Dose – Dependency of The Cardiovascular Risks of Non-Steroidal Anti-Inflammatory Drugs

by

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A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

In

Pharmaceutical Sciences

Faculty of Pharmacy and Pharmaceutical Sciences
University of Alberta

Abstract

Rheumatoid arthritis (RA) is a chronic, progressive, and systemic inflammatory condition leading to joint destruction, substantial pain, and functional disability. Patients with RA have a higher morbidity and mortality rate because of cardiovascular (CV) complications than the general population. Non-steroidal anti-inflammatory drugs (NSAIDs) are used to treat pain and inflammation associated with arthritis. However, NSAIDs are also known to elevate CV risks, but the mechanism by which this occurs is unknown. More attention has been paid recently to CV and the renal safety profile of NSAIDs, starting with the withdrawal of rofecoxib from the market because of increasing risk of thromboembolic events such as heart attack and stroke. However, not all NSAIDs are considered to cause major CV effects. The intrinsic pharmacological and pharmacokinetic properties of NSIADs could be involved in causing CV risks. Some data suggest of lack of CV complications at lower therapeutic doses of some NSAIDs.

Recently, ample recent preclinical data suggest that modulating of eicosanoids, the cytochrome P450 (CYP) metabolites of arachidonic acid (ArA), may be a potential clinical therapeutic strategy and achievable biomarker to manage different pathological disorders, in particular CV diseases. Taken together, we hypothesize that the CV risk NSAIDs is dose-dependent so that for diclofenac, an NSAIDs with known severe CV complications, lower therapeutic doses are void of the above side effect. We i) used adjuvant arthritis (AA) as an experimental model of arthritis; ii) dosed rats with a range of diclofenac doses to control AA; iii) identified the effective dosage range; and iv) euthanize the animals and assessed the effect of the treatments on CYP-ArA pathway, and determined drug concentrations in selected tissues.

Our results show i) a dose-dependent effect for the diclofenac dosage range used; ii) that extent of accumulation of diclofenac in the heart was dose-dependent; iii) that low therapeutic doses did not alter the CYP-mediated ArA metabolism, while high doses of diclofenac in inflamed rats suggest an increase in the 20-HETE/EETs (cardio-toxic/cardioprotective) metabolites concentration in the plasma and heart of AA rats. This was while.

In conclusion, within the therapeutic range, only the examined high doses of diclofenac caused imbalances in ArA metabolic patterns toward cardiotoxicity. This is suggestive of dose- and exposure-dependency of NSAID cardiotoxicity, and low therapeutic doses may be void of CV side effects. Human studies are needed to examine the safety of low but effective doses of NSAIDs.

Preface

This thesis is an original work by Surur Ali Ahmed. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, Project Name “Pharmacokinetics and Pharmacodynamics of anti-rheumatic and cardiovascular drugs”, No. AUP267.

Dedications

To my lovely mother, Amna, and my dear father, Ali, who always encouraged me

To my lovely husband, Mohammed, and our precious son, Kenan, for their understanding, patience, sacrifice and support

To my wonderful siblings for their love, encouragement, inspiration and support

To Dr Jamali, for his support and scientific inspiration

Acknowledgement

I would like to express my sincere special appreciation and deepest thanks to my supervisor Prof. Fakhreddin Jamali for his guidance, understanding, and encouragement throughout my Master's program.

A special thanks go to my examining committee members, Dr. Sherif Mahmoud and Dr. Raimar Loebenberg for their generous guidance and valuable comments.

I would also like to thank my colleagues and fellow lab mates Waheed, Hanan, and Zuhair for their help and for creating a friendly work environment.

My thanks are extended to Dr. Forughalsadat Sanaee for her love and support, and for sharing her knowledge with me.

I would like to thank my friends in Faculty of Pharmacy for their support, help and friendship.

A special thanks to my family: my son and husband, my parents, my brothers and sisters, for their love, support, and for always being a source of inspiration.

I would like to thank the Libyan Government for its financial support during my Master's program.

Finally, many thank go to all the academic and non-academic supporting staff at the Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta.

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LIST OF ABBREVIATION AND SYMBOLS

AA	Adjuvant-induced arthritis
AI	Arthritis index
APC	Antigen-presenting cell
ArA	Arachidonic acid
AUC	Area under plasma concentration-time curve
BHT	Butylated hydroxytoluene
Cmax	Maximum plasma concentration
CONT-0	Healthy control
COXs	Cyclooxygenases
CRP	C-reactive protein
CV	Cardiovascular
CYP 450	Cytochrome P450
DHTs	Dihydroxyeicosatrienoic acids
DM	Diabetes mellitus
DMARD	Disease modifying antirheumatic drugs
ED50	Median effective dose
EDHFs	Endothelium-derived hyperpolarizing factors
EETs	Epoxyeicosatrienoic acids
ESR	Erythrocyte sedimentation rate
HETEs	Hydroxyeicosatetraenoic acids
HPLC	High performance liquid chromatography
HR	Hazard ratio
IL	Interleukin

INF	Interferon
INF-0	Inflamed placebo
INF-15	Inflamed treated with diclofenac (15mg /kg/day)
INF-5	Inflamed treated with diclofenac (5mg /kg/day)
IS	Internal standard
LOX	Lipoxygenase
LPS	Lipopolysaccharide
MAPKs	Mitogen-activated protein kinases
mg	Milligram
MHC	Major Histocompatibility Complex
MI	Myocardial Infarction
mL	Milliliter
NE-TFM	2-(2,3-naphthalimino) ethyl-trifluoromethanesulphonate
NF-κB	Nuclear factor-kappa-B
ng	Nano gram
NO	Nitric oxide
NSAIDs	Non-steroidal anti-inflammatory drugs
PD	Pharmacodynamics
PG	Prostaglandins
PHS	Prostaglandin H synthase
PK	Pharmacokinetics
pKa	Ionization constant
PLA2	Phospholipases A2
PRRs	Pathogen recognition receptors
RA	Rheumatoid arthritis

RAS	Renin-angiotensin-system
RCTs	Randomized clinical trials
RF	Rheumatoid factor
ROS	Reactive oxygen species
RR	Relative risk
SD	Standard deviation
sEH	soluble epoxide hydrolase
SPE	Solid phase extraction
T-DHT	Total DHTs
T-EET	Total EETs
Tmax	Time to reach maximum concentration
TNF-α	Tumour necrosis factor-α
TX	Thromboxane
µg	Micro gram
µL	Micro liter

Chapter 1

1. Introduction

1.1. Rheumatoid arthritis

Rheumatoid arthritis (RA) is one of most common auto-immune disorders that mediate systematic inflammatory joint diseases. RA causes substantial personal and socioeconomic costs. It is characterized by chronic inflammation and the formation of antibodies against synovial joints, leading to swelling, stiffness, significant pain, and eventually symmetrical destructive arthropathy. Extra-articular manifestations such as neuropathy, ocular inflammation, splenomegaly, alteration in renal function and excessive CV risks are common in patient with RA. The pattern of this inflammatory disease ultimately is irreversible joint deformities, poor quality of life and functional disability [1, 2]. However, patient outcomes have been transformed in last two decades through an application of new therapeutics and management strategies. This has led to disease control and, in some cases, drug-free remission [3].

In Canada, approximately 4.6 million people have been negatively impacted by arthritis i.e., one in six Canadians age 15 and older. Patients between 40 and 70 year olds are the most affected. RA patients also face socioeconomic burdens since the disease is the first and third cause of disability as common chronic condition among Canadians female and male, respectively. Approximately 64 percent of RA patient are women. The prevalence of RA in women is 19 percent, while for men it is 13 percent. The number of RA cases increases with age; as such, the total number of cases is predicted to rise to a 7.5 million Canadian adults by 2030 [4].

The exact etiology of RA is not fully recognized. However, the epidemiological studies reveal a complex genetic component along with varying degrees of environmental factors. An analysis of twins studies shows that monozygotic twins have a higher rate of disease compared with the dizygotic twins. Moreover, the sibling of RA patient shows a risk that is approximately two to 17 times higher than that of the general population. One of the greatest associations in all genetic studies was found with the human leucocyte antigen (HLA) in the Major Histocompatibility Complex (MHC) region. The polymorphism in HLA gene was found to be the main genetic contributor to RA. The cumulative risk reported by HLA polymorphism is between 30% and 50 % [5, 6].

Understanding the associated risk factors is essential in order to assess overall risk of RA. Such risk factor could be non-modifiable, including age, sex, hormones particularly female hormones and genetic predisposition. Other factors reflect the environmental side and might be modifiable such as diet, physical activity, infections, excessive body weight and exposure to tobacco smoke. Furthermore, many studies have conducted with the purpose of liaison between RA risk and pharmacological field as some drugs can be linked with disease progression. For example, steroids can be used to treat RA, but they can also trigger the disease by affecting the function of the immune system [4, 7].

1.1.1. Pathogenesis of RA

The synovial membrane is the soft tissue that provides different lubricants such as collagen and hyaluronic acid to body joints. The attack of this membrane can lead to cartilage destruction and joint deformities.

Briefly, the full pathophysiology of RA can be divided into three main stages: initial phase starts with inflammation of synovial membrane with unknown causes: the second stage

involves complex cellular and process interaction, where T-cells accumulates in synovial cavity: and the last stage (amplification phase) is the joint destruction caused by progressive inflammation initiated by many mediators such as IL-1, TNF-alpha, and IL-6 [7].

The role of the immune system in the progression of RA is very critical as the disease begins resulting from losing the immune tolerance toward self-antigen. The activated B cells produce antibodies like rheumatoid factor (RF) against self-antigen in the joint tissue, leading to the recruitment of leukocytes, and the stimulation of complements and phagocytosis. Additionally, the activation of T-cells leads to infiltration of these cells into the synovium and secretion of lymphokines. Macrophages, on the other hand, can enhance the release of synovial enzymes such as collagenases and proteases. The activated lymphocytes, the pro-inflammatory mediators, and synovial enzymes invade the surrounding bone and cartilage, damaging and eventually destroying the joints [8].

The generation of reactive oxygen species (ROS) and nitrogen species can trigger the process of joint destruction. Additionally, the amplification phase of RA may also be linked to inflammation in the sclera, lungs, pericardium, and pleura; and with the formation of nodular lesions that are common in subcutaneous tissues [7].

1.1.2. Presentation, diagnosis and complication of rheumatoid arthritis

The clinical manifestation and disease outcomes vary among patients. Some experience a mild to moderate non-erosive RA, whereas other patients might experience a destructive, painful and persistent style of RA. Pain, joint swelling, redness and morning stiffness are the most classical signs and symptoms of RA. However, this disease is characterized by the concept of symmetry, where both sides of the body eventually will be affected. Over the half of RA patients experience

signs or symptoms in the upper part of the body (extremities). Large joints like the wrist, ankle, and knee are also involved in the RA progression [8].

The precise and accurate diagnosis of RA, especially in the early stages is still a big challenge. The primary tools for the diagnosis generally are the clinical presentation, laboratory tests and radiography examination.

Clinical symptoms are the primary criteria to diagnose RA. The main presentations are stiffness in multiple joints, accompanied by pain. At first, the symptoms only appear in a few joints, and it might develop over weeks or months to other joints in most patients. To illustrate, the nearest wrists, interphalangeal and metacarpophalangeal joints are initially involved and tend to be tender and warm to the touch. For morning stiffness, it needs at least 45 minutes to lasting. Low-grade fever, fatigue, malaise, anorexia, and involvement of the lymph nodes such as axillary and cervical lymph nodes might occur particularly in the acute presentation.

For a laboratory assessment, RA is marked by the existence of autoantibodies as any other autoimmune disease. Both (RF) and the anti-citrullinated protein play an important role in predating the onset of the disease, in which the former protein is not specific with some limitation to be a biomarker while the latter protein is more specific and presented as a diagnostic and prognostic marker. An increase in erythrocyte sedimentation rate (ESR) and the level of C-reactive protein are indicators for acute phase reactants, which is a characteristic feature of systemic inflammation.

Radiography, including ultrasonography, x-ray and magnetic resonance imaging, is used to determine the structural damage in joints and reaction to the therapeutic intervention. Radiography could be also the best option for the clinician as it is an available tool and commonly used [8, 9].

Although numerous inflammatory, immunological, and genetic markers are identified, further studies should be conducted to identify biomarkers that are more sensitive and specific for predicting, diagnosing and determining the prognosis of RA. .

Cancer and infection are serious complication of RA along with lung disease that is account for 6 % for death in RA cases [10]. RA patients have demonstrated higher mortality rates compared to general population. Specific determinants of mortality, generally, are the activity and duration of the disease, occurrence of extra-articular manifestation, reduction of functionality, and the number of joints involved with or without nodules. However, recent data suggest that increasing mortality in RA is mainly attributed to CV diseases (i.e., acute coronary syndrome, heart failure, peripheral arterial disease and cerebrovascular atherosclerosis) that seem to be increased by twofold in cases of RA in comparison to the general population. The occurrence of classical CV risk factors such as obesity, diabetes mellitus, dyslipidemia, hypertension, and smoking can-not entirely explain the excessive increase in mortality in RA patients [11].

Systemic inflammation is the crucial factor that contributes to the increased mortality rate and CV events in RA. It has been increasingly recognized that there are remarkable similarities in the responses in atherosclerosis and RA and the fundamental similarity is inflammation. Indeed, the chronic inflammatory state is the driving force of the formation of atherosclerotic plaque, starting with endothelial dysfunction, the accumulation of inflammatory cells and, finally, thrombosis. Furthermore, the inflammatory mediators such as TNF-α, IL-1, IL-6, CRP, and cellular adhesion molecules like intracellular adhesion molecule-1 (ICAM-1) are the main promotors that enhance the cascade of RA and the premature development of atherosclerosis [12,

13]. Therefore, the assessment of CV disease is inclined to be an important part of RA patient care with the purpose of decreasing CV-associated morbidity and mortality risks.

1.1.3. Anti-inflammatory therapy of RA

The overall aim of RA treatment is to control the progression of disease particularly the inflammatory process in order to maintain remission of symptoms, delay joint destruction and improve quality of life.

To determine the response of therapeutic intervention, both the patient and physician can use many clinical measures, including symptom assessment (swelling), inflammatory markers, assessment of disease activity and physical function. These measures are used by the American College of Rheumatology as criteria for patient improvement and assessment after treatment [14].

The current options for RA management are classified into pharmacological and non-pharmacological strategies (Figure 1). Physical activity, as an example of non-pharmacological intervention and according to Metsios et al., is a very effective in helping patients to maintain a healthy lifestyle and improve their CV profile as well [15].

From a pharmacological perspective, therapeutic agents used to treat RA are divided into three main categories: corticosteroids, disease-modifying antirheumatic drugs and NSAIDs (Table 1).

1.1.3.1. Steroidal anti-inflammatory therapy

Glucocorticoids are powerful anti-inflammatory and immunosuppressive agent that belong to steroidal hormones class. This class of therapy has several therapeutic indications including inflammatory conditions, autoimmune diseases and organ transplants due to its ability to inhibit the production of inflammatory mediators and suppress immune cells. The importance of

glucocorticoids in RA comes from the fact that systemic inflammation is a typical feature of RA and atherosclerosis and this drug is very potent in controlling the inflammation process. However, this class is usually used on short term because of the common predictable adverse effect such as cataracts, osteoporosis, diabetes, hypertension and hyperlipidemia. As a result, a follow-up plan should always be put in place to monitor and assess whether the benefits outweigh the drawbacks (i.e., side effects) [14].

Table 1: Therapeutic agents for treatment of rheumatoid arthritis [14].

Category	Example (s)
NSAIDs	Ibuprofen / diclofenac
Glucocorticoids	Prednisone / methylprednisolone
DMARDs	Methotrexate / hydroxychloroquine
Biological agents	Infliximab / etanercept / Ritiximab / adalimumab

1.1.3.2. Non-steroidal anti-inflammatory therapy

NSAIDs are one of the most commonly used drugs in the world due to their analgesic, antipyretic and anti-inflammatory properties. This class, as result, is used for a large perentage of the population diagnosed with RA or osteoarthritis. NSAIDs can be classified according to many criteria including COX selectivity and chemical and pharmacological properties. The mechanism of action of NSAIDs is related to the inhibition of PG biosynthesis by hindering the binding of ArA to the active site of the COX enzyme [16]. NSAIDs are discussed in a separate section.

1.1.3.3. Synthetic disease modifying antirheumatic drugs

Diseases modifying antirheumatic drugs (DMARDs) are defined as agents that suppress both inflammatory and destructive processes. DMARDs are able to reduce the radiographic progression and usually take from weeks to months to work [14]. Even though these immune modifying agents have a different mechanism of action, a meta-analysis of a clinical trials has shown that methotrexate, sulfasalazine, penicillamine and gold are similar in regard to their efficacy on RA [17]. However, the main obstacle in DMARD is their safety and toxicity profile. Penicillamine, for example, is rarely used because it has a high incidence of toxicity, while hydroxychloroquine is less effective.

1.1.3.4. Biologic disease modifying antirheumatic drugs

It is important to note that there is an enormous need to develop highly specific therapeutics that target specific molecules in the pathogenesis of RA. Biologic modifiers are a group of drugs produced to perform that role. The biological modifiers such as infliximab, etanercept, and adalimumab target tumour necrosis factor (TNF); anakinra and tocilizumab inhibit the action of interleukin; and rituximab that has anti CD20 mab. Biologics have revolutionized the treatment of RA, yielding substantial improvement in radiographic, clinical and functional outcomes. Hence, the expansion in biologics field has rapidly developed, providing more therapeutic choices for RA treatment. Biologics are generally well tolerated and approved by FDA. However, many safety concerns have been linked with this class, including application-specific issues such as injection site reactions and risks of infection such as tuberculosis. As result, general screening before treatment and monitoring during therapy is required [18]. Financial burden could be another obstacle due to the high cost of producing biologics. Consequently Biologics can be limited to patients who can afford or severe cases with high risk of disability.

1.1.4. Adjuvant arthritis animal model

Animals' models have been used for decades to understand disease pathways and develop therapeutic approaches. Such models are useful tools in many pharmaceutical research areas such as inflammation, despite the existence of predictable differences among animal species and humans. Such differences should be considered in the extrapolation of data.

The pathophysiology of RA is not limited to the joints; it includes complex pathways. Hence, it is important to choose an animal model that mimics the human state and can be translatable. The common features between the animal model and human conditions can provide useful experiment and reliable data for research hypothesis.

Joint swelling, bone erosion, cellular infiltration, and inflammatory mediator production are classical features of RA that should be exhibited at least in the chosen model. However, the main differences in all of the animal models are associated with the onset of disease, severity, resolution and histopathological changes [19, 20].

Adjuvant-induced arthritis (AA) is a widely used rat model for examining immuno-inflammatory processes, particularly RA. This model depends on utilizing the dried powder of heat-killed *Mycobacterium Butyricum* injected intra-dermally. This model has a rapid onset and involves typical symptoms of RA.

Sprague–Dawley rats are a commonly used strain in AA studies because the strain is more affordable and easily accessible. These valuable features allow this strain to be the first option for many studies including RA. Cai et al. reported that 100 percent of the Sprague-Dawley rats developed AA with significant increase in both ESR and CRP and overexpression of inflammatory mediators such as TNF-α, IL-1β, and IL-6., Cai et al. also stated that AA in Sprague-Dawley rats undergo the main histopathological changes in RA including cellular

infiltration, narrowing of joint space, swelling, and cartilage and bone destruction [21].

One of most achievable goals in the treatment of RA patient is to stabilize the cases with aim of stopping the disease progression and reducing swelling and pain sensation. In our lab, we use AA in Sprague–Dawley rats because these models mimic the clinical features of RA in humans. In addition, AA is widely used to investigate the eicosanoid pathway and conduct research on many therapeutic agents, in particular [20], which are the main core drug class in our hypothesis.

1.2. Inflammation

The word inflammation is derived from the Latin word inflammare (to set on fire) and it is a normal physiological response of the body toward noxious conditions [22]. Inflammation is also known as a complex process which is initiated by microbial infection, environmental pollution or chemical damage, resulting in local or systemic tissue injury or even cell death. In order to maintain homeostatic balance in tissue, a complicated system would identify the risks, restrict the extent of harmful stimuli and end with the healing or repair process. While a regulated inflammatory response might be beneficial, it is thought to be detrimental, as the inflammation is a common factor in numerous diseases or conditions such as inflammatory rheumatic disorders, neurodegenerative diseases, diabetes mellitus (DM), CV diseases, cancer and asthma [23]. An ample amount of research in regard to the impact of the inflammatory mediators on several body systems is considered to be an evolutionary direction toward a tremendous understanding of many unknown or incomplete aspects.

1.2.1. Chemical mediators of inflammatory response

The inflammatory response is highly diverse in term of molecular mediators and cell types, and it has also been directed by different processes of induction, regulation, and resolution. The field of

those regulators has expanded and several classes and mechanisms of action have been identified in recent decades. The chemical mediators are not only plasma protein, which is synthesized mainly in the liver in inactive forms, but could also be cells derived from preformed granules or produced immediately at the site of injury. These meditators have either a direct target action or produce a signal transduction by activating specific receptors [24].

