1.1. DRUG DESIGN, DISCOVERY AND DEVELOPMENT

Drugs are the chemicals used to restore the diseased condition in individuals. Pharmacologically, drugs are the substances, which can be utilised for the diagnosis, treatment, mitigation or prevention of any disease or disorder in human beings or animals (The Drugs and Cosmetic Act, 1940). The process of drug designing and discovery is an expensive, time consuming and very challenging task. As per the recent reports, the process of new drug discovery is expensive and takes around 12-15 years to develop. It is expected that around 1 billion US $ is required to develop and bring a single drug to the market (Adams and Bruntner, 2010). Thereby, many efforts have been made to develop and improve new drugs through various new technologies of drug discovery and development.

All the leading pharmaceutical and biotechnology companies have now implemented the use of molecular modelling, and computer-aided drug design (CADD) approaches. Molecular modelling allows us to do and teach chemistry better by providing superior and reliable tools for investigating, interpreting, explaining and discovering new phenomena (Nadendla, 2004). CADD also allows for studying the molecular similarity approaches such as comparative molecular similarity indices analysis (CoMSIA), comparative molecular field analysis (CoMFA), quantitative structure–activity relationship (QSAR), atom–based 3D QSAR and pharmacophore model generations.

Broadly, the drug design paradigm can be categorised into two types: direct drug design and indirect drug design. The direct approach, also known as structure-based drug design (SBDD) is based upon the known 3D structure of the biological targets. SBDD refers specifically to finding and complementing the 3D structure of biological target and discovering molecules that satisfy some geometric constraints and is also a good chemical match. The docking, molecular dynamics simulations, X-ray diffraction (X-RD), nuclear magnetic resonance (NMR) and homology modelling can be used as a tool in SBDD.

Development of SBDD against ambitious drug targets such as nucleic acids and proteins has led to exciting breakthroughs in the field (Anderson, 2003). Enzymes are good targets since their active site provides an excellent ligand

binding site for the drug. Successfully marketed drugs such as amprenavir (Agenerase®) and nelfinavir (Viracept®), which inhibits HIV protease were the first drugs to enter the market using SBDD design approach (Kaldor *et al.*, 1997). This was followed by zanamivir (Relenza®) which was developed as a neuraminidase inhibitor (Varghese, 1999), tomudex developed against thymidylate synthase (Rutenber and Stroud, 1996), and imatinib mesylate (Glivec®), the inhibitor of Abelson murine leukemia (Abl) tyrosine kinase (Schindler *et al.*, 2000).

The indirect drug design approach known as ligand-based drug design (LBDD) involves creating a lead molecule by comparing various structural characteristics of known active and inactive molecules. QSAR, CoMFA, CoMSIA, pharmacophore modelling, high throughput screening (HTS) and combinatorial chemistry techniques uses the LBDD approach in drug designing.

Indeed molecular modelling helps in the identification of moieties involved in the interaction with a particular protein and permits to understand the underlying molecular mechanism responsible for its specific biological activity. This knowledge of CADD could expedite the development of new active molecules to be successfully used as drugs. However, as simulation accuracy is limited to the precision of the constructed models, computational simulations have to be evaluated against *in vitro*/*in vivo* experimental outcomes to confirm the accuracy of the model and modify them if necessary to yield better representations of the system.

1.2. INFLAMMATION: AN OVERVIEW

Inflammation is a multifaceted process reflecting the response of a host, either localised or more generalised to various noxious stimuli. Pathogenesis of many mechanistically related disorders such as arthritis, carcinogenesis, neurodegeneration and autoimmune diseases are attributed directly or indirectly to an inflammatory response (Franks and Slansky, 2012).

In the recent years, pain and inflammation have resulted in an overwhelming burden to the healthcare status of our population and are the

underlying basis of a significant number of diseases (Edwards, 2005). Report from the Medical Expenditure Panel Survey puts the total cost incurred in the management of pain in the US market was $ 560 - $ 635 billion in 2010 (Gaskin and Richard, 2012). As per the forecasts from Global Business Intelligence Research, the anti-inflammatory drug market grew at a rate of 7.6% to $ 57.8 billion in 2010 and was expected to grow at the rate of 5.8% to generate revenues worth $ 85.9 billion in 2017 (Gaba *et al.*, 2014).

