume of 912.13 ± 99.06 mm^3 on day of termination. The tumor volume of animals treated by meloxicam with metformin was 231.06 ± 62.00 mm^3 on day 7, which slowly reached to 648.95 ± 99.23 mm^3 on day 21 and finally attained the volume of 1010.22 ± 69.47 mm^3 on day of termination. The tumor volume of animals treated by erlotinib along with both meloxicam and metformin was 182.17 ± 19.46 mm^3 on day 7, which slowly reached to 412.86 ± 43.93 mm^3 on day 21 and finally attained the volume of 805.98 ± 36.93 mm^3 on day of termination.

There was a considerable reduction in tumor volume of treatment group compared to the tumor control. It was observed that growing pattern of tumor was high during 3rd and 4th week. This finding is in agreement with finding of Xu and Glan (2010). The tumor size has shown the reducing trend in tumor volume growth on 1st, 2nd, 3rd and 4th week in treatment group compared to the tumor control group except animals treated with metformin alone. Such findings are in agreement with the findings of Howard *et al.* (1991) and Hu *et al.* (2004). On 28th day, the tumor size of control group was 1718.66 ± 561.01 mm^3 which is almost 11 times higher than its size on day 0 and represented as mean relative tumor volume (RTV) which is 11.53. The mean relative tumor volume (RTV) is calculated by dividing the mean tumor volume at any time by the mean tumor volume at the start of the treatment (Gourdeau *et al*., 2005; Nagano *et al*., 2009). The animals treated with all three drugs together showed the considerable reduction in mean relative tumor volume (5.33) as compared to tumor control (11.53) animals. The other treatment groups also showed the substantial reduction in mean relative tumor volume as compared to tumor control group. The tumor volume of animals treated with erlotinib, meloxicam and metformin as a single drug was observed as mean RTV of 6.61, 7.10 and 8.22, respectively. The tumor volume in the combinational treatment of erlotinib with meloxicam and metformin was observed with mean RTV of 5.75 and 6.28, respectively. Such findings are well supported by results of Clark *et al.* (2008) and Ouchi *et al.* (2010).The tumour volume in mice treated with meloxicam and metformin was observed with mean RTV of 5.96. Similar results of reduced tumor volume have been reported by Hristova *et al.* (2011). The mean RTV of treatment groups in comparison to tumor control group exhibited that, the mean RTV of animal treated with metformin as single drug was highest in comparison to other treatment groups. The animals treated with meloxicam as an

alone drug had less RTV than metformin treated animals and higher RTV than erlotinib treated animals as a single drug. Same way, the animals treated with erlotinib as single drug were observed with higher RTV than animals treated by erlotinib along with metformin, where as the RTV of animals treated by erlotinib along with meloxicam was lesser than animal treated by meloxicam along with metformin and higher than animals treated with all three drugs together. Individual drug efficacy studies on relative tumor volume reduction are available but combinational studies are very rare, few specific drugs combinational studies have been carried out by scientists like Saba *et al.* (2010) and Buchanan *et al*. (2007), who reported synergistic effect of combinational treatment on tumor volume reduction. Buchanan *et al*. (2007) also suggested that the inhibition of both COX-2 and EGFR may provide a better therapeutic strategy than either single agent through a combination of decreased cellular proliferation and prostaglandin signaling as well as increased apoptosis. However, combinational study has been done by Khuri *et al*. (2011), who reported that of everolimus and docetaxel are well tolerated but there was no clear signal of enhanced efficacy. The lowest RTV was observed in animals treated with all three drugs together. The trend of RTV from highest to lowest was control group > Metformin treatment group > meloxicam treatment group > erlotinib treatment group > erlotinib with metformin treatment group > meloxicam with metformin treatment group > erlotinib with meloxicam treatment group > erlotinib with both meloxicam and metformin treatment group.

The reduction in mean percentage tumor volume was calculated in comparison to tumor volume of control group for corresponding week. It has been observed that the tumor growth was considerably hampered in animal treated with erlotinib, meloxicam individually as well as in combination with each others. This observation

has been supported by different studies carried out separately by Bonomi (2003), Goldman *et al*. (1998), Fasolo and Sessa (2008), Yekaterina *et al*. (2012). The difference in efficacy between treatment group were expressed as percentage of tumor growth inhibition (TGI%) and calculated as per following formula.

$$[1- (T-T_0) / (C-C_0)] X 100$$

The tumor growth in initial two weeks was observed less in comparison to 3rd and 4th week. The tumor growth inhibition (TGI%) was observed in animals treated by metformin with erlotinib and/or meloxicam on 1st and 2nd week of the treatment, which is in accordance to study done by Hosono *et al.* (2010), who carried out experiment on balb/c mice by administering azoxymethane with or without metformin for 6 wk and concluded that metformin suppresses colonic epithelial proliferation via the inhibition of the mTOR pathway through the activation of AMPK. Hristova (2011) also reported that the combination of metformin and NSAID treatment is more effective than metformin alone on nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) production .The neurotrophins are nerve growth factors, it seems that their elevation in early stage metabolic syndrome is a risk factor for tumorigenesis. Treatment with metformin may cause reduction in neurotrophins during early stage metabolic syndrome.

More than 50 % tumor growth inhibition (TGI) was observed in animal treated with erlotinib alone, erlotinib and meloxicam, erlotinib and metformin, metformin and meloxicam as well as all three drugs together on 3rd week of the treatment whereas on 4th week, only animals treated with all three drugs showed more than 50 % tumor growth inhibition in comparison to tumor control group. The animals treated with all three drugs together have shown marked percentage reduction in growth of tumor volume on 1st, 2nd, 3rd and 4th week of the treatment as compared with tumor control.

