Chapter-3

Rationale, Objective, & Plan of Work

3.1 Rationale

Inflammation is a complicated biological mechanism comprising several different kinds of mediators originating from arachidonic acid metabolism. This metabolic pathway has two main branches: the cyclooxygenase (COX) pathway generates prostaglandins (PGs), and the lipoxygenase (LOX) pathway generates leukotrienes (LTs). Nonsteroidal anti-inflammatory drugs (NSAIDs) are non-selective COX inhibitors and selective COX-2 inhibitors (COXIBs) which efficiently suppress PG synthesis by blocking COX enzymes. This approach reduces inflammation and pain that accompanies along with it, but it has substantial disadvantages (Arfeen, Srivastava et al. 2024).

Conventional NSAIDs cause gastrointestinal (GI) adverse effects since they inhibit constitutive COX-1, while selective COX-2 inhibitors raise the risk of cardiovascular disease (CV) regardless of sparing the GI adverse effects. These side effects disturb the delicate equilibrium between thromboxanes and prostacyclins generated from the COX pathway. Furthermore, leukotriene-mediated inflammation remains unregulated since blocking the COX pathway alone overlooks the role of the LOX pathway. In fact, LOX expression can possibly spike in response to COX inhibition, which exacerbates inflammation (Ghosh, Alajbegovic et al. 2015).

Dual COX-2/5-LOX inhibitors offer a rationale to address such issues by inhibiting both pathways of the arachidonic acid mechanism (Fiorucci, Meli et al. 2001). It provides a broader range of anti-inflammatory effects than selective inhibitors since they reduce both PGs and LTs simultaneously. Dual inhibition not only minimizes the synthesis of inflammatory mediators, but it may also prevent the compensatory overexpression of the LOX pathways that is commonly found with COX inhibition. Furthermore, regulating both routes may lessen adverse effects by minimizing dependence on a single pathway of the biochemical cascade.

Dual COX-2/5-LOX inhibitors also have the benefit of potentially contributing in the resolution of inflammation. Although the 5-LOX pathway plays a role in the production of pro-inflammatory leukotrienes, it also produces lipoxins, which are the pro-inflammatory and pro-resolving mediators. Dual inhibitors may offer more comprehensive regulation of both acute and chronic inflammatory disorders by balancing proinflammatory mediator suppression alongside pro-resolving lipoxin production (Pergola and Werz 2010).

The fundamental structure of dual COX-2/5-LOX inhibitors emphasizes on development of hybrid molecules that incorporate pharmacophores that can target both families of enzymes. These novel molecules attempt to specifically inhibit COX-2 to mitigate GI toxicity while also efficiently reducing LOX activity to combat leukotriene-induced inflammation. Such hybrid designs offer the potential for reducing off-target impacts while enhancing overall safety profiles. As a result, dual COX-2/5-LOX inhibitors represent an innovative therapeutic approach to address inflammatory disorders such as arthritis, rheumatoid arthritis, allergies and asthma, and various other chronic forms of inflammation.

3.2.Objectives

The present research attempts to develop dual COX-2/5-LOX inhibitors as promising anti-inflammatory drugs rather than standard NSAIDs and selective COX-2 inhibitors. This entails designing an innovative molecular framework that combines pharmacologically potent ring systems, 1,2,4-triazine/benzhydrylpiperazine, and 1,3,4-oxadiazole, connected by a methylene bridge to increase their flexibility and biological activity. The objective is to develop molecules that specifically target inflammatory mediators associated with the COX-2 and 5-LOX pathways, resulting in

strong anti-inflammatory properties while minimizing the risk of adverse effects related to standard NSAIDs and selective COX-2 inhibitors.

