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Elucidation of Mechanism for Ligand Efficacy at Leukotriene B_4 Receptor 2 (BLT2)

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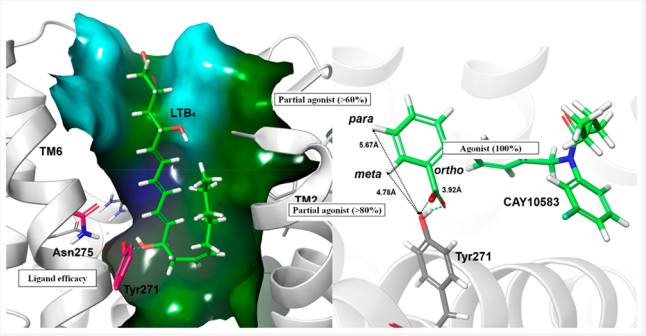
ABSTRACT: G protein-coupled receptors (GPCRs) have always been important drug targets in the pharmaceutical industry. One major question for the current GPCR drug discovery is how drugs have distinct efficacies at the same GPCR target. Related to this question, we studied how different ligands can have disparate efficacies at Leukotriene B_4 receptor (BLT2). By using molecular modeling studies, we predicted that Tyr271$^{6.51}$ located at TM6 of BLT2 performs as a key trigger for its activation and verified the prediction by site-directed mutagenesis, chemotactic motility studies, which included a chemical derivative of agonist CAY10583. We further identified Asn275$^{6.55}$ located at TM6 as a weak activation trigger in BLT2 and performed double mutation studies to confirm our computational results. Our results provide strong evidence for the exact mechanism of ligand efficacy at BLT2.



KEYWORDS: *Leukotriene B_4 receptor 2, ligand efficacy, molecular modeling, mutagenesis study, chemical study*

G protein−coupled receptors (GPCRs) are the first transmitters for cells to accept signals from the outside.[1] These receptors recognize various external stimuli such as extracellular chemical, sensory, and mechanical stimuli.[2] Drugs binding to GPCRs can promote or block physiological responses by modulating intracellular signal pathways.[3] The term ligand efficacy refers to the capability of a molecule to induce a specific physiological response of receptor activation.[4] The efficacy of a full agonist is by definition 100%, whereas that of a full antagonist is 0%. The efficacy of a partial agonist ranges from 0 to 100% while an inverse agonist has a negative efficacy.[5] Drugs that act on the same receptor can be used for different purposes based on their efficacies. Thus, the elucidation of efficacies of ligands at GPCRs has recently become a critical research subject in the GPCR drug discovery field.[6]

In the pharmaceutical industry, GPCRs are among the most extensively investigated drug targets.[7] Among them, leukotriene B_4 receptor 2 (BLT2) is a receptor for leukotriene B_4 (LTB_4), which is a pro-inflammatory lipid mediator, and is a recent promising GPCR drug target. It has been mainly recognized as a drug target for the management of inflammatory diseases[8] as well as cancer treatment.[9] BLT2 is minimally expressed in the homeostatic normal state and specifically overexpressed in response to stress environment-inflammation in asthma and cancer.[8,9] Recent studies suggest that blocking BLT2 is a potentially useful strategy for the treatment of several pulmonary inflammatory diseases, including asthma, acute respiratory distress syndrome (ARDS), acute lung injury (ALI), and chronic obstructive pulmonary disease (COPD).[10,11] Because of its association with various diseases, BLT2 has multiple feasibilities as a drug target.

However, due to the immune system-related role of BLT2, only ligands that exhibit antagonist efficacy are eligible for new drug candidates for BLT2. In this context, understanding the mechanism of ligand efficacy at the molecular level is necessary for the selective design of a compound. Based on recently solved GPCR structures,[12] it has been shown that the difference between agonists and antagonists can be explained by distinct interactions with a few binding site residues. In the case of the β2-adrenergic receptor, agonist BI-167107 forms a distinct hydrogen bond with S207$^{5.46}$ (the superscript indicates Ballesteros−Weinstein numbering.) The serine undergoes conformational changes in the agonist-bound active state and stabilizes the active state by forming a hydrogen bond with the agonist. However, the crystal structure has not been solved for

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BLT2 and the molecular mechanism responsible for ligand-dependent efficacy for BLT2 remains poorly understood.