Non-steroidal anti-inflammatory drugs (NSAIDs) are the mainstay in the therapeutic intervention of inflammation and pain. The key mechanism by which NSAIDs exert their anti-inflammatory activity is through inhibition of cyclooxygenase (COX) derived prostaglandin synthesis. However, the long-term use of these drugs are restricted due to gastrointestinal (James and Hawkey, 2003), renal (Schneider *et al.*, 2006), and hepatic side effects (Adebayo and Bjarnason, 2006).

Vane hypothesised that selectively blocking COX-2 can circumvent the side effects of the conventional NSAIDs and at the same time retaining all of the therapeutic effects (Vane, 1994; Vane and Botting, 1995). It led to the development and subsequent introduction of first selective COX-2 inhibitor celecoxib (Celebrex®) in 1998 for use in the treatment of rheumatoid arthritis, osteoarthritis and acute pain followed by rofecoxib (Vioxx®) in 1999. Shortly after their introduction in the market, the preliminary results of a Vioxx gastrointestinal outcomes research (VIGOR) trial was reported in 2000. The report raised concern about the cardiovascular safety of all selective COX-2 inhibitors (Howard and Delafontaine, 2004; Donge *et al.*, 2005; McGettigan and Henry, 2006; Graham, 2006).

Several additional coxibs including valdecoxib (Bextra®), etoricoxib (Arcoxia®), and parecoxib sodium (Dynastat®) were marketed worldwide in 2002. The consequences of potential cardiovascular risk were not seriously considered till 2004 when rofecoxib (Vioxx®) was voluntarily withdrawn worldwide following an additional risk assessment from a three-year randomised, placebo-controlled, double-blind clinical trial (Donge *et al.*, 2005). To date, all but celecoxib have been withdrawn from the worldwide market

depriving medical professionals of a rational choice of pain medications for arthritis patients who are at a higher risk of serious of gastrointestinal complications (Graham, 2006). Therefore, the challenge persists, to address the unmet medical need by developing effective anti-inflammatory agents with enhanced safety profile.

1.3. HISTORICAL BACKGROUND OF NSAID's

The anti-inflammatory and analgesic drugs have way back originated from the use of extracts of plants rich in salicylate content such as the bark of willow tree (*Salix alba* and other members of the Salix species), for the treatment of inflammation, fever and pain (Rainsford, 2004). These procedures which originated from early Chinese, Indian, African and American periods were firstly recorded in detail by Roman and Greek medical authorities.

Following the publication by the Reverend Edward Stone in the 17th century of probably what were the first clinical trials of willow bark extract for the treatment of fever, the popularity of these plant extracts became more evident. It was followed by the isolation of active salicylate components in the early 19th century owing much of its development to the advances made by the German chemical industry. In the mid-late 19th century, ushered the synthesis of salicylic and acetylsalicylic acids, the latter being highly successful commercial entity by Bayer AG known as Aspirin™ over a century ago (Rainsford, 2004).

During the 19th century, simultaneous development of antipyretic/ analgesic agent *viz* antipyrine, aminopyrine, phenacetin also took place. Later the recognition of paracetamol (acetaminophen) as the active metabolite of phenacetin, led to its commercial development as an analgesic and antipyretic agent in the 1950's (Prescott, 2001). Phenacetin fell out of favour around 1980 when it was found to cause renal and urinary tract tumours in experimental animal models (Peters *et al.*, 1999).

The development of the first of the category of non-steroidal anti-inflammatory drugs (NSAIDs) of which aspirin has now become recognised as the predecessor, was phenylbutazone in 1946 (by JR Geigy, Basel, Switzerland).

It was later followed by the advent of indomethacin in the 1960's (by Merck & Co, Rahway, NJ, USA) (Otterness, 1995).

Phenylbutazone, initially employed as a combination with antipyrine, with a belief to improve the actions of the latter, emerged to be a better anti-inflammatory and analgesic agent. It was successfully used for 30 years for the treatment of arthritis and other painful inflammatory conditions. Its popularity was however marred with life-threatening agranulocytosis and bone marrow suppression, upper gastrointestinal ulcers and bleeding and subsequent popularity of more advanced NSAIDs.

