Dual anti-inflammatory and selective inhibition mechanism of leukotriene A₄ hydrolase/aminopeptidase: insights from comparative molecular dynamics and binding free energy analyses

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Human leukotriene A₄ hydrolase/aminopeptidase (LTA₄H) is a zinc metalloenzyme with a dual catalytic activity; conversion of LTA₄ into LTB₄ and degradation of chemotactic tripeptide Pro-Gly-Pro (PGP). Existing inhibitors, such as SC-57461A, block both catalytic activities of the enzyme, leading to drug failures. Recently, a novel compound, ARM1, was reported to selectively inhibit the hydrolase activity of LTA₄H while sparing its aminopeptidase activity. However, the molecular understanding of such preferential inhibitory mechanism remains obscure. The discovery of ARM1 prompted us to further explore its binding theme and provide more insight into the structural and dual mechanistic features of LTA₄H protein. To accomplish this, we embarked on wide range of computational tools, including comparative molecular dynamics (MDs) simulations and postdynamic analyses for LTA₄H and in complex with ARM1, PGP, ARM1-PGP, and SC-57461A. MD analysis reveals that the binding of ARM1 exhibits a more stable active site and overall stable protein conformation when compared to the nonselective inhibitor SC-57461A. In addition, MM/GBSA-binding free energy calculation also reveals that ARM1 exhibit a lower binding affinity, when compared to the nonselective inhibitor SC-57461A – which is in a great agreement with experimental data. Per residue energy decomposition analysis showed that Phe314, Val367, Tyr378, Trp311, Pro382, and Leu369 are key residues critical for the selective inhibition of the epoxide hydrolase activity of LTA₄H by ARM1. Findings from this report will not only provide more understanding into the structural, dynamic, and mechanistic features of LTA₄H but would also assist toward the rational design of novel and selective hydrolase inhibitors of LTA₄H as anti-inflammatory drugs.

Keywords: dual LTA₄H hydrolase/aminopeptidase; ARM1; selective inhibition; molecular dynamics

1. Introduction

The human leukotriene A₄ hydrolase (LTA₄H) is a bi-functional zinc metalloenzyme, exhibiting dual aminopeptidase and epoxide hydrolase activities. LTA₄H employs the same zinc (Zn) coordinated active site that catalyzes the formation of leukotriene B₄ (LTB₄), a potent chemotactic agent in inflammatory responses (Grice et al., 2008). In LTB₄ biosynthesis, the free arachidonic acid (AA) is converted into an unstable epoxide leukotriene A₄ (LTA₄) by the enzyme 5-lipoxygenase (5-LO) assisted by 5-lipoxygenase-activating protein (FLAP). This unstable LTA₄ intermediate is then hydrolyzed into either natural immune response lipid mediator LTB₄ by LTA₄H or LTC₄ by leukotriene C₄ synthase (LTC₄S) (Rinaldo-Matthis & Haeggström, 2010). As an aminopeptidase, LTA₄H exhibits a higher affinity to N-terminal arginine of several synthesized tripeptides (Orning, Gierse, & Fitzpatrick, 1994). LTA₄H dual enzymatic activities are exerted through distinct; however, overlapping active sites, based on the catalytic zinc binding motif HEXXH-(X₁₈)E, of M₁ metallopeptidases (Haeggstrom, Wetterholm, Shapiro, Vallee, & Samuelsson, 1990).

The crystal structure of LTA₄H was first determined in complex with bestatin (Thunnissen, Nordlund, & Haeggström, 2001). As shown in Figure 1, the protein structure consists of three distinct domains, namely the C-terminal domain, a catalytic domain and an N-terminal domain, each consisting of approximately 200 amino acid residues. The active site is located within a cavity at the interface of the three domains. The catalytic domain is comprised of two lobes; a main alpha-helical and a mixed alpha-beta lobe. In between these lobes is the site of a zinc atom coordinated with amino acid residues His295, His299, and Glu318 (Thunnissen et al., 2001). There is a narrowing at the binding site of the zinc, which forms a nonpolar hydrophobic tunnel penetrating deep into the catalytic domain of the protein. The wider and opening parts of the L-shaped active site cavity are highly polar near the protein surface.

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The epoxide hydrolase activity of LTA₄H involves the conversion of LTA₄ into the potent chemotactic agent LTB₄, a key lipid mediator in the activation of inflammatory responses (Haeggstrom, 2004). LTB₄ plays a significant role in the pathogenesis and amplification of inflammatory diseases, such as rheumatoid arthritis (Shao, Del Prete, Bock, & Haribabu, 2006), inflammatory bowel disease (Rask-Madsen, 1998), chronic obstructive pulmonary disease (Gompertz & Stockley, 2002; Barnes, 2002), asthma (Luster & Tager, 2004; Lemiere et al., 2004), and atherosclerosis (Back et al., 2005), by regulating the recruitment and activation of leukocytes in the inflamed tissues mediated through BLT1 and BLT2 G-protein-coupled receptors (Mandal et al., 2008). Therefore, LTA₄H has been an attractive target for anti-inflammatory drug discovery due to its function in the biosynthesis of LTB₄.