The animals treated with erlotinib alone, erlotinib with meloxicam, erlotinib with metformin and meloxicam with metformin showed considerable reduction in growth of tumor volume in comparison to tumor control. Bonomi (2003) reported that patients treated with erlotinib have modest survival improvement in patients with stage III and IV non-small cell lung cancer (NSCLC)**,** moreover, Zerbe *et al.* (2006) also reported that there is two fold reduction in tumor volume after treatment with erlotinib in comparision to tumor control. Fenton *et al.* (2001) reported inhibitory effect of meloxicam and celecoxib on Murine MCa-35 mammary carcinomas. Both of these agents have shown similar reductions in tumor volume compared with untreated tumors. Goldman (1998) reported that meloxicam significantly inhibited HCA-7 colony and tumor growth but had no effect on the growth of the COX-2 negative HCT-116 cells. They also observed 51% reduction in tumor size after 4 weeks of treatment. Metformin was found to inhibit proliferation of most cultured breast cancer cell lines. Zhuang and Miskimins (2008) and Liu *et al.* (2011) have reported that metformin was able to induce G0/G1 cell cycle arrest and inhibit renal cell carcinoma (RCC) growth *in vitro* and *in vivo*, which suggest that metformin may be a potential therapeutic agent for the treatment of RCC.

As explained above all drugs i.e. erlotinib, meloxicam and metformin have antitumor effect by inhibiting EGFR, COX-2 and mTOR pathway, respectively. Many scientists have also tried the combination of EGFR and Cox-2 inhibitors for the treatment of cancer and they have found very good results as reported by Michele *et al.*, (2001); Tortora *et al.*, (2003); Shishodia *et al.*, (2004); Chen *et al.*, (2004); Buchanan *et al.*, (2007). They also suggested that cell growth inhibition induced by a combination of EGFR, TKIs and COX-2 were mediated through simultaneously blocking EGFR and COX-2 pathways. Same way the another combination with

mTOR inhibitors and COX-2 inhibitors/NSAIDs study done by Hristova (2011) have reported that the combination of metformin and NSAID treatment is more effective than metformin alone on nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), which is responsible for tumorogenesis through neurotrophin production and it is prevented by metformin. Vazquez *et al* (2009) have reported that metformin inactivates mTOR lead to have beneficial effect on lapatinib acquired resistance in chronic treatment of cancer and showed favorable combinational effect.

No references reporting combinational therapy of all the three drugs together are available. However, very recently Pal *et al.* (2010) and Khuri *et al.* (2011) have reported that the combination of everolimus and docetaxel was tolerated well and the TKIs gefitinib and erlotinib combination shown significant benefit in clinical trials evaluating insulin-like growth factor-1 receptor (IGF-IR)-targeting agents, cyclooxygenase-2 (COX-2) inhibitors, c-met inhibitors, irreversible pan-HER inhibitors, mammalian target of rapamycin (mTOR) inhibitors, and histone deacetylase (HDAC) inhibitors. This also described novel therapies for NSCLC including relevant biomarkers and determination of synergy with both cytotoxic therapy and other targeted agents.

5.2.3 Effect on body weight

Weekly body weight of all the animals including control and all treatment groups of erlotinib, meloxicam and metformin alone and in combination with each other and all three drugs together were recorded on 0, 1, 2, 3 and 4^{th} week of the study period. The body weight was significantly reduced ($p < 0.05$) in animal treated with metformin along with erlotinib in comparison to tumor control group in 1^{st}, 2^{nd}, 3^{rd} and 4^{th} week of the study. Abraham *et al*. (2011) reported that Mice treated with

erlotinib for 2 - 3 weeks experienced a 10–20% loss in body weight as compared to controls. Matsui *et al*. (2010) and Vladimir *et al*. (2011) also reported that the chronic treatment of metformin leads to reduced body weight in human and animals. The bodyweight gain of animal treated with erlotinib alone, erlotitinb with meloxicam, erlotinib with metformin and all three drugs together was significantly reduced ($p < 0.05$) in comparison to tumor control group on 3rd week of the study. On 4th week of study, the body weight of animals treated by erlotinib with meloxicam and erlotinib with metformin was significantly reduced ($p < 0.05$) in comparison to tumor control group. There is no significant effect of meloxicam therapeutic doses on body weight reduction as reported by the Granado *et al*., 2007 but the reduction in body weight of mice treated with erlotinib and meloxicam both may be due to the erlotinib side effect as well as the gastric irritation effect of meloxicam leading to decreased feed intake. Similar to the findings of Matsui *et al*., (2010) and Vladimir *et al*. (2011), in the present study there was significant reduction in body weight of mice upon treatment of erlotinib and metformin individually for more than 2-3 weeks and same synergistic effect has been observed in mice treated with erlotinib and metformin.

5.2.4 Effect on feed consumption

Feed consumption was measured weekly for all animals of control and treatment groups of erlotinib, meloxicam and metformin alone and in combination with each other and all three together on day 7,14 ,21 and 28 of the experiment. Feed consumption in SCID male mice of treatment groups was not changed significantly as compared to that of control animals. In accordance to our findings non significant change in feed consumption has been reported by Cooper *et al*. (2009) following administration of meloxicam in rabbits and Vladimir *et al*. (2011) have also reported non-significant change in feed consumption of mice after chronic treatment with

metformin. The effect of erlotinib on feed consumption has not been reported during any such studies however, the same group tyrosin kinase inihibitor like ematinib had no significant effect on feed consumption of mice in studies of 8 week duration as reported by Hagerkvist *et al*. (2008).

5.2.5 Effect on hematological parameters of SCID mice treated with erlotinib, meloxicam, metformin alone and in combination with each other and all drug together.

The blood samples of erlotinib, meloxicam, metformin alone and in combination with each other and all drugs together treated SCID male mice and its control were analyzed and mean values of total WBC count, total RBC count, haemoglobin, hematocrit, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration (MCHC), platelets and differential leukocyte count (neutrophil, lymphocyte, basophil, eosinophil and monocyte) were calculated.