Inflammatory pathways provoked under specific conditions rely on the nature and severity of harmful trigger. However, the fundamental features of the inflammation are almost similar: the same basic regulators are present in each inflammatory response, but the level of regulation is different. Nitric oxide (NO), vasoactive amines, reactive oxygen species, cytokines, complements, kinins, coagulation systems, transcriptional factors, lysosomal enzymes and eicosanoids play a vital role in tailoring the inflammatory response.

Nitric oxide, as single oxygen atom attached to one nitrogen atom, is a short-lived gas and freely diffusible intercellular messenger. NO synthase (NOS) is responsible for NO production, and it presents in three discrete isoforms: the constitutive neuronal, endothelial, and the inducible NOS. NO is also present in hepatocytes, heart muscle and smooth muscle cells. While NO plays an essential physiological role in regulating platelet aggregation, blood pressure and signaling neurotransmission, the activated macrophages and neutrophils produce NO during the inflammation reaction due to its vasodilation as well as its cytotoxic effect against the microbes [25, 26].

Histamine and serotonin are vasoactive amines produced mainly in mast cells and platelets in preformed granules. The secretion of histamine under the influence of many stimuli causes an increase in vascular permeability and is rapidly subjected to hydrolysis by histaminase, whereas serotonin acts as a vasoconstrictor and neurotransmitter in the central nervous system.

The activation of complement fragments C3a, C4a and C5a promote leukocyte chemotaxis in addition to enhancing both the phagocytosis process and mast cell degranulation. The proteolytic enzymes, involving collagenase, elastase and cathepsin, are vital system for degrading the phagocytized ingredients. However, releasing the lysosomal system at site of the inflammation may lead to tissue damage through proteins destruction [24, 27].

Reactive oxygen species (ROS) including superoxide anion ($O_2^{\bullet -}$), hydroxyl radical (OH), and hydrogen peroxide (H_2O_2), play a key role as signaling molecules at a low level. ROS are involved in the progression phase of inflammatory disease and cancer by oxidising lipids or proteins components and altering DNA at high concentration. The ability of endogenous antioxidants such as catalase, glutathione peroxidase and superoxide dismutase to overcome the ROS relies on both the severity of the inflammation and the level of ROS produced by neutrophils [28].

Each inflammatory trigger can activate different genes via different signal transduction pathways at the transcriptional or translational levels. Additionally, the long exposure to the harmful stimuli might continue to alter the genes and interrupt any negative feedback to control the transcription of certain pro-inflammatory genes. Such mechanisms could result in autoimmune disease and cancer [29]. NF-kB, for instance, belongs to a family of transcriptional factors which promote growth, activation, discrimination and maturation of lymphocytes and other immune cells [30].

TNF-α, IL-1, and IL-6 are the most renowned polypeptide molecules, known as cytokines, which are released mainly from macrophages and mast cells. The fundamental tasks of cytokines are activating the endothelium, increasing the expression of adhesion molecules, recruiting leukocytes in local inflammatory response and promoting the thrombus formation.

Cytokines are also considered to be a therapeutic target in treating inflammatory disease. Furthermore, the chemoattractant components of cytokines are responsible for migration of neutrophils, lymphocytes or eosinophils to the site of damage during the acute phase reactions of inflammation [24, 27].

Arachidonic acid (ArA) metabolites will be discussed later in a separate section.

1.2.2. Pathophysiology of inflammation

The scope of inflammation studies has broadened in recent decades in order to obtain a correct estimation of inflammatory process in health and disease conditions. The comprehensive pattern of inflammation has been involved a series of common pathways and modalities. While the classical pathway of inflammation consists of four major components (inducer, sensors, chemical mediators and target tissue), the mechanism of the inflammatory process can be vascular, cellular or immune.

The inflammation pathway starts with the presence of an inducer that can be endogenous or exogenous. The endogenous sources of induction might occur from a signal of stressed, injured or dead cells accompanied by the release of some of their contents such as uric acid and ions. The exogenous triggers generally are a microbial assault or could be a non-microbial such as allergen or toxic or irritant materials.

At the site of the attack, specific sensor cells such as macrophages, dendritic cells, and mast cells can recognize the inducers in a direct or indirect manner. The sensors react by producing inflammatory mediators at various levels or magnitudes, and some of tissue resident cells could yield a distinct marker for the specific inducer. Inflammasome, for example, is a complicated protein system in plasma that distinguishes the product of dead cells and activates the IL-1β. These mediators work on the target tissue in different approaches, including the

extravasation of leukocytes and vasodilation enhancement. Thus, the structural and functional status of a cell will be changed based on the length of exposure to the noxious stimuli. The ultimate strategy of tissue during inflammation is to adapt until the causative agent is removed and the homeostatic balance is restored [24, 27, 31, 32]

The four cardinal signs of inflammation have been reported by Celsus and they include *calor, dolore, tumor and rubor,* which represent heat, pain, swelling and redness, respectively. These signs represent the vascular changes of inflammation, which aim to cause vasodilation and form small spaces between the endothelium. Consequently, both vascular permeability and blood flow will increase, leading to accumulation of leukocytes and proteins.

In the inflamed area, leukocytes are infiltrated through multiple stages starting with rolling and adhering to the endothelium and ending with the migration process under the influence of chemoattractant molecules. The main action of leukocytes is phagocytosis and the elimination of triggers. This mechanism represents the cellular response to inflammation [22, 24].

The immune defense can be classified into innate and adaptive immunity. The innate response is a non-specific first-line defense where the macrophages, neutrophils, natural killer and dendritic cells can recognize the triggers by a set of pathogen recognition receptors (PRRs) and limit the immunogenic response. The adaptive immunity is interlinked with the innate response. However, the adaptive defense is individualized by antigenic specificity and memory of immunity. B lymphocytes produce specific antibodies that attach to a specific antigen and identify this antigen with any recurrent attack, whereas the T lymphocytes manage the resistant cases that are unreachable by antibodies. The balance between all components of immunity is

essential. Otherwise, immune homeostasis might be compromised, resulting in the development of several immune diseases [33].

1.2.3. Types of inflammatory conditions

The length of exposure to the stimuli is a critical factor in inflammation. Therefore, inflammatory response can be categorized into two types: acute and chronic.

Acute inflammation is symbolized by rapid onset and immediate action that continues from hours to days. Moreover, it consists mainly of vascular and cellular changes. The key part of acute phase is neutrophil accumulation. The chief results could be eradication of harmful trigger, scar formation or develop to chronic inflammation.

The chronic phase of inflammation is characterized by extended and recurrent exposure to the stimuli, lasting from days to years. The prolonged lymphocytes and plasma cells infiltration at the inflammatory area could be linked with the architectural and functional changes in tissues. A disorganized prolonged inflammatory response might become pathologic due to a loss of the regulatory processes and lack of tolerance. As result, a high grade chronic inflammation could occur along with a significant increase in the level of inflammatory markers, leading to many diseases such as inflammatory bowel diseases, rheumatoid arthritis (RA), atopic dermatitis, psoriasis and asthma. Low grade chronic inflammation, on the other hand, exhibits a negligible concentration of chemical mediators as in case of obesity [24, 34].

1.2.4. Local and Systemic effects of inflammation

The local response of inflammation is aimed to increase the blood supply to the injury site, resulting in occurrence of clinical manifestations such as heat, pain and redness. Inflammatory

mediators including TNF, IL-1, and IL-6 are still participating in the activation of leukocytes to restrict the inflammatory response and start the repair process as soon as possible.

The acute phase response is a characteristic feature of systemic inflammation that affects many organs and systems. Fever, hepatic acute-phase protein, hematological changes and leukocytosis are the main signs of systemic inflammation in order to restore body homeostasis.

Levels of C-reactive protein, serum amyloid A protein and fibrinogen are elevated in plasma under the influence of cytokines and reflect the hepatic acute-phase response. C-reactive protein attaches to microbes or to the damaged cells and directs the phagocytosis process, while the amyloid A protein plays an important role in immune cells recruitment and enzyme induction as in RA, atherosclerosis and amyloidosis. Fibrinogen and upon activation assists the erythrocytes to rapidly accumulate in units in process called ESR. Both C-reactive protein and ESR are nonspecific markers of systemic inflammatory response in of RA and vascular diseases such as stroke and myocardial infarction.

Fever is another indicator of the systemic effects of inflammation. The elevation of body temperature is due to presence of prostaglandin mediators in the hypothalamus as a response mainly to bioactive products. This response causes neurotransmitters with high levels of regulation to be released to correct disturbance of thermoregulatory center and control body temperature.

Leukocytosis is also a general feature of systemic inflammatory response where cytokines enhance the bone marrow to produce more leukocytes as a compensatory mechanism. The specificity of increasing the number of leukocytes relies on the type of trigger in which neutrophilia, lymphocytosis and eosinophilia are associated particularly with certain conditions as in bacterial infection, viral infection and allergic reaction, respectively.

Acute Phase Reaction is correlated with systemic diseases and has pathogenic role of tissue alteration. Such response acts as reflection for treatment effectiveness and an estimation of systemic disease progression [24, 35].

1.2.5. The effect of inflammation on pharmacokinetics and pharmacodynamics of drug

Inflammation could be a determining factor in the failure of therapy because inflammation affects the disposition, action and effectiveness of drugs. For example, the alteration in the responsiveness of CV agents might lead to an increase in the rate of morbidity or mortality in inflammatory conditions including RA [36]. Our laboratory is one of the pioneer groups working in the area of drug-disease interactions (inflammation- drug interaction).

Pharmacokinetics (PK) indices, including absorption, distribution, metabolism and elimination, in addition to the pharmacodynamics (PD) profile of drug could be subjected to changes during the inflammation, in which the alteration of hepatic metabolism, plasma protein and target receptors are most important consequences of inflammation effect [37, 38]. Sanaee et al. have studied whether Crohn's disease (an inflammatory disorder) can influence the PK and PD of verapamil (a calcium channel blockers) in two groups of patients: a healthy control group and patients with clinically active or quiescent Crohn's disease. The result of this study indicated that patients with severe Crohn's disease did not obtain an increase in response despite the pronounced elevated verapamil concentration. However, patient with quiescent disease state achieved better response even though verapamil concentration was decreased [39]. Mayo et al. has also studied the PK and PD of verapamil but did so comparing RA patients to a healthy control group. The increased verapamil concentration could be attributed to changes in hepatic blood flow or to decreases in the hepatic clearance and/or protein binding. However, the decrease

in the dromotropic effect of the drug was significant and this might be due to the down-regulation of receptors produced by the cytokines [40].

Similarly, PK and PD of propranolol, a β-blocker drug, yielded a finding similar to those of verapamil. Guirguis et al. studied changes in propranolol disposition in an RA inflammatory model. The same finding of verapamil was confirmed in which the reduction in the prolongation of the PR interval was significant in the arthritis model compared with control rat despite the increase in propranolol concentration due to the reduction of hepatic clearance [41]. In contrast, PD effect of sotalol, a β-adrenergic receptor and potassium channel antagonist, was reduced in term of QT interval and PR interval due to inflammatory conditions even though the concentration of sotalol does not change. These findings could be partially attributed to route of elimination where sotalol is renally excreted compared to hepatic cleared propranolol. In addition, Kulmatycki et al. concluded that both β-adrenergic and potassium channel receptors were altered as a result of the overexpression of pro-inflammatory mediators. Thus, the response of sotalol was decreased [42].

The severity of inflammation is another aspect that might influence the disposition and response of a drug. Piquette-Miller et al. reported that when arthritic rats were treated with ketoprofen, the changes in propranolol disposition were lessened. This led to the conclusion that it is essential to consider the activity of disease when examining drug-disease interaction [37] . Ling et al. also investigated the PK and PD of verapamil in RA patients taking infliximab as an anti-TNF-α compared with other antirheumatic therapy. Ling et al. found that remission from active RA tends to restore verapamil hepatic metabolism and plasma protein level. Therefore, the PK of verapamil was comparatively normal [36]. Moreover, Clements et al. stated that pravastatin which has anti-inflammatory, is able to reduce serum interferon-γ and restore

propranolol response in pre-AA model, but it does not influence the elevated concentration of propranolol [43]. Such findings suggest that control the inflammatory conditions can enhance the drug response including CV drug through observing the level of inflammatory mediators.

It appears that drug-receptor interaction is linked to target receptors' expression and that the response, as a result, will be affected. On the other hand, the response of losartan, an, angiotensin II type 1 receptor antagonist, does not change in patient with active or control RA even though the concertation of active metabolites was reduced in arthritis [44]. It seems that the receptors' expression in the inflammation does not follow the similar trend in which both the β-adrenergic receptor and calcium channel were down-regulated in contrast to behavior of the angiotensin II Type 1 receptor.

1.3. Arachidonic acid Pathways

Arachidonic acid (ArA), a precursor of many regulatory molecules in the body, is an n-6 (omega- 6) polyunsaturated fatty acid that is physiologically stored in phospholipid membrane. In general, ArA is not an essential fatty acid, and it can be synthesized from linoleic acid or obtained directly from dairy products [45]. In the presence of extracellular stimulation, free ArA is released by the activation of phospholipase A2, while insignificant levels of free ArA in cells are usually detected when stimulation is absent [46].

The free level of ArA reflects the balance between the re-esterification of ArA and its liberation. Such balance can be maintained through three important pathways, including the incorporation, remodeling, and release of ArA. The incorporation of ArA into phospholipids starts with the activation of the fatty acid coenzyme A (CoA) ligase, which converts ArA to CoA thiol-ester. ArA CoA is incorporated by remodeling mechanism into the *sn*-2 position of phospholipids, which involves the deacylation of the preformed phospholipid and the ATP-

dependent reacylation of ArA CoA by a fatty acyl-CoA acyltransferase. ArA transfers later from diacyl-phospholiplids to the phosphatidylethanolamine pool and into ether-linked phospholipids by a CoA-independent transacylase.

Phospholipase A2 (PLA2) is the enzyme responsible for releasing the ArA form of the phospholipid in order to control the level of free ArA and its metabolites. The catalytic activity of PLA2 can increase because of the phosphorylation of Ser-505 by mitogen-activated protein kinases (MAPKs). Another regulator of PLA2 is calcium which binds to the C2 domain and enhances the translocation of PLA2 from cytosol to the phospholipid. Many studies show that the cytokines increase the expression of PLA2 concomitantly with cyclooxygenase and that this can be linked with delay phase of ArA metabolites production, while the immediate phase production of ArA can occur as result of the post-translational regulation of the acute activation of PLA2 (29). Generally, the translocation of the PLA2 to the nuclear envelope or endoplasmic reticulum tends to be cell-specific and agonist-dependent.

There are three main metabolic pathways of ArA that generate metabolites that differ either in structure or bioactivity. cyclooxygenase, lipoxygenase, and cytochrome P450 are responsible for ArA metabolic cascade [47].

1.3.1. Prostaglandin H synthase and lipoxygenase dependent metabolism of arachidonic acid

Prostaglandins, prostacyclin, thromboxane, and leukotrienes are generated from the oxidative metabolism of ArA via cyclooxygenase, or lipoxygenase pathways. Both are dioxygenases and they catalyze the reaction by first abstracting hydrogen from allylic methylene carbon. Thereafter, through regio- and enantio-selection, radical carbon attach to molecular oxygen and return to the ground state [48] (Figure 3).

Cyclooxygenase (COX) occurs into two isoforms: COX-1 and COX-2. COX-1 is a basal and constitutive isoform, while COX-2 is an inducible enzyme especially in inflammatory disorders [49]. PGG2 is reduced into prostaglandin H2 (PGH2), which is responsible for generating further metabolites including prostaglandin E2 (PGE2), PGD2, PGF2α, PGI2 (prostacyclin), and thromboxane A2 (TXA2). During the reduction of PGG2 into PGH2 by peroxidase, many chemicals undergo bioactivation into free radical intermediates that might have mutagenic or carcinogenic activity. Such bioactivation occurs widely in extrahepatic tissues that might have low levels of cytochrome P450. Moreover, oxidation of xenobiotic by PHS can occur by several mechanisms including production of peroxyl radical, direct oxidation of the xenobiotic, or secondary oxidant species. These electrophilic metabolites are able to form adducts with critical cellular components such as proteins or DNA, leading to toxic response [50, 51]. It is notable that the production of PG is cell specific. For instance, endothelial cells contain prostacyclin synthase, producing PGI2 which is a vasodilator and inhibit platelet aggregation. Platelets contain thromboxane synthase, producing TXA2 which is vasoconstrictor and enhance platelets aggregation. During inflammation, PG amplifies the pain sensation and causes fever through a cytokine interaction [24].

Lipoxygenase (LOX) dependent ArA oxidation generates LTs, which are produced under the influence of immune complexes or microbial peptides and are formed by inflammatory cells. Leukotriene A4 is first LOX-generated product and yields further LTs. For example, leukotriene B4 is a neutrophil chemoattractant and leukotriene D4 causes vasoconstriction after being released from mast cells [24, 49].

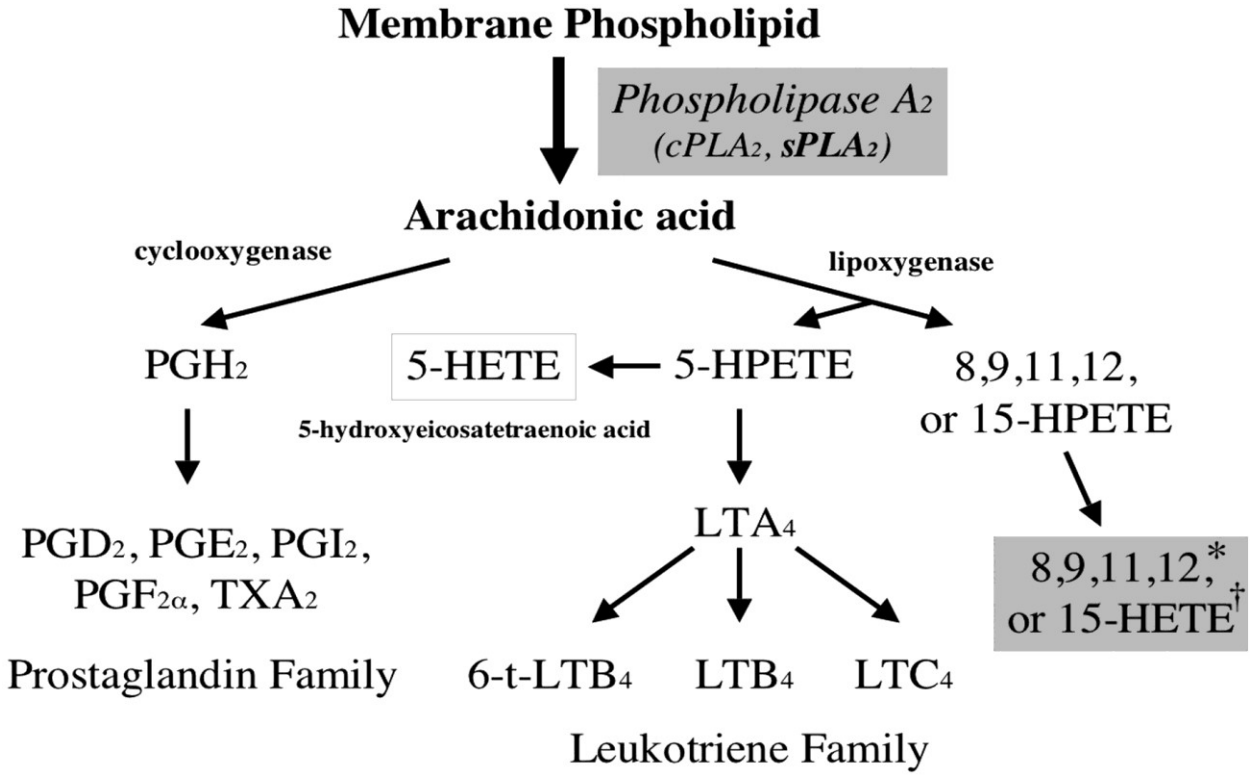


Figure 1: Production of arachidonic acid metabolites via cyclooxygenase and lipoxygenase pathways [52].

1.3.2. Cytochrome P450 dependent metabolism of arachidonic acid

Cytochrome P450 (CYP 450/ P450), an extraordinary enzyme, plays an established role in the metabolism of both endogenous and exogenous substrates. P450 contributes to the ArA metabolism as a monoxyganase endogenous pathway. Such pathways have gained enormous scientific attention on account of the importance of metabolites in cell physiology and pathophysiology.

CYP 450 is cysteino-heme enzyme that is distributed widely in animal tissues and it varies in both structural and functional homology. P450 isoforms are species, sex, age and organ specific. P450 is different from COX and LOX in that CYP involves an electron transport from NADPH into the heme part of CYP through flavoprotien activation. The typical sequence of the oxidative metabolism of CYP –ArA starts with a scission of molecular O2. Whereas one atom of

oxygen is introduced to substrate (ArA), the other atom is liberated as water. The insertion of oxygen into ArA depends on three main factors: the kind of the reaction, regioisomeric and enantiofacial selectivity of oxygen insertion [46, 48].