The physiological function of the aminopeptidase activity of LTA₄H is less known and was previously assumed to process peptides that are involved in inflammation and host defence (Haeggstrom, 2004; Tholander et al., 2005). Recently, the aminopeptidase activity of LTA₄H was reported and was found to catalyze the cleavage and degradation of chemotactic tripeptide Pro-Gly-Pro (PGP), a neutrophil chemoattractant responsible for inflammation, indicating a protective biological property of the aminopeptidase activity (Snelgrove et al., 2010; De Oliveira et al., 2011). These findings indicate that LTA₄H plays a dual and opposite roles in the inflammatory responses; (1) a protective effect by inactivating neutrophilic inflammation caused by the chemotactic PGP and (2) formation of chemotactic LTB₄ from LTA₄.

Insights from the functions of LTA₄H suggest that the development of LTA₄H inhibitors that can selectively block the synthesis of LTB₄ from LTA₄ while sparing its aminopeptidase activity would be beneficial; however, not much success has been made in this direction. Efforts over the years in drug discovery targeting LTA₄H has solely been based on the natural substrate of the aminopeptidase; nevertheless, no attempts have been made toward the development of inhibitors that selectively inhibit the epoxide hydrolase activity of LTA₄H enzyme. Until the recent discovery of ARM1 (Stsiapanava et al., 2014), a selective epoxide hydrolase inhibitor, all existing LTA₄H inhibitors block both the aminopeptidase and hydrolase activities (Haeggstrom & Funk, 2011). Nevertheless, the molecular understanding of the dual enzymatic activity of LTA₄H and preferential binding mechanism of ARM1 to this protein especially from a computational perspective, remain uncertain.

Recently, Thangapandian et al. conducted a 5 ns molecular dynamics (MDs) simulation study on four structurally similar nonselective inhibitors of LTA₄H to provide reasons for their different biological activities. The most active compound among these inhibitors was used in pharmacophore modeling to identify three novel compounds as potential inhibitors of LTA₄H (Thangapandian, John, Arooj, & Lee, 2012). In another study, Thangapandian et al. also performed a 5 ns MD simulation on the full enzyme in complex with LTA4 and Arg-Ala-Arg tripeptide. The study investigated structural and conformation changes conferred by E271Q, R563A, and K565A point mutations which lead to the loss of catalytic function of the enzyme (Thangapandian, John, Lazar, Choi, & Lee, 2012).

The main objectives of this study are to provide molecular insights into the structural and dynamic as well as inhibitor binding features of LTA₄H’s associated with its dual mechanism, i.e. the mechanism by which ARM1 inhibits the epoxide hydrolase activity of LTA₄H, while still retaining its aminopeptidase activity in comparison to a previous nonselective LTA₄H inhibitor (SC-57641A). To accomplish this, we performed a wide range of comparative MD analyses on four different systems: (1) LTA₄H enzyme in complex with the ARM1, (2) LTA₄H enzyme in complex with the PGP peptide analog substrate (OPB-Pro), (3) LTA₄H enzyme in complex with ARM1 in the presence of OPB-Pro and (4) LTA₄H enzyme in complex with the nonselective inhibitor SC-57641A.

Findings from this study will not only further help in the understanding of the molecular mechanism by which ARM1 exerts its selective inhibitory activity, but would also provide guidance towards the development of next generation of selective LTA₄H inhibitors as anti-inflammatory drugs.
2. Materials and methods

2.1. System preparation

The X-ray crystal structures of LTA₄H in complex with tripeptide analog OPB-Pro (PDB code: 4MS6), ARM1 inhibitor (PDB code: 4L2L), both OPB-Pro and ARM1 inhibitor (PDB code: 4MKT) and the nonselective inhibitor SC-57461A (PDB code: 3U9W) were obtained from the protein data bank (Stsiapanava et al., 2014). The delta nitrogen of the catalytic Histidine residues was protonated. Chimera (Pettersen et al., 2004) and MMV molecular modeling suites were used to prepare the protein structures. Table 1 shows all the simulated systems in this study, the PDB codes and corresponding abbreviations, whereas Figure 2 shows chemical structures of the inhibitors, and the PGP analog (OPB-Pro) used in the study as well as the original PGP peptide substrate.

2.2. MD simulation

All MD simulations were performed using the GPU version of the PMEMD engine provided with the Amber 14 software package (Götz et al., 2012; Case et al., 2012). Gaussian 09 at HF/6-31G* level was used to perform geometry optimization for the drugs and the peptide substrate (Swope et al., 2004). The ANTECHAMBER module was used to generate atomic partial charges for the drugs and peptide substrates by employing the Restricted Electrostatic Potential and the General Amber Force Field procedures (Wang, Wolf, Caldwell, Kollman, & Case, 2004). The protein system parameters were defined with the FF99SB force field in the Amber 14 suit (Lindorff-Larsen et al., 2010). Prior to MD simulation, the catalytic Zn metal coordination geometry was modeled using the nonbonded model approach. The Zn metal was assigned a van der Waals radius of 1.10 Å and a formal charge of +2.0 based on previously derived parameters (Stote & Karplus, 1995).