The results suggest that mean value of WBC of animal group treated by erlotinib with metformin was significantly increased in comparison to tumor control group. Europian medicines agency (2005) on erlotinib have reported that the WBC counts are increased after chronic treatment of erlotinib, same way the long term treatment of metformin in combination with sitagliptin also increased WBCs count as described in US Patent No. 6699871 and US Patent No. 7326708**.** However, individually treated group of erlotinib and metformin had little increased WBC count but no significant rise was observed. The WBC count of animal group treated by erlotinib with meloxicam was also considerably decreased in comparison to tumor control group. Similar results of non significant decrease in level of WBCs have also been reported by Al-Rekabi *et al.* (2009). The mean WBC count value of tumor

control group and all other treatment groups were little higher in comparison to normal control group, which is supported by the results of Shankar *et al*. (2006), who explained the higher count of WBCs in cancer mortality.

The neutrophil value of tumor control group was significantly higher in comparison to normal control, the observation in accordance to WBC count. The neutrophil % of tumor control and all treatment groups was considerably high in comparison to normal control, whereas the lymphocyte % and eosinophil % count were decreased in tumor control and treatment group in comparison to normal control. The monocyte % count in animal group treated by metformin alone and in combination with erlotinib was significantly higher than tumor control group. Similar to the findings of present study, increased count of WBCs and Monocytes are reported in European Medicines Agency (EMEA, 2005).

The mean value of all other hematological parameters of male SCID mice treated with erlotinib, meloxicam, metformin alone and in combination with each other and all three drugs together for 28 days did not differ significantly from the corresponding values observed in tumor control animals.

5.2.6 Effect on biochemical parameters

The blood samples of erlotinib, meloxicam, metformin alone and in combination with each other and all drugs together treated SCID male mice and its control were analyzed and mean ± SEM values for various biochemical parameters like glucose, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total bilirubin (TB), creatinine, creatine kinase (CK), blood urea nitrogen (BUN), lactate dehydrogenase (LDH) and acid phosphatase were calculated.

The mean value of creatine kinase (CK) was significantly decreased in animal group treated with meloxicam in comparison to tumor control group. In accordance to present study, Kluivers-Poodt and Robben (2008) have also reported the significant reduction in creatine kinase following treatment of meloxicam in piglet. The creatine kinase levels were significantly increased in animal group treated with all three drugs together in comparison to tumor control group. Maione *et al.* (2006) have reported elevation in creatine kinase following long term treatment with erlotinib in patients with non small cell lung cancer. Same way, chronic treatment of metformin elevated creatine kinase level in human with prior exposure to simvastatin followed by Rosiglitazone. Simultaneous treatment with erlotinib and metformin may give synergistic effect on elevation of creatine kinase level. In the present study, mice treated with all three drugs together have shown elevated level of creatine kinase which could be due to such synergistic effect. Whereas, the mean value of CK and blood urea nitrogen (BUN) of animal group treated with erlotinib with metformin was significantly increased in comparison to tumor control group. Chen *et al.* (2012) have reported elevation in BUN level following erlotinib treatment in mice. Silvestre *et al.* (2007) have reported raised level of BUN in case reports of lactic acidosis following treatment with metformin in human beings. Raised level of BUN in present study might be due to additive damaging effect of metformin and erlotinib on kidney. The mean values of AST, ALT, TB and acid phosphatase of tumor control group were considerably high than normal control group. The glucose level in animal group treated with metformin alone and in combination with erlotinib and meloxicam were considerably low, where as AST of animal group treated with meloxicam alone and in combination with erlotinib was also found markedly low in comparison to tumor control group. The mean serum ALT level were notably reduced in animal group

treated with meloxicam and metformin alone as well as erlotinib with metformin in comparison to tumor control group.

The LDH level in animal group treated with meloxicam and in combination with erlotinib was noticeably reduced in comparison to tumor control group. The mean values of alkaline phosphatase (ALP), total bilirubin (TBIL), creatinine, and acid phosphatase did not differ significantly from those of control animals.

5.2.7 Necropsy

Gross pathological examination of all animals did not reveal any abnormal lesions attributable to the 28 days administration of erlotinib, meloxicam, metformin alone and in combination with each other and all three drugs together.

5.2.8 Effect on organ weight

The absolute organ weights were recorded for tumor, heart, lung, liver and kidneys of SCID male mice of all groups on 28^{th} day of experiment at the time of necropsy. The results showed that there was a considerable reduction in mean tumor weight of all treatment groups except metformin treatment group in comparison to the tumor control group. The tumor weight of treatment group of erlotinib and meloxicam alone was 46.34 % and 42.73 % less compared to tumor control group whereas tumor weight of treatment group of erlotinib with meloxicam and metformin was 61.54 % and 37.40 % lesser than tumor control group. The tumor weight of treatment group of meloxicam with metformin was 29.24 % less than tumor control group. The tumor weight of the animal group treated simultaneously with all three drugs was 61.78 % lesser compared to tumor control group. In the present study, reduction in tumor weight can be straightforwardly correlated with reduction in tumor volume in respective experimental groups. The reduction in tumor size have been observed by

Goldman *et al*. (1998); Bonomi (2003) and Yekaterina *et al*. (2012) for the similar group drugs.

There was no change in mean organ weight of heart, lung and liver after 28 days administration of erlotinib, meloxicam, metformin alone and in combination with each other and all three drugs together. The kidney weight of animal group treated with metformin along with meloxicam was significantly reduced in comparison to the tumor control group. The reduction in kidney weight may be due to meloxicam chronic treatment, which reduces blood flow towards the kidney and the metfromin clearance done through kidney would have hampered, which may lead to lactic acidosis and kidney impairment. Such hypotheses are indirectly supported by individual toxicity reported by Mahaprabhu *et al.* (2011) and Jones *et al*.(2003).

