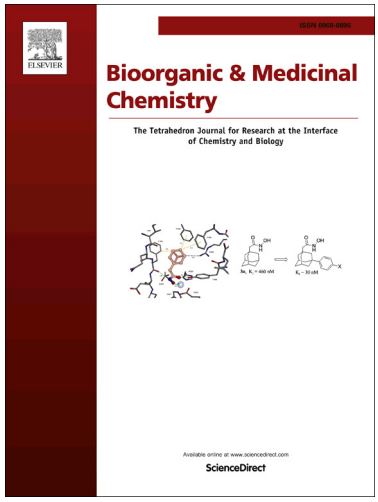

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Structure Based Library Design (SBLD) for New 1,4-dihydropyrimidine Scaffold as simultaneous COX-1/COX-2 and 5-LOX Inhibitors

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Abstract:

The various scaffolds containing 1,4-dihydropyrimidine ring were designed by considering the environment of the active site of COX-1/COX-2 and 5-LOX enzymes. The structure-based library design approach, including the focused library design (virtual combinatorial library design) and virtual screening was used to select the 1,4-dihydropyrimidine scaffold for simultaneous inhibition of both enzyme pathways (COX-1/COX-2 and 5-LOX). The virtual library on each 1,4-dihydropyrimidine scaffold was enumerated in two alternative ways. In first way, the chemical reagents at R groups were filtered by docking of scaffold with single position substitution, i.e. only at R_1, or R_2, or R_3….R_n on COX-2 enzyme using Glide XP docking mode. The structures that do not dock well were removed and the library was enumerated with filtered chemical reagents. In second alternative way, the single position docking stage was bypassed, and the entire library was enumerated using all chemical reagents by docking on the COX-2 enzyme. The entire library of approximately 15,629 compounds obtained from both ways after screening for drug like properties, were further screened for their binding affinity against COX-1 and 5-LOX enzymes using Virtual Screening Workflow. Finally, 142 hits were obtained and divided into two groups based on their binding affinity for COX-1/COX-2 and for both enzyme pathways (COX-1/COX-2 and 5-LOX). The ten molecules were selected, synthesized and evaluated for their COX-1, COX-2 and 5-LOX inhibiting activity.

Keywords: COX-2, COX-1, 5-LOX, Structure Based Library Design (SBLD), Virtual Combinatorial Library Design, Virtual Screening, Docking Study, 1,4-dihydropyrimidine

1. Introduction:

Non-steroidal Anti-inflammatory Drugs (NSAID's) are the most popular and well recognized drug for the treatment against inflammatory diseases.[1] These drugs prevent the metabolism of arachidonic acid to prostaglandins (PGs) by binding and inhibiting Cyclooxygenase (COX) enzymes and hence exert their therapeutic effects.[2] The two distinct isoforms (COX-1 and COX-2) of COX enzyme discovered in 1990, which led to the discovery of a new class of NSAID's, selective for COX-2, name coxibs.[3] COX-1 is constitutive and responsible for the biosynthesis of PGs, which protects the stomach lining from the secreted acid and maintains blood flow in gastric mucosa. The other isoform, COX-2 is triggered only in proinflmmatory conditions. Thus the long term use of traditional NSAID's cause adverse gastrointestinal effects, which are overcome by the use of selective COX-2 inhibitors.[4] Unfortunately, the withdrawal of rofecoxib from the market in the fall of the year 2004 after its clinical report of its linkage to increased risk of cardiovascular toxicity has widely affected the safety of coxib[5-6]. Studies suggest that selective COX-2 Inhibitors also cause GI injury like conventional NSAIDs and increased systemic blood pressure and thus have severe side effects on the cardiovascular system and promote thrombogenesis[7].

Arachidonic acid, but also be metabolized through lipoxygenase (LOX) pathway, leading to the production of leukotrienes (LTs), which are potent inflammatory mediators. Therefore, it has been shown that selective COX-2 inhibitors, beside of preventing side effect NSAIDs associated with inhibition of COX-1, lead to upregulation of arachidonic acid metabolism by the 5-LOX enzyme.[8, 9] This result in the increased levels of LTB_4 in the gastric mucosa of NSAIDs and selective COX-2 inhibitors treated patients. The high level of LTs may also contribute to the development of atherosclerosis, myocardial infarction. It is revealed from literature that

simultaneous inhibition of both enzymatic pathways (COX-1/COX-2 and 5-LOX enzymes) would possibly exhibit enhanced anti-inflammatory potency along with reduced GI tract damage and other inflammatory side effects.[10-12] Thus licofelone, a competitive inhibitor of COX-1, COX-2 and 5-LOX, is currently in clinical development for the treatment of osteoarthritis and is expected to possess clinical advantages over the conventional NSAIDs and selective COX-2 inhibitors with a safer GI profile.[13, 14] It is also noted from literature that the inhibition of more than one enzyme involved in different pathways of arachidonic acid metabolism lead to a synergistic anti-inflammatory effect with enhanced spectrum of activity.[15-17]

The virtual combinatorial library is generated routinely for small molecules during the drug discovery cycle and screened based on their predicted physicochemical properties, activities, specificities, etc.[18-20] The virtual screening is useful in selecting a smaller set of promising hits from a large set of compound libraries. The one of the major advantages of generating virtual combinatorial library is that one can utilize cost effective computational techniques to identify a diverse set of compounds with desired physicochemical properties and specificities.

Recently, we reported the design and synthesis of COX-2 inhibitors having scaffold containing dihydropyrimidine nucleus.[21-22] With the help of Literature data of COX-1/COX-2 and 5-LOX inhibitors and focused virtual combinatorial library design approach, we herein tried to design new dual enzyme pathways inhibitors by modifying/changing substitution pattern around 1,4-dihydropyrimidine scaffold. The **Fig. 1** showed the steps which we carried out for developing the new dual inhibiting compounds. The designed library was primarily screened by predicting ADME properties and then virtually screened for dual inhibition of COX-1/COX-2

and 5-LOX enzymes. Some of the hits were then selected and experimentally evaluated for COX-1, COX-2 and 5-LOX enzymes.

2. Result and Discussion:

2.1. Design of Scaffolds for Virtual Combinatorial Library Design:

The search for COX selective inhibitors is somewhat complicated by the close structural similarities between COX-1 and COX-2. Both COX-1 and COX-2 are homodimers of 70 kDa subunits composed of N-terminal epidermal growth factor like module, α-helical membrane binding domain and a large C-terminal globular catalytic domain with active site (Cyclooxygenase and peroxidase containing heme cofactor)[23]. COX catalytic domain in COX-2 is 20% larger and differs in the presence of side pocket when compared to COX-1. The main and important difference in side pocket is the presence of valine in COX-2 and Isoleucine in COX-1 at position 523 which enables interaction of the sulfonamide or a sulfate group of the coxibs with Arg 513 in the side pocket of COX-2.

The 5-LOX is iron containing non-heme enzymes which catalyze the dioxygenation of archidonic acid to lipid hydroperoxide, leukotrienes A_4 (LTA_4). The 5-LOX enzyme resides in the reduced inactive state (FE^{2+}) and need to be in a oxidized active state (FE^{3+}) for performing peroxidising activity[23]. The direct approaches for the inhibition of 5-LOX enzyme involve redox inhibitor or antioxidant, iron-chelating agents and non-redox competitive inhibitors. We mainly focused on the class of iron-chelating agents which include the compounds containing hydroxamic acid, *N*-hydroxyurea, carboxylate or ester groups, as powerful metal ligating groups.

Therefore, in an attempt to design new, potent and balanced dual inhibitors, we made the structural modifications to our previously reported selective COX-2 inhibitors,

dihydropyrimidine analogues[21-22] by substituting with the hydroxamic acid, N-hydroxyurea, carboxylate or ester groups. The ten new scaffolds (core structures) were designed and various reagents/building blocks at predefined connections (R groups) for each core structure were selected based on synthetic feasibility, strategy of maintaining the tricyclic (butterfly or 'V' shaped) pattern of COX-2 inhibitors, while the iron chelating groups were added to the side chain of compounds for 5-LOX activity **(Fig. 2).**

2.2. Virtual Combinatorial Library Design (VCLD):

The Virtual Combinatorial Library Design (VCLD) is regarded as a virtual reaction library, within which reagents (building blocks) undergo specific reaction and product is generated out as the result of all possible combinations of reagents. The concept of *in-silico* combinatorial chemistry of CombiGlide was used here to design focused combinatorial libraries for the inhibition of COX-2 enzyme. The Virtual combinatorial Screening Workflow of CombiGlide holds the core structure constant and changing the side chain composition.

The structure of 1,4-dihydropyrimidine template along with the predefined connection site (R_1, R_2, R_3, R_4,…R_n) are presented in **Fig. 2**. The reagents/fragment structures that used for the construction of virtual 1,4-dihydropyrimidine library were selected from building block chemical structures available at commercial vender databases (**Table S1, Supplementary material)**. Before enumerating the virtual library, all reagents were prepared with respect to their functional group for the identification of bond, which is then replaced when the reagent is added to core structure. Likewise, the attachment position was identified for each side chain site (connection site) in each core structure by identifying the bond that defines, where the

substitution will take place. Therefore the virtual library was enumerated by replacing selected side chain in the core with the other side chain from the reagents.

Each designed 1,4-dihydropyrimidine scaffold (various core) that is without substitution at R groups were first docked on the COX-2 enzyme. The resulting poses were clustered, and poses from each cluster were selected. This ensures the widest diversity of core poses while limiting their number. The core poses were then used as the initial positions of the substituted molecules in the subsequent docking stages. The virtual library was then enumerated in two alternative ways. In the first way, the fragment structures at R groups were filtered by docking of each core structure with single position substitution, i.e. only at R_1, R_2, R_3, R_4…R_n on COX-2 enzyme, using Glide XP docking mode. These structures are generated by adding the reagents, one at a time, to the core structure for each scaffolds and a "single position library" was generated for each attachment position with a side chain at that position and the core structures at the other positions. In this single-position docking, a selection algorithm was used to determine or predict the fully substituted cores having the highest probability of binding to the receptor. The selection algorithm takes into consideration the XP GlideScores of the docked poses from the single-position docking. It also checks that the side chains it has identified for a particular fully substituted core are not predicted to compete for the same part of the receptor binding site. The structures that do not docked well were removed from the reagent list for the appropriate attachment position and the library was enumerated for each core with filtered fragment structures that are identified by the selection algorithm.

In a second alternative way, the single position docking stage was bypassed, and the entire library was enumerated using all fragment structures at all R positions of core structures by docking of each generated on the COX-2 enzyme.