The LEAP module of AMBER 14 was used to add missing hydrogen and heavy atoms. The systems were then neutralized by adding nine sodium counter ions. The entire system including the divalent metal ion (Zn²⁺) were immersed within a box with TIP3P (Jorgensen, Chandrasekhar, Madura, Impey, & Klein, 1983) water molecules such that any solute atoms were within 10 Å of any box edge during the MD simulations. Particle mesh Ewald method (Harvey & De Fabritiis, 2009) was used to treat long-range electrostatic interactions with a direct space and van der Waals cutoff of 12 Å.

To eliminate bad contacts during the MD simulation, a total of 2000 steps of energy minimization were performed for each system. An initial 1000 steps of steepest descent was performed for all counter ions and water molecules followed by 1000 steps minimization carried out by conjugate gradients algorithm without restrain. A gradual heating of the systems from 0 to 300 K with a 5 kcal/mol Å harmonic restrain potential and a Langevin thermostat of collision frequency of 1/ps using a canonical ensemble constant volume and temperature (NVT) MD simulations were then carried out. All the systems were the equilibrated at 300 K in a constant pressure and

Table 1. The crystal structures of the simulated systems, PDB codes, and abbreviations.

| Simulated system          | PDB code | Abbreviation* |
|---------------------------|----------|---------------|
| LTA₄H-ARM1-OPB-Pro        | 4MKT     | LTARPEP       |
| LTA₄H-OPB-Pro             | 4MS6     | LTPEP         |
| LTA₄H-SC-57461A           | 3U9W     | LTSC          |
| LTA₄H-ARM1                | 4L2L     | LTAR          |

*These abbreviations are used throughout the entire text.

Figure 2. The 2D structures of LTA₄ Hydrolase lead ARM1 [4-(4-benzylphenyl) thiazol-2-amine] inhibitor (A), the nonselective inhibitor SC-57461A (N-[3-(4-benzylphenoxy)propyl]-N-methyl-β-alanine) (B) the original PGP peptide substrate (C) and its analog with nitrogen replace by a carbon (OPB-Pro) (D).
temperature (NPT) ensemble for 500 ps without restrain and the pressure of the systems were maintained at 1 bar pressure using Berendsen barostat. All hydrogen bonds (HBs) were constrained using the SHAKE algorithm (Ryckaert, Ciccotti, & Berendsen, 1977) in a time step of 2 fs and all MD runs were done using SPFP precision model (Le Grand, Götz, & Walker, 2013).

A 50 ns production run was performed without any restrain on the systems in an NPT ensemble at a temperature of 300 K with target coupling constant of 2 ps and pressure at 1 bar.

Trajectories from all system simulations were then saved and analyzed in every 1 ps. Post-MD analyses, such as root mean square fluctuation (RMSF), root mean square deviation (RMSD), principal component analysis (PCA), radius of gyration (Rg), and solvent accessible surface area (SASA), were performed using the CPPTRAJ and PTRAJ modules (Roe & Cheatham, 2013) of the Amber 14 suit. All plots were constructed using Origin data analysis tool (http://www.originlab.com/). Molecular modeling suite Chimera (Pettersen et al., 2004) was used for all visualizations.

2.3. Principal component analysis
Principal component analysis (PCA) also known as essential dynamics (ED) is one of the advanced methods for trajectory analysis in identifying prominent conformational changes through the extraction of conformational modes of the protein during MD simulation. The PCA was performed by constructing covariance matrix of the C-α atom displacement. PCA describes the eigenvectors and eigenvalues, which represents the direction of motions and the amplitudes in those directions of the protein, respectively (Cocco, Monasson, & Weigt, 2013). PCA was performed on C-α atoms on 1000 snapshots at time interval of 100 ps using the CPPTRAJ module in AMBER 14 in computing the first two principal components (PC1 and PC2) after stripping the 50 ns MD trajectories of ions and solvent. The corresponding PCA scatter plots were generated using Origin software (http://www.originlab.com/).

2.4. Dynamic cross correlation matrices (DCCM)
The calculation of cross correlation of dynamic trajectory is use to study the correlated motions of residual-based fluctuations during an MD simulation. The cross-correlation coefficient $C_{ij}$ or the pair of each C-α atoms $i$ and $j$ were computed according to the equation below.

$$C_{ij} = \frac{\langle \Delta r_i \cdot \Delta r_j \rangle}{\left[ \langle \Delta r_i^2 \rangle \cdot \langle \Delta r_j^2 \rangle \right]^{1/2}}$$  \hspace{1cm} (1)

where $\Delta r_i$ and $\Delta r_j$ is the displacement from the mean position of the $j$ and $i$ atom, respectively. The cross-correlation coefficient $C_{ij}$ has a range of $+1$ to $-1$ of which the upper and lower limits correspond to a fully correlated and anticorrelated motion during the simulation process. The dynamic cross-correlation analysis was calculated by the CPPTRAJ module of AMBER 14.