Ibuprofen, developed by Boots (UK) in the 1950–1960's after establishing its encouraging safety profile at dose ranges (up to 1200mg daily) for analgesic and antipyretic activity. It was the first NSAID other than aspirin to be approved for non-prescription over-the-counter sale in the UK in 1963, followed by the USA in 1964 and later in many other countries worldwide (Rainsford, 1999a). Post development of ibuprofen, some pharmaceutical companies scouted for the discovery and development of NSAIDs with a range of chemical and biological properties (Evans & Williamson, 1987; Otterness, 1995; Rainsford, 1999b, 2004; 2005). The chemical classifications and structures of these drugs are depicted in **Figure 1.1** and **1.2**, respectively.

Most of these drugs developed in the 1960's were discovered in the pre-prostaglandin era. Their anti-inflammatory, analgesic and antipyretic properties were determined using animal models with some supportive properties being established in some biochemical systems which were also known to be important in inflammation (e.g. mitochondrial oxidative, intermediary and connective tissue collagen and proteoglycan metabolism; stability of albumin; and later oxyradicals).

Based on the early success of phenylbutazone, mefenamic acid, ibuprofen and indomethacin, diclofenac was introduced as Voltaren®- an antirheumatic agent by Ciba-Geigy (now Novartis). It was first synthesised by Alfred Sallmann and Rudolf Pfister in 1973 with an aim to generate NSAID with high efficacy and tolerability (Sallamann, 1986). The structural elements of diclofenac include a phenacetin group, a secondary amino group and a phenyl ring containing

chloride atoms which imparted maximum flexibility to the ring. Experimental and clinical findings of more than 200 analogues have confirmed that diclofenac sodium possessed the most useful pharmacological properties (Sallamann, 1986). Diclofenac sodium was developed on well-founded principles of drug transport, the atomic and spatial structure of the molecule and the electronic structure (Sallamann, 1986). However, diclofenac sodium nearing the end of its useful life, with the cardiovascular issues placing the final nail in its coffin (McGettigan and Henry, 2011).