5.2.9 Drug Distribution in tissue

The extract of lung and tumor were analyzed for the presence of erlotinib, meloxicam and metformin in respective treatment group after 24 hrs of last administered dose of the drug but none of the drug was detected in either lung or tumor. This observation is also supported by the result of pharmacokinetic experiment under this study, which revealed that drugs are eliminated from blood within 24 hrs. The dosages were in therapeutic range, which indicatived that there is no detectable drug level is accumulated in either tumor or lung tissues.

5.2.10 Effect on gene expression.

The gene expression change in tumor tissue was analyzed for AKT, AMPK, P070S6K, PTEN and RAF. The expression of PTEN gene was markedly increased in all treatment groups in comparison to the tumor control group. There was significant increase in expression of PTEN gene in animal group treated with erlotinib along with meloxicam as well as metforimn in comparison to the tumor control group. This

observation was supported by the study of Yamamoto *et al.* (2010), who demonstrated that PTEN suppression leads to carcinogenesis and it is playing very important role in EGFR inhibitors therapy. Hensing *et al*. (2010) also reported that PTEN expression was the only factor significantly associated with improved overall survival, which is also supported by Mohiuddin *et al*. (2002*)* indicating over-expression of PTEN engenders apoptosis in MM by AKT hypophosphorylation. The expression of PTEN is increased with the treatment of erlotinib as reported by Mutter et *al*. (2000) and Fenner *et al*. (2010). The similar observations were found by Lee *et al*. (2006) with same group drug gefitinib ,which exhibits antitumor activity by regulation of cellular differentiation and growth in patient with non-small cell lung cancer (NSCLC) mediating through Peroxisome proliferators-activated receptor-gamma (PPAR-gamma) and Phosphatase and tensin homologue (PTEN) levels. PPAR-gamma and PTEN expression were found to increase in the gefitinib- and rosiglitazone-treated cells. Mustafa and Kruger (2008) reported that the COX-2 inhibitor celecoxib's efficacy was also linked with PPAR-gamma and PTEN expression. Leigh *et al*. (2010) reported that metformin also play role of potent inhibitor of cell proliferation in presence of PTEN and the effect of metformin on PTEN status required to study.

Maria *et al.* (2006) have demonstrated that coexpression of EGFRvIII and PTEN protein by glioblastoma cells was strongly associated with clinical response to EGFR kinase inhibitor therapy. They also demonstrated that mTOR inhibitor enhance the sensitivity of PTEN-deficient tumor cells to the EGFR kinase inhibitor erlotinib. So with above all references it was clear that increased PTEN expression was indicative of anti tumor effect of the drugs and results have shown that PTEN expression has considerably increased in all treaent groups in comparision to the tumor control group.

The expression of AKT is considerably decreased in animal group treated with erlotinib, meloxicam and metformin as a single drug as well as erlotinib with meloxicam and metformin and all three drugs together in comparison to the tumor control group. Results of this study is also in agreement with report of decreased expression of AKT following erlotinib treatment in patients with head and neck cancer. Similarly Jimeno *et al*. (2007) have also reported abrogating AKT activity in athymic nude mice following erlotinib and temsirolimus (mTOR inhibitors).

There is no significant alteration in expression of AMPK gene in this study. The AMPK expression is slightly increased in animal group treated with erlotinib, meloxicam and metformin alone as well as all combinational treatment in comparison to tumor control. This observation is also supported by Fenner *et al.* (2010), who indicated that the AMPK expression was increased with erlotinib treatment. The Sahra *et al.* (2010); Song *et al.* (2012); Shi *et al.* (2012) reported that the AMPK activated by the treatment of mTOR inhibitors or metformin. Niu *et al.* (2011) reported that AMPK activation lead to reduction in Cox-2 expression.

The expression of P070S6K gene was considerably reduced in animal group treated by erlotinib along with metformin and animal group treated with metformin along with meloxicam in comparison to tumor control group. It was slightly reduced in other treatment group in comparison to tumor control group. Vazquez *et al.* (2009) have also revealed similar reduction of P070S6K in patients with breast carcinoma upon concurrent admission of metformin and lapatinib supporting possible combinatorial therapy of erlotinib and metformin in non small cell lung carcinoma.

The expression of RAF gene was considerably reduced in animal group treated by meloxicam along with metformin in comparison to tumor control group. It was also decreased slightly in animal group treated with erlotinib, meloxicam and

metformin alone and in combinational treatment group in comparison to tumor control group. The similar finding like inhibition of raf expression by elrotinib were reported by Elizabeth *et al*. (2006). Teh *et al.* (2004) reported that bFGF-induced phosphorylation of c-raf was prevented by the COX-2 inhibitor meloxicam. Feng *et al.* (2012) also reported that Spry2 promoted apoptosis of cancer cells in association with activation of the PTEN pathway and the blockade of Ras-Raf-Erk signaling due to induced sensitization to fluorouracil (5-FU) and metformin.

5.2.11 Histopathology

The histopathological changes were observed in tissues like tumor mass, lungs, liver, heart and kidneys of all the animals of treated and control groups. Significant differences in histological changes were observed in tumor mass. The tumor tissue exhibited microscopically necrotic core, mitotic figure, hemorrhage, infiltration in surrounding tissue in minimal to moderate grade in all treatment groups except animal group treated with all three drugs together, where the mitotic figure observed was minimal to mild in comparison to tumor control group. Since the major intention was to score the degree of shrinkage of tumor mass due to administration of drugs, the severity of necrosis was main attribute to designate the positive response. The evaluation of drug effect by tumor mass necrosis grading is also supported by the Petty *et al*. (2004) and Schaake *et al*. (2012), who have evaluated the erlotinib effect on tumor regression and necrosis in tumor mass. Schaake *et al*. (2012) also reported that there was more than 50% necrosis observed in tumor mass of patient treated with erlotinib. The tumor mass of animals treated with erlotinib along with both meloxicam and metformin combination appeared to be reduced to a considerable size as necrosis of tumor mass was severe in these animals. This observation was also well supported by significant reduction in the size of tumor mass measured i.e. RTV

(Relative tumor volume). The tumor mass or necrosis of tumor cells was also seen with moderate severity in animals treated with combination of erlotinib along with meloxicam followed by mild to moderate severity in erlotinib along with metformin and meloxicam along with metformin. The minimal or minimal to mild necrotic changes in tumor was seen in animals treated with metformin alone or meloxicam or erlotinib. The tumor mass was intact and none of the animal revealed necrotic changes in the animals of tumor control group.