2.5. Binding free energy calculations
To estimate the binding affinities of ARM1 and SC-57461A bound to LTA$_4$H, the binding free energies were calculated using the molecular mechanics/generalized-born surface area method (MM/GBSA) (Kollman et al., 2000; Massova & Kollman, 2000; Tsui & Case, 2000; Onufriev, Bashford, & Case, 2000; Hou, Wang, Li, & Wang, 2011; Xu, Sun, Li, Wang, & Hou, 2013). Binding free energies were averaged over 1000 snapshots extracted from the 50 ns trajectory. The binding free energy ($\Delta G$) computed by this method for each molecular species (complex, ligand, and receptor) can be represented as follows:

$$\Delta G_{\text{bind}} = G_{\text{complex}} - G_{\text{receptor}} - G_{\text{ligand}}$$  \hspace{1cm} (2)

$$\Delta G_{\text{bind}} = E_{\text{gas}} + G_{\text{sol}} - TS$$  \hspace{1cm} (3)

$$E_{\text{gas}} = E_{\text{int}} + E_{\text{vdw}} + E_{\text{ele}}$$  \hspace{1cm} (4)

$$G_{\text{sol}} = G_{\text{GB}} + G_{\text{SA}}$$  \hspace{1cm} (5)

$$G_{\text{SA}} = \gamma \text{SASA}$$  \hspace{1cm} (6)

The term $E_{\text{gas}}$ denotes the gas-phase energy that consists of the internal energy $E_{\text{int}}$, Coulomb energy $E_{\text{ele}}$, and the van der Waals energies $E_{\text{vdw}}$. $E_{\text{gas}}$ was directly estimated from the FF99SB force field terms. The salvation free energy, $G_{\text{sol}}$, is estimated from the energy contribution from the polar states, $G_{\text{GB}}$ and nonpolar states, $G_{\text{SA}}$. The nonpolar solvation energy, $G_{\text{SA}}$, is determined from the SASA using a water probe radius of 1.4 Å, whereas the polar solvation, $G_{\text{GB}}$, contribution is estimated by solving the GB equation. The total entropy of the solute and temperature are denoted by $S$ and $T$, respectively.

2.6. Per-residue energy decomposition analysis
To obtain the contribution of each residue to the total binding free energy profile between the inhibitors ARM1 and SC-57461A, per-residue free energy decomposition was carried out at the atomic level for important residues using the MM/GBSA method in Amber 14.
3. Results and discussion

3.1. MD simulation and systems stability

The stability of the MD simulation was investigated by assessing the variations in the root mean square deviation (RMSD) with respect to their starting structures, as well as the potential energy (PE) of the structures during the simulation. Through the entire simulation process, there is negligible variation in the PE for all the systems (Supplementary Material S1). This reveals that all the systems under study were well equilibrated and stability was observed throughout the entire simulations.

RMSD analysis with respect to the backbone atoms of the 3D protein structure was employed in assessing its deviation from the starting structure over the simulation period. The RMSD results from the study for all the systems are shown in Figure 3. The RMSD plots of LTAR, LTPEP and LTARPEP as illustrated in Figure 3(A) indicates that LTAR attained a lower RMSD value than the LTARPEP and LTPEP systems throughout the simulation. The LTPEP system showed much higher RMSD pattern with much difference observed after 40 ns than both LTAR and LTARPEP systems. The overall RMSD values for the backbone atoms LTAR, LTPEP and LTARPEP were 1.298, 1.525, and 1.488 Å, respectively.

Furthermore, the LTAR and LTSC systems revealed significant difference in their RMSD pattern (Figure 3(B)), LTAR showed a lower RMSD pattern throughout the entire simulation with a lower RMSD value of 1.298 Å as compared to 1.801 Å of the LTSC system. LTAR system maintained a much more stable conformation throughout the entire simulation compared to LTSC, LTPEP and LTARPEP systems.

3.2. Root mean square fluctuation

The root mean square fluctuation (RMSF) of the protein backbone was calculated from the MD trajectories in order to evaluate and compare the amino acid residues flexibility for LTAR, LTPEP and LTARPEP as well as LTAR and LTSC. Figure 4(A), shows the RMSF values of LTAR, LTPEP and LTARPEP as a function of residue numbers. The plots clearly show that LTAR exhibits less protein flexibility than LTPEP and LTARPEP (Figure 4(A)). It was also observed that LTAR showed a slightly higher rigidity around its binding site residues (325–375).

LTPEP show an overall higher degree of flexibility when compared with the LTAR, and LTARPEP systems. Region 350–400 of LTPEP showed more flexibility when compared to LTAR and LTARPEP systems. A possible reason may be due to the absence of ARM1 since it forms direct interactions with amino acid residues in this region such as Phe362, Lys364, Leu365, and also interacts with residues, such as Leu369, Tyr378, Ala377, Asp375 (Figure 9 in Section 3.2.8). The aminopeptidase active site pocket residues in LTAR system showed similar RMSF patterns compared to LTPEP and LTARPEP. The overall average RMSF values for LTAR, LTPEP and LTARPEP were .763, .903 and .822 Å, respectively. LTAR is slightly less flexibility than LTSC, with much rigidity observed between residues 340–370 as highlighted in Figure 4(B). The extension of ARM1 beyond the position of SC-57461A’s phenyl ring to interact with Lys364, Leu365, and Phe362 with its thiazol-2-amine ring at the end of the hydrophobic cavity may explain the observed rigidity in this region.