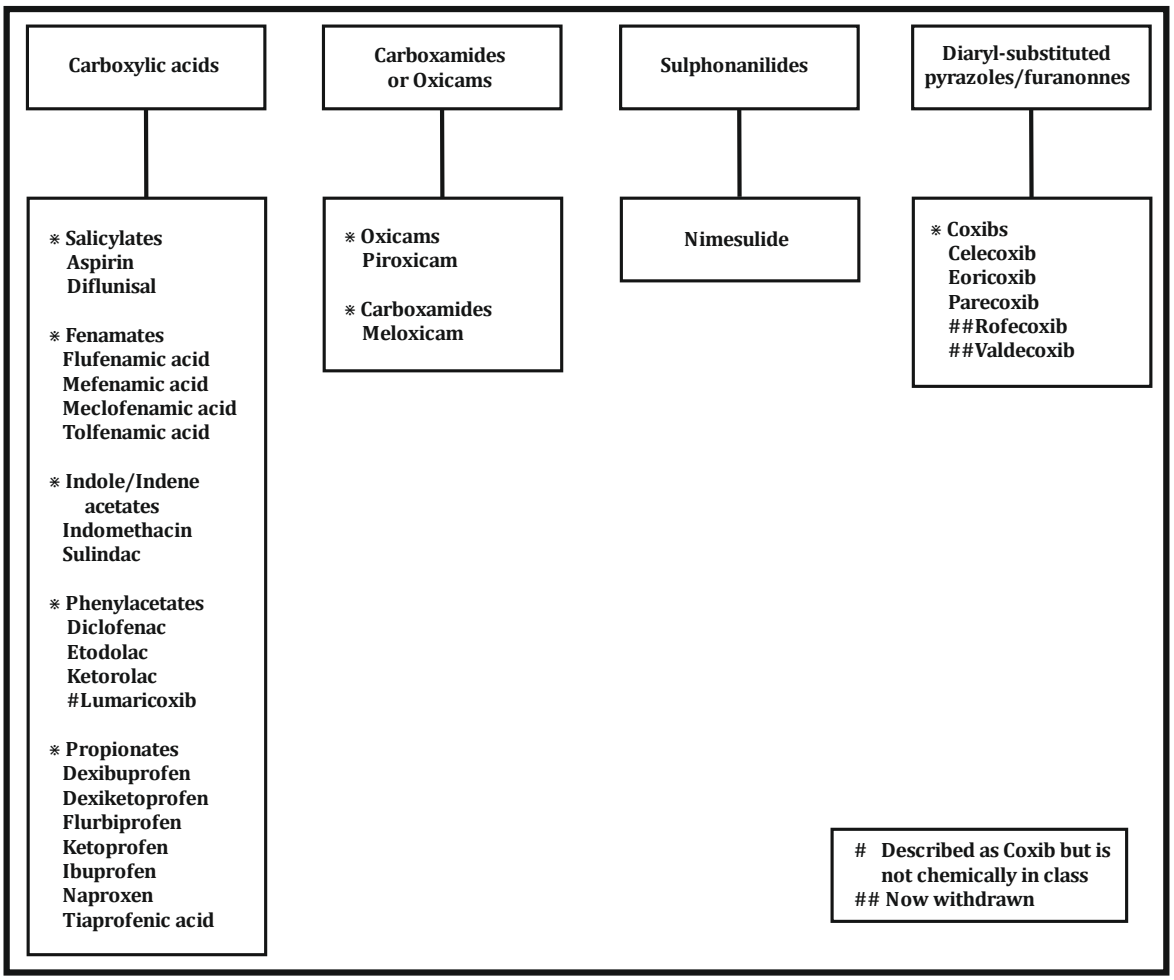

Figure 1.1. Chemical classification of NSAID's.