Depending on chemical reaction, CYP-ArA metabolism is classified into three main pathways. The first pathway is allylic oxidation, which is a lipoxygenase-like reaction, and can generate six hydroxyeicosatetraenoic acids (HETEs) as regioisomeric dienols. The hydroxylation of C7, C10 or C13 or the oxidation of the double bond with rearrangement leads to the formation of 5-HETE, 8-HETE, 9-HETE, 11-HETE, 12-HETE and 15-HETE (mid-chain HETEs). The second pathway is the hydroxylation reaction, another direction of the CYP-ArA metabolism, which is characterized by asymmetric carbon with an R or S enantiomer of regioisomeric HETEs. 20-HETE is formed by the hydroxylation of the terminal methyl group whereas sub-terminal hydroxylation generates 19-HETE. The third pathway is olefin epoxidation which yields 5, 6-, 8, 9-, 11, 12-, and 14, 15-epoxyeicosatrienoic acids (EETs) in a regio- and stereo-selective manner [45, 47]. Soluble epoxide hydrolase ultimately would hydrolyze the EETs to dihydroxyeicosatrienoic acids (DHTs), which are less biologically active than EETs [53] (Figure 4) .

CYP enzymes are expressed in many tissues and cells including the kidney, lung, heart, small blood vessels, liver, white blood cell and pancreas. As a result, the CYP-ArA metabolites are involved in the regulation of cardiac, pulmonary, renal and vascular functions and associated with the inflammatory modulation of various pathological conditions. The balanced production of lipid-derivatives of the CYP-ArA pathway is crucial for the maintenance of body homeostasis. The abundance of preclinical data suggests that the alteration of these mediators' production,

metabolism or action might be an achievable therapeutic benefit, particularly in CV diseases [54].

20-HETE is produced in a cell- and tissue-specific manner and plays a central role in the regulation of vascular tone in body tissue [55]. The chief subfamilies of CYP4, which is responsible for 20-HETE formation, are CYP4A, CYP4B and CYP4F in animals or humans [56]. 20-HETE is a powerful vasoconstrictor on vascular smooth muscle in the kidneys, heart, skeletal muscles and lungs. The stretch of cellular membranes and vasoactive amines such as angiotensin II or noradrenalin are able to stimulate the phospholipase C, leading to the synthesis of inositol trisphosphate and enhancing the release of intracellular Ca. The elevated level of Ca will activate PLA2 and enhance the formation of 20-HETE after releasing of ArA. 20-HETE will block the calcium-activated potassium channel in blood vessels, preventing the membrane hyperpolarization and stimulating the calcium influx via an L-type channel [57]. The level of 20-HETE is associated with the mediation of the myogenic response of cerebral, renal, and skeletal arteries or arterioles. Gebremedhin et al. reported that the concentration of 20-HETE is increased six-fold as the transmural pressure is elevated from 20 to 140 mm. Such data suggest that the role of 20-HETE is to autoregulate of both cerebral and renal blood flow [55, 58-60].

Many studies have investigated the mitogenic response to 20-HETE under the influence of growth agents. Chen et al. reported that inhibition of the formation of 20-HETE abolish the mitogenic response to the vascular endothelial growth factor (VEGF) in human umbilical vein endothelial cells (HUVECs) and the angiogenic response to VEGF and epidermal growth factor in vivo by 80-90%. Such data suggest the role of 20-HETE in angiogenesis and cell proliferation [54, 61]. Moreover, 20-HETE appears to have a dual action on the renal system. In proximal tubules, 20-HETE inhibits Na^+-K^+-ATPase through a protein kinase C-dependent

phosphorylation. Consequently, the sodium reabsorption will be inhibited. The same pattern will occur in the collecting duct, in which 20-HETE blocks the K^+ channel in the apical membrane and limits the availability for the Na^+-K^+-$2Cl^-$ cotransporter, leading to a reduction in required force for cation reabsorption. This action of 20-HETE will induce diuresis and produce antihypertensive effect. On the other hand, 20-HETE constricts the renal blood vessels particularly afferent arterioles [54, 60, 62]. This effect can be considered pro-hypertensive as the vasoconstrictor action of 20-HETE increases vascular resistance.

Several pathological conditions such as hypertension, diabetes, inflammation, tumor formation, metabolic syndrome, and kidney disease are associated with the alteration of the 20-HETE level and changes in the expression of CYP4A [63, 64]. 20-HETE is metabolized by the COX enzyme to 20-hydroxy PGs that are vasodilators. Escalante et al. investigated the effect of indomethacin, a COX inhibitor, on the contraction produced by 20-HETE, and concluded that the indomethacin abolish the contraction of 20-HETE in a concentration-dependent fashion [65]. β-oxidation is another metabolism pathway of 20-HETE, resulting in 20-carboxy-arachidonic acid (20-COOH-AA) and shorter chain length products. 20-COOH-AA might modulate coronary vascular function, while 20-HETE is inactive [66].

EETs are another family in the CYP-ArA pathway, and are produced by endothelial cells, astrocytes, cardiac myocytes, the glomerulus, proximal tubules, the collecting duct and the gastrointestinal tract. The main subfamilies of CYP, which have the epoxygenase activity, are of CYP1A, 2B, 2C, and 2J [54]. CYP2J2 is the key enzyme involved in the production of EETs in extrahepatic tissue, whereas CYP2C9, C8, and 1A2 have emerged as the basic generators of EETs in the liver [67]. There is growing evidence that total EETs are organ specific because of the heterogeneity of CYP enzyme expression [68].

EETs possess high potent biological effects in the renal and cardiovascular systems, where they contribute to the regulation of renal hemodynamic status and cardiovascular homeostasis [69]. To illustrate, CYP epoxygenase-derived EETs exert a direct vasodilatory effect. This action is mediated by the diffusion of EETs from endothelial cells to activate calcium-sensitive potassium channels located on vascular smooth muscle cells, resulting in their relaxation and hyperpolarization. EETs are also able to increase intracellular calcium, which promotes the release of NO and PG [54, 70]. Thus, elegant studies have shown that EETs are considered as endothelium-derived hyperpolarizing factors (EDHFs). EETs exhibit a broad spectrum of anti-inflammatory activity against acute and chronic inflammation by inhibiting leukocyte adhesion to the vascular wall, hindering the aggregation of platelets, enhancing fibrinolysis, and reducing vascular smooth muscle proliferation or angiogenesis. Such effect of EETs can be explainable for atherosclerosis prevention [71].

CYP epoxygenase-derived EETs reduce the contractility of myocytes by inhibiting both the sodium channel and L-type calcium channel openings. In addition, EETs protect the cardiac and cerebral regions from ischemia and reduce hypertension-induced renal damage by inhibiting sodium transport in the kidneys [54, 72]. The alteration of the EET pathway is linked with the pathophysiology of many conditions, including hypertension, vascular inflammation, atherosclerosis, ischemic injuries, heart hypertrophy, and renovascular disease [72]. EETs' valuable contribution to the variety of pathologies suggests that modulating the CYP-derived EET pathway can become a pharmacological strategy to prevent and treat several conditions.

All EETs are metabolized by soluble epoxide hydrolase (sEH) into corresponding diol derivatives, DHTs, via hydration reaction. Remarkably, the sum of EETs and DHTs or the ratio of EETs / DHTs is used to indicate the level of EETs production by CYP or reflect the level of

degradation by sEH. B-oxidation is another available pathway particularly for 11,12- and 14,15-EET , forming short chain 16-carbon or long chain 22-carbon, while both 5,6- and 8,9-EET are subjected to COX in order produce epoxy metabolites. Reincorporation into the phospholipid layer could be another channel of EETs as they can be released under action of PLA [54].

20-HETE	EETs
Vasoconstrictive	Vasodilator
Pro-inflammatory	Anti-inflammatory
Pro / Anti-hypertensive	Anti-hypertensive
Mitogenic / Thrombogenic	

Table 2: The functional properties of 20-HETE and EETs mediators [54] .

Both 20-HETE and EETs are lipophilic metabolites and can display their effect in an autocrine fashion. These metabolites can bind to plasma proteins, limiting their systemic distribution, and this could partially reveal the exact reason why ArA metabolites fail to deliver the predicted effect in vivo as in vitro [54, 73]. However, Widstrom et al. have reported that the process ArA metabolites influx, efflux, and their intracellular transport to target protein or organelles are very critical for these fatty acids, in which fatty acid binding protein may be involved in retention, metabolism or release of these metabolites [68].

It appears that EETs are anti-inflammatory meditators with antihypertensive and vasodilation properties. 20-HETE, on the other hand, has vasoconstrictive, pro-inflammatory, mutagenic and thrombogenic properties (Table 2). The functional diversity of both CYP hydroxylase and epoxygenase pathways exists in a balance at normal body homeostasis. Such parallels may be subjected to alteration and might contribute to the pathogenesis and progression of many disorders. Inflammation has a great impact on the physiological systems in the body by modulating the ArA pathway and alters pro-inflammatory (20-HETE) and anti-inflammatory (EETs) metabolite levels [74, 75] .

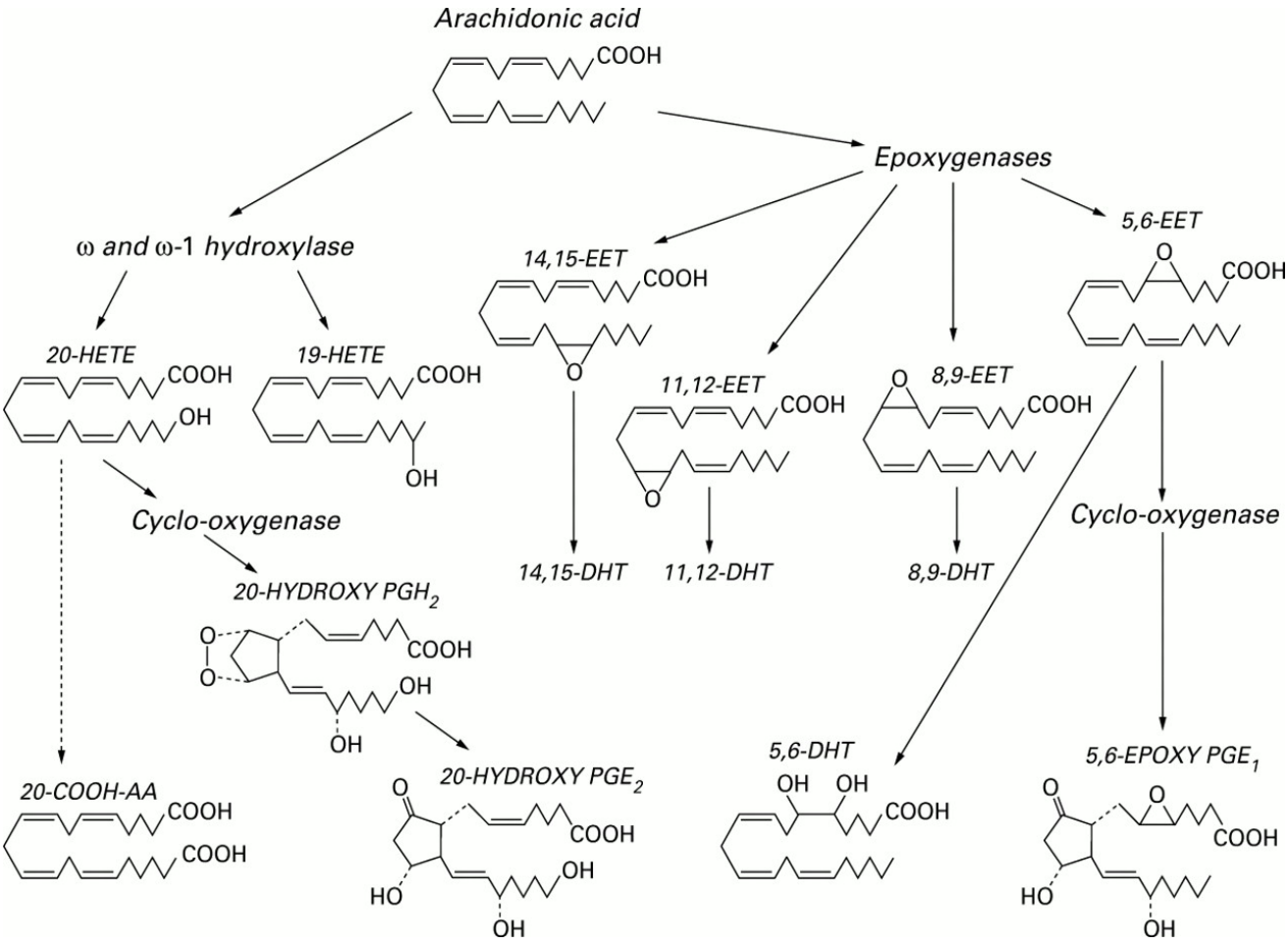


Figure 2: Cytochrome P450 dependent metabolism of arachidonic acid [76].

1.3.3. Effect of inflammation on cytochrome P450 dependent metabolism of arachidonic acid

The regulation of CYP 450 by inflammation can be differentiated into down-regulation or up-regulation. Animal models have been used to investigate how an inflammatory disorder can down-regulate the expression of these enzymes. While these models involve inflammation within the liver (e.g., dextran sulphate) or outside the liver (e.g., irritants or vaccines), the same impact (down-regulation) has been shown [77]. In addition, rat hepatocytes show results that are quite similar to those in cultured human hepatocytes. Both enzyme-selectivity and the nature of inflammatory stimulation are two key factors to determine how CYP expression might be affected differently. Administering endotoxin in rats causes rapid down-regulation in liver CYP 2E1 and 3A4, while the expression of 2C11 is down-regulated after 24-28 hours of injection. The most of published studies indicate that the down-regulation of CYP enzymes is predominant, but up-regulation effects might also occur. After the injection of lipopolysaccharide (LPS), the induction of genes encoding CYP4A1, 4A2 and 4A3 (mRNA) has been noted in the liver and even in the kidneys [78].

The main components involved in the modification of P450 might be cytokines or non-cytokines components. Using preparation of human recombinant interferon (INF) classes showed that INF depressed most CYP enzymes. Down-regulation has been involved in the expression of constitutive enzymes including CYP2C12, 3A4 and 2C11. Inflammation-mediated CYP down-regulation can also resulted from TNF and IL. Interleukins such as IL-1a, IL-1b, IL-6, and recombinant TNF have shown a significant suppression on the expression of CYP1A1, 1A2, 2B1, 2C12 and 3A3 [77]. Level of non-cytokine components could also be elevated in response

to inflammation. For example, many hormones such as glucocorticoids and noradrenaline can be increased as result of inflammation- mediate stress response [79].

The mechanism of inflammation-mediated regulation of CYP could be molecular or non-molecular. Most sites associated with a loss of CYP activities are pre-translational mechanism, leading to suppression in mRNA expression and consequently low enzyme synthesis. In response to IL-6, CYP1A1 in hepatoma cells have been regulated at a transcriptional level through hem oxygenase induction, whereas IL-1 inhibit the transcription of both CYP1A1 and CYP1A2 in isolated hepatocytes. IFN appear to down-regulate CYP1A1 mRNA formation in transfected lymphoblastoid cells and decreases the rate of transcription for CYP1A1 and CYP1A2. Several transcription factors such as natural factor (NF)-kB have been involved in the regulation of gene transcription by cytokine enhancement. [77].

Even though cytokines are the principle components of the alteration of CYP expression during inflammation, other pathways can be linked to such modulation, even with cytokine production. ROS is simultaneously elevated during inflammation, in which hydrogen peroxide, for instance, is responsible for the suppression of mRNA of CYP1A1 and 1A2 in isolated hepatocytes. The level of NO is increased substantially during inflammation by the induction of nitric Oxide synthase. NO can result in the CYP depression of mRNA or enzyme heme protein. This effect has been confirmed by NOS inhibition that prevents loss of CYP mediated by inflammation [77].

1.4. Non-steroidal anti-inflammatory drugs (NSAIDs)

In 1989, acetylsalicylic acid was marketed as the mainstay of NSAID and non-narcotic analgesic drug therapy. For decades, many drugs with aspirin-like action have been developed. This class

of therapy is considered crowded with pharmacological agents that have the same indications and mechanisms of action, but belong to various classes of organic acids [80]. In the United States (US), it was approximated that 100 million prescriptions for NSAIDs were written each year [81]. In addition, it is estimated that annually around seven billion dollars are expended on NSAID worldwide which represents about 2.5 % of total prescription dollars globally [82]. Diclofenac appears to be the most frequently prescribed among NSAIDs [83]. NSAIDs have acidic properties; highly bound to plasma proteins after comparatively high absorption (bioavailability); and are metabolized generally by liver as well as renal enzymes.

1.4.1. Classification of NSAIDs

NSAIDs can be classified based on various characteristics including chemical structure (Table 3), COX selectivity (Table 4) and half-life (Table 5). Many NSAIDs have chiral structures that are available as racemates [16]. It is notable that some chiral NSAIDs such as ibuprofen can be metabolized from R enantiomers into S enantiomers in humans [84]. The degree of selectivity in inhibiting the COX pathway is also another NSAID classification. This class has three main categories: non-selective COX inhibitors, and mild, moderate and high COX-2 selective inhibitors depending on IC_{80} ratio (COX-2 / COX-1) as reported in vitro analysis by Warner et.al [85]. From a pharmacokinetic perspective, NSAIDs have a different rate of absorption which can influence their appropriateness for certain indications, particularly pain. NSAIDs can be also categorized, depending on half-life, into short or long half-life and this determine the dosing schedule for particular indication [16].

1.4.2. Epidemiology of NSAID prescribing

NSAIDs are used by more than 30 million people annually and represent about 60% of US market of analgesic medication. There is a regional variation in the prescription of NSAIDs among countries; diclofenac is most commonly prescribed in the United Kingdom; ibuprofen and naproxen are the most commonly used in the US. Such variations might be explained by which drug was marketed first in a certain region. The sale of NSAIDs increased after the introduction of COX-2 selective inhibitors. However, there was a significant reduction in prescriptions of the new generation after rofecoxib was withdrawn from the market in 2004. On the other hand, the prescription of non-selective NSAIDs, in general, remained relatively stable or increased slightly depending on the geographical region [16]. Even though there is a history of withdrawal of NSAIDs such as bromfenac, carprofen and pirprofen from the market, NSAIDs are still among the most commonly used drugs globally.

Table 3: Chemical classification of NSAIDs [82].

Chemical Group	Example (s)
Salicylates	Acetyl salicylic acid
Propionic acid derivatives	Ibuprofen, / Naproxen
Heteroaryl acetic acid	Diclofenac / Ketorolac
Oxicams	Piroxicam / Meloxicam
Fenamates	Mefenamic acid,
Diaryheterocycles (COXIBs)	Rofecoxib / Celecoxib,

Table 4: Cyclooxygenase selectivity of NSAIDs [85].

COX Selectivity	Instance(s)
Highly (50-fold) COX-2 selective	Rofecoxib
Moderately (5 to 50-fold) COX-2 selective	Celecoxib / Meloxicam
Mild (< 5-fold) COX-2 selective	Diclofenac / Sulindac
Non Selective COX inhibitor	Naproxen / Ibuprofen

Table 5: Classification of NSAIDs based on half-life [82].

Short half-life (<6 h)	Long half-life (>6 h)
Ibuprofen	Meloxicam
Diclofenac	Celecoxib
Ketoprofen	Naproxen

1.4.3. Therapeutic Use of NSAIDs

In spite of the fact that NSAIDs have different chemical structures, these drugs all share the same therapeutic properties (i.e., they reduce fever, cure a headache, and alleviate the swelling, redness and pain of inflammation). Such great therapeutic properties make NSAIDs the first choice in the treatment of pain and discomfort in chronic conditions, including RA [86], osteoarthritis [87], ankylosing spondylitis, gout, gouty arthritis, psoriatic arthritis, systemic lupus erythematosus, rheumatic fever, patent ductus arteriosus and dysmenorrhea [82]. Epidemiological studies have found a link between NSAIDs and the prevention of both colon cancer [88] and Alzheimer's disease [89]. However, more research should be conducted in order to investigate such associations and the mechanisms behind them.

1.4.4. Mechanism of action of NSAIDs

Vane and Piper described the mechanism of action of NSAIDs in 1971. NSAIDs exert their effects through the inhibition of the biosynthesis of PG by blocking the active site of COX enzymes and preventing ArA from the binding to this site (Figure 5). In general, COX-1 and COX-2 catalyze the conversion of ArA into the intermediate metabolite PGH_2, which is the rate-limiting step. The activity of different PG in a tissue depends on the cell type–specific expression of PG isomerase enzyme. Non selective NSAIDs and coxibs act by selectively inhibiting COX-1–dependent and/or COX-2. Hence, PGD2, PGE2, PGF2α, PGI2, and thromboxane A2 are the end products of a PG cascade. It is well-known that COX enzymes exist in two isoform: COX -1 and COX-2, characterized in 1976 and 1991, respectively. COX-1 is expressed in a constitutive manner and plays a role in a variety of physiological functions including the kidneys, gastrointestinal tract, and platelets, whereas COX-2 is expressed through induction by inflammatory mediators and has a part in pain, fever and inflammation. Another variant of COX,

COX-3, has recently emerged, but its role is not fully understood [90, 91].