In this study, we aim to elucidate the mechanism of ligand efficacies at BLT2 using a series of molecular modeling techniques and experimental verification. First, we predicted the three-dimensional (3D) structures of BLT2 in *apo* and agonist- or antagonist-bound forms at an atomic scale using molecular modeling. Based on the predicted structures, we identified residues that could determine ligand efficacy. Subsequently, we verified our predictions through site-directed mutagenesis and cell motility experiments. By chemically modifying a known agonist, we further confirmed our proposed ligand efficacy mechanism thus enabling the design of partial agonists. Additionally, we discuss in detail our molecular modeling procedures of class A GPCR structure prediction and GPCR ligand docking used to identify the mechanism for ligand efficacy at BLT2.

First, we predicted the 3D structure of human BLT2 (Figure S1) using the GPCR homology modeling method we have developed (Supporting Information). The crystal structure of guinea pig BLT1 (PBD ID: 5X33) recently revealed was used as the template for homology modeling. BLT2, like BLT1, belongs to the leukotriene receptor family and recognizes the same native ligands including LTB_4. Human BLT2 and BLT1 exhibit 39.5% sequence identity and have similar amino acid compositions in the ligand binding sites (Figure S2).

Seven transmembrane (TM) helices of GPCRs show considerable variations in the helix shape because of kink structures.[13] Because conformations of TMs determine the shape and volume of the ligand-binding site, accurately predicting the helix conformation is crucial. Therefore, we added two additional modeling steps. First, we ensured that the position of proline or glycine that can form a kink in the helix structure is consistent between template-BLT1 and target-BLT2 (Figure S2). Second, we performed molecular dynamics (MD) simulation to optimize the predicted structure of BLT2 under explicit membrane and solvent environments. MD results showed that the predicted BLT2 structure rapidly entered the equilibrium state at 200 ns and maintained stable conformations to 1 μs (Figure S3). For comparison, we performed another MD calculation on the crystal structure of BLT1. At the equilibrium state, average heavy atom RMSD values of the crystal structure of BLT1 and the predicted structure of BLT2 were 4.84 and 4.86 Å, respectively.

Considering the thermal fluctuation of the BLT1 crystal structure, one can conclude that the predicted BLT2 structure did not undergo significant structural changes during the MD simulation. This result also implies that the initial predicted BLT2 structure is as stable as the crystal structure of BLT1. The only notable structural difference between the predicted-BLT2 and template-BLT1 is the conformation of extra-cellular loop 2 (ECL2). Among the six loop structures in GPCRs, ECL2 was found to be in direct interactions with the bound ligand.[14] The predicted BLT2 structure has a distinct parallel β sheet in ECL2 like other members of γ-branch GPCRs including BLT1 and opioid receptors.[15] However, the ECL2 of BLT2 is four residues shorter and located further inside the ligand binding site than that of BLT1 (Figure S1).

Following the structure prediction of BLT2, we performed a docking simulation on the BLT2 structure to predict the binding pose of ligands. GPCRs have various conformational ensembles between active and inactive states, and the ligands form complexes with their respective states of receptors according to their efficacies.[16] Our predicted structure of BLT2 is based on an antagonist-bound structure of BLT1; therefore, to accommodate the possibility of conformational changes when an agonist binds BLT2, we introduced a structure optimization step for protein−ligand complex after the docking. It is well-known that the overall conformational changes of GPCRs in active and inactive forms are difficult to simulate.[17] On the other hand, the structure optimization was performed only on the binding site residues within 5 Å of the ligand using the Prime[18] module of the Schrödinger suite.

We first predicted a binding pose of a native agonist leukotriene B_4 (LTB_4). In the binding pose, LTB_4 adopts a U-shaped conformation in the binding site of BLT2 (Figure 1).

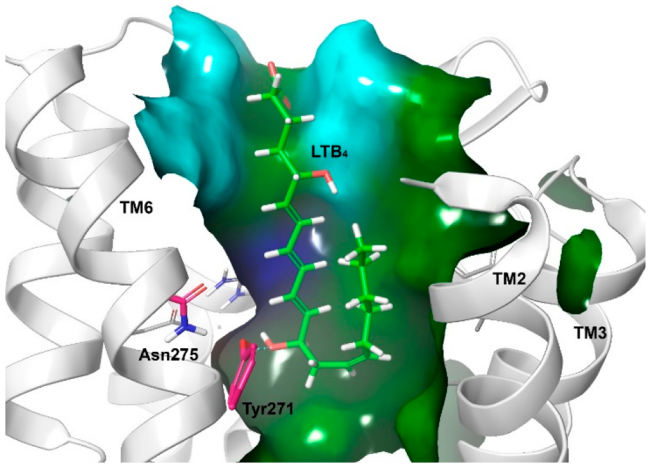

Figure 1. Structural features of agonist LTB_4 binding in BLT2 (green surface: hydrophobic, cyan surface: polar uncharged).