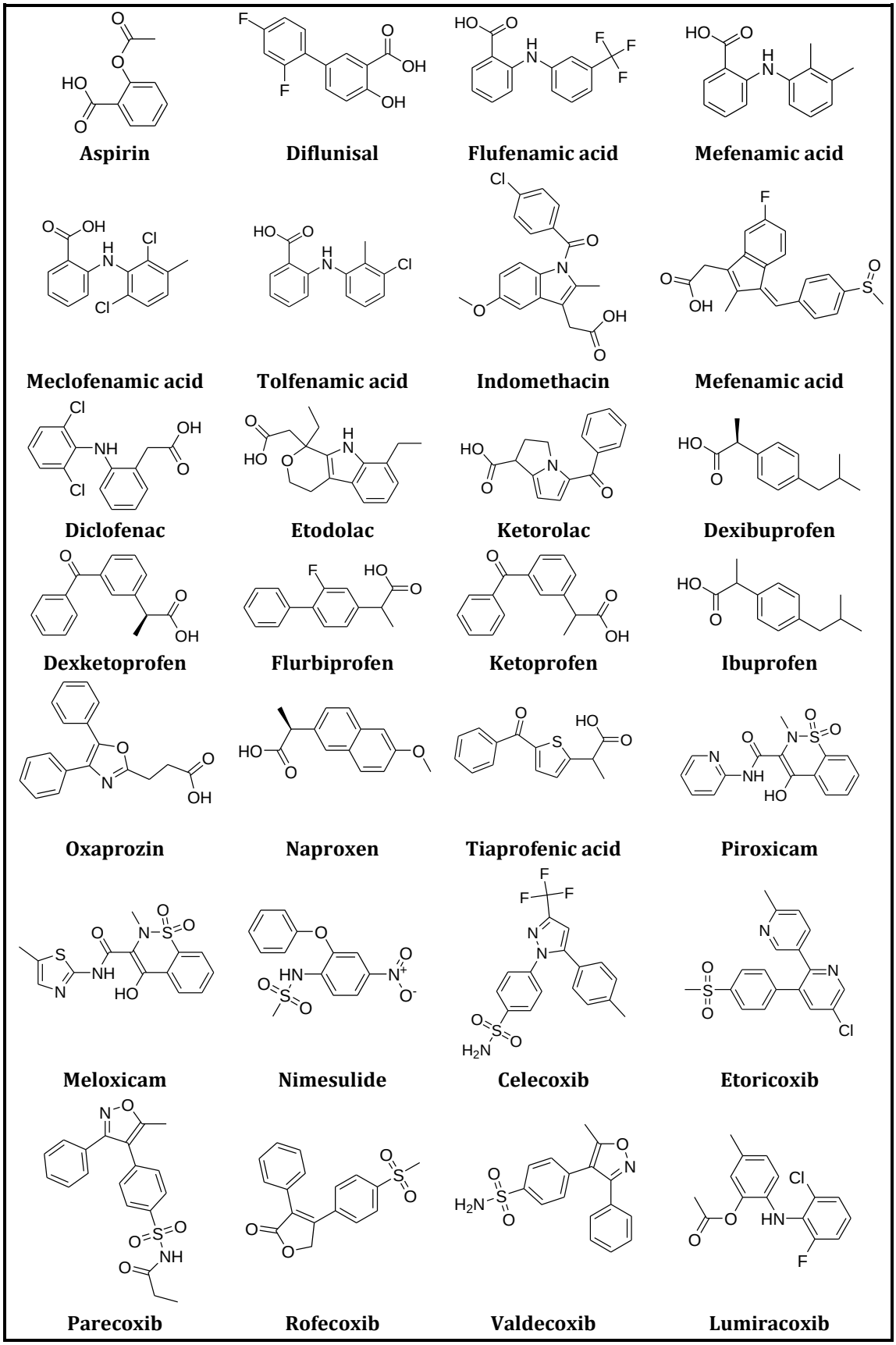

Figure 1.2. Chemical structures of NSAID's.

Piroxicam (Feldene®) was developed as a novel anti-arthritic and anti-inflammatory agent by Pfizer and was launched in 1980. Before the advent of piroxicam drugs such as aspirin, indomethacin, diclofenac and ibuprofen, all belonging from the carboxylic acid class dominated the market. However, members of this chemical class underwent rapid metabolism necessitating multiple doses which lead to poor patient compliance. It also increased the potential for gastrointestinal, hepatic and renal toxicity. This formed the basis of development of piroxicam wherein the introduction of a carboxamide functional group proved to be a cornerstone which was a key factor in increasing anti-inflammatory activity. Piroxicam (Feldene®) provided round-the-clock symptomatic relief from arthritis with just 20mg daily dose making it one of the most successful drugs of the 1980s (Lombardino and Lowe, 2004).

1.4. CYCLOOXYGENASE ISOENZYME

The cyclooxygenase was first identified as the therapeutic target of NSAIDs by Vane in 1971. The anti-inflammatory substances that block the biosynthesis of prostaglandins (PGs) *via* inhibition of COX could contribute to a number of physiological and pathophysiological functions (Vane, 1971).

COX-1 and COX-2 are isoenzymes and COX-2, discovered in the early 1990s was distinct from the first one (Fu *et al.*, 1990). The COX isoenzymes are membrane bound enzymes in the endoplasmic reticulum (ER). Since isoenzymes are genetically independent proteins, the genes in humans responsible for expression of both the enzymes are located on different chromosomes and exhibit different properties (Tazawa *et al.*, 1994). The COX-1 gene is located on chromosome 9, and COX-2 is encoded by a gene on chromosome 1 (Tazawa *et al.*, 1994; Kosaka *et al.*, 1994). Both genes also differ in size; the human COX-1 gene with 22 Kb comprises of 11 exons, whereas the human COX-2 gene contains only ten exons with a relatively small genomic size of 8.3 Kb (Yokoyama and Tanabe, 1989; Kraemer *et al.*, 1992).

COX-1 is constitutively expressed in many tissues and PGs produced by COX-1 facilitates the “housekeeping” functions such as protection of gastric mucosa, platelet aggregation and regulation of renal blood flow. In divergence,

COX-2 remains undetected in most normal tissues. However, it is expressed rapidly when induced by stimuli such as proinflammatory cytokines (IL-1B, TNFα), lipopolysaccharides, mitogens and oncogenes, growth factors (fibroblast growth factor, FGF; platelet-derived growth factor, PDGF; epidermal growth factor, EGF), hormones (luteinizing hormone, LH) and disorders of water-electrolyte homeostasis, resulting in augmented synthesis of prostaglandins (PGs) in inflamed and neoplastic tissues. This inducible isoenzyme (COX-2) has been implicated in pathological processes such as inflammation and several types of cancer (Williams and DuBois, 1996; Konturek *et al.*, 2005).