The libraries generated by both ways, before docking studies were pre-filtered for drug like properties using Lipinski's rule of five. The tool QikProp v3.9 was used to generate ADME properties of each compound within each library. The properties like molecular weight (> 500), logp (>5), hydrogen bond donor (>5), hydrogen bond acceptor (>10) and rotatable bond (>10) were set to filter the compounds in each library. The **Fig.3** shows the number of compounds generated and screened by ADME and docking by both ways of combinatorial library enumeration process. The entire library of roughly 15,629 compounds having good binding affinity for COX-2 enzyme, obtained from both ways of combinatorial library enumeration, was further screened for specifying their binding affinity against COX-1and 5-LOX enzymes using Virtual Screening Workflow.

2.3. Virtual Screening Workflow:

Virtual screening is the most efficient way of identifying small-molecule inhibitors from a large drug-like database. The entire library of compounds obtained from VCLD was first screened by removing the ligands with reactive functional groups and then for their potential affinity against COX-1 and 5-LOX enzymes using Virtual Screening Workflow. For the selection of dual inhibitors, the hit structures were simultaneously docked on both enzymes (COX-1and 5-LOX) using three different precisions (HTVS, SP and XP) of Glide docking. The retained compounds after docking through one precision were further docked through another higher precision for accuracy and further screening of compounds. The retention percentage was set for HTVS, SP and XP to keep the 50%, 20% and 10 % of compounds after docking. The compounds retained for COX-1 and 5-LOX after all Glide docking levels were 1614 and 454 (HTVS docking), 322 and 90 (SP docking), 32 and 9 (XP docking) respectively. In last, all the structures retained from

SP and XP docking studies for both COX-1 and 5-LOX enzyme were combined, merged for duplicates and cross docking was done against all three enzymes (COX-1, COX-2 and 5-LOX) for selecting compounds with dual binding affinity. Finally 142 hits were obtained and divided into two groups based on their binding affinity for COX-1/COX-2 and for both enzyme pathways (COX-1/COX-2 and 5-LOX).

Table 1 and Table 2 shows the structure of compounds selected for experimental studies and their corresponding docking scores respectively. These 10 compounds were selected based on their potential binding affinity for both enzyme pathways (COX-1/COX-2 and 5-LOX enzymes), similar binding pose as that of standard and distance between metal ligating groups of compounds and iron in the active site of 5-LOX enzyme.

2.4. Synthesis of Compounds:

The synthetic routes of selected, designed, final compounds are outlined in **Scheme 1** and **Scheme 2**. The final compounds were synthesized in major two or three steps. The compounds **1a-7a** were synthesized using one pot Biginelli reaction of substituted acetophenones, substituted aldehyde and thiourea in the presence of sodium t-butoxide and ethanol as solvent. Condensation of ethyl 3-oxo-3-phenylpropanoate /ethyl 4,4,4-trifluoro-3-oxobutanoate with thiourea in the presence of sodium ethoxide in boiling ethanol gave Compounds **8a-10a**. The compounds **1b-10b** were then synthesized by the reaction of compounds **1a-10a** and ethyl chloroacetate/Ethyl 3-bromopropionate which undergone S-alkylation in the presence of triethylamine and dimethylformamide. The compounds **1c, 2c-4c** and **7c-c9** were then synthesized by reaction of compounds **1a, 2b-4** and **7b-9b** with hydroxylamine hydrochloride in methanol:dichloromethane (3:1) in the presence of sodium hydroxide.

2.5. *In-vitro* COX-2 and 5-LOX inhibiting activity:

All 10 compounds were assayed *in-vitro* for their inhibitory activity against COX-1, COX-2 and 5-LOX enzymes. This initial *in-vitro* assay was done with a single concentration (µM) equivalent to 100 µg/ml, for all 10 compounds. The eight compounds except **4c** and **9c**, nine compounds except **9c** and nine compounds except **6b** showed some inhibitory activity with 3.97-88.43%, 0.33-35.2% and 0.5-51.84% inhibition range respectively against COX-1, COX-2 and 5-LOX enzymes respectively **(Table 3)**. The compound **2b** was found to be potent and inhibited all three enzymes activity COX-1, COX-2 and 5-LOX with inhibitory activity of 70.08%, 35.12% and 51.84% respectively. This compound was further assayed with varying concentrations to determine their IC_{50} against COX-1 and 5-LOX, showing the IC_{50} of 254.82µM and 19.12µM respectively. The same *in-vitro* activity protocol for determining IC_{50} was also performed for compounds showing inhibition of activity of enzymes more than 50% and we obtained IC_{50} of 22.57µM, 18.41µM, 259.68µM and 31.54µM against COX-1 enzyme for compounds **1c, 5b, 8c** and **10b** respectively. The *in-vitro* data showed that the predictions from the virtual screening were not perfect, but it did lead to the identification of the new hits with inhibitory activity against all three enzymes and prepared a base for design of new compounds for simultaneous inhibition of both enzyme pathways.

2.6. Analysis of Binding Mode:

Based on the identification of the new hits with respect to *in-vitro* activity, we further analyzed detailed binding mode of compound **2b** in the active site of all the three enzymes **(Fig. 4, Fig. 5 & Fig.6)**. The binding mode of compound **2b** was compared with that of corresponding reference standards for all three enzymes.

It is seen from **Fig. 4** that the compound **2b** exhibit the similar binding mode as that of standard celecoxib and formed 'V' shaped binding pose in the active site of the COX-2 enzyme. The overlapping of compound **2b** and celecoxib in the active site of the COX-2 enzyme showed that $–CF_3$ group and phenyl ring with $-CH_3$ group of celecoxib is overlapped by phenyl rings of compound **2b**. However, phenyl ring with $-SOCH_3$ group of celecoxib is overlapped by aliphatic chain containing $-COOC_2H_5$ group of compound **2b**. Hence, it is revealed that two phenyl rings of compound **2b** are positioned at the same hydrophobic region where $–CF_3$ and phenyl ring of celecoxib bind. Whereas $-COOC_2H_5$ group of compound **2b** bind at the electrostatic region as similar to $-SOCH_3$ group of celecoxib and form the H-bond with Arg 513. The middle core ring 1,4-dihydropyrimidine of compound **2b** overlapped and form H-bond with Tyr 355 as that by imidazole of celecoxib.

Similarly, the binding pose of compound **2b** is visualized and compared with that of standard atreleuton and tepoxalin in the active site of 5-LOX enzyme (**Fig. 5**). It was noted that both phenyl rings and the middle core ring of all three compounds atreleuton, tepoxalin and compound **2b** orient toward same region and exhibit hydrophobic interaction with surrounding active site amino acids Phe 177, Trp 147, Leu 141, Ala 410 and Phe 151, in the hydrophobic region of 5-LOX. However, the side chain containing metal binding group of all three compounds posed region near to Fe^{2+} and established interactions with it through either carbonyl or hydroxamte group.

We focused our interest primarily for the design of dual inhibitors of COX-2 and 5-LOX enzymes, but as compound 2b showed higher in-vitro inhibiting activity and less virtual binding affinity toward COX-1 enzyme. Therefore, we analyzed and compared binding pose of compound **2b** with that of standard ibuprofen and compound **10b** which showed a higher binding

affinity and comparable *in-vitro* activity (**Fig. 6)**. It was observed that compound **10b** bind and pose similar to ibuprofen. Both these compounds exhibit strong electrostatic interaction with Arg 120. However, compound **2b** bind at a region in the active site of the COX-1 enzyme, slightly away from the region where ibuprofen bind and fit into the pocket by establishing electrostatic interaction with Arg 83. Due to this, compound **2b** showed less docking score as compared to that of compound **10b**. However, it is seen that the presence of carbonyl or hydroxamte group in the side chain of all compounds, forming strong electrostatic interaction, may be the reason for *in-vitro* COX-1 inhibiting activity.

3. Conclusion:

The structure-based library design approach, including the focused library design (virtual combinatorial library design) and virtual screening was applied to identify simultaneous dual inhibitors of COX-1/COX-2 and 5-LOX enzymes. The ten 1,4-dihydropyrimidine scaffold were designed based on substitution pattern, about 15,629 compounds after the enumeration of combinatorial library were generated and finally 142 hits with dual inhibiting activity were obtained. The combined computational and observational studies have led to identification new of hit, compound **2b** with inhibitory activity against all three enzymes. The experimental outcome and analysis of the binding mode of compounds revealed that for the good dual enzyme pathways (COX-1/COX-2 and 5-LOX) inhibiting activity, the compound must have 'V' shaped structure to bind perfectly in the active site of the COX-2 enzyme, a metal binding group that binds to Fe^{2+} in the active site of 5-LOX enzyme and must have an electronegative group to bind electropositive amino acid in the active site of the COX-1 enzyme. So the overall studies reveal

the valuable clues for future rational design of new, more potent dual inhibitors of COX-1/COX-2 and 5-LOX enzymes starting from the identified new hit.

4. Experimental Studies:

4.1. Computational Methods:

The process of virtual combinatorial library design was carried out using CombiGlide v3.2 (Schrödinger, LLC, New York, NY, 2014). The fragment structures used for building library compounds were extracted from reagents structures, selected from Sigma Aldrich. All structures of reagents were drawn using drawing tools in Maestro v9.4. The structures of reagents were prepared by selecting a reagent type (a functional group), identifying the functional group that is replaced when the reagent is added to the core, and performing 2D-to-3D conversion, structure variation and clean up. This reagent preparation was carried using LigPrep v 2.9. Likewise, core containing molecule was drawn in maestro v9. 4 and prepared using LigPrep v2.9. The different attachment position on the core was defined and reagent files for the attachment positions were assigned. The pre-generated grid file of COX-2 enzyme was used for the docking and screening of a library. The coordinate for the COX-2 enzyme (PDB Id: 1CX2) was taken from RCSB Protein Data Bank and prepared for docking using 'protein preparation wizard' in Maestro v9.4. Water molecules in the structures were removed and termini were capped by adding ACE and NMA residue. The bond orders and formal charges were added for heterogroups and hydrogens were added to all atoms in the structure. Side chains that are not close to the binding cavity and do not participate in salt bridges were neutralized. After preparation, the structure was refined to optimize the hydrogen bond network using OPLS_2005 force field. This helps in reorientation of the side chain hydroxyl group. The minimization was terminated when the energy converged or

the RMSD reached a maximum cutoff of 0.30 Å. Grids were then defined around refined structure by centering on ligand using default box size. First core containing molecule was docked and then both single-position and combinatorial docking and fully enumerated docking was done for each system separately. The maximum number of structures to be docked was set to 3000 using SP precision docking mode of Glide. Similarly, the coordinates for COX-1 enzyme (PDB Id: 1Q4G) and 5-LOX enzyme (PDB Id: 3V99) were taken from RCSB Protein Data Bank and prepared using 'protein preparation wizard' in Maestro v9.4 for virtual screening of compounds obtained from virtual combinatorial library design.