The histopathological changes such as foci of leucocytic infiltration, necrotic foci in heart, liver, lungs and nephropathy in kidneys, respectively did not differ from the normal back ground lesions in the animals of this age and strain therefore considered to be spontaneous lesions and not related to treatment.

CHAPTER – VI

SUMMARY AND CONCLUSIONS

Pharmacokinetics of erlotinib (30 mg/kg, p.o.), meloxicam (20mg/kg, i.p.) and metformin (100 mg/kg, p.o.) alone and in combination with each other as well as all three drugs together at same dose rate by single administration was investigated in SCID male mice. The plasma samples were assayed for drug concentration using liquid chromatography with mass spectrometry (LC-MS/MS) procedure. Furthermore, Pharmaco-toxicological effect of erlotinib (30 mg/kg, p.o.), meloxicam (20mg/kg, i.p.) and metformin (100 mg/kg, p.o.) alone and in combination with each other and all three drugs together were evaluated by repeated administration for 28 days in xenograft tumor model. The pharmaco-toxicological effect was evaluated by measuring tumor volume, hematological and serum biochemical analysis, gene expression study, tissue distribution study and histopathological examination.

The mean observed peak plasma concentrations of erlotinib following its administration (30 mg/kg, p.o.) as single drug and in combination with meloxicam (20mg/kg, i.p.) and metformin (100 mg/kg, p.o.) separately, as well as meloxicam (20mg/kg, i.p.) and metformin (100 mg/kg, p.o.) together in SCID male mice were 16372.63 ± 846.59, 17741.60 ± 2918.54, 13400.85 ± 882.54 and 13600.97 ± 1176.92 ng/ml, respectively, which were observed at 0.167 hr after administration of drug. The drug concentration was not detected after 24 hrs post oral administration of erlotinib alone or in combination with meloxicam and/or metformin.

The mean observed peak plasma concentrations of meloxicam following its administration (20mg/kg, i.p.) as single drug and in combination with erlotinib (30 mg/kg, p.o.) and metformin (100 mg/kg, p.o.) separately, as well as erlotinib (30 mg/kg, p.o.) and metformin (100 mg/kg, p.o.) together in SCID male mice were

44858.46 ± 3486.54, 49994.17 ± 2928.14, 46540.83 ± 3879.53 and 39648.94 ± 2190.42 ng/ml, respectively, which were observed at 0.33 hr after administration of drug. The drug concentration was not detected after 24 hrs post intra peritoneal administration of meloxicam (20mg/kg) alone or in combination with erlotinib and/or metformin.

The mean observed peak plasma concentrations of metformin following its administration (100 mg/kg, p.o.) as single drug and in combination with erlotinib (30 mg/kg, p.o.) and meloxicam (20mg/kg, i.p.) separately, as well as erlotinib and meloxicam together in SCID male mice were 5782.29 ± 441.77, 6307.71 ± 577.57, 7724.44 ± 384.36 and 9490.58 ± 963.64 ng/ml, respectively, which were observed at 0.33 h in all treatment groups except in case of metformin along with meloxicam was 0.67 h. The drug concentration was not detected after 12 hrs post oral administration of metformin alone or in combination with erlotinib and/or meloxicam.

Following oral administration of erlotinib (30 mg/kg, p.o.) as single drug and in combination with meloxicam (20mg/kg, i.p.), in combination with metformin (100 mg/kg, p.o.) and in combination with both meloxicam and metformin together in SCID male mice, the calculated pharmacokinetic parameters mean value of erlotinib were found as t_{max} 0.29 ± 0.09 h, 0.21 ± 0.04 h, 0.27 ± 0.09 h and 0.21 ± 0.04 h; C_{max} 17272.59 ± 640.80 ng/ml, 19865.31 ± 2154.82 ng/ml, 13840.11 ± 797.51 ng/ml, 15080.29 ± 828.43 ng/ml; half-life ($t_{½}$) 6.08 ± 0.59 h, 8.24 ± 1.48 h, 4.79 ± 0.71 h, 6.63 ± 0.72 h; area under plasma drug concentration-time curve ($AUC_{0-\infty}$) 84784.69 ± 3209.24 h.ng/ml, 91056.92 ± 5850.21 h.ng/ml, 66493.10 ± 3418.82 h.ng/ml, 62452.70 ± 2400.15 h.ng/ml; volume of distribution (V_z) 3083.37 ± 247.42 ml, 3865.44 ± 584.29 ml, 3052.14 ± 279.98 ml and 4559.44 ± 446.48 ml; clearance (Cl) 356.48 ± 14.03 ml/h , 335.50 ± 18.90 ml/h, 456.74 ± 21.69 ml/h and 483.92 ± 18.55 ml/h; mean retention time (MRT) 5.14 ± 0.36 h, 6.12 ± 0.56 h, 4.63 ± 0.53 h and 4.79 ± 0.71 h,

respectively. The $AUC_{0-\infty}$ of erlotinib was significantly decreased and the clearance was significantly increased when given in combination with metformin and in combination with both metformin and meloxicam together.