The structure-based drug design (SBDD) approach was utilized to analyse the target enzymes active binding site and amino acid interactions guiding the design of ligands with optimal binding affinity and specificity. This was followed by molecular hybridization, where pharmacophoric features of different bioactive scaffolds were strategically combined into a single hybrid molecule to enhance activity. For instance, a 5,6-diphenyl-1,2,4-triazine core selective for COX-2 was hybridized with 1,3,4-oxadiazole moiety, ensuring synergistic binding in both COX-2/5-LOX active binding pockets. Molecular docking studies were planned to evaluate the binding interactions and efficacy of the designed compounds with the target enzymes, providing insights into their mode of action and guiding structural optimization. These studies were aimed at identifying the crucial interactions, such as hydrogen bonding and hydrophobic forces, that contribute to the binding strength and specificity of the compounds. Based on the molecular docking results, the series of novel heterocyclic compounds synthesis was planned. Furthermore, biological evaluations were planned to assess the pharmacological activity of the promising compounds. This approach is designed to streamline the drug development process, ensuring that only the most rationally designed and potent therapeutic agents progress further in the discovery pipeline.

3.2.1. Designing of Dual COX-2/5-LOX Inhibitors (Part-I)

The development of dual COX-2 and 5-LOX inhibitors with better therapeutic index is still a big challenge to the medicinal chemist for treating inflammatory disorders in various diseases. Recently, 1,2,4-triazine scaffolds have been utilized to design dual COX and LOX inhibitors, and a few molecules containing 1,2,4-triazine scaffold are also in the final stages of clinical investigations due to their enhanced therapeutic efficacy and

pharmacological properties. Diphenyl structural frameworks like selective COX-2 inhibitors, e.g., celecoxib, and the presence of 1,2,4-triazine scaffold in azapropazone drug as available in some European countries have suggested that these scaffolds can be further utilized.

Molecular docking and complex studies utilizing computer-aided drug design (CADD) have evidenced that the 5, 6-diphenyl nucleus facilitates alignment of 1,2,4-triazine within the COX-2 enzymes binding site. The 1,3,4–oxadiazole is another scaffold that confers promising pharmacological potential. The molecule siteleuton, which is under clinical trials as a 5-LOX inhibitor for treating inflammatory disorders including cancer, contains a substituted oxadiazole moiety(Sinha, Manju et al. 2019). Furthermore, Zibotentan is another drug containing 1,3,4-oxadiazole and is under clinical trials as a 5-LOX enzyme inhibitor to treat cancer. It has also been reported that oxadiazole linked to phenyl ring substituted with various electron-withdrawing groups (EWGs) and electron-donating groups (EDGs) influence enzyme inhibitory activity.

Our research group has already reported 5,6-diphenyl-1,2,4-triazin-3(2H)-one derivatives as selective COX-2 inhibitory agents (A, B and C depicted in Figure 3.1). Moreover, recently, the 5,6-diphenyl-1,2,4-triazin-3(2H)-one derivative has been granted a US patent for treating cancer and inflammatory conditions by modulating inflammatory cytokines (Srivastava, Shankar et al. 2021). Based on these promising outcomes and continued efforts to develop novel dual COX-2 and 5-LOX inhibitors, we selected marketed as well as drugs under clinical investigations to design novel hybrids Figure 3.1 as COX-2 and 5-LOX enzyme inhibitors with enhance pharmacological efficacy and also to subside the adverse effects of the existing drug molecules.