4.2. Synthesis:

The purities of all the synthesized compounds have been checked by thin-layer chromatography (TLC) using various non-aqueous solvents. Melting points were determined on SRS OPTIMELT and were uncorrected. ^{1}H NMR spectra and ^{13}C NMR were recorded on a Bruker DXM-400 spectrometer (400MHz) with DMSO as a solvent. Mass spectra were recorded on Time of flight mass spectrometer

4.2.1. General Procedure for synthesis of 4,6-disubstituted-1,4-dihydropyrimidine-2-thiol (1a-7a)

A mixture of acetophenones (5 mmol), thiourea (7.5 mmol), and aldehydes (6 mmol) in ethanol was heated to a clear solution. To this, t-sodium butoxide (2 mmol) was added slowly and the reaction mixture was refluxed for around 10-14 h. The completion of the reaction was monitored on TLC. After the completion of the reaction, the solvent was evaporated under vacuum and ice cold water was added to the mixture. The residue obtained was filtered, dried and purified either

by recrystallization from ethanol or by column chromatography using Ethyl acetate/n-hexane as mobile phase to obtain intermediate **1a-7a**.

4.2.2. General Procedure for synthesis of 2-mercapto-6-substitutedpyrimidin-4(1H)-one (8a-10a)[24]

To the 10 ml absolute ethanol, sodium (6 mmol) was added. When sodium was disappeared, ethyl acetoacetate derivatives (3 mmol) and thiourea (4.5 mmol) were added to the solution. The mixture was refluxed for 20 h. The solvent was evaporated under vacuum and the residue obtained was dissolved in water and acetified with glacial acetic acid. The product was then recrystallized from ethanol.

4.2.3. General Procedure for synthesis of ethyl 2-((4,6-disubstituted-1,4-dihydropyrimidin-2-yl)thio)acetate/propanoate (1b-10b)[25]

The Compounds (1a-10a) (2 mmol) and triethylamine (3 mmol) was added to dimethylformamide and heated to 80-90 °C to a clear solution. The ethyl chloroacetate (3 mmol) or Ethyl 3-bromopropionate (3 mmol) was then added drop wise to the reaction mixture. After the completion of the reaction, the reaction mixture was poured to ice-water and residue obtained was extracted with 3 × 25ml of ethyl acetate. The organic layer was then washed with water and evaporated to give the product which is finally purified by column chromatography using Ethyl acetate/n-hexane as mobile phase.

4.2.4. General Procedure for synthesis of Final Compounds (1c, 3c-4c, and 7c-9c)

Compounds **(1b, 3b-4b and 7b-9b)** (2.4 mmol) were dissolved in 30 ml of methanol: dichloromethane (3:1). To this mixture, hydroxylamine hydrochloride (9.6 mmol) in 3 ml of water was added at 0 °C and stirred for 10 min. Sodium hydroxide (19.4 mmol) in 3-4 ml of water was then added and stirred for 30 min. The reaction mixture was warmed to room temperature, stirred for 3-6 h and TLC was checked at each interval. After the complete consumption of starting materials, the solvent was evaporated. Residue obtained was diluted with 20 ml of water and acidified with acetic acid. Finally the product obtained was extracted with dichloromethane (2 × 20 ml), washed with water, dried and evaporated to give the product and purified by column chromatography using Ethyl acetate/n-hexane as mobile phase.

4.2.4.1. 2-((4,6-diphenyl-1,4-dihydropyrimidin-2-yl)thio)-N-hydroxyacetamide (1c)

Yield: 69%; ^{1}H NMR (400 MHz, DMSO): δ = 4.05 (s, 2H), 5.26-5.27 (d, 1H), 5.74 (s, 1H), 5.76-5.77 (d, 1H), 7.06 (s, 1H), 7.32-7.38 (m, 3H), 7.43-7.52 (m, 7H), 9.11 (s, 1H).; ^{13}C NMR (400MHz, $CDCl_3$): δ = 169.38, 153.72, 144.49, 143.23, 135.57, 134.43, 128.52, 126.70,125.89, 111.94, 73.85, 33.46; AP-MS: *m/z* =340.1 $[M+H]^+$; Elemental Anal. Calcd for $C_{18}H_{17}N_3O_2S$ (339.41): requires (Found): C, 63.70 (63.57); H, 5.05 (5.18); N, 12.38 (12.36).

4.2.4.2. ethyl 3-((4,6-diphenyl-1,4-dihydropyrimidin-2-yl)thio)propanoate (2b)

Yield: 56%; ^{1}H NMR (400 MHz, DMSO): δ = 1.17 (t, 3H), 2.94-2.96(t, 2H), 3.46-3.48 (t, 2H), 4.15-4.18 (q, 2H), 5.20-5.22 (d, 1H), 5.75-5.76 (d, 1H), 7.29-7.33 (m, 3H), 7.45-7.51 (m, 7H), 9.17 (s, 1H).; ^{13}C NMR (400MHz, $CDCl_3$): δ = 171.19, 154.99, 144.49, 143.23, 135.58, 128.52, 128.48, 126.70, 125.89, 111.94, 73.85, 60.81, 33.80, 26.85, 14.21; AP-MS: *m/z* =367.3 $[M+H]^+$;

Elemental Anal. Calcd for $C_{21}H_{22}N_2O_2S$ (366.48): requires (Found): C, 68.82 (68.80); H, 6.05 (6.15); N, 7.64 (7.77).

4.2.4.3. 2-((6-(4-fluorophenyl)-4-(pyridin-2-yl)-1,4-dihydropyrimidin-2-yl)thio)-N-hydroxyacetamide (3c)

Yield (solid): 42%; ^{1}H NMR (400 MHz, DMSO): δ = 4.21 (s, 2H), 5.05-5.06 (d, 1H), 5.52-5.53 (d, 1H), 5.72 (s, 1H), 7.08-7.16 (m, 3H), 7.23 (s, 1H), 7.35-7.42 (m, 3H), 7.65-7.70 (m, 2H), 9.13 (s, 1H).; ^{13}C NMR (400MHz, $CDCl_3$): δ = 169.38, 165.55, 163.53, 161.51, 160.67, 156.17, 147.58, 145.09, 141.98, 138.31, 136.90, 131.89, 127.12, 115.88, 113.76, 111.39, 66.34, 33.46; AP-MS: *m/z* =359.1 $[M+H]^+$. Elemental Anal. Calcd for $C_{17}H_{15}FN_4O_2S$ (358.39): requires (Found): C, 56.97 (56.85); H, 4.22 (4.25); N, 15.63 (15.78).

4.2.4.4. 2-((4-(4-fluorophenyl)-6-phenyl-1,4-dihydropyrimidin-2-yl)thio)-N-hydroxyacetamide (4c)

Yield (solid): 45%; ^{1}H NMR (400 MHz, DMSO): δ =: 3.96 (s, 2H), 5.13-5.14 (d, 1H), 5.74-5.75 (d, 1H), 5.83 (s, 1H), 6.11 (s, 1H), 7.10-7.13 (m, 2H), 7.42-763 (m, 7H), 9.12 (s, 1H).; ^{13}C NMR (400MHz, $CDCl_3$): δ = 169.38, 167.02, 163.13, 161.12, 153.72, 143.23, 137.09, 135.57, 133.65, 128.52, 128.08, 125.89, 115.66, 114.10, 111.94, 73.85, 33.46; AP-MS: *m/z* =358.2 $[M+H]^+$; Elemental Anal. Calcd for $C_{18}H_{16}FN_3O_2S$ (357.40): requires (Found): C, 60.49 (60.5); H, 4.51 (4.59); N, 11.76 (11.73).

4.2.4.5. ethyl 3-((6-(4-fluorophenyl)-4-phenyl-1,4-dihydropyrimidin-2-yl)thio)propanoate (5b)

Yield (solid): 59%; ^{1}H NMR (400 MHz, DMSO): δ = 1.18-1.21 (t, 3H), 2.87-2.91 (t, 2H), 3.59-3.64 (q, 2H), 5.17-5.18 (d, 1H), 5.70-5.77 (d, 1H), 7.05-7.08 (m, 2H), 7.31-7.35 (m, 3H), 7.39-7.44 (m, 4H), 9.17 (s, 1H); ^{13}C NMR (400MHz, $CDCl_3$): δ = 171.19, 163.53, 161.51, 154.99, 144.48, 143.23, 128.17, 126.70, 115.89, 111.94, 73.85, 60.81, 33.80, 29.76, 26.86, 14.21; AP-

MS: *m/z* =385.4 $[M+H]^+$; Elemental Anal. Calcd for $C_{21}H_{21}FN_2O_2S$ (384.47): requires (Found): C, 65.60 (65.69); H, 5.51 (5.58); N, 7.29 (7.36).

4.2.4.6. ethyl 3-((6-(4-fluorophenyl)-4-(thiophen-2-yl)-1,4-dihydropyrimidin-2-yl)thio)propanoate (6b)

Yield (solid): 52%; ^{1}H NMR (400 MHz, DMSO): δ = 1.15-1.19 (t, 3H), 3.30-3.32 (t, 2H), 4.06-4.08 (t, 2H), 4.58-4.62 (q, 2H), 5.30-5.32 (d, 1H), 5.79-5.80 (d, 1H), 6.91-6.99 (m, 4H), 7.20-7.27 (m, 3H), 8.96 (s, 1H).; ^{13}C NMR (400MHz, $CDCl_3$): δ = 171.19, 163.53, 161.51, 150.75, 146.97, 144.30, 139.12, 128.19, 125.92, 125.59, 125.34, 115.88, 112.00, 77.43, 60.81, 33.80, 26.85, 14.21; AP-MS: *m/z* =391.5 $[M+H]^+$; Elemental Anal. Calcd for $C_{19}H_{19}FN_2O_2S_2$ (390.49): requires (Found): C, 58.44 (58.52); H, 4.90 (4.87); N, 7.17 (7.20).

4.2.4.7. 2-((4-cyclopropyl-6-(p-tolyl)-1,4-dihydropyrimidin-2-yl)thio)-N-hydroxyacetamide (7c)

Yield (solid): 38%; ^{1}H NMR (400 MHz, DMSO): δ =: 0.71-0.80 (m, 5H), 2.30 (s, 3H), 4.33 (s, 2H), 4.61-4.63 (d, 1H), 5.36-5.38 (d, 1H), 5.67 (s, 1H), 7.10-7.14 (m, 2H).; ^{13}C NMR (400MHz, $CDCl_3$): δ = 169.38, 149.41, 144.04, 137.73, 134.48, 130.06, 129.00, 127.01, 112.11, 67.28, 33.46, 21.41, 19.87, 5.14.; AP-MS: *m/z* =318.2 $[M+H]^+$; Elemental Anal. Calcd for $C_{16}H_{19}N_3O_2S$ (317.41): requires (Found): C, 60.54 (60.52); H, 6.03 (6.01); N, 13.24 (13.31).