1.4.1. The Cyclooxygenase Pathway

The biosynthesis of prostaglandins (PGs) and thromboxanes, occurs in three steps: (a) the mobilisation of arachidonic acid (AA) substrate, from membrane phospholipids through the action of a phospholipase A_2; (b) biotransformation of AA by cyclooxygenase in a bifunctional action which leads to the generation of unstable PGG_2 by the cyclooxygenase reaction, and its immediate conversion into PGH_2 by the same enzyme in a peroxidase reaction; (c) the conversion of PGH_2 to specific prostanoids through the action of synthases and specific isomerases (Smith and Song, 2002) (**Fig. 1.3**).

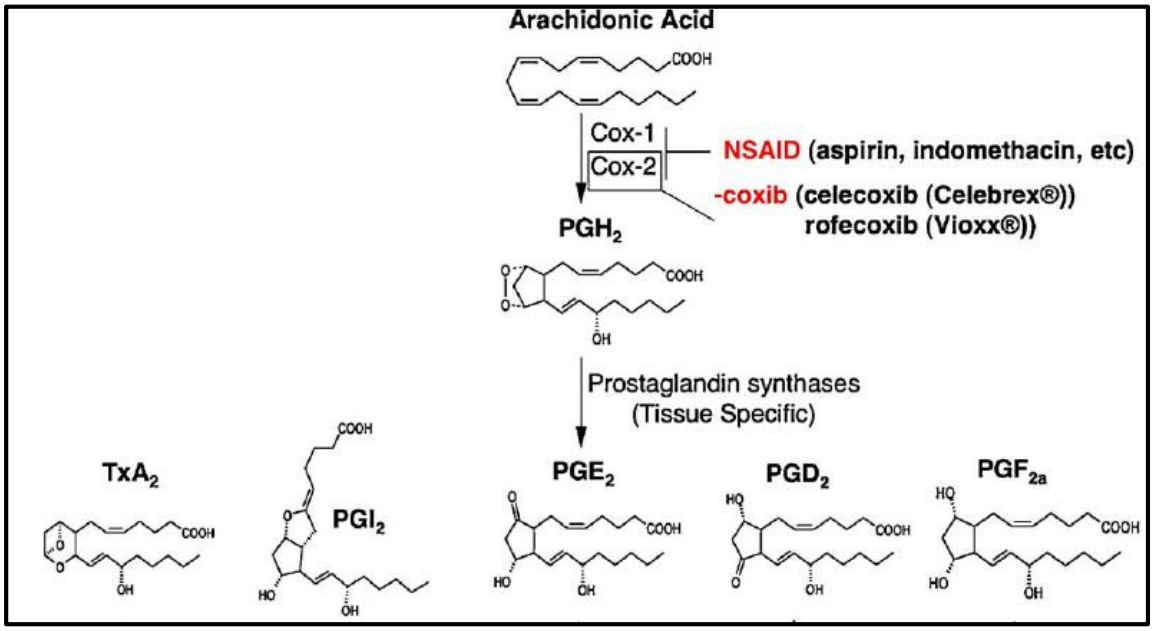

Figure 1.3. The conversion of arachidonic acid to prostaglandins.

NSAID's inhibits the prostaglandin synthesis at the stage of oxidative cyclisation of arachidonic acid (AA), catalysed by the rate limiting enzyme, cyclooxygenase (or PGH synthase), to hydroperoxy-endoperoxide (prostaglandin G_2, PGG_2). It is followed by its subsequent reduction to key intermediate prostaglandin H_2 (PGH_2) needed for all prostaglandin biosynthesis (Vane *et al.*, 1998). Blockage of PGH_2 production halts the further downward cascade where tissue specific terminal PG synthases or isomerases convert PGH_2 into different biologically active PG's including PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2 (prostacyclin), and thromboxane A_2 (TXA_2) (Vane *et al.*, 1998; Charlier and Michaux, 2003).

1.4.2. Enzymatic Structure of Cyclooxygenases

The crystal structures of the COX isoforms are structurally homologous and consistent with a high sequence identity (ca. 60%). The overall structures of COX-1 and COX-2 are highly conserved. The COX monomer comprises of three domains: an *N*-terminal epidermal growth factor (EGF) like domain, a membrane binding domain (MBD) of about 48 amino acids in length which anchors the protein on the lipid bilayer, and a large C-terminal globular catalytic domain with the COX active site which houses the substrate or the inhibitors and the peroxidase one which containing the heme co-factor. Though distinct in their appearance, these sites are functionally and structurally interconnected (Garavito *et al.*, 2002).