1.4.5. Adverse Effects of NSAIDs

Many patients tolerate NSAIDs very well for short durations, in therapeutic doses and under the supervision of a physician or pharmacist. However, NSAIDs are linked to a variety of adverse effects including renal, gastrointestinal and CV complications. In addition to the comorbidity risk factor, using NSAIDs for long durations might be associated with an increase in such side effects [82].

1.4.5.1. Renal adverse effects

The majority of PGs is produced in the kidney and has an important role in renal functions. For example, PGE2 exists in nephrons and collecting ducts where its vasodilatory effect perform a role in renal blood flow, glomerular filtration rate, and salt and water excretions through binding to prostaglandin-E (EP) receptor [91]. Such effect of PG also can antagonize the vasoconstriction effect of both norepinephrine and angiotensin II [92]. NSAIDs, which block PG biosynthesis, can cause renal function abnormalities. Such abnormalities are uncommon and are usually temporary and reversible upon discontinuation of NSAIDs. The renal risk increases in the presence of co-existing risk factors including heart failure, diabetes, and renal disorder. The range of renal adverse effects is broad: it ranges from fluid retention and electrolyte disturbance to interstitial nephritis and chronic renal failure. From a clinical perspective, such side effect appears with 1% of patients both those who are over-the-counter users and those who use NSAIDs on a prescription basis [93, 94]. NSAID-associated renal side effects are inclined to be dose-, duration- and exposure-dependent. Harirforoosh et al. reported that there is no association between reported COX-2–COX-1 selectivity and urinary electrolytes excretion as rofecoxib, celecoxib, diclofenac, and flurbiprofen significantly reduced the excretion rate of sodium and

potassium [95]. It appears that both COX isoforms are involved in renal physiology as studies showed that sodium retention is mediated mainly by COX-2 while the reduction of the glomerular filtration rate is mediated mainly by COX-1 [96]. NSAID-induced renal changes can be linked to the CV risk of NSAIDs and can impact the therapeutic level of renal-excreted drugs. Furthermore, it could be also valuable to investigate the effect of NSAIDs on the ArA pathway in kidneys and find other ways to observe renal function to identify negative side effects caused by NSAIDs.

1.4.5.2. Gastrointestinal adverse effects

Both PGE2 and PGI2 are produced by COX-1 in GI mucosa and exert cytoptrotective effects by inhibiting gastric acid secretion and enhancing the release of viscous mucus and blood flow [96, 97]. The gastrointestinal side effects of NSAIDs are noticeable by patients and range from dyspepsia to gastric ulceration and perforation [97]. COX-2 inhibitors were introduced in order to prevent such side effects. However, recent meta-analysis studies show that all NSAID regimens, including coxibs, increase upper gastrointestinal complications [98]. To illustrate, celecoxib appears to cause less GI complication than traditional NSAIDs [99], but the extrapolation of such observation to whole COXIB is biased from some authors. For example, revision of unpublished studies failed to confirm the safety of rofecoxib over non-selective NSAIDs. This could be considered as misleading from pharmaceutical industry fields [82].

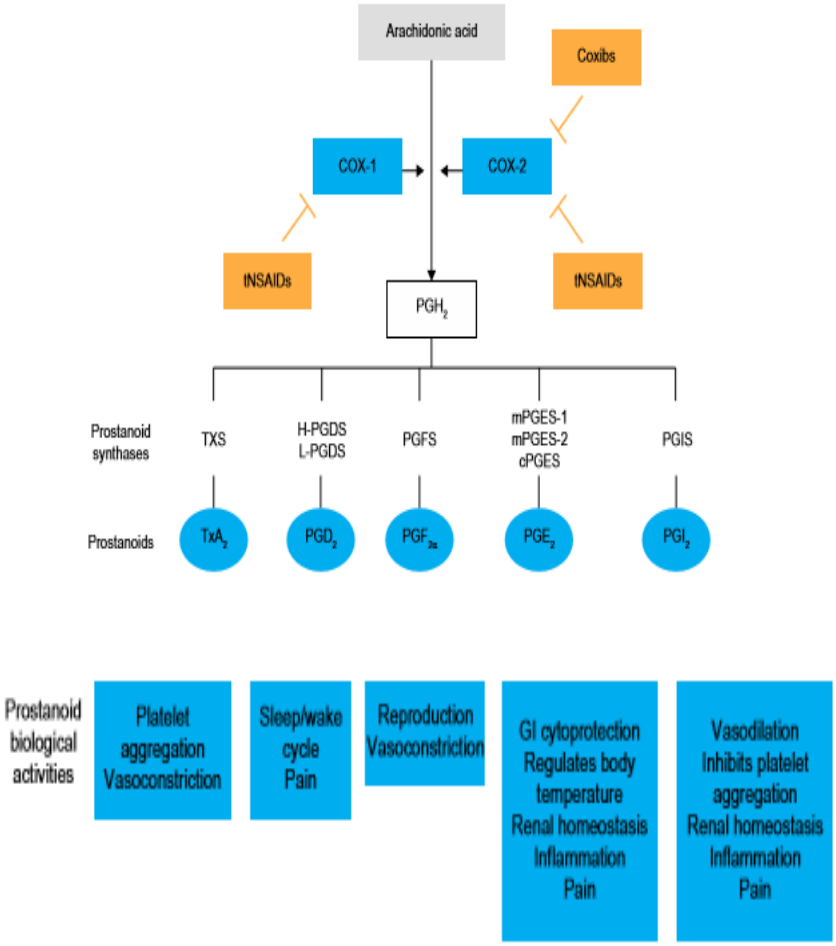


Figure 3: Mechanism of action of NSAIDs[100].

A number of risk factors, such as old age, a history of ulcers, helicobacter pylori infection and the concomitant use of anticoagulants, can worsen NSAID-associated gastrointestinal injuries [16]. On the other hand, persistent exposure of the gastrointestinal tract to circulating NSAIDs should be also considered in particular long half-life drugs [101]. It appears that high doses of NSAIDs are linked to an approximately three-fold increase in gastrointestinal injuries, in in comparison with low doses [100].

Lower gastrointestinal side effects of NSAIDs are not readily visible, but NSAIDs might cause the distal gastrointestinal side effect with varying intensity. It is essential to note that

NSAID-associated lower gastrointestinal injuries could be related to a slow release formulation. Enteric-coated NSAIDs, for instance, are designed to release the drug in the intestine in order to reduce gastric irritation, but the distal part of the gastrointestinal tract is still exposed to the same irritants in a slow-release-manner, especially if the NSAIDs are highly potent. It is important to emphasize that there is not a strong rationale for using such formulations and cause a shift in side effects from the visible segments (stomach) to less detectable parts of the gastrointestinal tract [82].

1.4.5.3. Cardiovascular adverse effects

CV risks of NSAIDs were not extensively appreciated until selective COX-2 inhibitors were introduced into clinical practice. On September 30, 2004, Merk and Co. instituted an urgent and immediate global withdrawal of rofecoxib (Vioxx®), a potent selective COX-2 inhibitor that had exhibited a significantly increased risk of myocardial infarction and stroke [102]. The Vioxx Gastrointestinal Outcomes Research (VIGOR) was a study that looked at 8076 RA patients, some of whom took rofecoxib 50 mg/day and others who took naproxen 1000 mg/day. None took aspirin. The VIGOR study showed a five-fold increase in the incidence of CV for those who took rofecoxib [103]. In addition, the Adenomatous Polyp Prevention on Vioxx® (APPROVe) revealed an increase in the relative risk of thrombotic events in patients taking 25 mg /day of rofecoxib compared with a placebo group [104]. As a result of these findings, rofecoxib was removed from the market first, followed by valdecoxib in 2005. It is well-known that there is an equilibrium between several anti- and pro-thrombotic factors including thromboxane (COX-1 isoform product) and PGI2 (COX-2 mediated product), which demonstrate pro- and antithrombotic actions, respectively. It has been suggested that the inhibition of COX-2 might disturb the well-balanced state between the COX isoform systems, which could partially explain

such side effects. However, both COX-2 inhibitors and traditional NSAIDs (non-selective COX) inhibit COX-2 at the therapeutic dosage range and are potentially toxic for CV system [16].

The Celecoxib Long Term Arthritis Safety Study (CLASS) compared 8059 patients with osteoarthritis and RA and concluded that there are no significant differences in CV disease among the observed NSAIDs; celecoxib (400 mg, twice daily), diclofenac 75 mg (twice daily) and ibuprofen 800 mg (three times a day) [105]. Multinational Etoricoxib and Diclofenac Arthritis Long-term (MEDAL) study compared etoricoxib and diclofenac in randomized clinical trials of RA patients. This study also showed no difference between the examined NSAIDs [106]. Additionally, Nissen et al. have compared celecoxib (daily dose = 209±37 mg) with naproxen (daily dose = 852±103 mg), and ibuprofen (daily dose = 2045±246 mg) and concluded that celecoxib is noninferior to naproxen and ibuprofen regarding CV safety [107].

Most of the focus was primarily on CV risk of COX-2 inhibitors. However, there is an obvious concern related to the use of nonselective NSAIDs. Kearne et al. have reported that in a meta-analysis of randomized trials, high dose regimens of both ibuprofen and diclofenac are associated with a moderate increase in the risk of vascular events (similar to COX-2 inhibitors) compared to a placebo [108]. McGettigan et al. have investigated cardiovascular events with individual NSAIDs derived from meta-analyses of randomized trials and controlled observational studies in 15 countries. The outcomes of this study show that diclofenac, rofecoxib and etoricoxib are consistently linked with the highest CV events, while naproxen has a lower risk. Both celecoxib and ibuprofen are associated with elevated relative risk values when used in high doses but not in the lower doses [83].

The safety profile of NSAIDs remains a clinical issue, in which a high risk of mortality for NSAIDs has been reported. A Danish clinical trial has used death as an end point and showed

a link between the dose and mortality because of MI and heart failure. The hazard ratios were detected to be 1.70 (1.58-1.82) for rofecoxib, 1.75 (1.63-1.88) for celecoxib, 1.31 (1.25-1.37) for ibuprofen and 2.08 (1.95-2.21) for diclofenac [109].

Myocardial infarction (MI) is the most studied among CV conditions although the mechanism of this adverse effect is unclear. Grimaldi-Bensouda et al. have reported that NSAIDs are associated mainly with non-ST elevation [110]. In observational study of OA patients taking NSAIDs, Lonas et al. found that a high risk of CV events was obvious in patients with previous MI who used NSAIDs both short and long term [111]. In Canada, a cohort study was conducted on subjects aged 40-85 and found that there was small elevation of acute MI risk for naproxen, ibuprofen, diclofenac, indomethcin, celecoxib and rofecoxib users. However, higher CV risks were linked with higher doses of those drugs [112]. It appears that patients with previous MIs are at more risk to have another attack when they take high doses of some NSAIDs. .

The risk of thromboembolic events is correlated with NSAIDs regardless of the selectivity of COX-2. In a retrospective study, Layton et al. found that there is a small elevation in cerebrovascular thromboembolic events for celecoxib users as compared to meloxicam users [113]. Kearney et al. reported that serious vascular events increased by about 42% in COXIB users compared to study subjects given a placebo [108]. The reason is related to a disruption in the prostacyclin pathway. The association between NSAIDs and cardiogenic stroke is not yet known and still controversial. More studies are needed to build a reliable conclusion.

In general, it is now known that NSAIDs are heterogeneous in their potency to increase CV risks. There is great need for long-term studies to evaluate CV events, particularly for non-

selective NSAIDs. Such studies may help us to better understand the true benefits and risks of individual NSAIDs.

1.4.5.3.1. Mechanism of cardiovascular risks of NSAIDs

There are some suggested mechanisms that attempt to govern the cardiotoxicity profile of NSAIDs. Understanding all the possible mechanisms that cause CV events is critical and required for best evaluating the pros and cons of the prescribed NSAIDs, as well as for developing safer derivatives.

COX inhibition might alter the balanced state between COX-1 and COX-2. COX-2 selectivity, in addition, is another proposed mechanism as the degree of COX-2 inhibition increases, more CV events can be detected. However, both COX-2 inhibitors and traditional NSAIDs can cause CV side effects. The concept of prostacyclin–thromboxane imbalance alone is not satisfactory to define the risk of NSAIDs-related complication. In addition, the notion of COX2 selectivity as a cause of cardiovascular complication can be excluded.

Physicochemical properties such as solubility, the partition coefficient and the ionization constant (pka) may have a role in the distribution of NSAIDs in body tissues. Harirforoosh et al. have shown that the relatively higher distribution of NSAIDs into the kidneys suggests that these drugs interfere more with renal functions [114]. The preference of certain NSAIDs to accumulate in kidney could be linked to their physicochemical properties. Moreover, Davies and Jamali have proposed that physicochemical parameters of rofecoxib might be responsible for the CV events caused by this drug [102].

The role of transporters in regulating the safety and efficacy of NSAIDs has not been highly investigated. Reid and Wielinga reported that in addition to inhibiting PG synthesis, NSAIDs might also act by inhibiting PG release by blocking the multidrug resistance protein

(MRP4) [115]. Mandery et al. concluded that NSAIDs differ in their impact on the function of PG transporters OATP2A1 [116]. Hence, the impact of NSAIDs on the transporter system or vice versa could have an important role in determining a toxicity profile for NSAIDs.

Cell integrity could be affected by many factors including oxidative modification. The extended use of rofecoxib appears to be associated with the production of oxidative stress in cardiomyocytes that might contribute to plaque instability and thrombus formation [117]. Such mechanism could be drug specific or could be produced by more than a therapeutic agent in this class.

Interestingly, metabolomics is a promising tool that has many applications, including diagnosis, biomarker detection, toxicity determination and research development. Using this approach suggests that rofecoxib acts to increase of ArA metabolites (20-HETE) in murine plasma and this, subsequently, might enhance atherothrombotic vascular events [118].

1.4.5.3.2. Effect of dose and duration of treatment on cardiovascular adverse effects

According to clinical evidence, the comprehensive recognition of the difference between individual NSAIDs is an alternative approach to explaining the safety profile of NSAIDs. One of strongest concepts that need to be considered is the relationship between the dose, the duration of treatment, and NSAID-associated CV risks.

Many of the available reports about CV side effects of NSAIDs suffer from generalization, in which the observed data from certain drugs is extrapolated to all NSAIDs. This also applies to COX-2 inhibitors as many are assuming that the toxicities are a class issue. Furthermore, some reports pool all NSAIDs together, which masks the risk assessment associated with individual NSAIDs [119, 120]. This while others ignore the used dose or formulation as data generated from high doses not essentially reflect the low therapeutic doses

and data obtained from sustained release formulations do not certainly show the comparable side effect profile of immedaite release folrmulation [121]. Additionally, inflammatory conditions are associated with increasing both the morbidity and mortality rates that are mainly attributed to CV complications. However, many studies have ignored the effect of inflammatory conditions on the CV system [122]. As result, the presence of any underlying conditions should be strongly considered, especially in high-risk patients.

On the other hand, some studies consider only the high doses, while they ignore the effect of low doses. Others may only compare one drug to another and not to a placebo. Ibuprofen, for example, at a high dose ((≥1,200 mg/day) is linked with CV events, whereas naproxen tends to be neutral at high doses. Similarly, high doses of celecoxib, rofecoxib and diclofenac have also been connected to a high risk of death or MI [82]. Olsen et al. have found that diclofenac and rofecoxib have highest CV death in dose-dependent fashion with hazard ratio (HR) 1.96 [1.79–2.15] and 1.66 [1.44–1.91], respectively, while naproxen has been correlated with the lowest risk with (HR) of 1.27 [1.01–1.59][123]. This indicates that it is crucial to take into account the importance of dosage when conducting clinical trials or animal research. Hence, dose-dependency as a pharmacological factor should be strongly considered, especially since patients may increase NSAID doses regardless of their physician's or pharmacist's advice.

Duration of treatment is another controversial topic as there is uncertain evidence as to whether the side effects of NSAIDs appear with a short or long duration of treatment. Chao et al. showed that NSAID users had increased risks of atrial fibrillation, particularly those who had recently taken these drugs (new users) with an odd ratio of 1.651 [124]. On the other hand, Liu et al. reported that there is no correlation between preoperative treatment with NSAIDs and increased risk of MI after knee replacement [125].

In general, little is known about the association between NSAID treatment duration and risk of CV disease as it might be neglected in some studies. Schjerning Olsen *et al*, for example, who divided the treatment periods intervals into 0, 7, 14, 30, and 90 days reported that most studied NSAIDs are associated with increased CV risk within short –term treatment and concluded that neither short- nor long-term treatment with NSAIDs is advised in recurrent MI population.

Overall, it is unknown whether such risks persist during or even after the course of treatment or whether a case of tolerance can develop with some NSAIDs. It is worthy to consider that both short and long-term adverse effects of NSAIDs might occur through several mechanisms that need further investigations.

1.4.5.3.3. Biomarkers for NSAID-induced cardiotoxicity

RA is tremendously heterogeneous disorder including a many mediators with extremely complicated network of pathophysiological pathways. Biomarkers are key biological indicators involved in measuring disease activity and severity. From a clinical practice perspective, biomarkers are used for many purposes such as risk stratification, disease prevention, screening, diagnosis, prognosis, therapeutic monitoring, and post-treatment surveillance.

Many biomarkers are used currently in clinical and health fields, including blood pressure for hypertension, blood sugar for diabetics, and arrhythmia suppression exercise tolerance in congestive heart failure. The most reliable biomarkers are characterized by specificity, sensitivity, consistency and cost-effectiveness. However, in many cases, using combinations of biomarkers from different perspectives is important to determine the disease state and the response to certain pharmacological classes.

The ArA pathway is an important system in regulating body functions and it involved in CV system homeostasis. Cytochrome P450 metabolizes ArA to HETEs and EETs. Recent studies indicate that inflammatory conditions disrupt the balance between cardioprotective (EETs) and cardiotoxic (HETEs) metabolites [74]. Ample recent preclinical data suggest that modulation of eicosanoids, the cytochrome P450 (CYP) metabolites of arachidonic acid (ArA), may be a potential clinical therapeutic strategy and achievable biomarker for the management of different pathological disorders, in particular CV diseases [54].

Our lab recently suggested plasma ArA metabolites as surrogate biomarkers for NSAID-induced cardiotoxicity [126].

1.5. Selection of NSAIDs for this study

Our lab has reported the relationship between NSAIDs, their kidney-to-plasma concentration and their effect on kidney functions. In addition, we have studied several NSAIDs at PK equivalent doses, their heart to plasma concentration and, subsequently, their influence on ArA metabolites. Our observations suggest that the tissue distribution of NSAIDs correlates with their potential CV and renal complications. We would like to broaden the horizons regarding the cardiotoxicity profile of NSAIDs from a dose-dependency perspective. We limited ourselves to diclofenac, a well-known cardiotoxic drug, because diclofenac's cardiotoxic effect appears to be as high as that of rofecoxib, even though diclofenac is much less of a COX-2 selective than rofecoxib [85, 108]. The HR of diclofenac is 1.96 compared to 1.66 for rofecoxib [108]. The additional concern about the risk associated with diclofenac is in its availability in low-dose forms as a non-prescription medication. Furthermore, diclofenac is the most prevalent NSAID: its market alone is equivalent to that of ibuprofen, naproxen and mefenamic acid together, based on an examination of essential medicine lists from that examination of the sales in 15 countries [83].

1.6. Diclofenac

1.6.1. Chemistry

Diclofenac is a phenyl acetic acid derivative and is known chemically as [2-(2,6-dichloroanilino) phenyl] acetic acid (Figure 6). Diclofenac has a molecular formula of $C_{14}H_{11}Cl_2NO_2$ and molecular weight of 296.1 g/ mol [127].

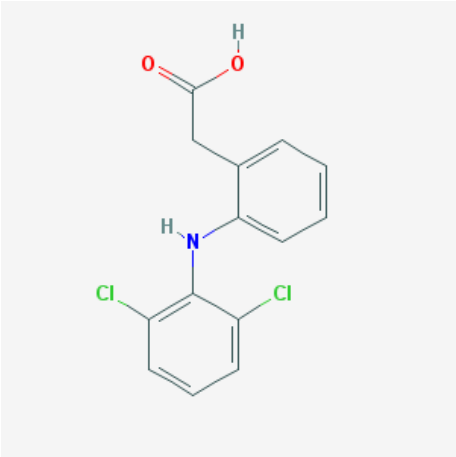


Figure 4: Chemical structure of diclofenac.

1.6.2. Physicochemical properties

Diclofenac is white to light beige and exists in crystalline form. It is freely soluble in alcohol and methanol, sparingly soluble in water and acetone and practically insoluble in sodium hydroxide (1M). For storage, diclofenac should be protected from light in an airtight container. The dissociation constant (pKa) of diclofenac is 4.15 with a log P of 4.51 [127, 128].

1.6.3. Pharmacokinetic properties

1.6.3.1. Absorption

Diclofenac is usually given orally, and it is also administered in topical, intravenous, intramuscular, intraocular, and rectal form. Diclofenac is available in many dosage formulations such as tablets, capsules, suppositories, ampoules, gels and optic drops. Moreover, Diclofenac is accessible in sodium or potassium oral forms as enteric-coated and slow-release tablets. Immediate-release diclofenac potassium sugar-coated is intended to release the drug to be rapidly absorbed in the stomach, while the slow release is designed to release diclofenac over a period of time. The latter formulation prevents the drug from dissolving in the stomach at a lower pH and permits a quick release in the intestine at a higher pH.