4.2.4.8. N-hydroxy-2-((4-oxo-6-phenyl-1,4-dihydropyrimidin-2-yl)thio)acetamide (8c)

Yield (solid): 40%; ^{1}H NMR (400 MHz, DMSO): δ = 3.56 (s, 2H), 5.69 (s, 1H), 7.36 (s, 1H), 7.63-7.72 (m, 5H), 9.25 (s, 1H), 7.12 (s, 1H).; ^{13}C NMR (400MHz, $CDCl_3$): δ = 169.38, 167.22, 165.74, 149.20, 135.19, 128.63, 126.66, 114.57, 33.46; AP-MS: *m/z* =278.2 $[M+H]^+$; Elemental Anal. Calcd for $C_{12}H_{11}N_3O_3S$ (277.30): requires (Found): C, 51.98 (51.91); H, 4.00 (4.05); N, 15.15 (15.20).

4.2.4.9. N-hydroxy-3-((4-oxo-6-phenyl-1,4-dihydropyrimidin-2-yl)thio)propanamide (9c)

Yield (solid): 42%; ^{1}H NMR (400 MHz, DMSO): δ = 2.63-2.66 (t, 2H), 3.05-3.09 (t, 2H), 5.28 (s, 1H), 7.43 (s, 1H), 7.66-7.74 (m, 5H), 7.12 (s, 1H), 8.27 (s, 1H).; ^{13}C NMR (400MHz, $CDCl_3$): δ = 168.29, 165.74, 164.46, 149.20, 148.07, 135.19, 128.63, 126.65, 114.57, 35.78, 27.33; AP-MS: *m/z* =292.2 $[M+H]^+$; Elemental Anal. Calcd for $C_{13}H_{13}N_3O_3S$ (291.33): requires (Found): C, 53.60 (53.68); H, 4.50 (4.55); N, 14.42 (14.40).

4.2.4.10. 2-((3-oxopentyl)thio)-6-(trifluoromethyl)pyrimidin-4(1H)-one (10b)

Yield (solid): 48%; ^{1}H NMR (400 MHz, DMSO): δ = 1.10-1.14 (t, 3H), 2.47-2.50 (q, 2H), 3.12-3.15 (d, 2H), 3.16-3.19 (d, 2H), 7.06 (s, 1H), 8.20 (s, 1H).; ^{13}C NMR (400MHz, $CDCl_3$): δ = 211.21, 168.89, 167.05, 135.00, 134.75, 134.24, 124.63, 122.48, 121.90, 120.34, 118.19, 41.26, 36.04, 27.58, 8.59; AP-MS: *m/z* =281.1 $[M+H]^+$; Elemental Anal. Calcd for $C_{10}H_{11}F_3N_2O_2S$ (280.27): requires (Found): C, 42.85 (42.80); H, 3.96 (3.88); N, 10.00 (10.03).

4.3. *In-vitro* COX-2 and 5-LOX inhibiting Assay:

4.3.1. Reagents and chemicals

Commercially acquired reagents: TMPD (N, N, N', N'-tetramethyl p-phenylenediamine), hematin and Tween 20 were purchased from Sigma, Arachidonic acid purchased from Nu-check Prep, Inc (MN, USA) and Dimethyl sulfoxide (DMSO) of HPLC grade. All solutions were prepared in deionised distilled water. All other reagents were of standard quality commercially available

4.3.2. Extraction and isolation of cyclooxygenase-1 (COX-1) from Ram seminal vesicles:

We had collected Ram seminal vesicles from the local slaughter house and stored in (−80 °C). Before starting the experiment, It was then taken out from deep freezer (−80 °C) and kept overnight at 4°C in refrigerator. Process of Extraction and isolation were carried out below 5°C in cold room.

4.3.2.1. Preparation of microsomes as a source of COX-1

Preparation of microsome was carried out, according to the method of Hemler et al.[26], with some modifications. Ram seminal vesicles were minced and homogenized in buffer containing 0.05 M Tris–HCl (pH 8), 5 mM EDTA disodium salt, 5 mM diethyl dithiocarbamate and 0.01% sodium azide. The homogenate was centrifuged at 10,000 × g for 20 min, at 4°C. The supernatant was filtered through cheese cloth. The filtered supernatant was further centrifuged at 33,000 RPM for 1 h 30 min, 4°C by using ultracentrifuge (Himac, CP-100-α HITACHI) to obtain microsomal pellet. This microsomal pellet was solubilised in a solubilising buffer containing 0.05 M Tris–HCl (pH 8), 0.1 mM EDTA disodium salt, 0.1 mM diethyl dithiocarbamate and 0.01% sodium azide. This microsome fraction was stored at −80°C, and used as enzyme source.

4.3.3. Preparation of microsomes as a source of cyclooxygenase-2

Microsome as a source of COX-2 was carried out according to the method by Reddy et al. [27], with some modifications. Human recombinant COX-2 expressed in Spodoptera frugiperda (Sf9) cell. Sf9 cells were maintained at 28°C in Grace's insect culture medium. Inscet Cells at 70% confluency were infected with recombinant baculovirus containing human COX-2. After 72 h of infection, the cells were collected by centrifugation at 3000 × g for 5 min at 4°C. The pellet was

suspended in a minimum volume of Tris–HCl buffer (50 mM, pH 8.0) containing 5 mM EDTA, 300 mM sucrose, 5 mM diethyl thiocarbamate, 1 μg/ml pepstatin, 1 mM phenol and sonicated for 3 min. The cell lysate was centrifuged at 100,000 × g for 1 h 30 min at 4°C by using ultracentrifuge (Himac, CP-100 α HITACHI), and the microsomal pellet obtained was suspended in Tris–HCl buffer (2.5 mM, pH 8.0) containing 0.5% glycerol, 0.8% Tween 20 and 1 mM phenol. This solubilized microsomal fraction was stored at −80°C and used as enzyme source.

4.3.4. Cycloxygenase (COX-1 and COX-2) assays

Enzymatic activities of both, COX-1 and COX-2 were measured according to the method of Copeland et al.[28], with slight modifications using a chromogenic assay based on the oxidation of N,N,N',N'-tetramethyl-*p*-phenylene diamine (TMPD), during the reduction of PGG2 to PGH2[29, 30]. The assay mixture contained Tris–HCl buffer (0.5 M) pH 8.0, hematin (5 mM), EDTA (0.5 M), enzyme (COX-1 or COX-2) and the test compound. The mixture was pre-incubated at 25°C for 5 min and then the reaction was initiated by the addition of substrate AA and TMPD, in total volume of 1 ml reaction mixture. The enzyme activity was determined by estimating the rate of TMPD oxidation for the first 60 s of the reaction by following the increase in absorbance at 610 nm. A low rate of non-enzymatic oxidation, observed in the absence of COX-1 and COX-2, was subtracted from the experimental value while calculating the percent inhibition.

4.3.5. Purification and assay of 5-lipoxygenase (5-LOX)

5-Lipoxygenase from potato tubers was purified and assayed as per the method described by Reddanna et al.[31]. Enzyme activity was assessed using the polarigraphic method with a Clark's oxygen electrode on Strathkelvin Instruments, model 782, RC-300. Reaction mixture contained

50-µl of enzyme and 10 µl, 40mM of the substrate, (Arachidonic acid) and final volume made with 100 mM phosphate buffer pH 6.3 to 3 ml. Since lipoxygenases are oxygen-consuming enzymes the concentration of oxygen decrease in the reaction mixture, the rate of decrease in oxygen was taken as a measure of enzyme activity. The reaction was allowed to proceed at 25°C and the maximum slope generated was taken for calculating enzyme activity. The activity was expressed as units/mg protein, where one unit is defined as one micro mole of oxygen consumed per minute. Assay standardization was done with NDGA (Nordihydrogutaric acid), a selective 5-LOX inhibitor.

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Fig. 1: Flow chart of Virtual Combinatorial Library Design and Virtual Screening shows the phases involved in the search of new simultaneous COX-1/COX-2 and 5-LOX Inhibitors.

Fig. 2: Designed 1,4dihydropyrimidine scaffold for dual inhibition of COX-1/COX-2 and 5-LOX enzyme.

Fig. 3: Virtual Combinatorial Library Design and Screening of compounds. (a) Enumeration of library by docking of each core structure with single position substitution for the filtering of fragments and (b) Enumeration of entire library by selecting each fragment or substitution, without performing docking of each core structure with single position substitution.

Fig. 4: Binding pose of compounds (a) Overlap of celecoxib (Orange Color Carbon Atoms) and compound 2b (Green Color Carbon Atoms), (b) Celecoxib (c) compound 2b in the active site of COX-2 enzyme

Fig. 5: Binding pose of compounds (a) Overlap of *tepoxalin* (Orange Color Carbon Atoms) and compound 2b (Green Color Carbon Atoms), (b) *tepoxalin* (c) compound 2b in the active site of 5-LOX enzyme

Fig. 6: Binding pose of compounds (a) Overlap of *Ibuprofen* (Orange Color Carbon Atoms) and compound 10b (Green Color Carbon Atoms), (b) *tepoxalin* and compound 2b in the active site of COX-1 enzyme