Following intra peritoneal administration of meloxicam (20mg/kg, i.p.) as single drug and in combination with erlotinib (30 mg/kg, p.o.), in combination with metformin (100 mg/kg, p.o.) and in combination with both erlotinib and metformin together in SCID male mice, the calculated pharmacokinetic parameters mean value of erlotinib were found as $t_{max,}$ 0.40 ± 0.09 h, 0.33 ± 0.00 h, 0.39 ± 0.06 h and 0.33 ± 0.00 h; C_{max} 45715.22 ± 3343.07 ng/ml, 49994.17 ± 2928.14 ng/ml, 46725.46 ± 3798.13 ng/ml and 39648.95 ± 2190.42 ng/ml; half-life ($t_{½}$) 3.79 ± 0.34 h, 3.51 ± 0.37 h, 4.66 ± 0.30 h and 4.10 ± 0.4 h; area under plasma drug concentration-time curve ($AUC_{0-\infty}$) 123999.94 ± 4181.79 h.ng/ml, 196948.88 ± 6206.02 h.ng/ml, 113819.23 ± 1751.21 h.ng/ml and 137189.18 ± 2845.93 h.ng/ml; volume of distribution (V_z) 873.90 ± 59.61 ml, 524.79 ± 75.53 ml, 1184.20 ± 87.65 ml and 858.22 ± 69.29 ml; clearance (Cl) 162.24 ± 5.67 ml/h, 102.10 ± 3.52 ml/h, 175.92 ± 2.66 ml/h and 146.10 ± 3.02 ml/h; mean retention time(MRT) 3.32 ± 0.15 h, 4.33 ± 0.19 h, 3.80 ± 0.08 h and 3.51 ± 0.27 h, respectively. The $AUC_{0-\infty}$ and MRT of meloxicam were significantly increased whereas Vz and Cl were significantly decreased when given in combination with erlotinib. The Vz of meloxicam was significantly increased when given in combination with metformin and clearance of meloxicam was significantly decreased in combination with both metformin and meloxicam together.

Following oral administration of metformin (100 mg/kg, p.o.) as single drug and in combination with erlotinib (30 mg/kg, p.o.), in combination with meloxicam (20mg/kg, i.p.) and in combination with both erlotinib and meloxicam together in SCID male mice, the calculated pharmacokinetic parameters mean value of erlotinib

were found as $t_{max,}$ 0.61 ± 0.10 h, 0.39 ± 0.09 h, 0.56 ± 0.07 h and 0.33 ± 0.00 h; C_{max} 6277.75 ± 404.25 ng/ml, 7250.65 ± 487.30 ng/ml, 8663.34 ± 464.89 ng/ml, and 9490.58 ± 963.64 ng/ml; half-life ($t_{½}$) 3.36 ± 0.27 h, 2.33 ± 0.30 h, 1.79 ± 0.21 h, and 2.58 ± 0.31 h,; area under plasma drug concentration-time curve ($AUC_{0-\infty}$)17218.83 ± 885.71 h.ng/ml, 17672.48 ± 454.97 h.ng/ml, 23339.74 ± 1314.84 h.ng/ml, and 24658.04 ± 1303.61 h.ng/ml; volume of distribution (V_z) 28950.33 ± 3629.19 ml, 18957.40 ± 2289.64 ml, 11329.62 ± 1581.91 ml, and 15338.18 ± 2005.80 ml; clearance (Cl) 5897.32 ± 350.95 ml/h, 5678.84 ± 158.06 ml/h, 4356.26 ± 255.94 ml/h and 4124.21 ± 261.29 ml/h; mean retention time (MRT) 2.64 ± 0.12 h, 2.35 ± 0.14 h, 2.20 ± 0.06 h and 2.59 ± 0.11 h, respectively. The C_{max} and $AUC_{0-\infty}$ of metformin were significantly increased whereas the Vz and Cl was significantly decreased when given along with both erlotinib and meloxicam. The $AUC_{0-\infty}$ of metformin was significantly increased where as the $T_{1/2}$, Vz and Cl was significantly decreased when given in combination with meloxicam. The Vz of metformin was also significantly decreased when given in combination with erlotinib.

The Pharmaco-toxicological effect of repeated administration of erlotinib (30 mg/kg, p.o.), meloxicam (20mg/kg, i.p.) and metformin (100 mg/kg, p.o.) for 28 days were evaluated by recording the clinical symptoms, mortality, tumor volume, body weight, feed consumption, hematological parameters (Hb, RBC, WBC, MCV, MCH, MCHC, HCT and DLC) and serum biochemical parameters (AST, ALT, ALP, Total Bilirubin, Creatine kinase, Serum Creatinine, BUN, LDH and Acid Phosphatase), tissue distribution, organ weight, gene expression, and histopathological changes in xenograft tumor model of SCID male mice.

Few animals of group treated with erlotinib along with meloxicam and in combination with both meloxicam and metformin together revealed symptoms like

piloerection, staring eyes, hunched posture, incordinating gait etc attributable to administration of erlotinib (30 mg/kg) in combination with meloxicam (20mg/kg) and two animals each from same treatment groups died after 20-22 days treatment, where as one animal died on 23rd day of treatment in group treated with erlotinib (30 mg/kg) in combination with metformin. Animals in other treated groups were found clinically normal throughout the study period.

The body weight was significantly reduced ($p < 0.05$) in animal group treated with erlotinib in combination with metformin during experiment period. The bodyweight of animal group treated with erlotinib, erlotinib with meloxicam, erlotinib with metformin and erlotinib with both meloxicam and metformin was significantly reduced ($p < 0.05$) in comparison to untreated tumor control group during 3rd week of the study. The body weight of animal groups treated with erlotinib in combination with meloxicam and erltoinib with metformin were significantly reduced ($p < 0.05$) as compared to untreated tumor control group during fourth week of study. The Feed consumption in SCID male mice of treatment groups was found to be comparable with control group animals.