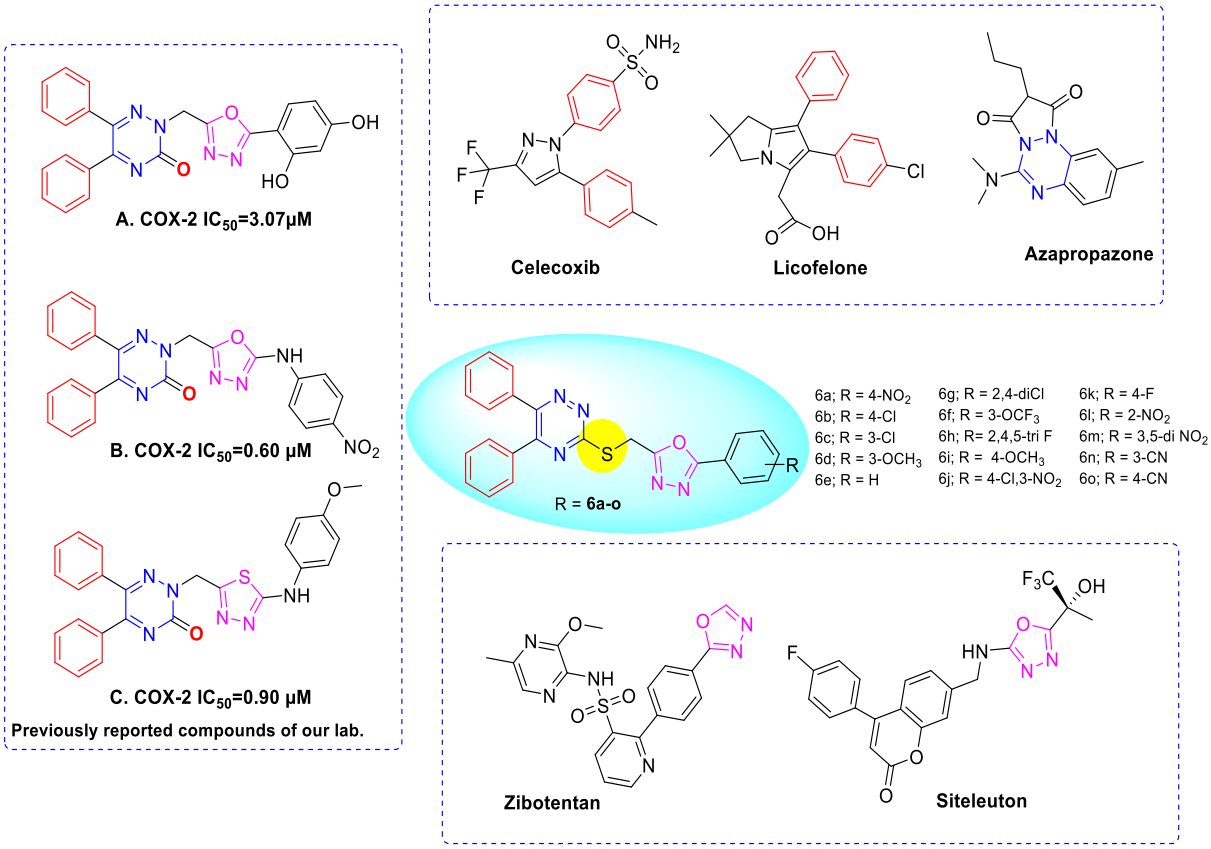

Figure 3.1. Design of novel derivatives as dual COX-2/5-LOX inhibitors.

In the present work, the novel series of compounds was designed by replacing the divalent oxygen (O) of 5,6-diphenyl-1,2,4-triazin-3(2H)-one with a sulfur (S) to convert it into 5,6-diphenyl-1,2,4-triazine-3-thiol considering it as bioisostere with an assumption that it would provide a defense mechanism against reactive oxygen species (ROS) as well as pathologic conditions caused by oxidative stress. The sulphur-methylene bridge aims to offer flexibility in the molecular structure when coupled with the 2-(chloromethyl)-5-phenyl-1,3,4-oxadiazole ring via nucleophilic substitution reaction to obtain the final series of compounds, allowing the molecule to bend or expand to occupy the COX-2 active pocket as well as 5-LOX enzymatic cleft. The substitution of the phenyl ring of 2-(chloromethyl)-5-phenyl-1,3,4-oxadiazole with various EWGs and EDGs along with an unsubstituted ring was planned to study the effects of electronic distribution around the ring on its inhibitory potential.

3.2.2. Designing of Dual COX-2/5-LOX Inhibitors (Part-II)

The piperazine framework represents a vital core having a medicinal significance which has shown potential for drug discovery and development of novel lead compounds (Jain, Chaudhary et al. 2020, Sharma, Wakode et al. 2020). Piperazine framework has been reported to aid in the improvement of anti-inflammatory activity. Furthermore, several investigations have identified similarities between NSAIDS and benzhydrylpiperazine containing antihistamines (e.g. cetirizine, hydroxyzine) that might be explored to develop novel molecular hybrids with promising pharmacological properties. Based on these presumptions, initially, we have performed the virtual screening (VS) of all piperazine-containing molecules obtained from the ChEMBL database against COX-2 enzymes.