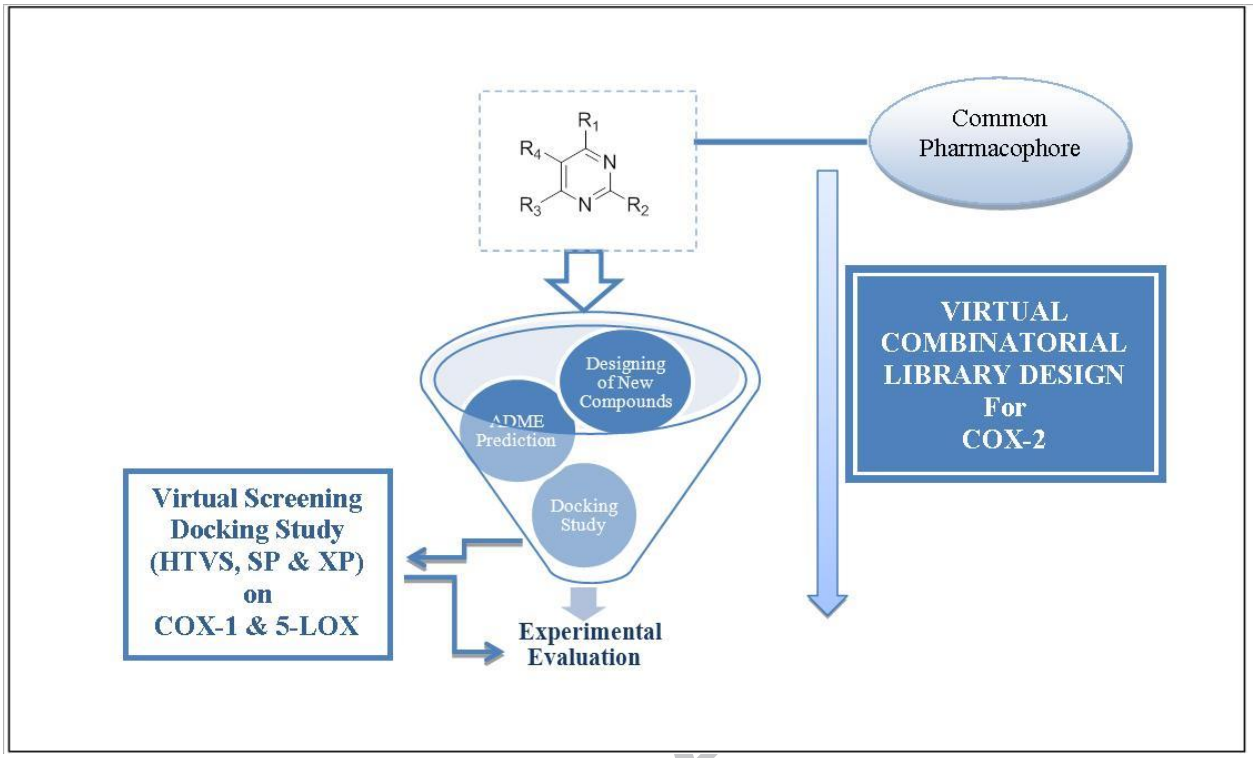


Fig. 1

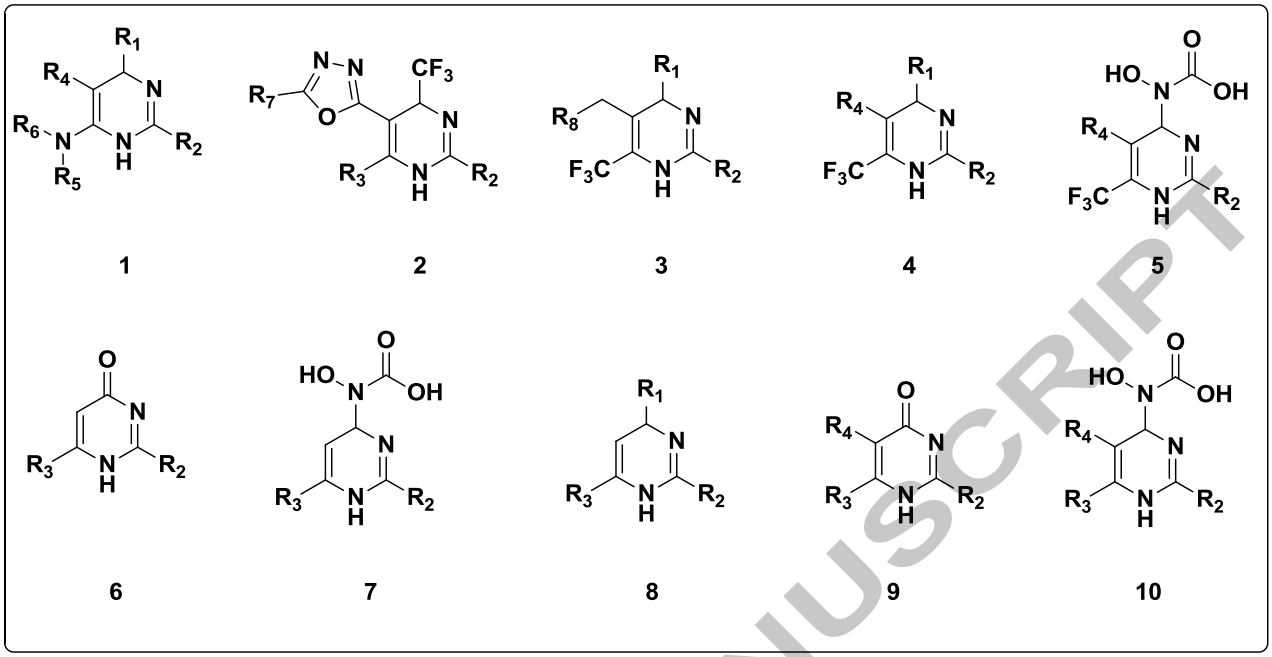
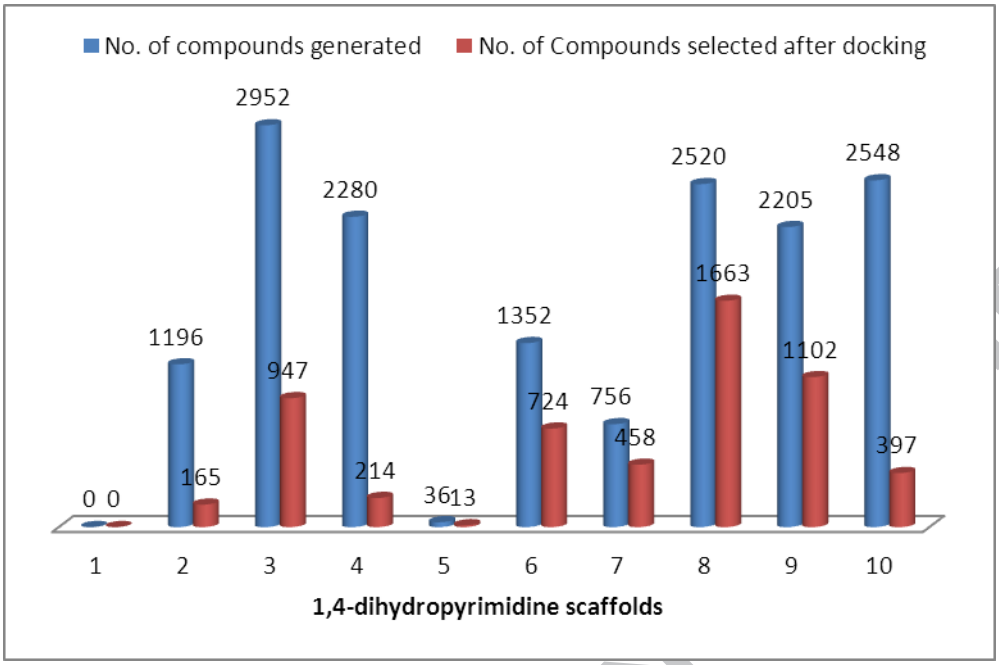
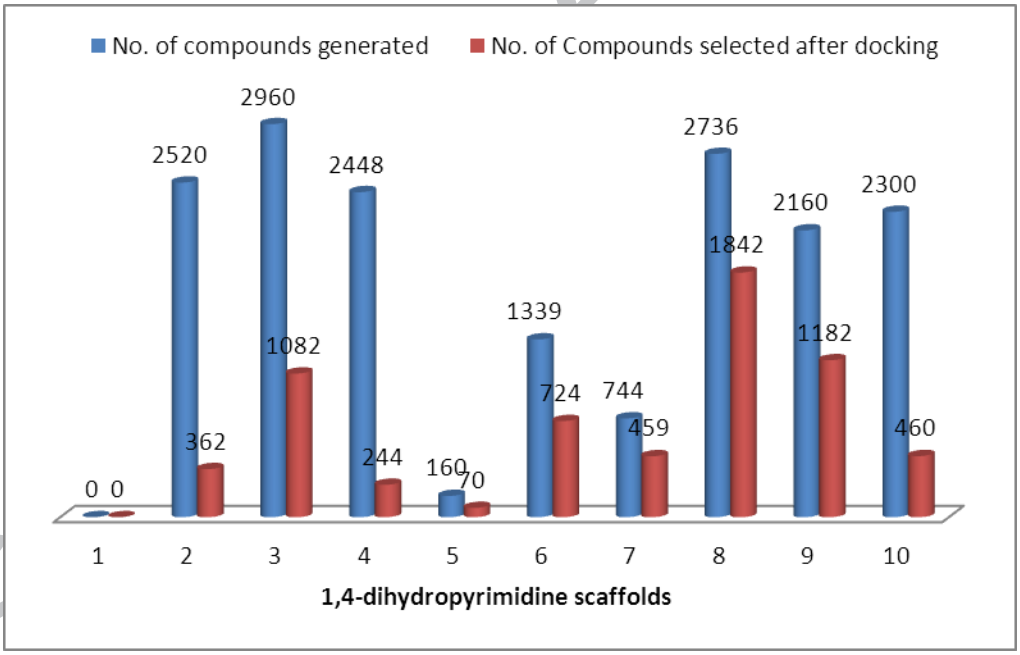