The systemic absorption of diclofenac is linear with the dose from of 25 to 150mg. The absorption behavior of multiple doses is similar to that single dose. The absolute bioavailability of diclofenac is still controversial as it is ranges from 60% to 90% ± 11.6%. Such a conflict might be attributed to the fact that diclofenac is susceptible to first-pass metabolism. The absorption process generally is not affected by age or the disease state in the gastrointestinal tract. However, in elderly patients, a reduction in the hepatic metabolism might decrease diclofenac's biotransformation, leading to an increases area under curve (AUC) and peak plasma drug concentration (Cmax).

Diclofenac tends to have a fast absorption in capsule, tablet and suspension forms. After the oral administration of the solution, Cmax can be obtained within 10 to 40 minutes. However, the controlled release has a lower Cmax and delay in the time that it takes to reach peak concentration (tmax), but this dosage form has relatively equivalent bioavailability to enteric-

coated formulations. In addition, the AUC of this drug is not significantly affected by food intake, but there is a slow absorption (delay tmax) in the presence of food [129, 130].

1.6.3.2. Distribution

The apparent volume of distribution ranges from 0.1 to 0.2 L / kg. Such values indicate that diclofenac has low tissue bindings in comparison with plasma protein binding and it is mainly limited to the central compartment. This drug is extensively bound to plasma protein (>99.7%) as it has high affinity at therapeutic rang to albumin.

The initial site of action of diclofenac is proposed to be the synovial fluid, in which the drug is detected in considerable concentrations. Diclofenac's Cmax in synovial fluid is between approximately two to four hours after the appearance of the plasma Cmax in addition to having longer elimination half-life in this fluid. On the other hand, limited data is available in regard to distribution of such drug to other tissues as low volume of distribution give an explanation why the peripheral presence of diclofenac is small [129].

1.6.3.3. Metabolism

The main pathway of metabolism of diclofenac are acyl glucuronidation and phenyl hydroxylation, with the former reaction catalyzed primarily by uridine 5'-diphosphoglucuronosyl transferase, and the latter catalyzed by cytochrome P450 [131] .

The Phase I metabolites of diclofenac are 4′-hydroxydiclofenac, 5-hydroxydiclofenac, 3′-hydroxydiclofenac, and 4′, 5-dihydroxydiclofenac. The 4'-hydroxy derivative is catalyzed by CYP2C9, while the 5-hydroxy derivative is a product of CYP3A4. In in vitro studies, CYP2C8 might be responsible for other hydroxylation reactions of diclofenac. The 4'-hydroxy metabolite accounts for 30% of diclofenac dose in urine. These metabolites lack the anti-inflammatory or analgesic effect in animal models. Phase II metabolism could be a conjugated form of diclofenac

itself or its metabolites, representing 12.6 % and 22 % of dose excreted in urine, respectively [129].

Reactive metabolites, p-benzoquinone imines, o-imine methide and arene-oxide, are thought to play a role in the idiosyncratic adverse effect of diclofenac. The inactivation of these intermediate reactive depends on the presence of glutathione S-transferase [132]. Furthermore, genetic polymorphism in cytochrome P450 could be responsible for interindividual variability in drug metabolism or in toxicity profile. Such area of research in needed for more evaluation of drug efficiency and their suspected adverse effect [133].

1.6.3.4. Elimination

Diclofenac occurs in urine, bile and faeces. Approximately 1 % of diclofenac is eliminated in unchanged form, while about 60% and 35% of conjugated metabolites are excreted mainly in urine and bile, respectively. The terminal half-life is reported to be between 1 to 2 hours [127].

1.6.4. Pharmacodynamics

As with other NSAIDs, diclofenac has analgesic, antipyretic and anti-inflammatory properties and is indicated mainly for osteoarthritis; RA; ankylosing spondylitis; soft-tissue disorders such as sprains and strains; and other painful conditions such as renal colic, acute gout, dysmenorrhea, migraine. Diclofenac inhibits the synthesis of pro-inflammatory and nociceptive prostaglandins in blood and synovial tissue. This therapeutic agent believed to be a COX-2 inhibitor even though it has dual suppression of COX-1 and COX-2 activity [130]. However, diclofenac might have other mechanisms of action independent of COX inhibition that explain its effects or side effect. The effect of diclofenac on the ion channel has been recently investigated. This effect could be activation, inhibition or change in ion channel expression, including voltage-gated sodium, calcium or potassium channels and ligand-gated potassium channels. Such effect could

be related to interference with second messenger system , direct interaction with channel or agonist /antagonist interaction with other channel modulator [134].

1.6.5. Precautions and interactions

Diclofenac should be used with caution in patients with CV risks such as hypertension, smoking, diabetes mellitus, and hyperlipidemia. It should also be used with caution in patients with moderate or severe renal impairment, hypovolemia, or dehydration and hepatic impairment. It is clear that this drug should also be avoided by patients with moderate to severe heart failure, ischemic heart disease, peripheral arterial disease, or cerebrovascular disease [127].

As for general interactions with drugs, cholestyramine tends to decrease the bioavailability of diclofenac when both are taken together. As with other NSAIDs, diclofenac increases the plasma level of lithium and methotrexate. Concomitant use of diclofenac with tacrolimus, diuretics and cyclosporine can lead to kidney toxicity [127].

1.6.6. Adverse effect of diclofenac

Even though diclofenac is generally well tolerated, several safety considerations should be measured against the clinical benefits associated with this drug. The US Food and Drug Administration’s label requirements focus on the increased risk of serious gastrointestinal adverse events and serious cardiovascular thrombotic events, MI, and stroke. Furthermore, renal failure and hepatotoxicity are potential complications of treatment with diclofenac and, therefore, should be included as an integral part of toxicity when evaluating safety consideration [130].

The incidence of side effect is reported to be around 12 %. The most commonly reported adverse effect in generally reported cases is gastrointestinal (7.6%), followed by central nervous

system-associated side effects (0.7%) and hypersensitivity (0.4%). Diclofenac is also linked to a syndrome of inappropriate antidiuretic hormone secretion [127].

GI toxicity of diclofenac is one of the most critical adverse effects and ranges from such issues as bleeding, ulceration and perforation in the upper part of the gut to potentially life-threatening effects in the lower part of the GI tract [93, 135]. Because it is eliminated rapidly through the hepatic metabolism, resulting in a short half-life, diclofenac was the best candidate for sustained-release preparations. As result, the lower GI might have a greater residence time of exposure to sustained formulations [136]. Furthermore, this might lead to shifting GI toxicity to lower GI tract [82, 137, 138].

Health Canada stated that the recommended maximum daily dose of diclofenac is 100 mg/day as it is linked with a 60% increase in critical CV side effects such as MI and stroke [139, 140]. The dose dependency was analyzed in some retrospective cohort and observational studies. Van Staa *et al.* reported that increasing the daily dose was associated with high risk of MI, the relative risk (RR) was 1.13 with <150 mg /day and 2.03 with >300 mg /day compared with control patients [141]. Gislason et al. showed that high dosages of diclofenac increase mortality in patients with previous MI, with an HR of 0.89 for death and 1.27 for Re-MI at ≤100mg/day, whereas HR of 4.44 for death and 1.89 for Re-MI at > 100 mg/day [142]. Diclofenac's CV profile appears to be similar to that of rofecoxib. As a result, national health agencies and regulatory authorities have been required to remove diclofenac from essential drug lists as it is removed from the list in World Health Organization (WHO).

Overall, diclofenac has been associated with increased rates of CV risks among patients with RA as well as among healthy populations. Diclofenac's side effects could be based on the drug's specific pharmacology. More seriously, the time and dose of diclofenac must be

controlled by physicians and pharmacists as in many countries diclofenac is available as an over-the-counter medication. It is compulsory to raise the awareness of diclofenac's CV risk and toxicity profile.

1.7. Thesis rationale, hypotheses, and objectives

1.7.1. Rationale

Rheumatoid arthritis (RA) is one of most common auto-immune disorders that mediates systematic inflammatory joint diseases [122]. Non-steroidal anti-inflammatory drugs (NSAIDs) are used to treat the pain and inflammation associated with arthritis. In spite of NSAIDs' beneficial effects in RA treatment, this class of drugs is likely to be associated with many adverse effects, including renal/cardiovascular (CV) events. Much awareness has been raised regarding the CV safety of NSAIDs since rofecoxib (a highly selective COX-2 inhibitor) was withdrawn from the market in 2004 [102].

Recent studies have revealed that more than one mechanism might be associated with CV risks of NSAIDs; they include the cardiorenal mechanism, prostacyclin–thromboxane imbalance and the extent of inhibition of COX-2 [143]. However, a meta-analysis of randomized clinical trials (RCTs) is to some extent conclusive as CV risks are not only associated with selective COX-2 inhibitor but also linked with non-selective NSAID [144, 145]. Additionally, some NSAIDs seem to be more toxic than others, so even ranking this class based on COX-2 selectivity was pointless. Therefore, the notion of COX2 selectivity as a cause of CV complications was excluded.

Many of the available reports about NSAIDs' CV side effects are suffering from heterogeneity not only of the involved patients but also due to their methodology. Other studies

may ignore the importance of the pathophysiological conditions. To illustrate, inflammatory conditions are associated with increasing both the morbidity and mortality that are mainly attributed to CV complications [122]. As result, the presence of any underlying conditions should be strongly considered, especially in high-risk patients. In addition, many studies have only compared one drug to another and not to the placebo, or else they have pooled data, assuming that the toxicities are a class issue [119, 120].

Furthermore, some of these studies are lacking any information about doses. The data attained from using high doses in a study does not essentially represent the effect of low doses. In fact, there is a possibility that low doses of some NSAIDs, such as ibuprofen and naproxen, provide cardioprotection [82]. It would be valuable to consider whether the adverse effects of NSAIDs are dose- dependent. The pharmacological factor should be largely considered and investigated since patients may increase NSAID doses regardless of their physician's or pharmacist's advice and in presence of a limited animal studies that have done to be consistent with human clinical trials.

Based on our previous observation in experimental animals, CV related adverse effects of both rofecoxib and flurbiprofen could be attributed to their high heart tissue exposure compared to meloxicam [126]. Moreover, studies have shown that a higher kidney-to-plasma ratio of celecoxib might have a role in producing renal side effects [114]. Hence, the diversity in NSAIDs' pharmacokinetics (PK) and the subsequent pharmacological outcomes might be responsible in part for CV adverse effects.

Eicosanoids, CYP 450 metabolites of arachidonic acid (ArA), would involve in the regulation of cardiac, pulmonary, renal and vascular function [54]. These metabolites are one of the fundamental mediators of various biological processes. CYP epoxygenases and hydroxylases

are the two main balancing arms of the CYP-ArA pathway, producing the cardioprotective component (EETs) and cardiotoxic metabolites (20-HETE). Inflammation modulates the CYP-ArA metabolites and alters their production and metabolism [79].

For this study, a widely used animal model of arthritis was chosen to evaluate the dose-dependence of the CV effect of NSAIDs. This will assist both patients and health care providers who are in dilemma of whether all NSAID are toxic. As the model drug, we used diclofenac because of its known high CV toxicity. Various tissue concentrations of P450-mediated metabolites of ArA would be measured as markers of CV risk.

1.7.2. Thesis Hypothesis

A CV risk of NSAIDs is dose-dependent as the avoidance of such adverse effect can be prevented by using a sufficient and a lowest effective dose of NSAIDs.

1.7.3. Thesis Objectives

- A dose escalation study to determine the therapeutic dosage range of diclofenac using an experimental animal model.
- The effect of diclofenac treatment on cytochrome P450 metabolism of ArA in plasma, kidney, and heart in rat with adjuvant arthritis.
- The tissue accumulation of diclofenac following therapeutic doses.

Chapter 2

2. Materials and Methods

2.1. Chemicals and reagents

Diclofenac sodium salt was purchased from Alfa Aesar (Tewksbury, MA, USA). Methylcellulose was obtained from BDH Chemicals (Edmonton, Alberta, Canada).Glacial acetic acid, phosphoric acid and HPLC grade water used to prepare different methanol–water solutions were acquired from Caledon Laboratories LTD. (Georgetown, ON, Canada).

HPLC grade acetonitrile, acetone, hexane, methanol, anhydrous acetonitrile, diethyleehter, N,N-di-iospropylethylamine, formic acid(96%), 16-hydroxydecanoic acid, butylhydroxytoluene, potassium fluoride, Mefenamic acid, indomethacin, Aspergillus nitrate reductase, flavin adenine dinucleotide (FAD), β-nicotinamide adenine dinucleotide phosphate (NADP), L-lactic dehydrogenase, pyruvic reagent, and a grisess reagent were obtained from Sigma–Aldrich (Oakville, ON, Canada). A solid phase extraction (SPE) cartridge of Oasis HLB 1 ml (30 mg) was purchased from Waters Corporation (Milford, MA, USA). Standards of ArA metabolites were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). The metabolites consisted of analogs of DHTs, 20-HETEs, and EETs. 2-(2, 3-naphthalimino) ethyl-trifluoromethanesulphonate, a fluorescent label (NE-TFM) was acquired from Molecular Probes (Eugene, OR, USA).

2.2. Methods

2.2.1. Dose calculation

Based on literature studies, we searched first for the area under the plasma concentration-time

curve (AUC) for the rheumatoid arthritis (RA)-recommended dose of diclofenac (100 mg /day) in humans [140]. Then we obtained a weight-normalized rat dose and its relevant AUC. We worked under the assumption that both the absorption process and protein binding would be comparable in rats and humans, and that the AUC was a reliable indicator of the drug's systemic exposure. Based on this, we calculated the proportion of the pharmacokinetic (PK) equivalent rat dose to be 5 mg/kg/day. This would yield an AUC of a rat that would be equivalent to that of an AUC in a human [146]. As a result, the doses that we used were diclofenac 2.5 mg/kg, 5 mg/kg, 10 mg/kg, and 15 mg/kg per day.

2.2.2. Animals

The study protocol, adjuvant arthritis (AA), was approved by the Health Sciences Animal Care and Use Committee at the University of Alberta, Edmonton, Canada. Healthy adult male Sprague-Dawley rats, weighing 230–250 g, were obtained from Health Sciences Laboratory Animal Services. Animals were housed in hygienic conditions under standard temperature and ventilation, and they had free access to drinking water and food (rat chow) with 12 hours light and dark cycle. For acclimatization purpose, rats were permitted for adequate time before starting the experiments.

2.2.3 Diclofenac dose / effect correlation study

We divided the rats (n = 4-5/ group) in a random manner into groups of healthy control (CONT-0), inflamed placebo (INF-0) and inflamed who would receive the respective dose of diclofenac (INF-2.5, INF-5, INF-10, and INF-15). Rats in the control group were anaesthetized with isoflurane/oxygen and injected intra-muscularly at the tail base with a pyrogen-free sterile solution of normal saline, while the rats in the inflamed groups were injected with 0.2 mL of the

adjuvant containing 50 mg/mL of *Mycobacterium butyricum* suspended in squalene (Difco Laboratories, Detroit, MI, USA).

2.2.4. Assessment of experimental adjuvant arthritis

After the arthritis induction, the daily change in the body weight was recorded using a regular animal balance, and the changes in the paw and joint diameters were measured daily by using a caliper with 25 µm sensitivity (Mitutoyo Canada Inc., Toronto, ON) [37]. Arthritis index (AI) as macroscopic scoring system was used to monitor the adjuvant arthritis (AA) progression as described in published data [37]. To illustrate, for each forepaw on a 0-3 scale, where is 0 = no sign; 1= involvement of single joint; 2= involvement of > 1 joint and/or wrist; and 3 = involvement of wrist and joints with moderate-to-severe swelling. Each hind paw involved in swelling was assessed on a 0-4 scale, where is 0 = no sign; 1= involvement of single joint; 2= involvement of >1 joint and/or ankle; 3= involvement of several joints and ankle with moderate swelling; or 4=involvement of several joints and ankle with severe swelling. By adding the all scores from both hind paws and both forepaws, the AI was calculated with a maximum score of 14 (Table 6). Serum nitrates and nitrites were quantified in plasma using Griess reagent as biochemical assessments of arthritis according to a published method [147].

2.2.5. Treatment regimens

When AI has score of ≥ 5, it would be considered as significant emergence of the disease and the signs of inflammation were visible. The study was blinded and the identity of the rats concealed until the completion of the analysis. Rats in the INF-2.5, INF-5, INF-10, and INF-15 groups received a dose of 2.5, 5, 10, 15 mg/kg of diclofenac suspended in methylcellulose, respectively, through an oral gastric gavage once daily for seven days. The rats in the inflamed placebo (INF-0)

and healthy control (CONT-0) groups received daily doses of blank methylcellulose (Figure 7). The daily doses for all rats were adjusted according to body weight. After identifying the therapeutic dosage range of diclofenac, we determined the two main doses, the PK equivalent dose (5 mg/kg) and double PK equivalent dose (15 mg/kg), to investigate our hypothesis.

2.2.6. Sample collection

Using 1.5–2.0 L/min oxygen and 0.25%–1.50% isoflurane [148], rats were subjected to euthanization process after 8 hours of last dose. Both the heart and kidneys were removed by surgically and divided into portions, while the blood was collected with an 18-gauge needle pushed in the ventricle using cardiac puncture technique.

For each analytical procedure, the samples were processed differently as following:-

A) For nitrate and nitrite measurement, a blood was collected in heparinized tube ((BD Diagnostics, NJ, USA), gently mixed and then centrifuged for 10 minutes at 250 g at 4° C. The plasma was separated in an eppendorf tube, put directly into liquid nitrogen to be frozen and later stored at -80 ° C until analyzed by simple Griess reaction.

B) For the ArA metabolites measurement, 200 µL of normal saline, including 10 uM of indomethacin and 0.113 mM of butylated hydroxytoluene (BHT) in order to prevent enzymatic and chemical degradation of fatty acids, respectively, was added to each 1 ml of blood. The blood sample was gently mixed and subjected to centrifugation at 10,000 × g for 10 minutes at 0◦. Then the plasma was separated in Eppendorf tube. For heart and kidney, they were washed with normal saline containing indomethacin and BHT and snap frozen together with plasma into liquid nitrogen and stored at -80 ° C until they could be analyzed later.

Table 6: Assessment of adjuvant arthritis using arthritis index system [37].

Fore Paw	Hind Paw
0 = no sign	0 = no sign
1 = involvement of single joint	1 = involvement of single joint
2 = involvement of > 1 joint and/or wrist	2 = involvement of >1 joint and/or ankle
3 = involvement of wrist and joints with moderate-to-severe swelling	3 = involvement of several joints and ankle with moderate swelling
	4 =involvement of several joints and ankle with severe swelling
Sum ≤ 6	Sum ≤ 8
Total ≤ 14	

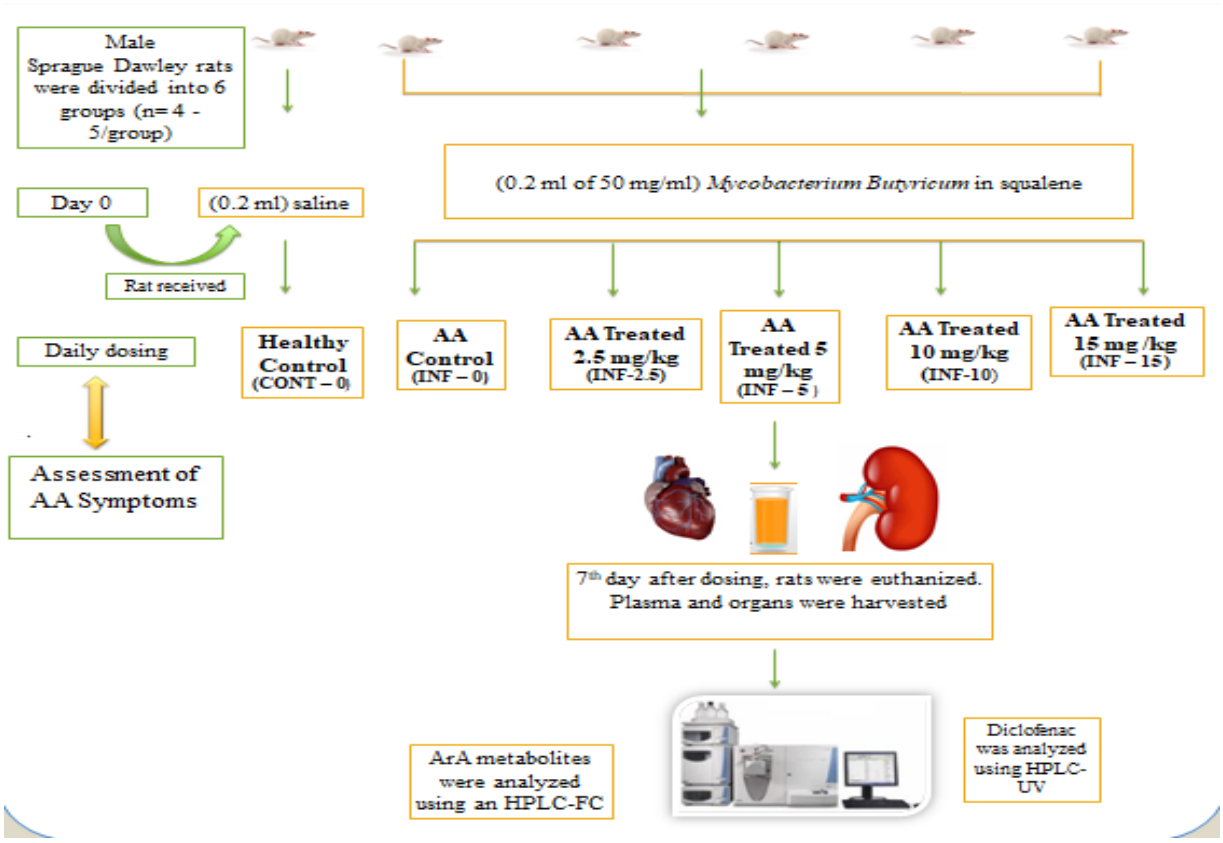


Figure 5: Flow chart of experimental design and methods.