The structure-based drug design approach was utilized and 59,090 piperazine-containing ligands from the ChEMBL database (https://www.ebi.ac.uk/chembl/, accessed on April 2022) were screened using the crystal structure of COX-2 (PDB code: 3LN1). The identified hit was employed to rationally design novel multifunctional anti-inflammatory compounds. The top two hits; ChEMBL342253 and ChEMBL4794855 displayed the common presence of benzhydrylpiperazine and displayed favourable docking results for the COX-2 enzyme, but, they did not demonstrate potential binding against the 5-LOX enzyme. They failed to exhibit acceptable molecular stability in dynamic simulation studies.

The molecular hybridization approach was further employed to develop multifunctional ligands with improved molecular binding and stability against both COX-2 and 5-LOX enzymes by incorporating the pharmacophoric features of some known compounds, including hit molecules. The biphenyl rings are usually evident in coxibs, such as celecoxib, as they are necessary to bind the active COX-2 pocket. This characteristic is also evident in licofelone, a dual COX/LOX inhibitor. The hit compounds from the

ChEMBL database shared similarities with the scaffold found in licofelone and antihistamines, such as levocetirizine and hydroxyzine. Thus, considering several therapeutic benefits of the scaffold, it is retained in the designed molecules.

Subsequently, 1,3,4-oxadiazole was selected as an auxiliary scaffold considering its multifunctional potential. We identified dual COX-2/5-LOX inhibitory potential in recently developed molecular hybrids containing 1,3,4-oxadiazole (A) (Saraf, Tripathi et al. 2022). Also, the experimental drug zibotentan, containing 1,3,4-oxadiazole is under investigation for its anti-cancer activity. Based on the aforementioned information, a novel series of molecular hybrids has been developed (Figure 3.2), and their molecular binding and stability were confirmed through *in-silico* studies.

The envisioned compounds were planned to be synthesized through a series of well-planned chemical reactions, followed by thorough characterization using techniques such as NMR, HPLC, and mass spectrometry to confirm their structure and purity. The synthesized compounds were aimed to be evaluated to assess their efficacy in inhibiting inflammatory mediators, specifically targeting both COX-2 and 5-LOX enzymes. In addition to their biological activity, the study may provide valuable insights into the structure-activity relationships of the compounds, identifying key structural features responsible for their inhibitory action. These findings offer a solid foundation for further optimization, guiding modifications to enhance potency, selectivity, and drug-like properties, and ultimately paving the way for the development of more effective anti-inflammatory therapies.