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Figure 3. Root mean square deviation (RMSD) of the backbone atoms relative to the starting minimized structure over 50 ns simulation for LTAR, LTPEP and LTARPEP (A) and LTAR and LTSC (B).
3.2.2. Radius of gyration

To measure the overall protein dimension, Rg was analyzed and plotted. Rg provides insight into the compactness as well as the stability of a biomolecular structure such as protein structure (Lobanov, Bogatyreva, & Galzitskaya, 2008). The Rg of LTAR, LTPEP and LTARPEP were compared (Figure 5(A)). A significant difference is observed after 30 ns. LTAR shows a lower average Rg of 24.911 Å when compared to LTARPEP (24.976 Å). This indicates that LTA4H exhibits more structural stability when it binds to ARM1 alone than to both ARM1 and PGP. On the other side, average values of 25.01 and 24.97 Å for LTPEP and LTARPEP respectively, indicates less structural differences between the two systems.

We further compared the Rg pattern of LTAR and LTSC. Figure 5(B) clearly shows distinguishable overall Rg pattern during the entire simulation. LTAR shows an overall lower degree of oscillation than the LTSC system, indicating that LTAR has more compact packing with less flexibility than LTSC.

To further obtain more insight into the stability of the active site of the studied systems, the Rg of Cα atoms between the active site residues was analyzed. The Rg the active site residues of LTAR, LTPEP and LTARPEP was compared (Figure 6(A)). Comparing the three systems, LTAR confers a much more stable active site when

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Figure 4. RMS fluctuations (RMSF) of C-α atoms for LTAR, LTPEP and LTARPEP (A) and LTAR and LTSC (B) over 50 ns of simulation.

Figure 5. Rg of Cα atoms of LTA4H for LTAR, LTPEP and LTARPEP (A) and LTAR and LTSC (B).
compared to LTPEP and LTARPEP, with LTARPEP showing the least stable active site.

We further analyzed and compared the centre of mass distance between the active site residues of LTAR and LTSC. Figure 6(B) clearly shows that LTSC system exhibit much more unstable active site residues compared to LTAR.

### 3.2.3. Solvent accessible surface area

SASA of biomolecule such as proteins defines the proteins surface that interacts with its solvent molecules and provides insight in relation into the compactness of the protein structure (Richmond, 1984). The SASA for LTAR and LTARPEP were compared (Figure 7(A)). The overall SASA showed that LTAR had a slightly lower accessibility to solvent molecules as compared to LTARPEP. The average SASA values for LTAR and LTARPEP were 23,819.894 Å² and 23,831.821 Å², respectively. For LTPEP and LTARPEP, the SASA showed a less significant difference (Figure 7(A)), although LTPEP has a lower average SASA value of 23,306.866 Å² compared to 23,831.8213 Å² for LTARPEP. This shows that overall LTPEP has less accessibility to solvent molecules than LTARPEP.

The SASA of LTAR and LTSC were also compared. Figure 7(B) clearly shows that overall LTAR has a lower SASA than LTSC throughout the entire simulation. LTA4H accessibility to solvent molecules is also relatively lower when either only ARM1 or PGP binds, compared to when both bind. These findings suggest that the hydrophobic cavity of the protein structure may unfold upon binding of both ARM1 and PGP, allowing the

![Figure 6. Rg of Cα atoms of active site residues of LTA4H for LTAR, LTPEP and LTARPEP (A) and LTAR and LTSC (B).](image)

![Figure 7. SASA of LTAR, LTPEP and LTARPEP (A) and SASA of LTAR and LTSC (B) over the 50 ns simulation time.](image)
3.2.4. Total number of hydrogen bonds (H-bonds) in the systems

The number of intermolecular hydrogen bond for LTAR, LTPEP and LTARPEP as well as LTAR and LTSC were analyzed and plotted (S2). By comparing the number of HBs formed between LTAR, LTPEP and LTARPEP (S2.a), there is significantly less difference between HB formations for the three systems. The order of increasing HB formation is LTARPEP > LTPEP > LTAR with values 316.05 (~316), 315.23 (~315), and 314.99 (~315), respectively. For LTAR and LTSC systems (S2.b), LTSC shows fewer HBs than LTAR. The average H bonds formed by LTAR is 314.985 (~315) compared to 308.894 (~309) of LTSC.

3.2.5. Analysis of hydrogen bonding interactions between inhibitors (ARM1 and SC-57461A) and active site residues

To obtain further information on hydrogen bonding, HB interactions between ligands and active site residues and their percentage occupancy throughout simulation time were analyzed and the results are listed in Table 2.

Experimental findings indicate that ARM1 adopts two possible conformation (Figure 8) (Stsiapanava et al., 2014). The percentage HB occupancy analysis of ARM1 in LTAR and LTARPEP systems seems to suggest that the probability of ARM1 to be anchored with Lys364 and Phe362 might be much more probable as compared to Leu365 and Lys364.

In the SC-57461A-LTA4H complex, the NE2HE21 and NE2HE22 of Gln136 forms HB with the 03 and N of SC-57461A with a HB probability of 41.97 and 2.56%, respectively.

3.2.6. Conformational motions revealed by PCA

The application of PCA or ED can provide considerable insight into the nature of conformational changes in protein structures by identifying large as well as concerted conformational motions and underlying fluctuations (Jolliffe, 2002). The major constituents of correlated motions of the protein structure were obtained from covariance matrix diagonalization for the Cα atoms. In order to gain insight into the significance of such conformational motions, the conformational behavior of LTAR, LTARPEP, LTPEP and LTSC systems were projected along the first two principal components or eigenvectors (ev1/PC1 vs. ev2/PC2) directions (Figure 9).