C) For tissue distribution purpose, a blood was collected in heparinized tube, gently mixed, centrifuged for 10 minutes at 250 g at 4° C and then separated in an eppendorf tube. Both heart and kidneys were washed with normal saline and snap frozen along with plasma in liquid nitrogen and stored at -80 ° C until they could be analyzed.

2.2.7. Determination of arachidonic acid metabolites by reversed-phase HPLC method using fluorescence detection.

The concentrations of ArA rat plasma, heart and kidneys were measured using a previously published reverse-phase high performance liquid chromatography (HPLC) method [149]. Standard ethanolic solutions were prepared to obtain a concentration range for each metabolite from 0.01 to 2.5ug/ml using serial dilution. 16-hydroxydecanoic acid, internal standard (30µL of 1 mg/ml), was added to each standard tube, and the standards were evaporated to dryness under nitrogen stream.

Approximately 90 mg of frozen kidney or heart were precisely weighed and added to a glass tube with 200 µL of methanol and 0.4µL of 96% formic acid surrounded with crushed ice and homogenized at 15,000 rpm for 1 min using a tissue homogenizer (Omni-TH Thomas Scientific, NJ, USA) on ice. 0.4 µL of 96% formic acid was added to an aliquot of 300 µL of plasma samples. All samples of the tissue and plasma were spiked with internal standard and followed by centrifuging at 10,000 g for 10 min at 0◦C. The supernatant layer was transferred to new glass tube, diluted with 1.8 ml of 10% methanol and loaded on 1 ml Oasis HLB C18 SPE cartridge on a vacuum manifold. The SPE cartridge was previously conditioned with 1 mL methanol, 1 mL acetone, 2 mL hexane, 1 mL acetone, 1 mL methanol and 2 mL water. The loaded cartridge was then washed 3 mL of water and 1 mL of 10% methanol and later dried under a nitrogen stream, while the ArA metabolites were collected by elution with 2 ml of

anhydrous acetonitrile. The eluted anhydrous acetonitrile was evaporated to dry under stream of nitrogen.

The dried standard along with tissue and plasma were reconstituted with 136 µL of anhydrous acetonitrile. A freshly prepared derivatizing agent solution (NE-TFM) (10 µL of 2 mg/mL in a saturated potassium fluoride solution in anhydrous acetonitrile) and 4 µL of catalyst (N,N-di-iospropylethylamine) were added to each tube and maintained at 4 ◦C for 30 min in desiccator. In order to stop the reaction, all samples were dried under stream of nitrogen, afterwards reconstituted with 1 mL of 20% methanol, and loaded onto pre-conditioned SPE cartridges. Subsequently, the SPE cartridges were washed with 3 ml of water and 1 ml of 30% methanol and dried under nitrogen. The derivatized ArA metabolites were collected by elution with 2 ml of anhydrous acetonitrile. The eluted anhydrous acetonitrile was evaporated to dry under stream of nitrogen. 10 µL of each sample was injected on to HPLC after reconstituted with 100 µL 90% acetonitrile in water.

To validate this method in our lab and analyze the samples, we used a Shimadzu Prominence HPLC system (Mandel Scientific, Guelph ON, Canada), including a DGU-20A5 degasser, a LC-20AT dual pump, a SIL-20A auto-sampler, a RF-10AxL fluorescence detector, a CBM-20A communication module and a CTO-20AC column oven. The reversed phase chromatographic separation were accomplished using a two columns of C18 ((100 × 4.6 mm, 3.0 mm I.D.) linked to C18 Guard Cartridge (4.0 mm × 3.0 mm I.D.) obtained from ((Phenomenex, Torrance CA, USA). The mobile phase as gradient system comprised of 0.1% formic acid in HPLC-grade water (A) and 0.1% of formic acid in acetonitrile (B), in which the ratio between the both A and B changed during 124 min run time with flow rate 0.8 ml/min at 30◦C and maximum pressure of 3500. At initial time, the ratio was 45:55 between A and B, then the

mobile phase B rose in a linear manner to 65% over 40 min and kept on plateau for 25 min. Subsequently, the gradient increased to 75% over 20 min, followed by 95% over 10 min, and maintained a plateau for 22 min. The system was dropped back to the initial ratio as pre-equilibration period 7 min before the run of next sample. Shimadzu Class VP 7.4 version software was used for data acquisition, and both excitation and emission wave-length were adjusted at of 260 and 396 nm, respectively, for detection purpose.

Intra- and inter-run accuracy and precision of the method were evaluated based on calibration standards, at low, middle, and high analyte concentrations (n = 3). Accuracy was calculated using the following equation: Accuracy (%) = (Measured Concentration / Nominal Concentration) × 100, while the precision was estimated as percentage relative standard deviation (R.S.D.).

2.2.8. Diclofenac assay

Diclofenac plasma concentrations and its amount in heart or kidney were determined using reversed-phase HPLC method previously reported by our laboratory [150]. Briefly, a standard stock solution of diclofenac (20 mg /100 ml in methanol (wt. /v %)) and mefenamic acid as internal standard (10 mg/100 methanol wt. /v %) were spiked with blank rat plasma and blank homogenate of heart and kidney tissue in order to achieve a standard dilution of 0.066, 0.33, 3.3, 16, and 30 µg/ml containing 0.04, 0.2, 2, 10, 20 µg/gram of diclofenac, respectively.

A two fold of water was added to accurately measured weight of treated kidney or heart tissue, and followed by homogenization on ice for maximum 1 min. 200 µL of plasma treated rat and 200 µL of tissue homogenate were spiked with 100 µL of internal standard, 100 µL of methanol, and acidified with 100 µL of 1M of phosphoric acid. The standards and samples matrix were double extracted with 3 ml of diethyl ether. The organic layer then centrifuged,

collected, and later dried using evaporatory drier. The standards and samples residue were reconstituted with 100 µL of mobile phase.

Shimadzu Prominence HPLC system (Mandel Scientific, Guelph ON, Canada) were used to analyze the samples, involving of SPDA-6A variable UV spectrophotometer adjusted at 280 nm. The reversed phase chromatographic separation were accomplished using a C18 column (100 × 4.6 mm, pore size: 3 µm) and a mobile phase was acetonitrile: water - acetic acid with ratio of 50: 50 – 0.2 % and maximum pressure of 1500. The run time was approximately 9 min with flow rate and injection volume of 1 ml/min and 20 µL, respectively.

Intra- and inter-run accuracy and precision of the method were evaluated based on calibration standards, at low, middle, and high analyte concentrations (n = 3). Accuracy was calculated using the following equation: Accuracy (%) = (Measured Concentration / Nominal Concentration) × 100, while the precision was estimated as percentage relative standard deviation (R.S.D.).

2.2.9. Statistical methods

The results of drug concentration assays are presented as mean ± standard deviation (SD) of different doses of diclofenac in tissue (ug/g) or in rat plasma (µg/mL). The tissue to plasma ratios are calculated individually for each rat, for each diclofenac dose concentration in the kidney over plasma (ratio) and heart over plasma (ratio) and then mean ± SD was calculated.

The results of ArA metabolites are reported as concentration (mean ± SD) of the respective metabolites (20-HETE, EETs and DHETs) in heart (ng/mg), kidney (ng/mg) and in the rat plasma (ng/mL). The 20-HETE/EETs ratios were calculated individually for each rat (plasma, heart and kidney) samples and then mean ± SD was calculated. The multiple comparison among all groups were performed using a parametric method, One Way Analysis of

Variance on Rank (ANOVA), followed by Bonferroni adjustment utilizing GraphPad Prism® statistics software 2015 (GraphPad Software, Inc. La Jolla, CA 92037 USA). Results are considered significant at $p < 0.05$.

Chapter 3

3. Results.

3.1. Validation of arachidonic acid metabolites methods.

The acceptable limits for accuracy were 80–120% for the high, middle and low concentration. The acceptable precision was ≤20% for all concentrations. This means that the accuracy values were within 80–120 % for all concentrations. Additionally, the precision values (R.S.D.) were ≤20% for all concentration (Table 7).

3.2. Validation of diclofenac Assay.

The acceptable limit of accuracy was 80–120% and the acceptable precision for all concentrations was ≤20% (Table 8).

3.3. A dose escalation study to determine the therapeutic dosage range of diclofenac using an experimental animal model.

The signs of adjuvant arthritis (AA) were observable 10 - 12 days post-adjuvant injection. Such sign exhibited itself by early ascending of redness of paw sole, erythema of ankle joints and later both metatarsal and interphalangeal joints were involved. These symptoms extended with time into other areas of hind and forepaws. INF-0 showed a significant appearance of AA symptoms with AI equal to 5.2 ± 0.83. Therefore, those rats (INF-0) were euthanized as AI was ≥ 5.

The AA rats that had been injected with adjuvants but received different doses of diclofenac responded in a dose-dependent manner. Their paw and joint diameters were substantially less than those rats that were not treated. Although the dose of 2.5 mg/kg /day of diclofenac showed a 30% reduction in AI, the reduction was only significant at doses of ≥5

mg/kg/day. The AI was inversely correlated in a linear trend with different doses of diclofenac ($p < 0.05$, $R^2 = 0.657$) (Figure 6). A non-linear regression approach with sigmoidal model was used to demonstrate the relationship between percent reliefs of AI symptoms and log dose (Figure 9). This model revealed ED50 of ~ 4.962 mg /kg/day (log ED50 ~ 0.694). As result, we decided to choose both pharmacokinetic equivalent dose 5 mg/kg/day and high dose of diclofenac of 15 mg/kg/day to continue further in our study.

Changes in the percentage of body weight gain, AI and serum nitrite observed 18 days post adjuvant injection were used to determine the extent of inflammation in comparison to INF-0 (Table 9, Figure 7 & 8).

3.4. The effect of diclofenac treatment on cytochrome P450 metabolism of ArA in plasma, kidney, and heart in rat with adjuvant arthritis

The eicosanoid levels were measured in the plasma, heart and kidneys (Figure 10, 11 &12, Table 10,11 & 12) in rats with AA using the HPLC-fluorescence detection method. The statistical analysis was based on a comparison of all the groups using the parametric approach.

3.4.1. ArA metabolites concentration in the plasma

The results of the present study demonstrated that the total CYP-epoxygenase-derived EETs plasma significantly declined in inflamed control (INF-0) in comparison to healthy control (CONT-0) ($p < 0.05$). Treatment with diclofenac increase plasma levels of total EETs compared to inflamed rats, but only the rat treated with 5 mg/kg/day diclofenac (INF-5) were significantly different compared to inflamed rats ($p < 0.05$) and restore the T-EETs to comparable level with healthy group. Individually, both 14,15-EET and 11, 12-EET followed the same trend of T-EET (Figure 10 & 11).

The plasma levels of 20-HETE were comparable between the arthritic rats (INF-0) and inflamed that had received 5 mg/kg/day of diclofenac. Nevertheless, a significant ($p < 0.05$) increase in 20-HETE concentration was observed in the rats treated with high dose of diclofenac (INF-15) in comparison with healthy control and inflamed rats (Figure 10).

A significant difference was observed in the total T-DHT concentration between the inflamed and healthy rats; however, those groups that received ED 50 (INF-5) and high doses (INF-15) of diclofenac showed a significant decline in T-DHTs in comparison to the inflamed group ($p < 0.05$). Individually, while 14,15- DHT was approximately similar to T-DHTs, 11,12-DHT levels were similar among all groups (Figure 10 & 11).

From a ratio perspective, 20-HETE / T-EETs, 20-HETE / 14,15-EET and 20-HETE / 11,12-EET as marker of vasoconstrictive/ vasodilator components of ArA cascade were significantly higher in inflamed rats (INF-0) compared to healthy control (CONT-0). Interestingly, treatment of high dose of diclofenac (INF-15) significantly resulted in even a higher ratio of 20-HETE / T-EETs and 20-HETE / 11,12-EET in comparison to all groups ($p < 0.05$), whereas the rats treated with ED50 dose decrease the ratio of 20-HETE / T-EETs and 20-HETE / 14,15-EET. The ratio of T-EETs/T-DHTs reflects sEH enzyme index activity that is responsible for EETs hydroxylation. This index appears to be highest in 5 mg/kg/day treated group among all groups (Figure 12).

3.4.2. ArA metabolites concentration in the heart

The increase in 20-HETE level was found to be a trend in inflamed control (INF-0) and in the rats that received diclofenac (INF-5 & INF-15), but only the group that received the high dose of

diclofenac (INF-15) presented significantly ($p < 0.05$) higher concentrations of 20-HETE when compared with the other groups (Figure 10).

Likewise, the heart tissues samples from rats in inflamed and treated groups showed increase in T-EETs when compared to healthy rats (CONT-0). Such compensatory increased mechanism of T-EETs occurred significantly in inflamed rats (INF-0) and treated with high dose of diclofenac (INF-15) compared with healthy one ($p < 0.05$). On the individual level, On an individual level, findings for 14,15-EET were similar to those for T-EETs, while the 11,12-EET level was comparable among all groups. However, there was no significant difference in observed T-DHTs, or even in the individual 14, 15-DHT and 11,12-DHT concentrations among all of the rats in the study (Figure 10 & 11).

The proportion of the cardio-toxic/cardio-protective (20-HETE/T-EETs, 20-HETE/14,15-EET and 20-HETE/11,12-EET) concentrations of the ArA metabolites seems to follow the same pattern as the observations showed increase in these ratios in both inflamed rats and those which treated with diclofenac in comparison to o health control. Remarkably, a high dose of diclofenac exhibits a highest value with significant increase of these entire ratios among all groups ($p < 0.05$). Additionally, an index of the sEH enzyme activity, T-EETs/T-DHTs, tends to be similar among all groups (Figure 12).

3.4.3. ArA metabolites concentration in the kidney

The tissue concentrations of 20-HETE metabolites in the kidneys were lower than those in the plasma and heart. The decline style of 20-HETE emerged in arthritic group along with treated rats. A highly significant decrease in 20-HETE was detected in renal samples of inflamed animals (INF-0) (Figure 10).

There was no significant difference in observed total or individual EET concentration between rats in healthy, inflamed condition as well as treated groups. The overall total or individual DHT was not inclined to be significantly difference among all groups as it appeared that DHT in general was not altered by inflammation or treatment (Figure 10 & 11).

On the ratio scale, the ratio of 20-HETE/T-EETs, 20-HETE/14,15-EET and 20-HETE/11,12-EET had changed on the contrary to the plasma and heart. Such decline trend occurred in rats with inflammatory condition and those which received diclofenac treatment. However, a highly significant reduction in these ratios were noticed in inflamed group ($p < 0.05$) compared to healthy one. In addition, the ratio of T-EETs /T-DHTs was comparable among all groups (Figure 12).

3.5. The tissue accumulation of diclofenac following therapeutic doses

High-performance liquid chromatography (HPLC) method was used for determination of diclofenac concentration in the plasma and tissue samples. In spite of the fact that pharmacokinetic equivalent dose (5 mg/kg/day) tends to accumulate more in heart, we found that the distribution of eight hours post-dose concentration of diclofenac (5 mg/kg/day) seven days post-dose is not significantly different among plasma, heart, and kidney of 0.04± 0.01, 0.04±0.01 and 0.03±0.01, respectively. In addition, heart-to-plasma ratio was 1.28±0.71 and kidney-to-plasma ratio was 0.97±0.41, suggesting also the absence of accumulation discrepancy of diclofenac dose 5mg/kg/day (Figure 13 & 14, Table 13).

On the other hand, the accumulation of high dose of diclofenac (15mg/kg/day) in rat heart was significantly higher and greater by approximately four-fold than its amount in kidney. This was also evident from high heart-to-plasma ratio. Heart-tissue concentration after 8-hours post-dose and

after seven days of dosing was 0.27±0.07 with heart-to-plasma ratio of 1.72±0.52, while kidney-tissue concentration of 15mg/kg/day diclofenac was found 0.06±0.01 with a kidney-to-plasma ratio of 0.46±0.24. We were observed the same trend for diclofenac in plasma, in which 8-hours post-high dose concertation of diclofenac in plasma after seven days of treatment is lower but not significantly different from its concertation in heart likewise the findings of 5 mg/kg/day of diclofenac (Figure 13 & 14, Table 14).

Table 7: Intra- and inter-run accuracy and precision of ArA method.

Concentration µg/ml	Intra-run		Inter-run	
	Accuracy	R.S.D. %	Accuracy	R.S.D. %
14,15-DHT				
2.5	99.1	2.2	99.8	2.5
1	103.3	11.1	113.3	10.1
0.1	86.6	6.6	113.3	18.3
11,12-DHT				
2.5	103.4	3.9	98.9	1.2
1	106.6	14.3	100	10
0.1	110	9.1	100	17.3
8,9-DHT				
2.5	98	3.5	97.7	2.3
1	103.3	20.1	103.3	11.1
0.1	103.3	11.1	106.6	19.5
20-HETE				
2.5	99.1	1.5	100.5	1.3
1	96.6	20.0	100	20
0.1	106.6	14.3	116.6	4.9
14,15-EET				
2.5	100.1	2.0	101.1	1.9
1	110	9.1	86.6	13.3
0.1	93.3	6.1	120	16.6
11,12-EET				
2.5	100.2	2.8	100.6	0.8
1	90	11.1	110	9.1
0.1	100	17.3	106.6	14.3
8,9-EET				
2.5	98.2	0.8	100.5	0.2
1	110	9.0	110	9.1
0.1	113.3	18.3	83.3	13.8

Table 8: Intra- and inter-run accuracy and precision of diclofenac assay.

Concentration µg/ml	Intra-run		Inter-run	
	Accuracy	R.S.D. %	Accuracy	R.S.D. %
30	100	0.47	99.1	0.23
16	99.6	1.32	98.7	0.8
0.33	100	4.28	95.4	2.2

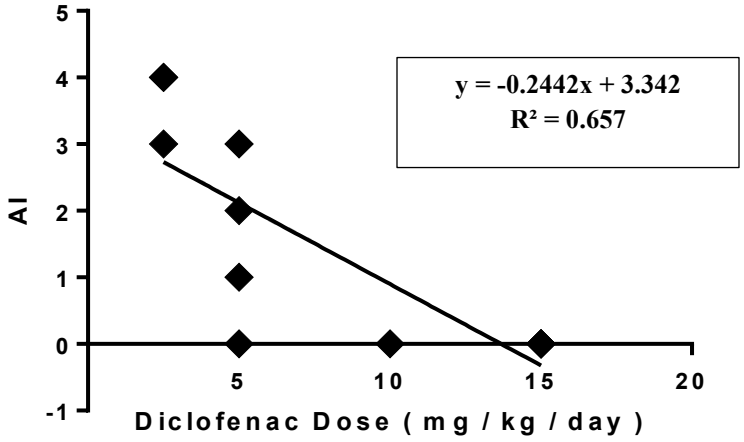


Figure 6: The therapeutic dosage range of diclofenac. Inverse correlation between different doses of diclofenac (n=4-5/ group) and arthritis index ($p < 0.05$).

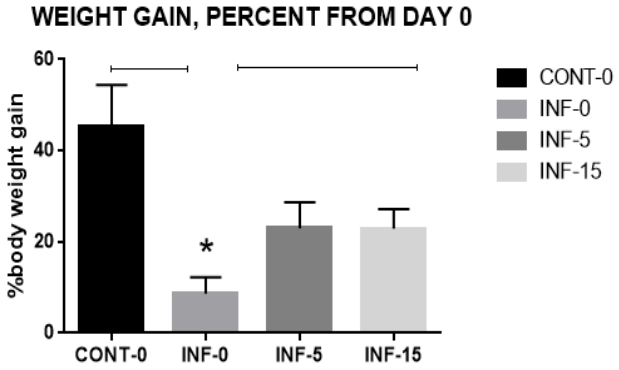


Figure 7: Effect of different daily doses of diclofenac on percentage of body weight gain. * Significantly different from other groups (P< 0.05).