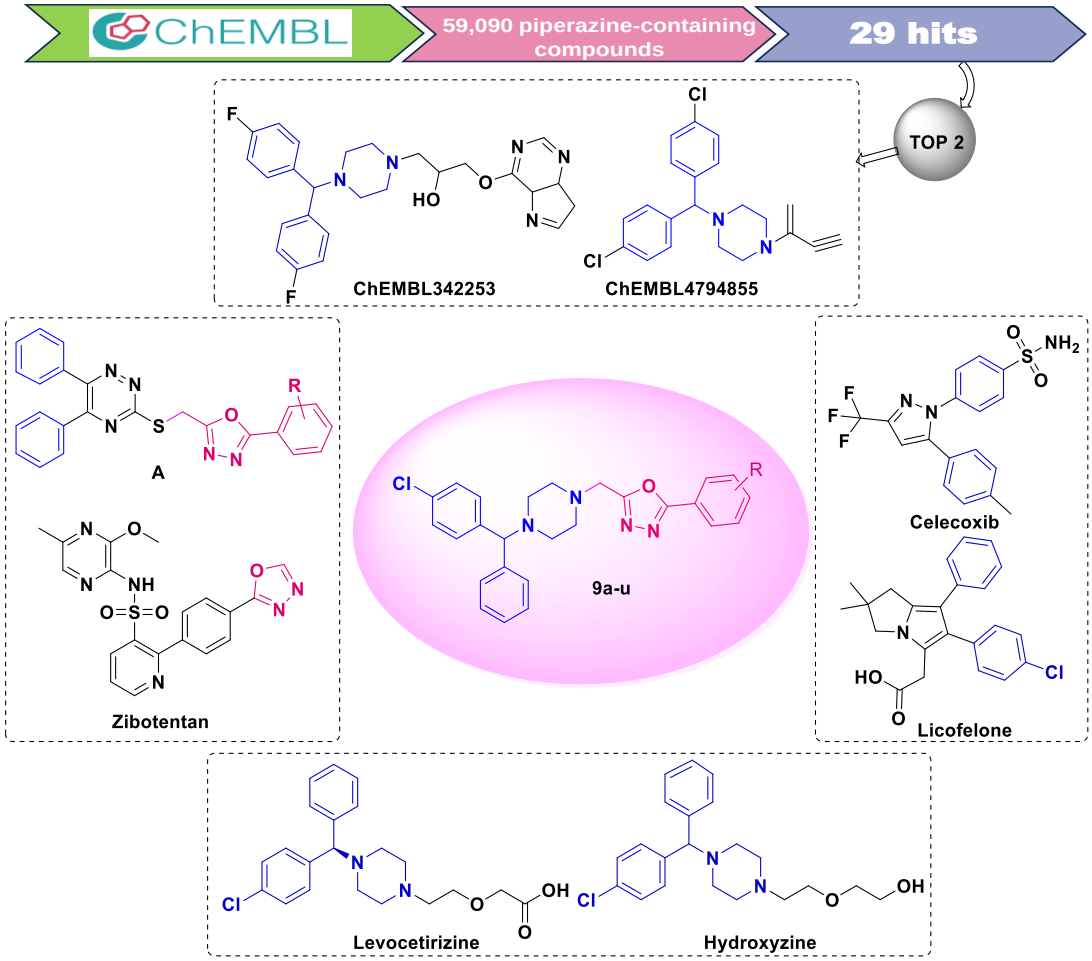

Figure 3.2. Design strategy for novel benzhydrylpiperazine-based molecular hybrids.

3.3. Plan of Work

3.3.1. In-silico optimization studies

- Protein Preparation
- Grid generation
- Structure-based virtual screening workflow (HTVS and VSW)
- Docking-post processing (DPP) and pose filtration
- Molecular Docking Studies
- Molecular Dynamics and simulation
- *In-silico* drug likeliness

3.3.2. Synthesis of novel heterocyclic compounds

- **Series-I:** 5,6-diphenyl-1,2,4-triazine-3-thiol derivatives
- **Series-II:** Benzhydrylpiperazine with substituted phenyl oxadiazole derivatives

3.3.3. Characterization of synthesized compounds

- Physicochemical characterization including melting point and R_f using TLC
- Estimation of % purity by HPLC
- Structural characterization using state of art techniques ^{1}H NMR, ^{13}C NMR, FT-IR and Mass spectrometry.

3.3.4. In vitro evaluation

- IC_{50} Determination

3.3.5. In-Vivo Evaluation

- Acute oral toxicity
- Carrageenan-induced rat paw edema model
- Effect on prostaglandin E_2 (PGE_2)
- Effect on cytokines levels
- Arachidonic acid-induced rat paw edema model
- Ulcerogenic risk assessment
- Biochemical analysis
- Assessment of liver and kidney functions
- Effect on platelet aggregation
- Assessment of cardiotoxic liability
- Assessment of analgesic activity

3.3.6. Anti-cancer activity in human cell lines

- *In vitro* anti-cancer activity

3.3.7. Anti-cancer activity in in vivo Drosophila cancer model

- Toxicity assay
- Therapeutic experiment

3.3.8. Pharmacokinetics studies

3.4. Significance of the studies

The significance of the study lies in its potential to contribute to the development of novel, selective, and potent therapeutic agents targeting the COX-2/5-LOX enzymes involved in the inflammatory process associated with various diseases such as arthritis, CVDs, and cancer. By employing SBDD and molecular hybridization, this research aims to develop compounds that specifically inhibit COX-2/5-LOX while minimizing off-target effects. The molecular docking studies provide valuable insights into the binding interactions between the designed compounds and the enzyme, helping to optimize their structure for enhanced binding affinity and specificity. This approach leads to the development of safer, more effective anti-inflammatory drugs, streamlining the drug discovery process and offering newer and potential treatments for inflammatory diseases.