Lipophilicity has been proved to be a major physicochemical parameter for effective COX inhibitors (Dannhardt and Kiefer, 2001). The cyclooxygenase active site comprises of a long hydrophobic channel which is the binding site for non-steroidal anti-inflammatory drugs. This active site extends from the membrane binding domain to the core of the catalytic domain (Kurumbail *et al.*, 2001; Picot *et al.*, 1994). The binding site for the arachidonate is located in the upper half of the channel, from Arg 120 to near Tyr 385. Ser 530 occupies a position in the middle of the channel and is the site of acetylation by aspirin (Loll *et al.*, 1995). Three amino acid differences result in a larger (about 20%) and more accessible channel, in COX-2. The interchange of a valine at the position of

523 in COX-2 for a relatively bulky isoleucine (Ile) residue in COX-1 active site causes a structural modification.

This modification in the COX-2 enzyme opens up an additional side pocket, which is a pre-requisite for COX-2 drug selectivity. Entry to this side pocket is constrained in the case of COX-1. Additionally, the exchange of Ile 434 for a valine in COX-2 allows a neighbouring phenylalanine 518 (Phe 518) residue to swing out of the way, thereby further increasing access to the side cavity. One more essential amino acid difference between the two isoforms is prevalent which changes its chemical environment rather than altering the shape of the drug-binding site. Located within the side pocket of COX-2 is an arginine in place of histidine 513 (His 513) in COX-1, which can interact with polar moieties. The abovementioned differences between the COX active sites have significant implications on the selectivity profile of inhibitors (Charlier and Michaux, 2003; Dannhardt and Kiefer, 2001; Kurumbail *et al.*, 1996) (**Fig. 1.4**).

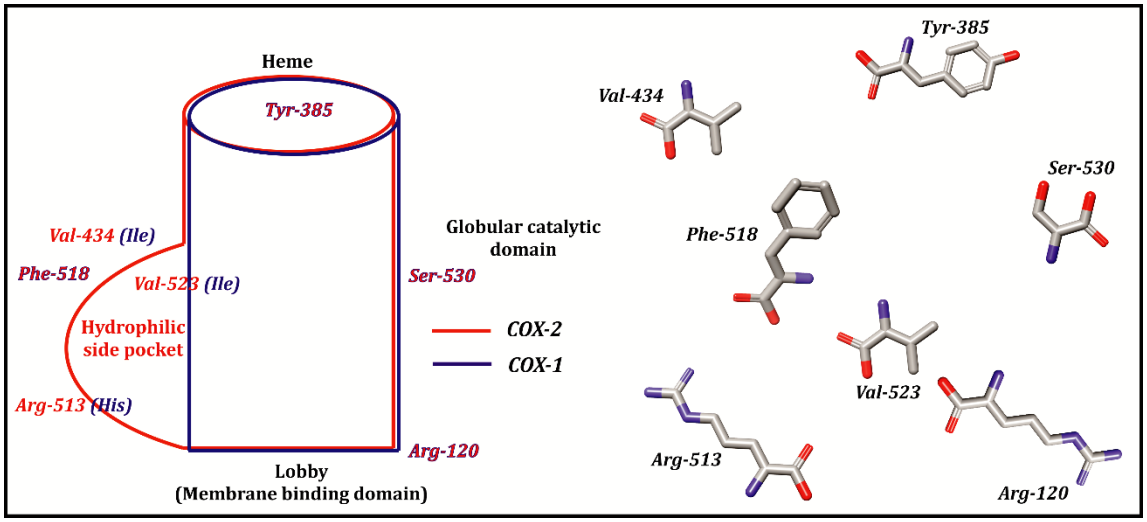

Figure 1.4. Schematic representation of the COX-2 active site.

1.5. HETEROCYCLIC COMPOUNDS AS POTENTIAL NSAID's

Heterocyclic compounds play a major role in the untiring effort to develop new anti-inflammatory and analgesic agents. Various synthetic approaches based on chemical modification have been undertaken with an aim to improve the safety profile of NSAIDs.