The plot of LTAR and LTARPEP reveals different and clear conformational motions in the essential subspace along the two principal components (Figure 9(A) and (B)). In the LTPEP and LTARPEP system plots, a clear separation of motion is observed in both systems, with LTPEP showing a much more correlated motion along the first principal component compared to LTARPEP (Figure 9(B) and (D)). As observed for the other systems, LTAR and LTSC also depict a clear separation in their motion along the two principal components (Figure 9(A) and (C)).

3.2.7. Dynamic cross correlation matrices analyses

To further investigate the conformational dynamics of LTA4H upon ligand binding, the differences in internal correlation motions of all systems were analyzed by cross-correlation matrices of the Cα atom fluctuations. Figure 10 shows the DCCM plots of all studied systems;

| Complex | Acceptor | Donor | Occupancy (%) | Distance (Å)* | Angle (°)* |
|---------|----------|-------|---------------|---------------|------------|
| LTAR    | LYS364-O | 1V6-N2-H13 | 62.80 | 2.8384 | 162.2297 |
|         | LYS364-O | 1V6-N2-H12 | 21.08 | 2.8338 | 161.9408 |
|         | PHE362-O | 1V6-N2-H12 | 9.589 | 2.8637 | 142.2661 |
|         | PHE362-O | 1V6-N2-H13 | 3.03 | 2.8705 | 142.5678 |
|         | ALA377-O | 1V6-N2-H13 | .43 | 2.8671 | 157.5786 |
|         | LEU365-O | 1V6-N2-H12 | .01 | 2.819 | 158.7357 |
|         | 1V6-N2  | SER379-OG-HG | .01 | 2.8343 | 146.9423 |
| LTARPEP (ARM1) | LYS364-O | 1V6-N2-H12 | 84.21 | 2.8377 | 162.1784 |
|         | PHE362-O | 1V6-N2-H13 | 24.08 | 2.8658 | 144.5361 |
|         | LEU365-O | 1V6-N2-H12 | .24 | 2.8682 | 156.3504 |
| LTSC    | 28P-O3   | GLN136-NE2-HE21 | 41.97 | 2.8237 | 151.9451 |
|         | 28P-N1   | GLN136-NE2-HE22 | 2.56 | 2.9119 | 155.8339 |

*The HBs were determined by the acceptor … donor atom distance of <3.0 Å and acceptor … H-donor angle of >140 Å. 1V6 = ARM1 and 28P = SC-57461A.
Figure 8. Binding of ARM1 at the hydrolase active site of LTA₄H in two conformations. Conformation A ~ the thiazol-2-amine interacts with Leu365 and Lys364; and conformation B ~ with the thiazol-2-amine interacting with Lys364 and Phe362.

Figure 9. PCA projection of C-α atoms motion constructed by plotting the first two principal components (PC1 and PC2) in conformational subspace. PCA plots of LTAR (A), LTARPEP (B), LTSC (C) and LTPEP (D).
highly negative regions (blue–black) and highly positive regions (yellow–red) are associated with strong anticorrelated motions and strong correlated movements of specific residues, respectively. The four systems in the present study exhibited much overall correlated residual motions compared to anticorrelated motions.

An overall strong correlated residual motion is observed in LTARPEP (Figure 10(B)) than LTAR (Figure 10(A)) and LTPEP (Figure 10(D)). Comparison of LTAR and LTSC complexes (Figure 10(A) and 10(C)) shows that the binding of ARM1 leads to an overall increase in the extent of correlated residual motions as opposed to those observed in SC-57461A binding. Anticorrelated residual motions in LTSC complex occurs between residues 250–600 relative to 1–100, whereas strong correlated motions occurs between residues 200–600 relative to each other. In the ARM1 system, strong correlated residual motions occur between the residues 125–450 relative to each other whereas anticorrelated motion observed 475–600 relative to 1–100.

3.2.8. MM/GBSA binding free energy calculation

To gain insight into the energetics of LTA4H binding to SC-57461A and ARM1, the relative binding free energy and the various energy components contributions of the protein–ligand complexes were averaged over 1000 snapshots extracted from the 50 ns MD trajectories at an interval of 50 ps using MM/GBSA method. The contributions of electrostatic interaction, vdW interaction, and solvation to the relative binding free energies of SC-57461A and ARM1 to LTA4H were estimated to obtain a better view of which energy component that has more impact on the overall binding energy and are listed in Table 3.