Fig. 2

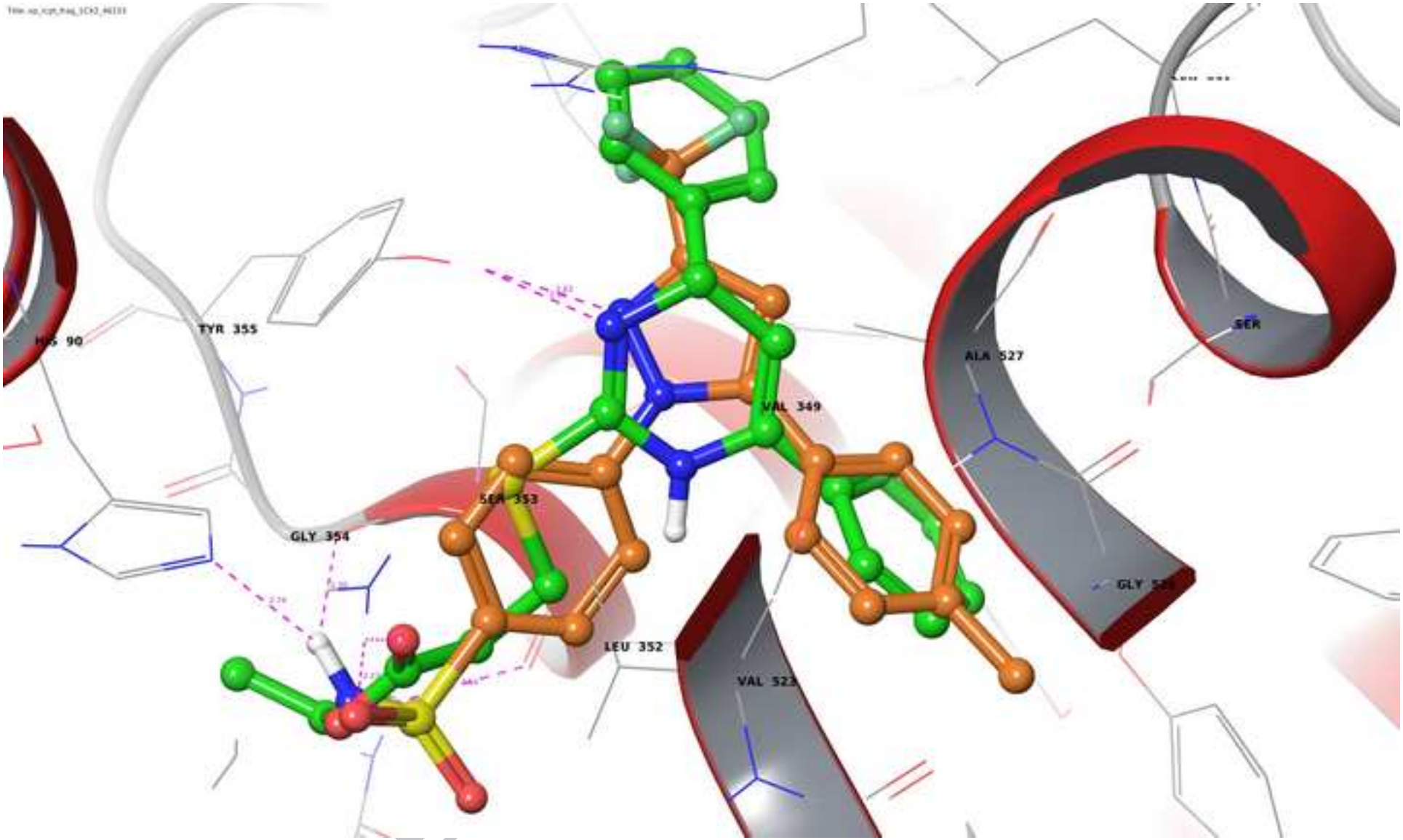


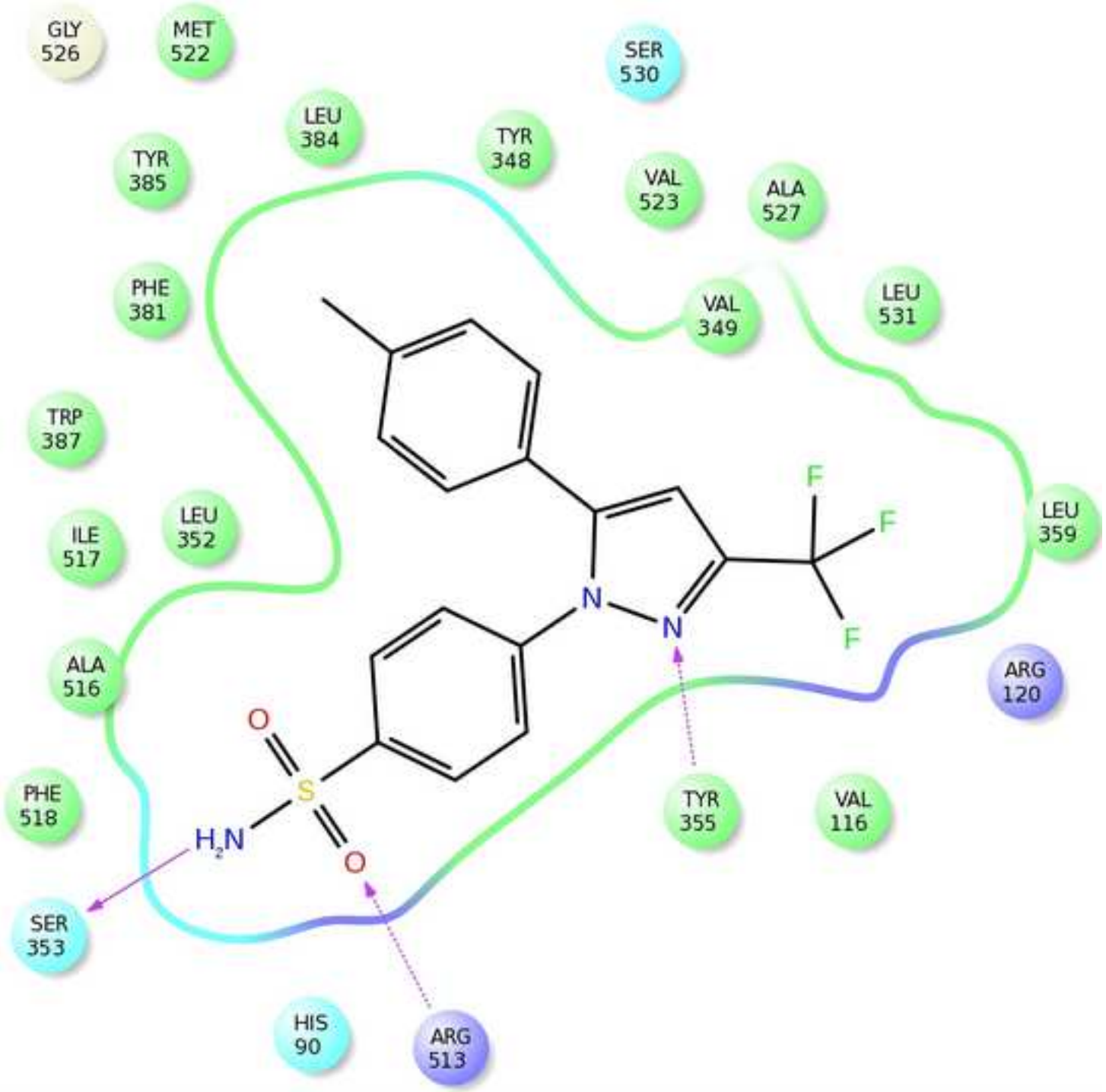
(a)

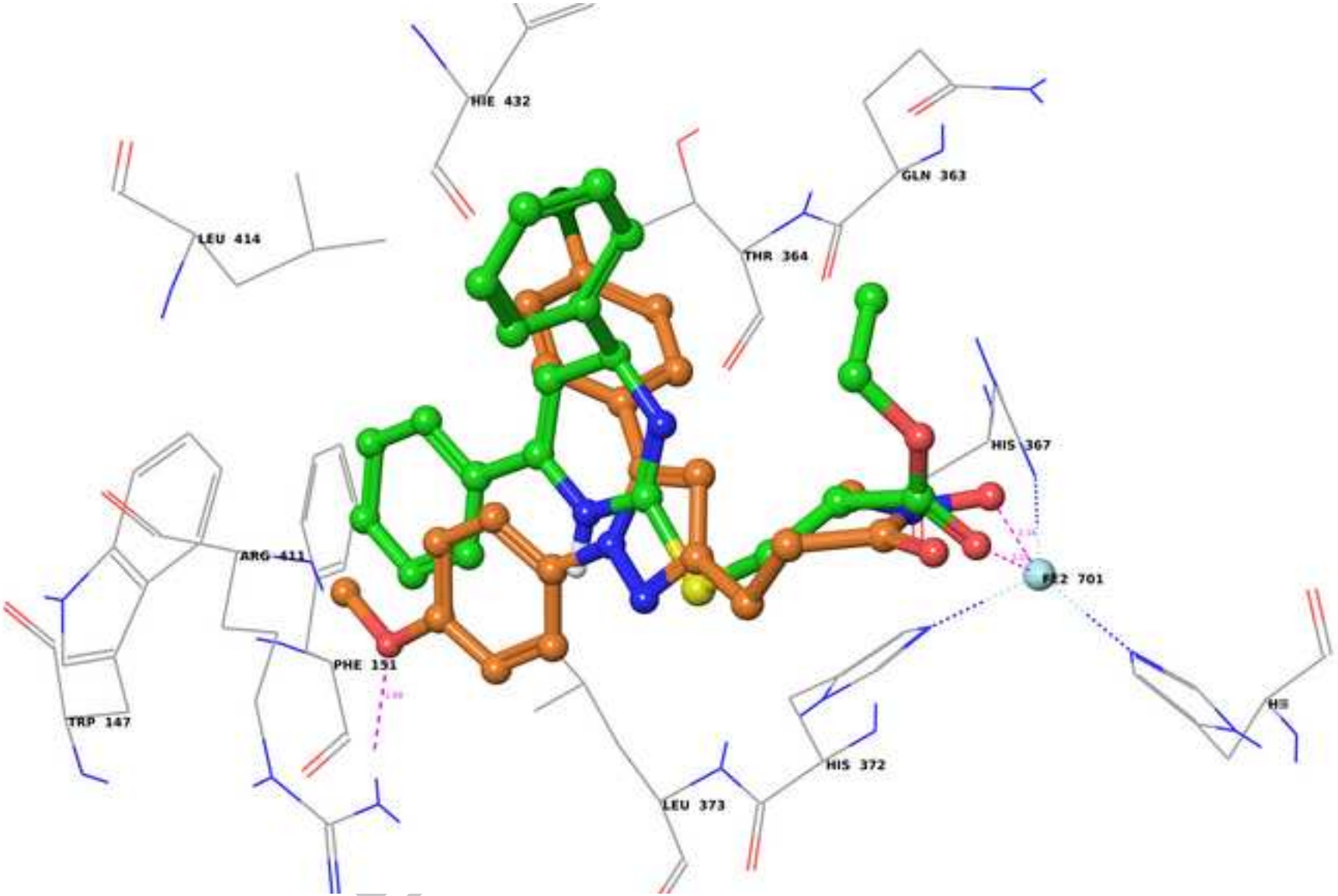


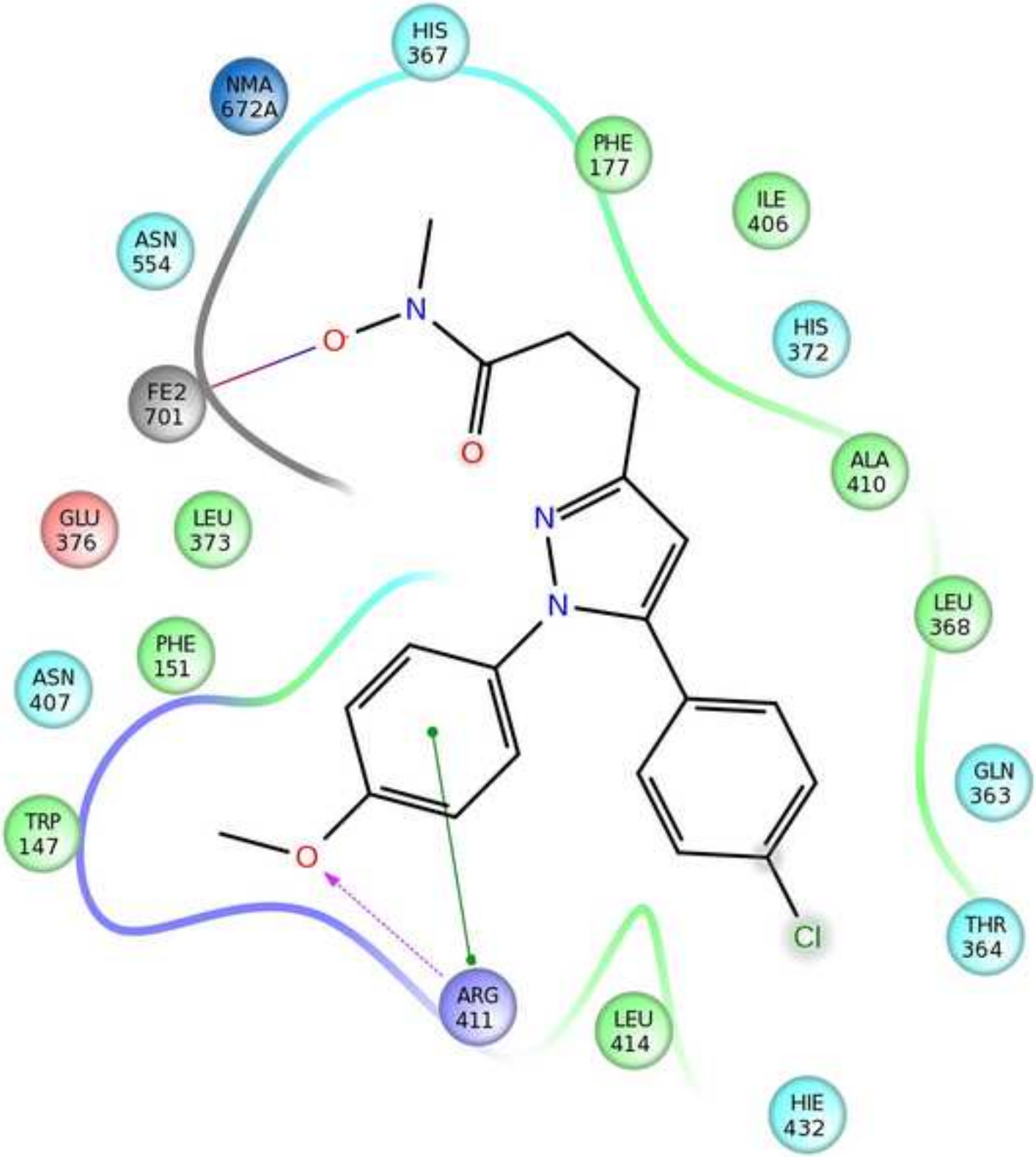
(b)