The tumor volume was measured on day 0, 7, 14, 21, and 28th day during the treatment period. The grouping of animals was done based on mean tumor volume on day 0. It was observed that growing pattern of tumor was high during 3rd and 4th week. A considerable reduction was observed in tumor volume in treatment group compared to the tumor control. The efficacy of drugs was evaluated by observing reduction in tumor size, which was expressed as percentage reduction in tumor volume and relative tumor volume (RTV). On termination day the mean tumor volume of untreated control group was 1718.66 ± 561.01 mm^3 where as the mean tumor volume of animals of groups treated with erlotinib, meloxicam, metformin, erlotinib with meloxicam, erlotinib with metformin, meloxicam with metformin and all three drugs together was 939.99 ± 10.70, 1262.03 ± 5.80, 1535.54 ± 251.80, 869.72 ± 54.51, 912.13 ± 99.06, 1010.22 ± 69.47 and 805.98

± 36.93, respectively. The mean percentage reduction in tumor volume was calculated in comparison to tumor volume of control group for corresponding week. Results showed that, the tumor growth was considerably affected in animal treated with erlotinib, erlotinib with meloxicam, erlotinib with metformin, meloxicam with metformin and all three drugs together during 1st and 2nd week of the treatment. The reduction in tumor volume was observed more than 50 % in animal groups treated with erlotinib, erlotinib with meloxicam, erlotinib with metformin, meloxicam with metformin and all three drugs together on 3rd week of the treatment whereas, on 4th week of treatment animal group treated with all three drugs together showed more than 50 % tumor volume reduction in comparison to tumor control group. The animal group treated with all three drugs together showed marked percentage reduction in tumor volume through out the study period as compared with tumor control.

The mean relative tumor volume (RTV) was calculated by dividing the mean tumor volume on any day by the mean tumor volume at the start of the treatment. All treatment group showed the substantial reduction whereas all three drugs together treated animal group showed the considerable reduction in mean relative tumor volume as compared to tumor control group. The RTV was observed in descending pattern in treatment group Metformin > meloxicam > erlotinib > erlotinib with metformin > meloxicam with metformin > erlotinib with meloxicam > all three drugs together. The lowest RTV was observed in animal treated with all three drugs together.

The mean value of WBC of animal group treated with erlotinib in combination with metformin was significantly increased and the Monocyte % of animal group treated with metformin was also significantly higher in comparison to untreated tumor control group. All other hematological parameters in SCID male mice of all treatment groups were found to be similar with control group animals.

The mean value of creatinine kinase (CK) of animal group treated with meloxicam alone was significantly decreased whereas animal group treated with all three drugs together was significantly increased in comparison to untreated tumor control group. The mean value of blood urea nitrogen (BUN) in animal group treated with erlotinib in combination with metformin was significantly increased in comparison to untreated tumor control group. The mean values of AST, ALT, TBIL and Acid Phosphatase of tumor control group were considerably high than normal control group. The glucose level in animal group treated with metformin alone and in combination with erlotinib and meloxicam were considerably low, where as AST of animal group treated with meloxicam alone and in combination with erlotinib was also found markedly low in comparison to tumor control group. The mean serum ALT levels were notably reduced in animal group treated with meloxicam and metformin alone as well as erlotinib with metformin in comparison to tumor control group. The LDH level in animal group treated with meloxicam and in combination with erlotinib were noticeably reduced in comparison to tumor control group. The mean values of alkaline phosphatase (ALP), total bilirubin (TBIL), creatinine, and acid phosphatase were found to be similar with control group animals.

The absolute organ weights were obtained for tumor, heart, lung, liver and kidneys of SCID male mice of all groups at the time of necropsy on termination day of experiment. The tumor weight of animal groups treated with erlotinib, meloxicam, metformin , erlotinib with meloxicam, erlotinib with metformin, meloxicam with metformin and all three drugs together were 1.70 ± 0.49g, 0.91 ± 0.22g, 0.97 ± 0.21g, 1.76 ± 0.05g, 0.65 ± 0.31g, 1.06 ± 0.16g, 1.20 ± 0.41g and 0.65 ± 0.23g, respectively. The results showed that there was a considerable reduction in mean tumor weight of animal groups treated with erlotinib, meloxicam, erlotinib with meloxicam, erlotinib with metformin, meloxicam with metformin and all three drugs together; which were 46.34 %, 42.73 %, 61.54

%, 37.40 %, 29.24 % and 61.78 % respectively. The mean kidney weight of animal group treated with metformin in combination with meloxicam was significantly reduced in comparison to the untreated tumor control group. The mean organ weights of heart, lung and liver of all other treatment group were found to be comparable with control group animals.

The extracts of lung and tumor tissues were analyzed for the presence of erlotinib, meloxicam and metformin in respective group of treatment but none of the drug was detected in either lung or tumor tissues.

The gene expression change in tumor tissue was analyzed for AKT, AMPK, P070S6K, PTEN and RAF. The expression of PTEN gene was markedly increased in all treatment groups in comparison to the tumor control group. There was significant increase in expression of PTEN gene in animal group treated with erlotinib along with meloxicam as well as metforimn in comparison to the tumor control group. The expression of AKT is considerably decreased in animal group treated with erlotinib, meloxicam and metformin as a single drug as well as erlotinib with meloxicam and metformin and all three drugs together in comparison to the tumor control group.

The AMPK expression was slightly increased in animal group treated with erlotinib, meloxicam and considerably increased in animal group treated with metformin alone as well as all combinational group of metformin treatment in comparison to tumor control. The expression of P070S6K gene was considerably reduced in animal group treated with erlotinib along with metformin and animal group treated with metformin along with meloxicam in comparison to tumor control group. It was slightly reduced in other treatment group in comparison to tumor control group. The expression of RAF gene was considerably reduced in animal group treated with meloxicam in combination with metformin in comparison to tumor control group. It was also decreased slightly in animal

groups treated with erlotinib, meloxicam and metformin alone and in combinational treatment group in comparison to tumor control group.