The relative binding free energies without entropy contribution for SC-57461A and ARM1 were found to be −42.79 kcal/mol and −31.15 kcal/mol respectively, after introducing contribution from conformation entropy (−TΔS_{tot}), the predicted relative total binding free energies of SC-57461A and ARM1 were −19.089 and −12.369 kcal/mol respectively. The computed binding energies correlate well with their experimental $K_{i}$, IC$_{50}$

![Figure 10. Cross-correlation matrices of the fluctuations of coordinates for Cα atoms around their mean positions during the entire 50 ns simulation. DCCM plots of LTAR (A), LTARPEP (B), LTSC (C) and LTPEP (D).](image-url)
Table 3. Binding free energy analysis (kcal/mol) for inhibitor-LTA₄H complexes.

| Energy component (kcal/mol) | SC-57461A       | ARM1         |
|-----------------------------|-----------------|--------------|
| ΔE_{ele}                    | 61.62 ± 2.05    | -8.94 ± 2.78 |
| ΔE_{vdw}                    | -50.63 ± 4.20   | -40.52 ± 3.41|
| ΔG_{nonpolar}               | -7.15 ± .34     | -4.84 ± .301 |
| ΔG_{polar}                  | -46.63 ± 7.21   | 23.15 ± 1.93 |
| ΔG_{gas}                    | 10.99 ± 8.50    | -49.45 ± 4.54|
| ΔG_{sol}                    | -53.78 ± 7.35   | 18.31 ± 1.98 |
| -ΔTAS_{tot}                 | 23.70 ± 7.35    | 18.77 ± 6.86 |
| ^aΔG_{binding}              | -42.79 ± 6.01   | -31.15 ± 4.29|
| ^bΔG_{binding}              | -19.0899 ± 6.01 | -12.3698 ± 4.29|
| ^cΔG_{exp}                  | -10.41          | -7.68        |

Notes: ΔE_{ele}, electrostatic energy; ΔE_{vdw}, van der Waals energy; ΔG_{polar}, polar solvation energy; ΔG_{nonpolar}, nonpolar solvation energy.

^aΔG_{binding}, predicted total binding free energy without entropy effect.

^bΔG_{binding}, predicted total free binding energy with entropy effect.

^cThe experimental values were derived from the experimental kᵢ values in reference using the equation ΔG_{exp} = RTlnkᵢ.

Figure 11. A 2D Ligand residue interactions using Ligplot analysis for SC-57461A-LTA₄H (A) and ARM1-LTA₄H (B) complexes.
reported values (Stsiapanava et al., 2014; Askonas et al., 2002) and the estimated experimental binding energies. The entropy contribution SC-57461A-LTA4H (23.7037 kcal/mol) is much more unfavorable compared to that of ARM1-LTA4H (18.7768 kcal/mol).

As evident from Table 3, van der Waals, electrostatic, and nonpolar interactions were found to be the most favorable energy contributors to ARM1 binding to the protein. On the other hand, the favorable energies contributing to the binding of SC-57461A were found to be van der Waals interaction, polar, and nonpolar interactions with a strong unfavorable contribution from electrostatic interactions.

3.2.9. Per-residue interaction energy decomposition analysis

The total binding free energies for SC-57461A and ARM1 were further decomposed into individual residual contributions using the MM/GBSA method to understand inhibitor binding at an atomistic level. Figure 11 shows the interacting amino acid residues with inhibitors. Per-residue energy decomposition analysis is shown in Table 4 and Figure 12.

As evident from Table 4 and Figure 12(A), residues Phe314, Val367, Tyr378, Trp311, Pro382, Leu369, Leu365, Ala 377, Lys 364, Ala137, and Phe362 were found to contribute the most toward ARM1 binding to LTA4H. The larger residual energy contributions (|ΔGbinding| > -1 kcal/mol) were from Phe314 (-2.204), Val367 (-1.466), Tyr378 (-1.300), and Trp311 (-1.257). On the contrary, an unfavorable residual energy contribution was afforded by Asp375 (+0.82).

In the SC-57461A-LTA4H complex, the observed residues favoring binding were Tyr267, Tyr376, Phe314, Gln136, Ala137, Gln134, Leu369, Val367, Ala377, and His295. The residues contributing largely to SC-57461A binding (|ΔGbinding| < -1 kcal/mol) were Tyr267 (-4.530), Tyr376 (-2.726), Phe314 (-1.795), Gln136 (-1.731), and Ala137 (-1.022). However, residues Glu318 (+3.902), Glu296 (+1.788), Glu271 (+0.907), and His299 (+0.778) showed unfavorable energy contribution to SC-57461A binding (Table 4 and Figure 12(C)). The observed favorable residual energy contributions in both systems appear to be from Phe314, Val367, Leu369, Ala377, and Ala137. Phe314 was found to be the highest and third highest contributor to inhibitor binding for ARM1 and SC-57461A, respectively.

3.2.10. Binding mode analysis of ARM1 and SC-57461A

To obtain the difference in the binding of SC-57461A and ARM1, binding modes of these inhibitors in the active sites of LTA4H were analyzed. In the binding mode of SC-57461A, SC-57461A is anchored in the aminopeptidase active site pocket, extending into the hydrolase active site to interact with the hydrophobic pocket with its phenyl rings. Hence, SC-57461A binds at an interface between the hydrolase/aminopeptidase pockets (Figure 13). Figure 14(A) reveals that SC-57461A is anchored in the aminopeptidase active site pocket with its carbonyl oxygen forming a strong coordination bond with the catalytic zinc ion (Figure 14(A)).

HB analysis reveals that SC-57461A accepts two hydrogens from Gln136 forming two possible HBs with its nitrogen and carbonyl oxygen with a 2.56 and 41.97% probability, respectively.