Fig. 3





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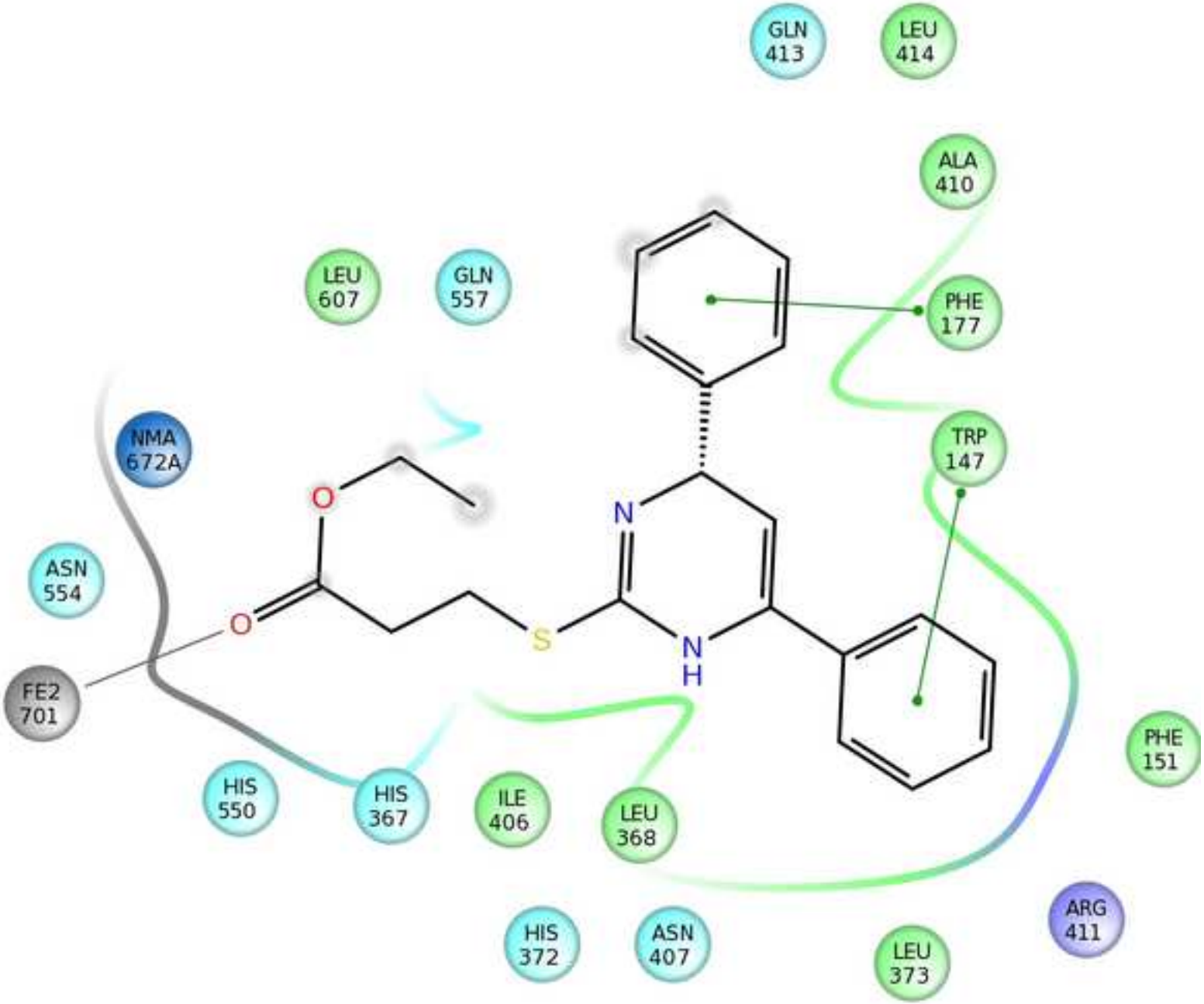
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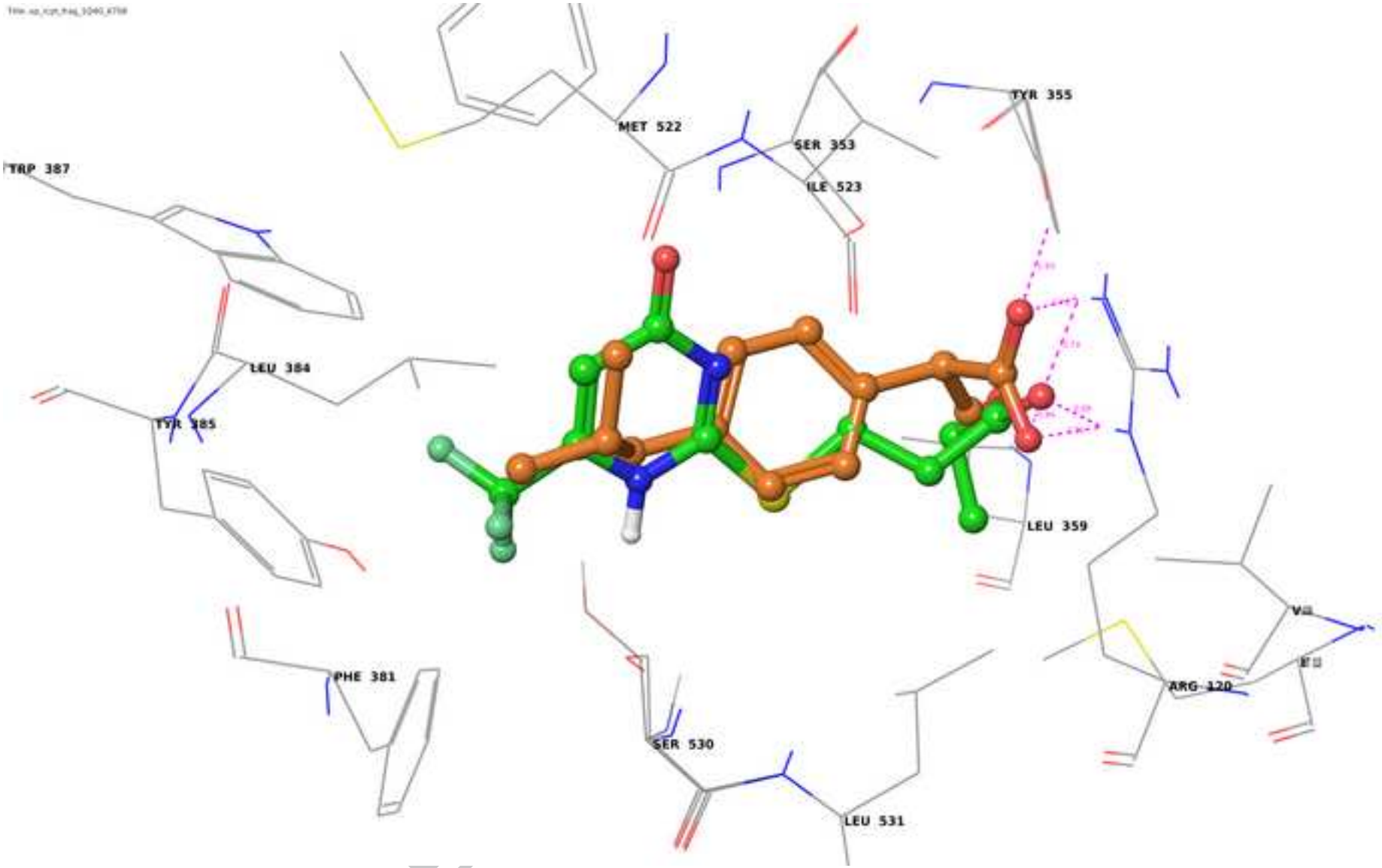
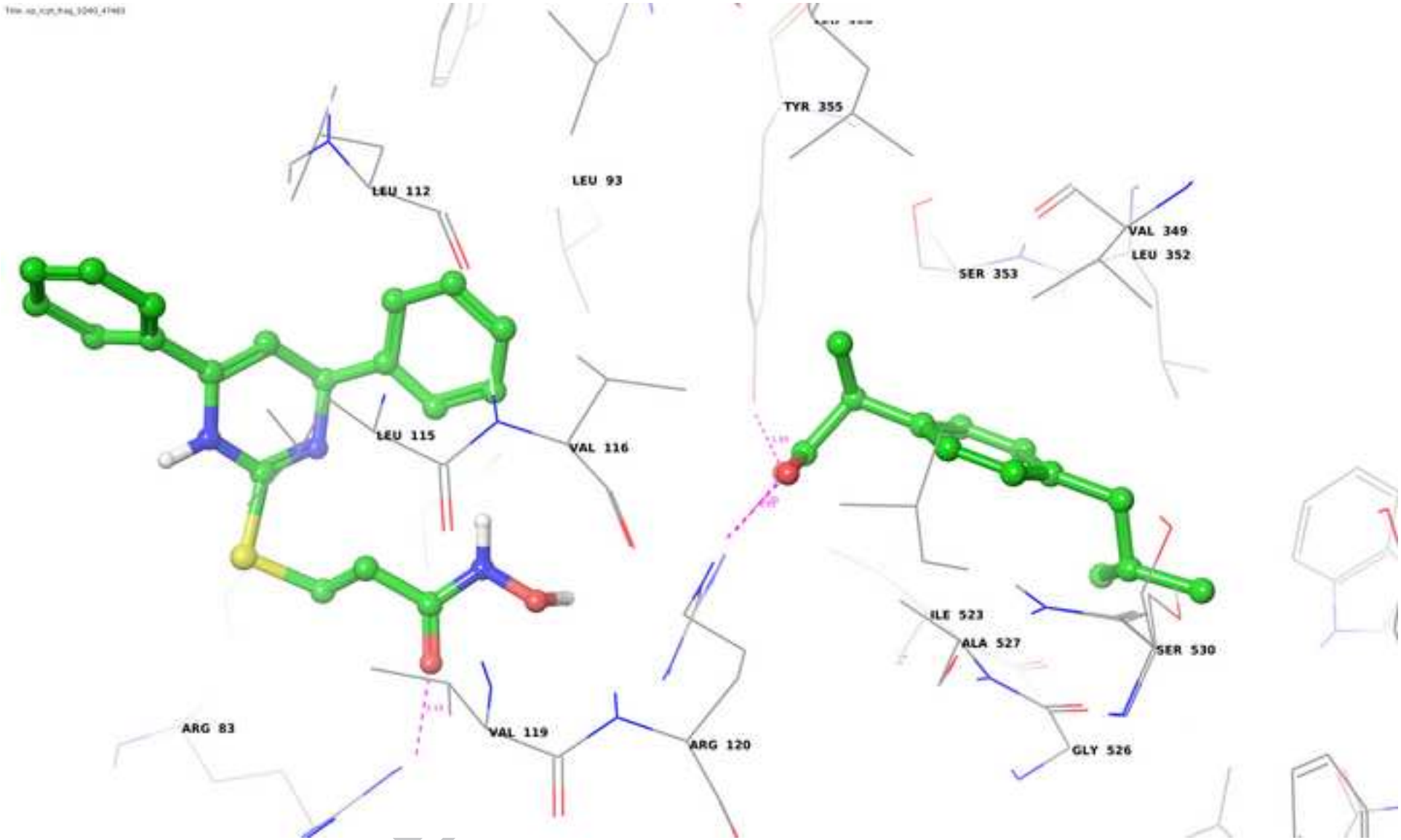
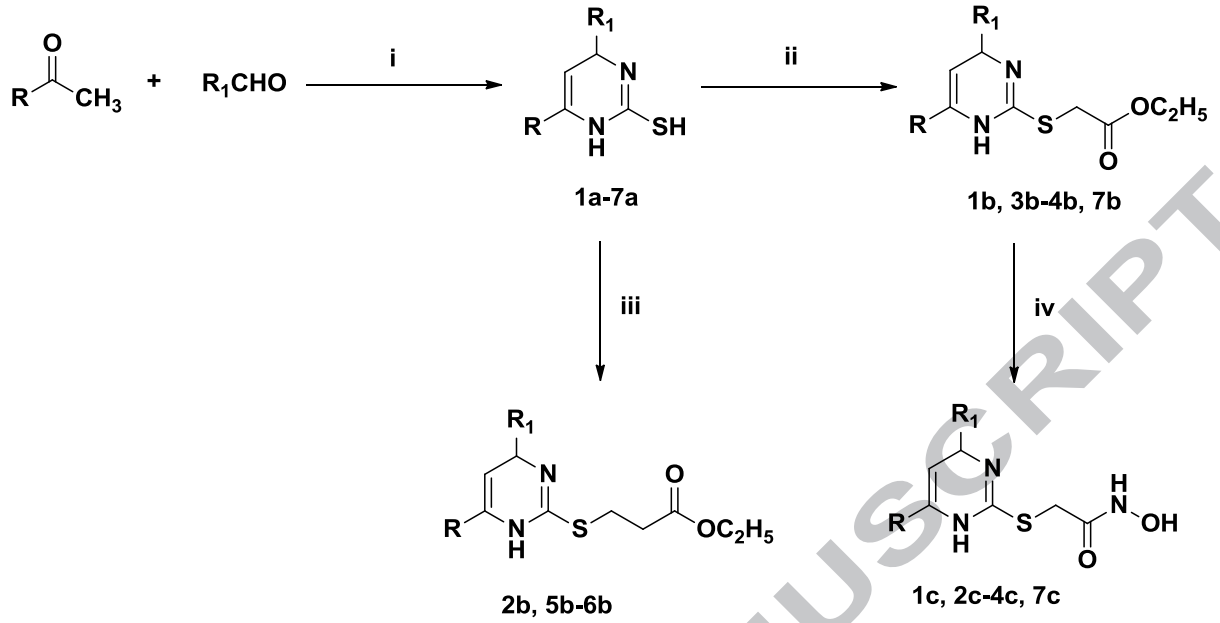
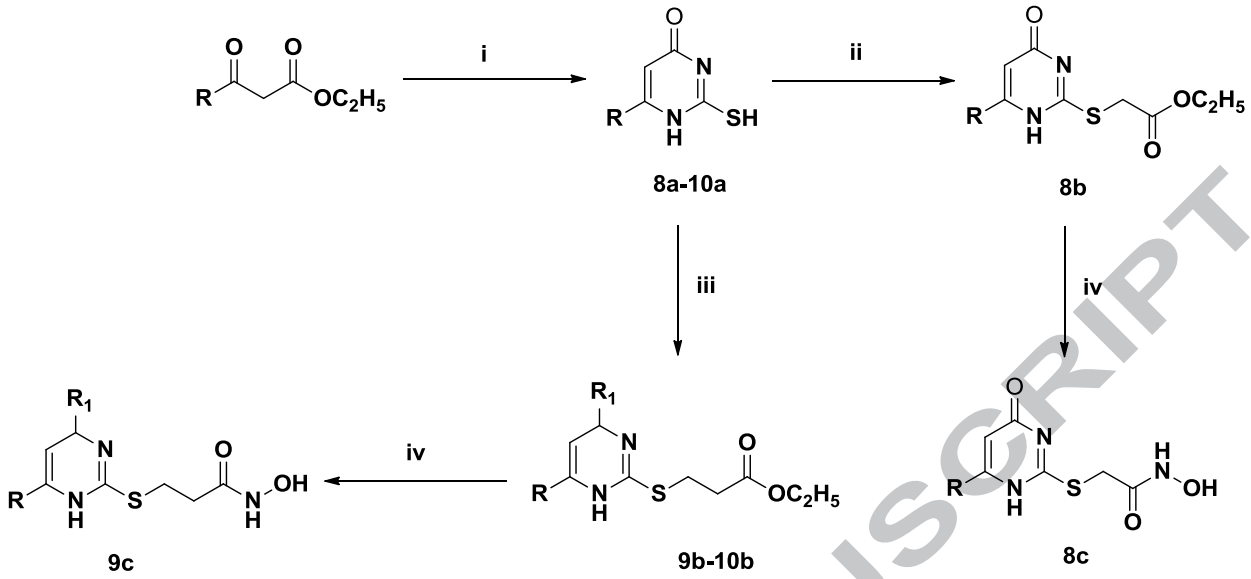