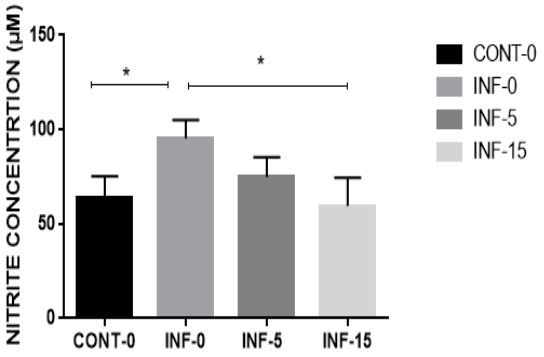


Figure 8: Changes in nitrite plasma concentration. The data are expressed as mean (SD) and * represents significant difference between each other using ANOVA (p<0.05).

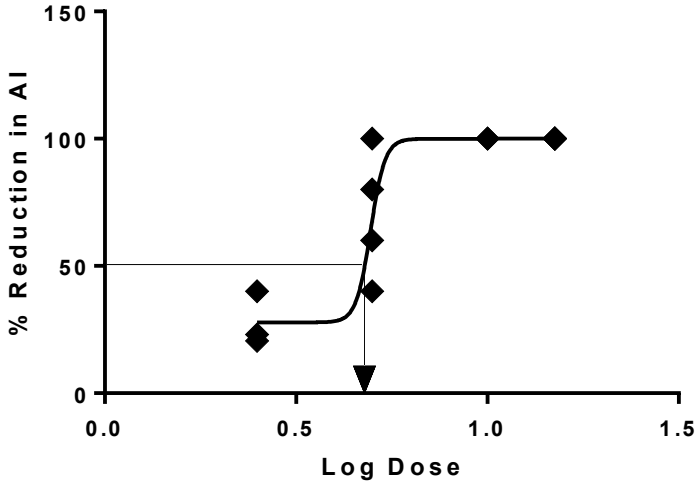


Figure 9: The log dose – response curve (n=4-5/ group). The data are estimated using non-linear regression approach. ED50 = 4.962 mg /kg/day (log ED50 ~ 0.694).

Table 9: Changes in percentage of body weight gain, arthritis index and serum nitrite observed in healthy control, inflamed placebo, and diclofenac treated rats, (CONT-0 = healthy control, INF-0 = Inflamed placebo, INF-5 / INF- 15 = Inflamed treated with diclofenac 5 / 15 mg/kg, respectively,), (n=4-5/group).

I- Percent change of body weight gain

Group	Mean(SD)	Vs. INF-0,%
CONT-0	45.2 (9.0)	* (36.5)
INF-0	8.6 (3.5)	-
INF-5	22.9 (5.7)	* (14.3)
INF-15	22.9 (4.2)	* (14.3)

The data are expressed as mean (SD). * Significantly different from INF-0 (p <0.05).

II- Arthritis Index

Animal Group	Arthritis Index		Paw Diameter (µm)	Joint Diameter (µm)
	Pre-Dosing	Post-Dosing	Post-Dosing	Post-Dosing
CONT-0	0(0)	-	69 (24)	412 (210)
INF-0	5.2(0.8)	-	1442 (501) *	3924 (490) *
INF-5	4.80(0.4)	1.60(1.1)	203 (113)	342 (72)
INF-15	4.75(0.5)	0(0)	190 (89)	317 (89)

The data are expressed as mean (SD) and * significantly different from other groups (p < 0.05).

III- Serum Nitrite

Group	Mean(SD)	Vs. INF-0
CONT-0	63.7 (11.4)	*
INF-0	95.3 (9.7)	-
INF-5	74.8 (10.5)	-
INF-15	59.4 (15.1)	*

Changes in nitrite plasma concentration. The data are expressed as mean (SD) and * represents significantly different from INF-0 (p <0.05).

Table 10: Concentration of EETs (mean± SD) in adjuvant arthritis rat plasma, heart, and kidneys (n=4-5/ group).

I- Plasma (ng/mL)

GROUP	14,15-EET	11,12-EET	8,9-EET	TOTAL EETS
CONT-0	3.80±1.74	6.75±2.55	3.10±0.37	13.66±1.90
INF-0	0.42±0.13	2.67±0.63	1.18±0.18	4.28±0.67
INF-5	4.89±1.38	5.47±1.35	4.01±1.04	14.37±2.34
INF-15	2.18±0.34	2.43±0.44	2.14±0.63	6.76±1.12

II – Heart (ng/mg)

GROUP	14,15-EET	11,12-EET	8,9-EET	TOTAL EETs
CONT-0	0.41±0.12	0.41±0.07	0.49±0.12	1.33±0.21
INF-0	1.57±0.47	0.43±0.30	0.86±0.41	2.87±0.78
INF-5	0.69±0.20	0.53±0.11	0.54±0.13	1.78±0.29
INF-15	1.42±0.35	0.67±0.20	0.77±0.18	2.88±0.65

III- Kidney (ng/mg)

GROUP	14,15-EET	11,12-EET	8,9-EET	TOTAL EETS
CONT-0	0.30±0.10	0.13±0.07	0.17±0.04	0.55±0.15
INF-0	0.43±0.38	0.33±0.07	0.21±0.07	0.98±0.46
INF-5	0.61±0.32	0.25±0.05	0.24±0.10	1.11±0.31
INF-15	0.50±0.32	0.46±0.44	0.31±0.15	1.28±0.29

Table 11: Concentration of DHT (mean± SD) in adjuvant arthritis rat plasma, heart, and kidneys ((n=4-5/ group).

I- Plasma (ng/mL)

GROUP	14,15-DHT	11,12-DHT	8,9-DHT	TOTAL DHTs
CONT-0	13.78±4.62	4.68±3.06	8.45±3.77	26.91±7.85
INF-0	21.38±5.53	3.95±1.67	15.20±2.66	40.53±7.33
INF-5	5.16±1.32	6.64±3.31	4.64±1.50	16.46±3.95
INF-15	6.93±2.75	4.18±2.42	3.81±2.56	14.93±3.62

II – Heart (ng/mg)

GROUP	14,15-DHT	11,12-DHT	8,9-DHT	TOTAL DHTs
CONT-0	4.29±1.19	2.99±1.42	2.25±1.84	9.53±3.48
INF-0	5.11±0.84	1.60±0.73	3.55±1.20	10.28±2.27
INF-5	6.20±1.30	1.35±1.02	1.09±0.76	8.65±2.19
INF-15	6.06±1.60	3.20±2.30	2.78±1.65	12.05±1.83

III- Kidney (ng/mg)

GROUP	14,15-DHT	11,12-DHT	8,9-DHT	TOTAL DHTs
CONT-0	2.60±1.98	0.50±0.17	0.66±0.38	3.78±1.86
INF-0	1.23±0.99	0.67±0.13	1.08±1.74	2.99±2.62
INF-5	4.38±3.28	1.27±1.40	1.02±1.04	6.69±2.44
INF-15	2.68±1.94	0.52±0.32	0.66±0.69	3.87±1.96

Table 12: Concentration of 20-HETE and its ratio (mean± SD) in adjuvant arthritis rat plasma, heart, and kidneys (n=4-5//group).

I- Plasma (ng/mL)

GROUP	20-HETE	20-HETE/T-EETs	20-HETE/14,15-EETs	20-HETE/11,12-EET
CONT-0	11.2±2.05	0.82±0.10	3.30±1.01	1.98±1.23
INF-0	52.6±4.20	12.42±0.99	136.40±47.82	20.25±3.21
INF-5	51.1±12.5	3.86±1.21	11.54±5.02	9.74±3.19
INF-15	163±15.7	24.61±4.51	75.27±5.06	68.78±14.38

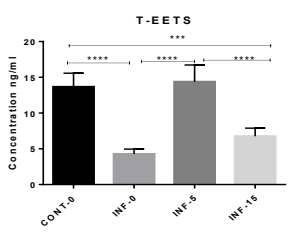
II – Heart (ng/mg)

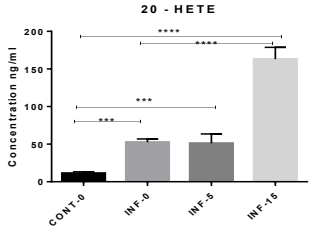
GROUP	20-HETE	20-HETE/T-EETs	20-HETE/14,15-EETs	20-HETE/11,12-EET
CONT-0	0.44±0.31	0.33±0.23	1.25±0.99	0.99±0.61
INF-0	2.11±1.04	0.79±0.49	1.38±0.71	5.96±2.76
INF-5	1.84±1.20	1.00±0.59	2.57±1.56	3.42±2.26
INF-15	8.89±0.77	3.18±0.61	6.30±2.12	13.41±3.99

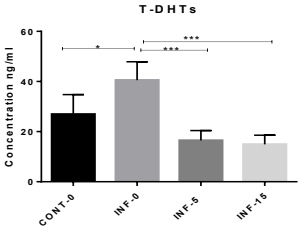
III- Kidney (ng/mg)

GROUP	20-HETE	20-HETE/T-EETs	20-HETE/14,15-EETs	20-HETE/11,12-EET
CONT-0	0.95±0.33	1.56±0.45	3.23±0.81	9.00±4.90
INF-0	0.09±0.11	0.10±0.11	0.26±0.19	0.30±0.37
INF-5	0.28±0.37	0.32±0.26	0.36±0.37	1.07±1.38
INF-15	0.41±0.18	0.35±0+.21	1.27±1.25	2.50±2.81

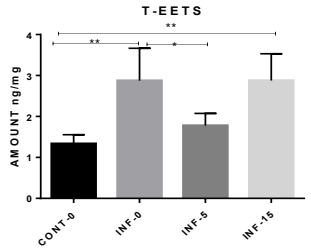
I – Plasma

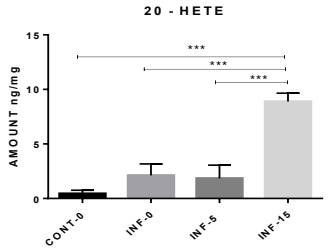


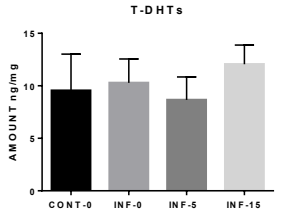




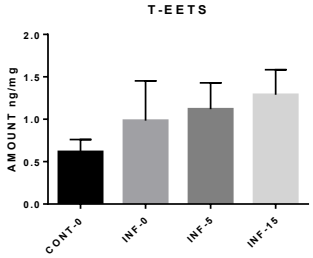
II – Heart

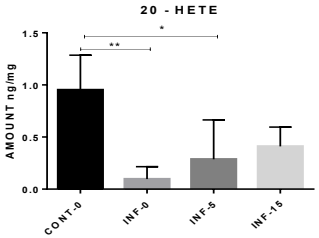






III - Kidney





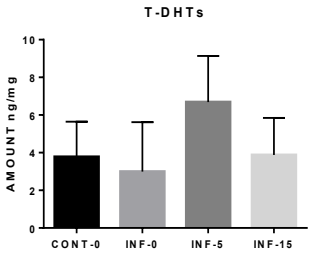
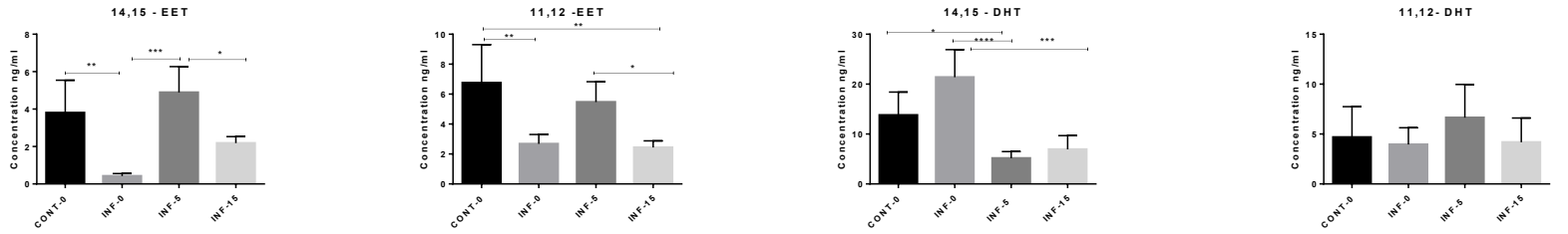
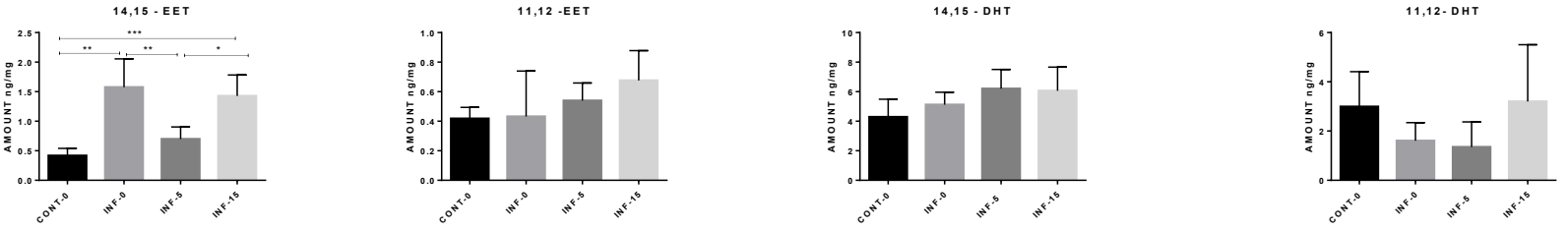


Figure 10: Concentrations of total EETs, 20-HETE, and total DHTs in tissue and plasma of healthy control, inflamed placebo, and diclofenac treated rats,(CONT-0 = healthy control, INF-0 = Inflamed placebo, INF-5 - INF- 15 = Inflamed treated with diclofenac 5 - 15 mg/kg, respectively,). The values are expressed as mean ± standard error. *, significantly different from each other ($p < 0.05$). **, significantly different from each other ($p < 0.01$). ***, significantly different from each other ($p < 0.001$). ****, significantly different from each other ($p < 0.0001$).

I- Plasma



II – Heart



III - Kidney

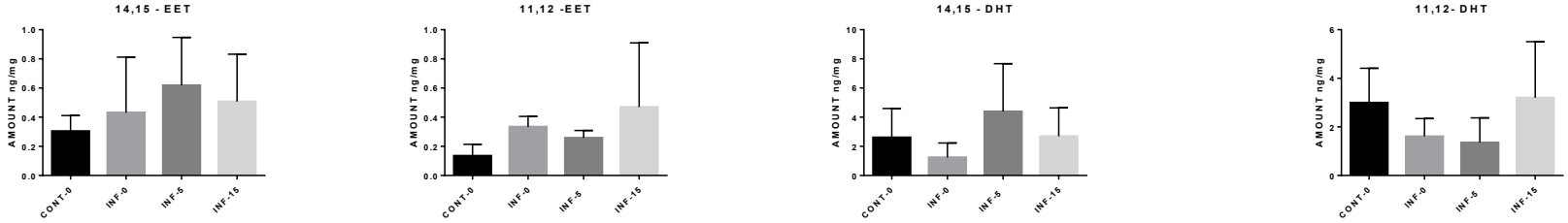
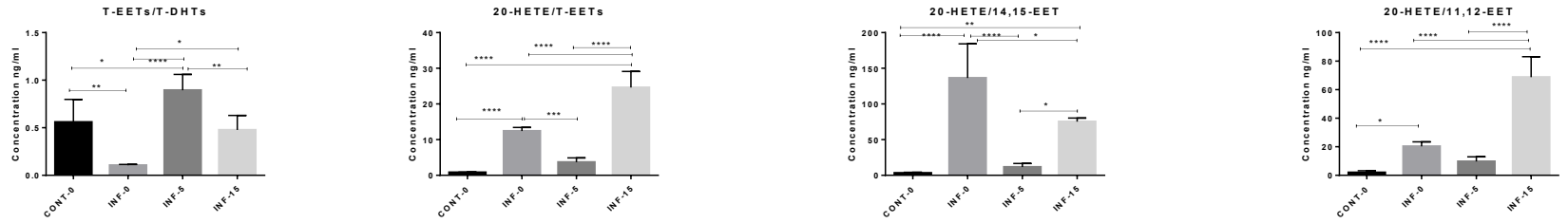
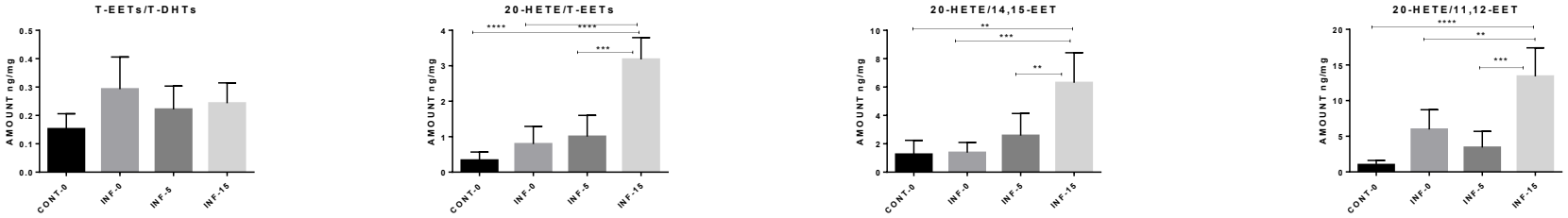


Figure 11: Concentrations of 14, 15-EET, 11,12-EET , 14,15-DHT, and 11,12-DHT in tissue and plasma of healthy control, inflamed placebo, and diclofenac treated rats, (CONT-0 = healthy control, INF-0 = Inflamed placebo, INF-5 - INF- 15 = Inflamed treated with diclofenac 5 - 15 mg/kg, respectively,). The values are expressed as mean ± standard error. *, significantly different from each other ($p < 0.05$). **, significantly different from each other ($p < 0.01$). ***, significantly different from each other ($p < 0.001$). ****, significantly different from each other ($p < 0.0001$).

I – Plasma



II – Heart



III - Kidney

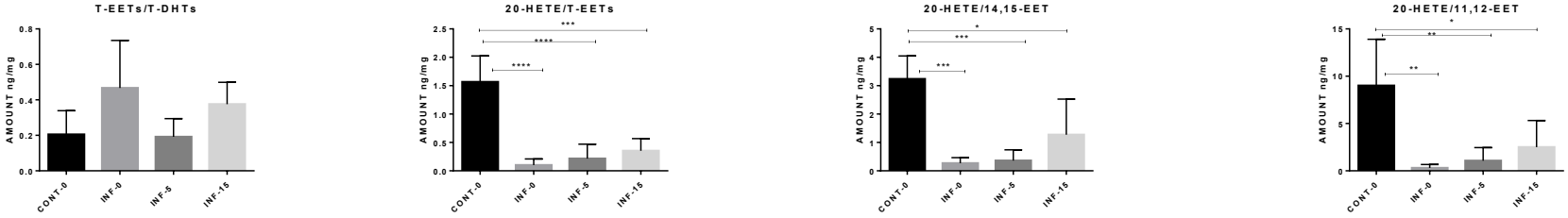


Figure 12: T-EETs/T-DHTs, 20-HETE/T-EETs, 20-HETE/14,15-EET and 20-HETE/11,12-EET ratio in tissue and plasma of healthy control, inflamed placebo, and diclofenac treated rats, (CONT-0 = healthy control, INF-0 = Inflamed placebo, INF-5 - INF- 15 = Inflamed treated with diclofenac 5 - 15 mg/kg, respectively,). The values are expressed as mean ± standard error. *, significantly different from each other ($p < 0.05$). **, significantly different from each other ($p < 0.01$). ***, significantly different from each other ($p < 0.001$). ****, significantly different from each other ($p < 0.0001$).

Table 13: Concentrations of diclofenac orally adminstrated 5mg/kg daily for 7 days in rat plasma, heart and kidneys in addition to the ratio of heart or kidney to plasma concentrations.

Rat #	PLASMA µg/ml	HEART µg/g	KIDNEY µg/g	HEART /PLASMA	KIDNEY/PLASMA
1	0.04	0.05	0.03	1.14	0.80
2	0.03	0.07	0.02	2.29	0.78
3	0.03	0.03	0.05	1.09	1.59
4	0.05	0.03	0.03	0.61	0.71
Mean	0.03	0.04	0.03	1.28	0.97
SD	0.009	0.01	0.01	0.71	0.41

Table 14: Concentrations of diclofenac orally adminstrated 15mg/kg daily for 7 days in rat plasma, heart and kidneys in addition to the ratio of heart or kidney to plasma concentrations.