The binding mode of ARM1 in the hydrolase active pocket is shown in Figure 14(B). The observed molecular interactions of ARM1 included HB network between its amino group in the thiazol-2-amine ring and the carbonyl oxygens of Lys364 and Phe362 or Lys364 and Leu365. ARM1 binds in the hydrophobic cavity by

| ARM1     | ΔEvdw ± SE | ΔDele ± SE | ΔGpolar ± SE | ΔGnonpolar ± SE | ΔGbinding ± SE |
|----------|------------|------------|--------------|-----------------|-----------------|
| TRP 311  | -1.849 ± .416 | -0.484 ± .365 | 1.194 ± .469 | -0.118 ± .036 | -1.257 ± .481 |
| PHE 314  | -2.635 ± .514 | -0.997 ± .401 | 0.784 ± .267 | -0.256 ± .040 | -2.204 ± .511 |
| VAL 367  | -1.469 ± .463 | -0.308 ± .246 | 0.474 ± .303 | -0.163 ± .034 | -1.466 ± .459 |
| TYR 378  | -1.339 ± .415 | 0.056 ± .244 | 0.135 ± .178 | -0.152 ± .038 | -1.300 ± .523 |
| PRO 382  | -0.996 ± .371 | -0.391 ± .203 | 0.477 ± .144 | -0.090 ± .021 | -1.000 ± .417 |

| SC-57461A | ΔEvdw ± SE | ΔDele ± SE | ΔGpolar ± SE | ΔGnonpolar ± SE | ΔGbinding ± SE |
|-----------|------------|------------|--------------|-----------------|-----------------|
| GLN 136   | -1.747 ± .385 | 0.028 ± .692 | 0.127 ± .460 | -0.139 ± .025 | -1.731 ± .803 |
| ALA 137   | -0.826 ± .251 | -0.337 ± .136 | 0.265 ± .147 | -0.124 ± .032 | -1.022 ± .292 |
| TYR 267   | -2.859 ± .385 | -5.260 ± .738 | 3.807 ± .522 | -0.217 ± .057 | -4.530 ± .680 |
| PHE 314   | -2.417 ± .431 | 0.001 ± .248 | 0.876 ± .248 | -0.253 ± .063 | -1.795 ± .453 |
| TYR 378   | -1.731 ± .780 | -3.554 ± 3.437 | 2.743 ± 1.270 | -0.184 ± .072 | -2.726 ± 2.043 |

Notes: ΔEvdw, electrostatic energy; ΔDele van der Waals energy; ΔGpolar, polar solvation energy; ΔGnonpolar, nonpolar solvation energy; ΔGbinding, total binding free energy.
Figure 12. Total residual binding free energies (A); van der Waals (vdW) and electrostatic (Ele) energy components (B) for ARM1-LTA₄H complex; and total residual binding free energies (C); van der Waals (vdW) and electrostatic (Ele) energy components (D) for SC-57461A-LTA₄H complex.

Figure 13. 3D superimposing of ligand residue interactions for PGP (cyan), SC-57461A (green), ARM1 (magenta) and Zinc metal (slate gray). The aminopeptidase active site pocket residues and hydrolase pocket residues shown in pink and orange, respectively.
engaging hydrophobic interactions with Gln136, Ala137, Phe314, Val367, Leu369, Ala377, and Tyr378. In addition, $\pi-\pi$ stacking interactions between Trp311 and the benzene ring of ARM1 can also be observed. Contrary to the observed binding mode of SC-57461A, ARM1 do not extend into the aminopeptidase active site pocket to interact with the Zinc ion as observed with the nonhydrolase selective inhibitors SC-57461A.

4. Conclusions

A compilation of computational analytical tools was used to understand the dual anti-inflammatory mechanism of LTA$_4$H and the selective inhibition by ARM1, a recently discovered anti-inflammatory compound. To this end, comparative MD simulations and analyses were performed for LTA$_4$H in complex with selective inhibitor (ARM1) as well as non-selective inhibitor (SC-57461A) in order to explore the binding landscape and dynamic and structural features associated with the binding of LTA$_4$H with different inhibitors. Results showed that, the binding of ARM1, a selective hydrolase inhibitor of LTA$_4$H, induces a more stable compact protein structure, when compared to the nonselective inhibitor SC-57461A. MM/GBSA calculations showed that ARM1 has lower binding affinity ($\sim$31.15 kcal/mol) compared to the nonselective inhibitor SC-57461A ($\sim$42.79 kcal/mol) – this is in great accordance with experimentally reported values. Per-residue energy decomposition analysis revealed that, amino acid residues Trp311, Phe314, Val367, Tyr378, and Pro382 are key residues largely contributing to the binding of ARM1, whereas residues Gln136, Ala137, Tyr267, Phe314 and Tyr378 largely contribute to the binding of SC-57461A. Furthermore, the HB and per-residue energy decomposition analyses show that Gln136 is an important residue towards the binding of SC-57461A. HB occupancy analysis also reveals that the probable stable configuration adopted by ARM1 in the hydrolase active site might be when it is anchored with Lys364 and Phe362 by its thiazol-2-amine ring.

Based on the information obtained from binding energy analyses (per-residue and binding forces), we believe that a more rational pharmacophore model- as well as structure-based design of novel and potent selective hydrolase inhibitors of LTA$_4$H as anti-inflammatory drugs is possible.

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Disclosure statement

No potential conflict of interest was reported by the authors.

Supplementary material

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