(i) Thiourea, t-BuONa, EtOH (ii) TEA, DMF, ethyl chloroacetate (iii) TEA, DMF, Ethyl 3-bromopropionate (iv) Methnol:DCM (3:1), $NH_2OH.HCl$ in H_2O, NaOH in H_2O

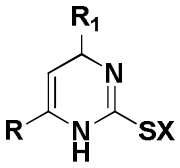
Scheme 1



(i) Thiourea, Na, EtOH (ii) TEA, DMF, ethyl chloroacetate (iii) TEA, DMF, Ethyl 3-bromopropionate
(iv) Methnol:DCM (3:1), $NH_2OH.HCl$ in H_2O, NaOH in H_2O

Scheme 2

Table 1: Structures of compounds which are selected for experimental validation



Comp Code	R	R_1	X
1c	$-C_6H_5$	$-C_6H_5$	$-CH_2CONHOH$
2b	$-C_6H_5$	$-C_6H_5$	$-CH_2CH_2COOC_2H_5$
3c	$-4F-C_6H_4$	-2-pyridine	$-CH_2CONHOH$
4c	$-C_6H_5$	$-4F-C_6H_4$	$-CH_2CONHOH$
5b	$-4F-C_6H_4$	$-C_6H_5$	$-CH_2CH_2COOC_2H_5$
6b	$4F-C_6H_4$	-2-thiophene	$-CH_2CH_2COOC_2H_5$
7c	$-4CH_3-C_6H_4$	$-C_3H_5$	$-CH_2CONHOH$
8c	$-C_6H_5$	=O	$-CH_2CONHOH$
9c	$-C_6H_5$	=O	$-CH_2CH_2CONHOH$
10b	$-CF_3$	=O	$-CH_2CH_2COOC_2H_5$

Table 2: Docking score of compounds

Comp Code	Docking Score (G-Score)		
	COX-1	**COX-2**	**5-LOX**
1c	-7.43	-10.48	-7.65
2b	-4.37	-8.94	-6.48
3c	-3.14	-10.82	-6.99
4c	-3.80	-8.40	-7.37
5b	-6.52	-5.19	-4.71
6b	-5.59	-6.50	-4.52
7c	-9.14	-11.07	-7.18
8c	-9.75	-8.40	-6.95
9c	-6.52	-8.00	-6.68
10b	-9.23	-5.02	-5.230

Table 3: *In-vitro* COX-1, COX-2 and 5-LOX inhibiting activity of compounds

Comp Code	Final Conc (µM)	% Inhibition		
		5-LOX	COX-1	COX-2
1c	294.9	13.54	78.46	7.78
2b	272.5	51.84	70.08	35.12
3c	279.32	18.59	40.31	14.55
4c	280.11	35.97	0	10.36
5b	260.41	21.43	80.0	5.85
6b	256.41	0	3.97	27.67
7c	315.45	14.63	4.92	5.94
8c	361.01	13.93	70.88	29.29
9c	343.64	0.5	0	0
10b	357.14	0.80	88.43	0.33

- COX-1 activity level : 0.0767µ mol product formed/min x ml
- Std. Inhibitor Indomethacin (COX-1) IC_{50}: 0.384 µM
- 5-LOX activity level : 0.039 µ mol product formed/ min x ml
- Std. Inhibitor NDGA IC_{50}: 7.31µM
- COX-2 activity level : 144.79 n mol product formed/min x ml
- Std. Inhibitor Celecoxib (COX-2) IC_{50}: 0.235µM

Graphical Abstract

Structure Based Library Design (SBLD) for New 1,4-dihydropyrimidine Scaffold as simultaneous COX-1/COX-2 and 5-LOX Inhibitors

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