Synthesis of nitrogen-containing heterocyclic compounds has been attracting increasing interest because of their utility for targeting various biological receptors with a high degree of binding affinity (Singla *et al.*, 2015). The 1,2,4-triazine nucleus is considered an important chemical synthon exhibiting a broad range of therapeutic activities including COX-2 inhibition (Dadashpour *et al.*, 2015) (Irannejad *et al.*, 2014) and anticancer activities (Yurttaş *et al.*, 2014). An array of established biological activities associated with the 1,2,4-triazine nucleus ensures that the synthesis of novel chemical entities (NCE's) containing this critical ring system remains a topic of current interest (Kumar *et al.*, 2014).

Interest on the 1,2,4-triazine nucleus has stemmed from the fact that apart from azapropazone, which is available only in some parts of Europe, no other NSAID consisting of the central 1,2,4-triazine heterocycle is currently available in the market. Azapropazone (**Fig. 1.5**) now stands discontinued in the British National Formulary (BNF). It is an anti-inflammatory, analgesic, antipyretic and a potent uricosuric agent used in the treatment of rheumatoid arthritis, osteoarthritis, ankylosing spondylitis, and gout. Its use is marred by the gastrointestinal side-effects such as nausea, epigastric pain, and dyspepsia. However, the use of azapropazone is restricted only to cases where other NSAID's have failed (Roberts and Morrow, 2001).

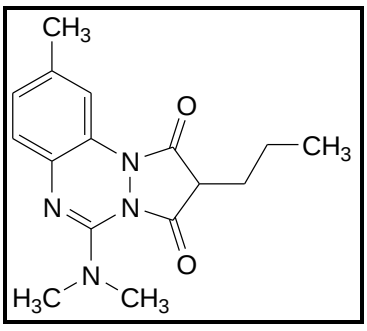

Figure 1.5. The structure of Azapropazone.

1,3,4-oxadiazole is another important stable and neutral heteroaromatic nucleus that is associated with potent pharmacological activity due to the presence of an -N=C-O- linkage (Rigo and Couturier, 1985). Compounds containing 1,3,4-oxadiazole have been marked with exceptional chemical behaviour with a plethora of varied biological activities reported in the literature

(de Oliveira *et al.*, 2012). They have also stirred interest as potential bioisosteres for carboxylic acids, esters, and carboxamides (Boström *et al.*, 2012). Many molecules based upon substituted 1,3,4-oxadiazoles template have been investigated for their COX-2 inhibitory effects (Dekhane *et al.*, 2011; Akhter *et al.*, 2011; Bansal *et al.*, 2014; Grover *et al.*, 2015).

Further, it is reported that the compact and a highly polarizable 1,3,4-thiadiazole mesoionic system can easily permeate through cellular membranes to interact with diverse biological targets with distinct COX-2 inhibitory activity (Song *et al.*, 1999; Gadad *et al.*, 2008; Kumar *et al*. 2010 Li *et al.*, 2013; Shkair *et al.*, 2016). 1,2,4-triazoles and their derivatives are also reported to exhibit an array of potential therapeutic properties (Maddila *et al.*, 2013), and due to the polar nature of triazole ring, it significantly improves the pharmacological profile by improving the solubility of the drug (Kaur *et al.*, 2016). There is an extensive literature on 1,2,4-triazole derivatives reported possessing selective COX-2 inhibitory property (Jiang *et al.*, 2010; Jiang *et al.*, 2014; Cai *et al.*, 2016).

On the basis of above-mentioned outcome and in continuation of research endeavour towards the development of safer anti-inflammatory agents (Banerjee *et al.*, 2011; Kulshreshtha *et al.*, 2014), the present work envisaged the design and synthesis of some new 5,6-diphenyl-1,2,4-triazine-3(2*H*)-ones assembled into a structural hybrid with the 5-substituted 1,3,4-oxadiazole/thiadiazole or 1,2,4-triazole nucleus. The study thus intends to investigate the benefits of such an approach on the anticipated anti-inflammatory and analgesic effects devoid of the undesirable effects associated with traditional NSAIDs.

The outcome of the design approach would be supported by *in vitro* and *in vivo* bioassay models. Further, the relative safety profile of the promising compounds to the standard drugs will be evaluated concerning gastric, hepatic, renal and cardiac parameters. Finally, their consensual binding mode to the COX-2 active site shall be validated by *in silico* docking and binding stability assessed using *in silico* molecular dynamics studies.