The histopathological changes were observed in tissues like tumor mass, lungs, liver, heart and kidneys in all the animals of treated and control groups. The tumor tissue exhibited microscopically necrotic core, mitotic figure, hemorrhage, infiltration in surrounding tissue was minimal to moderate grade in all the treatment groups except animal treated with all three drugs together, where the mitotic figures observed was minimal to mild in comparison to tumor control group. The tumor mass of animals treated with erlotinib along with both meloxicam and metformin combination appeared to be reduced to a considerable size as necrosis of tumor mass was severe in these animals. The tumor mass or necrosis of tumor cells was also seen in moderate severity in animals treated with combination of erlotinib along with meloxicam followed by mild to moderate severity in erlotinib along with metformin and followed by meloxicam along with metformin treated animals. The minimal or minimal to mild necrotic changes in tumor mass was seen in animals treated with metformin alone or meloxicam or erlotinib. The tumor mass was intact and none of the animal revealed necrotic changes in the animals of tumor control group. The histopathological changes such as foci of leucocytic infiltration, necrotic foci in heart, liver, lungs and nephropathy in kidney, respectively did not differ from the normal back ground lesions in the animals of this age and strain therefore considered to be spontaneous lesions and not related to treatment.

Results of this study suggest that repeated use of erlotinib (30 mg/kg, p.o.), meloxicam (20mg/kg, i.p.) and metformin (100 mg/kg, p.o.) at twenty-four hours interval for 28 days seems to be safe and having significant therapeutic effect in SCID male mice.

Following conclusions can be drawn from the present study:

1) The mean peak plasma concentration of erlotinib alone and in combination with meloxicam and/or metformin was observed at 0.17 h; The mean peak plasma concentration of meloxicam alone and in combination with erlotinib and/or metformin was observed at 0.33 h. The mean peak plasma concentration of metformin alone and in combination with erlotinib and all three drugs together was observed at 0.33 h, whereas in combination with meloxicam it was observed at 0.67 h.
2) The drug concentration in plasma was detectable till 24 h for erlotinib, meloxicam where as metformin drug concentration was detected till 12h post administration.
3) The $AUC_{0-\infty}$ of erlotinib was significantly decreased and the clearance was significantly increased when given in combination with metformin and in combination with both metformin and meloxicam together.
4) The $AUC_{0-\infty}$ and MRT of meloxicam were significantly decreased whereas Vz and Cl were significantly increased when given in combination with erlotinib. The Vz of meloxicam was significantly increased when given in combination with metformin and clearance of meloxicam was significantly decreased in combination with both metformin and meloxicam together.
5) The C_{max} and $AUC_{0-\infty}$ of metformin were significantly increased whereas the Vz and Cl were significantly decreased when given along with both erlotinib and meloxicam. The $AUC_{0-\infty}$ of metformin was significantly increased where as the $T_{1/2}$, Vz and Cl was significantly decreased when given in combination with meloxicam. The Vz of metformin was also significantly decreased when given in combination with erlotinib.

6) The body weight was significantly reduced ($p < 0.05$) in animal group treated with erlotinib along with metformin during all 4 weeks of study. The Feed consumption in SCID male mice of treatment group was found to be similar with control group animals.

7) The tumor growth was considerably affected in animal groups treated with erlotinib, erlotinib with meloxicam, erlotinib with metformin, meloxicam with metformin and all three drugs together. The animal groups treated with erlotinib, erlotinib with meloxicam, erlotinib with metformin, meloxicam with metformin showed more than 50 % reduction in tumor volume during 3rd week of treatment.

8) The animal group treated with all three drugs together exhibited more than 50 % tumor volume reduction during all 4 weeks of treatment.

9) The mean relative tumor volume (RTV) was substantially reduced in all treatment groups. The RTV was observed in descending pattern in treatment group metformin> meloxicam> erlotinib> erlotinib with metformin> meloxicam with metformin> erlotinib with meloxicam > all three drugs together. The lowest RTV was observed in animal group treated with all three drugs together.

10) The mean value of creatine kinase (CK) was significantly decreased with meloxicam treatment and significantly increased in animal group treated with all three drugs together. The mean value of blood urea nitrogen (BUN) was significantly increased in animal group administered erlotinib with metformin.

11) Based on evaluation of the hematological, biochemical parameters and histopathological examination, concurrent administration of erlotinib (30 mg/kg, p.o.), meloxicam (20 mg/kg, i.p.) and metformin (100 mg/kg, p.o.) at twenty-four hour interval for 28 days was found to be safe.

12) There was significant increase in expression of PTEN gene in animal group treated with erlotinib in combination with meloxicam and erlotinib with metformin. The expression of AKT gene is considerably decreased in all treatment groups. The AMPK expression is increased in all treatment groups with considerably high increase in alone or combinational with metformin.
13) The expression of P070S6K gene was reduced in all treatment groups, with considerable reduction in erlotinib with metformin and metformin with meloxicam treatment groups. The expression of RAF gene was reduced in all treatment groups, with considerable reduction in meloxicam with metformin treatment group.
14) The histopathological changes in tumor mass revealed that all the drugs have more or less therapeutic value against cancer treatment with most effective treatment when all the three drugs erlotinib, meloxicam and metformin were administered together.
15) The present study revealed that administration of erlotinib, meloxicam and metformin in combination with each other and all three drugs together altered pharmacokinetic profile of each other. Therefore, concomitant use of these drugs requires close therapeutic monitoring for potential pharmacokinetic drug interaction.
16) The present study revealed that administration of erlotinib, meloxicam and metformin in combination with each other and all three drugs together has better therapeutic value than individual drug in treatment of cancer.
17) The present study revealed that administration of meloxicam and metformin in combination has better effect than single TKIs (i.e. erlotinib), which can be studied further in future.
18) All three drugs have synergistic effect when given in combination for effect on tumor progression.

CHAPTER – VII

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