Rat #	PLASMA µg/ml	HEART µg/g	KIDNEY µg/g	HEART /PLASMA	KIDNEY/PLASMA
1	0.18	0.37	0.08	2.04	0.43
2	0.08	0.2	0.07	2.29	0.81
3	0.16	0.22	0.06	1.35	0.39
4	0.22	0.27	0.05	1.22	0.23
Mean	0.16	0.26	0.06	1.72	0.46
SD	0.05	0.07	0.01	0.52	0.24

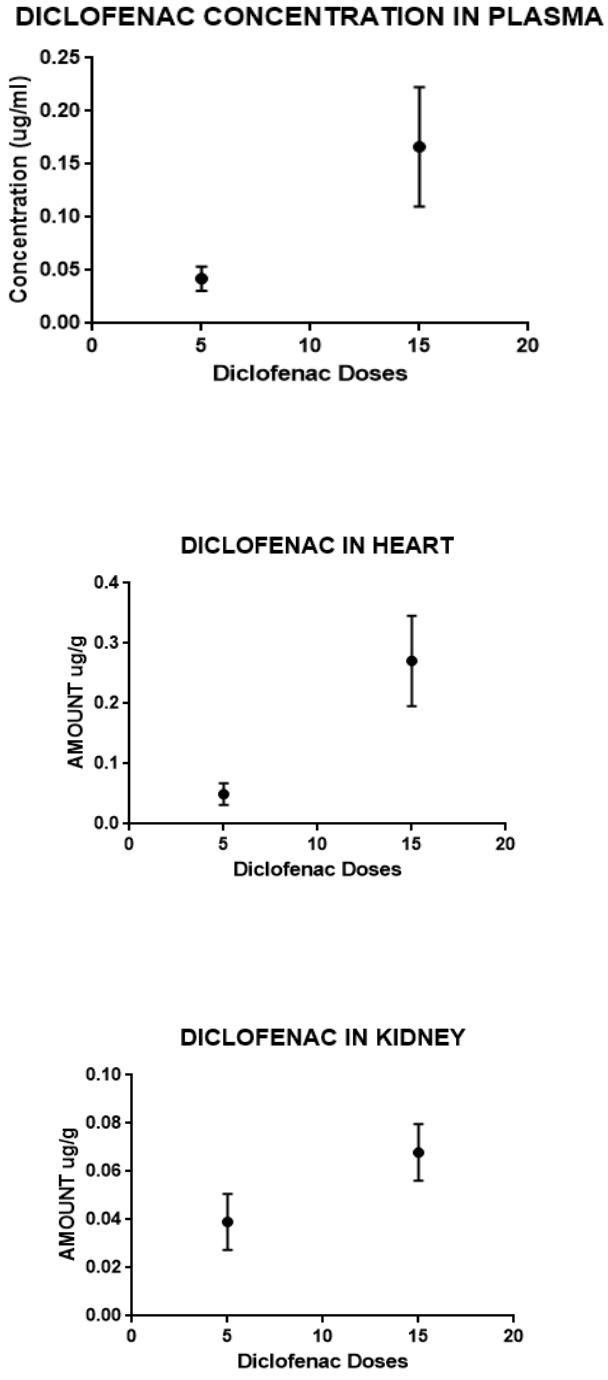


Figure 13: Concentrations of different doses of diclofenac in rat plasma and tissues (15 mg/kg/day is significantly different from 5 mg/kg/day ($p < 0.05$)). Data are expressed as arithmetic means (SD) (n = 4 /group).

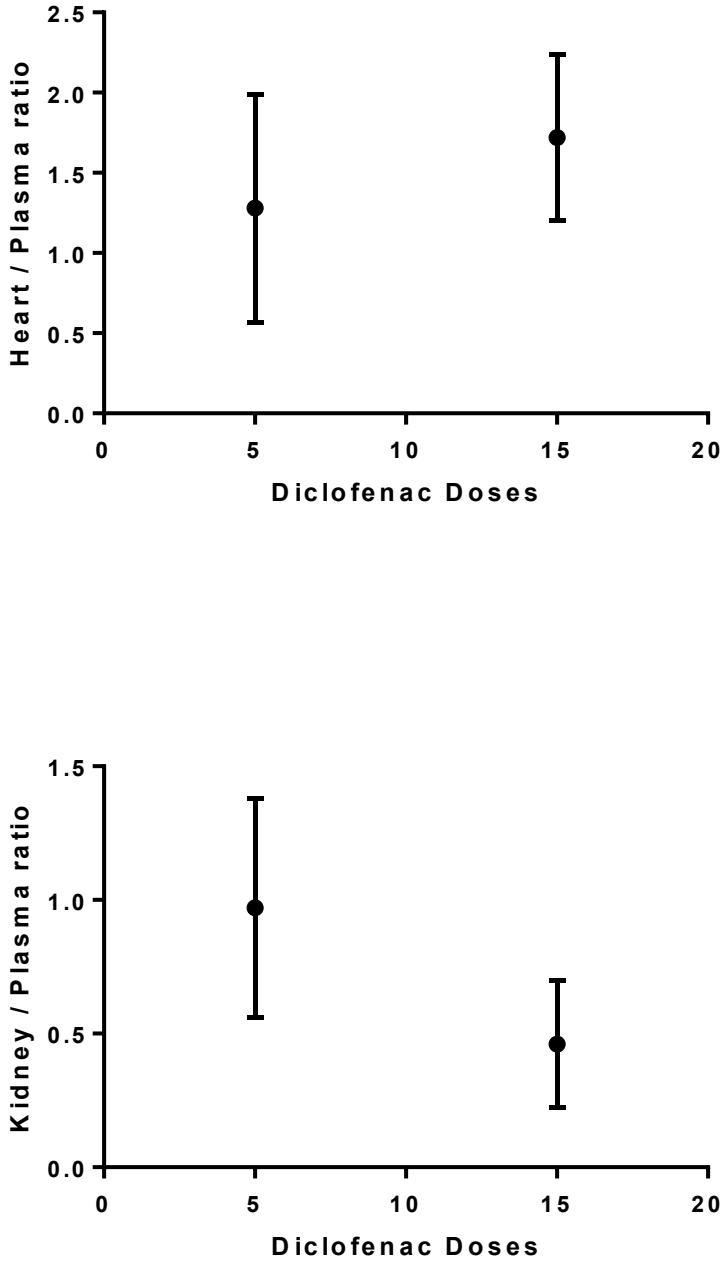


Figure 14: Ratio (Heart / plasma, Kidney / Plasma) of different doses of diclofenac in AA rats (15 mg/kg/day is not significantly different from 5 mg/kg/day ($p > 0.05$). Data are expressed as arithmetic means (SD) (n = 4 /group).

Chapter 4

4. Discussion

The rationale behind drug therapy is built on the assumption that there is a fundamental relationship between the dosing regimen (drug exposure) and the therapeutic response in addition to the adverse effects. Thus, it has been one of the leading objectives of clinical therapy to identify therapeutic dosing regimens as we did for our examined drug, diclofenac, which produced a clinically relevant ameliorating effect in adjuvant arthritis (AA) in a dose-dependent fashion [151]. In general, the evaluations of efficacy of NSAIDs have been constructed based on an index of pain or inflammation such as the arthritis index (AI), which is a hybrid measure of disease with intrinsic variability as compared to a metric measure such as paw and ankle diameter.

In the present study, we observed the suppressing effect of diclofenac on AA after repeated daily doses between 2.5 to 15 mg/kg/day with signs of dose-dependency. A significant effect was not detected until 5 mg /kg/day was used even though doses as low as 5 mg/kg showed some positive results on the metric scale. Despite the fact that such indices (AI) are subjective as a method to illustrate the dose-effect correlation, the therapeutic dosage range of diclofenac in animal studies is in line with randomized crossover human trials. Doubling the dose of diclofenac in AA rats produced a 3 –38 % increase in relief of inflammation symptoms until reaching the maximum response at a dose of 10 mg/kg/day, while doses above 10 mg/kg were equally effective in suppressing arthritis symptoms with the maximum attainable effect. Likewise, Giagoudakis *et al* have reported that doubling the dose of diclofenac from 50 mg to 100 mg in adult volunteers enhance the inhibition of PG concentration by 60 -65% [152]. Our

data suggest that an increase in the dose will increase the beneficial effects for treating the progression of inflammation. This exposure dependency of diclofenac observed in the AA model might provide evidence as to what extent increasing dose increments produces analogous increases in the relief of symptoms. Within the examined dosage regimen, ED50 is 5 mg/kg/day.

NSAIDs plasma concentrations have not correlated well with their therapeutic response especially with short half-life drugs like diclofenac. This may be related to protein binding, when total drug concentration is measured, and/or cellular uptake [153]. It is likely that the drug accumulates in the deep tissues, and returns to the systemic circulation with a very slow pace; so slow that measurement of the plasma concentration becomes problematic using routine assays. Indeed, the half-life of diclofenac may be substantially longer that what is generally believed; The more sensitive the assay, the longer the half-life [154]. This may explain the observed efficacy of single daily diclofenac despite its reported half-life of approximately 2 h.

Although diclofenac has been long in clinical use, the reported information regarding its tissue distribution is sparse for any species and most studies have focused on plasma measurements. To help clarify the regional sites of actions of diclofenac, we demonstrated both plasma and tissue concentration eight hours post-dose and after seven days of dosing in the AA model. Our data observed that diclofenac in therapeutic doses has no preferences regarding tissue selectivity. The ED 50 dose (5 mg/kg/day) suggests generally equal distribution throughout the body. This acidic drug, at high doses, shows high concentrations in the heart, which differs significantly from the concentrations found in the kidneys. Indeed, the extent of accumulation of diclofenac in heart generally appears to be trend for this drug as it has a ratio of concentration more than 1. The theory of high accumulation of certain compartment might be associated with drug toxicity. Herein, it might explain the CV/ renal adverse effect of diclofenac.

Shedding the light on degree of COX inhibition, diclofenac is an inhibitor of the COX-2 enzyme. Hence, reaching the target tissues in sufficient concentration is responsible for therapeutic activity while the higher concentration could be responsible for side effect as long as COX isoform are blocked in healthy and inflamed sites. The multiple doses regimen of diclofenac that we applied in our study leads to the persistence of tissue exposure and, hence, might mean that the tissues receive a relatively high concentration of this drug compared to plasma. Consequently, the recovery of the COX enzyme could be slower than single dose, resulting in more hemodynamics changes in inflamed and healthy tissues [155, 156]. Such hint might meet with fact that sustained exposure to high concentration of drug at abnormal condition such as inflammation might relatively attributed to drug effect and side effects in the peripheral tissues.

The correlation between adverse effects and tissue exposure to NSAIDs has been shown in animal studies. The adverse renal effect marked by a reduction in electrolyte excretion is associated with higher kidney-to-plasma drug concentrations as reported by Harirforoosh [114]. The same author also reported that rofecoxib, celecoxib, diclofenac, and flurbiprofen were able to significantly reduce sodium and potassium excretion in comparison with a placebo, whereas meloxicam, a selective COX-2 inhibitor, had no substantial effect on electrolyte excretion [95]. On same trail, our lab has found that concentration of rofecoxib and celecoxib in rat heart and kidneys were significantly higher than in the plasma, where accumulation of rofecoxib in the heart almost doubles the amount in the kidney, while the amount of celocoxib had almost the same concentrated in both heart and kidney tissue. Meloxicam appears to accumulate in the kidneys and heart much less than it does in plasma. Flurbiprofen enantiomers have also exhibited significantly higher heart-tissue concentrations than plasma concentrations [126]. These observations exclude the notion of COX selectivity as main cause of CV/ renal side effect. The

strongest evidence is that the deep compartment has filled with sufficient concentration of drug to be able produce effect or side effect. The concept of the exposure-dependent toxicity is also observed with other drugs. For example, the risk of cardiotoxicity of anthracyclines including daunorubicin and doxorubicin is directly linked with dose as the cardiomyopathy occur at low doses and increases considerably at dose of .550 mg/m^2 [157]. Gentamicin-related nephrotoxic effects have also been linked with that drug's pharmacokinetic behavior, where it has high renal tissue accumulation [158, 159].

CV risks are elevated among RA patients. Systemic inflammatory tends to be a driving force of such risk, in which the survival of the post-myocardial infarction (MI) patients correlate well with the degree of inflammation determined as the concentrations of pro-inflammatory mediators [160]. Anti-inflammatory therapy in RA is expected to normalize any imbalance caused by inflammation because of their inflammatory-dampening effects. However, meta-analysis of clinical studies has shown that NSAIDs that are frequently used in patients with RA are associated with increased risk of death and cardiovascular morbidity despite the fact that NSAIDs are heterogeneous in their CV safety [83, 109].

NSAIDs are supposed to treat inflammatory conditions, but they increase the CV/renal risk in a manner similar to inflammation [82]. There are multiple theories that have been suggested to explain the entire magnitude of CV morbidity and mortality in RA. The notion of some hypotheses, such as the involvement of the renin-angiotensin system (RAS), are excluded [161], whereas others such as pharmacokinetic properties and tissue distribution are succeeded at least in part to associate with CV/ renal events [82, 114], in which the extent of drug exposure might interfere with many homeostatic components and intracellular mechanisms.

Nevertheless, the dose-dependency of the well-acknowledged elevated CV risks of NSAIDs has not been well-studied. Additionally, the relationship, to the best of our knowledge, among inflammation, NSAIDs and the CYP-ArA pathway is not well-identified. Our null hypothesis was that a high dose of diclofenac, the model drug of NSAIDs, shares with inflammation the same mechanisms involved in disturbing the CV/renal system (i.e., an imbalance of the CYP-ArA system in the AA animal model, while low but effective therapeutic dose can void of any significant CV toxicity. The current observations support our hypothesis. Inflammation altered the ArA pathway in tissue dependent trend, changing the balance between anti-inflammatory and proinflammatory eicosanoids in AA rats. A high dose of diclofenac in inflamed rats imposed a cardio-renal risk by further increasing the 20-HETE/EETs cardio-toxic/cardioprotective metabolite concentrations in the plasma and hearts of AA rats. At the same time, low therapeutic doses did not alter the CYP-mediated ArA metabolism.

It is worthy to consider that dose-related increases in CV risk have been reported in clinical studies. Van Staa et al. reported that increasing daily dose was associated with high risk of MI for diclofenac, the relative risk (RR) was 1.13 with <150 mg /day and 2.03 with >300 mg /day compared with control patients [141]. Gislason *et al* showed that high dosages of diclofenac increase mortality in patients with previous MI, with a hazard ratio (HR) of 0.89 for death and 1.27 for Re-MI at ≤100mg/day, whereas there was an HR of 4.44 for death and 1.89 for Re-MI at > 100 mg/day [142]. NSAIDs are not homogenous in their CV/ renal outcomes. The rofecoxib, for example, appears to elevate such risk with dose elevation, while meloxicam tend to void these risks in low and high dose. Low therapeutic doses of some NSAIDs appear to be safe for patients who do not have any CV co-morbidities. This could provide cardioprotection as low doses of both ibuprofen and naproxen [109, 162]. The dose-dependency concept of causing side

effects has also been demonstrated in other therapeutic agents such as mitomycin C and diphenhydramine and in non-therapeutics like carbon tetrachloride [163-165].

The effect journey is starting from exposure to chemical molecules to appearance of biological response, involving cycle of interrelated pathways in correlation with dose or concentration. Such a concept might have a direct contribution in interpreting the CV toxicity of NSAIDs. Eicosanoids, CYP metabolites of ArA, play a role in the physiological and pathophysiological processes and can be considered as biomarkers of NSAIDs induced cardiotoxicity. Our study observed that AA model of inflammation modulate CYP-ArA metabolites. This is in line with the model of lipopolysaccharide (LPS) induced inflammation that demonstrates alteration CYP enzyme expression and eicosanoid metabolism [74, 75]. High dose of diclofenac is in preference to amplify the inflammation changes in ArA metabolite that could be translated in to changes in cardiac and renal functions.

20-HETE concentrations were revealed in increases in the plasma and heart but reduced in the kidneys (Figure 11). The action of 20-HETE seems to be tissue dependent, while the level of 20-HETE is a reflection of production or the degradation pathway. The CYP4A and CYPF that are responsible for 20-HETE synthesis are expressed in the kidneys, heart and vasculature. However, 20-HETE shows diversity in action and production between renal tubules and vasculature, producing anti- and pro-hypertensive effects respectively. The first action results from enhancing natriuresis, while the second occurs by promoting vasoconstriction [54, 166, 167]. The development of hypertension in Lewis rats fed a high salt was reported following infusion of inhibitors for 20-HETE formation [168]. Roman RJ et al. also found that reducing the CYP4A protein and consequently 20-HETE production contributed to the development of hypertension in Dahl salt-sensitive rats [169]. It is believed that in the proximal tubules, 20-

HETE inhibits Na^+-K^+-ATPase through a protein kinase C (PKC)-dependent phosphorylation and thus, sodium reabsorption will be inhibited. The same pattern would result in the collecting duct, in which 20-HETE blocks the K^+ channel in the apical membrane and limits the availability for the Na^+-K^+-$2Cl^-$ cotransporter, leading to a reduction in the required force for cations reabsorption. This action of 20-HETE will induce diuresis and produce an antihypertensive effect [54, 170-172]. In vasculature, CYP4A2 and CYP4A3 are mainly expressed in blood vessel and formed 20-HETE that exerts vasoconstrictive effects, resulting in development in vascular resistance in peripheral vessels [54, 173]. In the heart, it was reported that the level of CYP4A protein was induced in heart microsomes in rats treated with LPS, and that the mRNA of CYP4F significantly increased in inflamed hearts [75]. This might be linked with our findings in which levels of 20-HETE increased in AA rats. Moreover, the inhibition of the CYP omega-hydroxylase pathway and consequently, the reduced 20-HETE level provide an evidence to inhibit myocardial apoptosis in heart model of ischemia/reperfusion [174] .High dose of diclofenac tend to mimic the inflammation regarding the elevated level of 20-HETE in heart that might be associated with thrombogenic events and increase in blood pressure and consequently CV events [175].

EETs, on the other hand, as key eicosanoids, represent the cardioprotective arm in the CYP-ArA pathway due to their anti-inflammatory, vasodilatory, antihypertensive, and antiplatelet aggregation properties [54, 176, 177]. High doses of diclofenac as well as inflammation alter the level of EETs, causing reductions in plasma and increases in the heart and kidneys. The increase of T-EETs production in the heart is in contrast to what has been previously reported, and could be a compensatory mechanism by which an inflamed heart attempts to counterbalance the higher level of 20-HETE [75]. Additionally, in our AA study, the

ratio of plasma and heart 20-HETE/EETs of high dose of diclofenac was significantly increased compared to the control group and the level of DHTs in the heart was comparable among all groups. These observations imply that the balance between the pro-and anti-inflammatory properties of ArA components is biased toward pro-inflammatory metabolites in the AA model, even though the level of EETs in the heart increased. The EET/DHT ratio reflects the activity of the sEH enzyme, which is generally comparable in the tissues among all of the groups in the study. This might indicate that a high dose of diclofenac tends to mitigate the effects of inflammation on the sEH enzyme.

It is worth determining the extent of tissue drug exposure relative to the plasma concentration when studying the adverse effect profiles of NSAIDs. A direct relationship was found between renal function and concentrations of NSAIDs in rat kidneys. It was concluded that both rofecoxib and celecoxib have a relatively higher distribution into the kidneys compared with meloxicam, and this suggests the involvement of direct drug exposure in the kidneys, causing an adverse renal effect [114]. In our laboratory, unpublished data showed that high CV-related adverse effects in rofecoxib and flurbiprofen might be linked to their significant exposure to the heart. In the present study, we report a similar trend in heart tissue exposure to high doses of diclofenac, which might be linked to CV events. The concept of exposure dependency is also involved with toxicity of many drugs such as doxorubicin in the heart or gentamycin and cyclosporine in the kidneys [157, 158, 178, 179].

Chapter 6

5. Summary and Suggestions

5.1. General conclusion

Our results indicate that diclofenac in the therapeutic dosage range possesses ameliorating effects on adjuvant arthritis (AA) in a dose-dependent manner, in which only doses greater than 5 mg/kg controlled AA (ED50 ~ 5 mg/kg/day). This study confirmed that diclofenac tends to accumulate in the heart, where the observed heart over plasma ratio is above than one for the examined doses. Such tissue accumulation might prone to disrupt the homeostatic balance which consequently could result in CV complications.

The estimation of plasma and tissue concentrations of ArA metabolites in inflammatory conditions is essential for understanding their role in pathophysiological processes. We found that only the high dose of diclofenac (15 mg/kg) intensifies the deleterious effect of inflammation on the ArA pathway and favors cardiotoxicity by increasing 20-HETE / T-EETs ratio in plasma and heart, while a low therapeutic dose (5 m g /kg) did not cause imbalances in the ArA metabolic profile toward cardiotoxicity and keep the 20-HETE / T-EETs ratio as influence of inflammation. This was along with that different doses of diclofenac modulates ArA pathway similar to inflammation in kidney by decreasing the 20-HETE / T-EETs ratio.

We concluded that within the therapeutic range, only the examined high doses of diclofenac caused imbalances in the ArA metabolic patterns toward cardiotoxicity, while low therapeutic doses did not alter the CYP-mediated ArA metabolism. This suggests of the dose- and exposure-dependency of the cardiotoxicity of the non-steroidal anti-inflammatory drug

(NSAID). Hence, extrapolating the results of animal studies to humans, low but effective therapeutic doses of these drugs may be void of CV side effects. Human studies are needed to examine the safety of therapeutics doses of NSAIDs in association with eicosanoid plasma level.

5.2. Future directions and studies

For further elaboration of NSAIDs mechanism of action relating to CV risks, several studies can be design with goal of:-

- ✓ A study of effect of different doses of NSAIDs on mRNA expression and cytochrome P450 (CYP) enzymes level in particular tissue.
- ✓ A study of dose dependency effect of NSAIDs on functional performance in heart (echocardiography) and kidney (Urinary electrolytes).
- ✓ A study of dose dependency of NSAIDs on RAS system.

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