The position 1 carboxyl group located in the binding site entrance participates in various interactions with residues in ELC2; the position 4 hydroxyl group forms hydrogen bonds with $Gln298^{7.33}$; and the position 12 hydroxyl group forms a hydrogen bond with residue $Tyr271^{6.51}$, located deep in the binding site pocket. Long hydrophobic chain position 13−20 forms van der Waals contacts with the hydrophobic region in the inner binding site. The predicted binding pose of LTB_4 is similar to that obtained from the nuclear magnetic resonance (NMR) experiment.[19] This agreement strongly suggests that our predicted BLT2 structure and modeling methods are adequate for studying BLT2 and ligand interactions.

We conducted additional docking simulations using the agonists 12(*S*)-HETE, 12-HHT, and CAY10583[20] to find distinct interactions of agonists. 12(*S*)-HETE and 12-HHT are native BLT2 agonists derived from arachidonic acids such as LTB_4, whereas CAY10583 is a synthetic agonist. Prior to the interaction analysis, we calculated binding free energies of the four agonists based on the docking results using the molecular mechanics/generalized Born surface area (MM/GBSA) method, and compared the calculation results with the experimentally measured binding affinities to ensure the reliability of the docking results. Experimentally, 12-HHT exhibited the highest binding affinity for BLT2, followed by LTB_4 and 12(*S*)-HETE, respectively.[21] Our calculated binding energies were in the order consistent with the experimental results (Table S1). The two native BLT2 agonists commonly adopt a U-shaped conformation similar to LTB_4 and CAY10583 was bound in the transverse form of the binding

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site. Analysis of the interactions revealed that all agonists commonly formed hydrogen bonds with residue Tyr271$^{6.51}$ in helix 6 (Figure 2). As the TM6 participates in the activation mechanism of GPCR,[22] we hypothesized that Tyr271$^{6.51}$ serves as an activation trigger for BLT2.

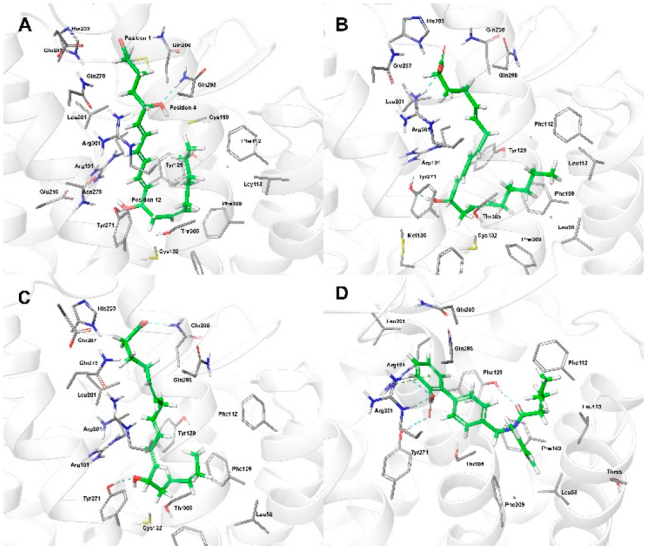

Figure 2. Binding poses of four agonists. (A) LTB_4, (B) 12(*S*)-HETE, (C) 12-HHT, (D) CAY10583 (white ribbon: BLT2, gray carbon tube: binding site residues of BLT2, green carbon tube: agonists, dotted-cyan line: hydrogen bond, dotted-green line: $\pi-\pi$ stacking interaction).

To test the above hypothesis, we performed further docking simulations using antagonists SC-41930,[23] CGS-25019C,[24] LY255283,[25] and CP-195543.[26] The docking results showed that all four antagonists do not form hydrogen bonds with Tyr271$^{6.51}$ (Figure S4), which confirms our hypothesis that agonists selectively form hydrogen bonds with Tyr271; thus, Tyr271 acts as an activation trigger for BLT2.

The simulation results were verified using a site-directed mutagenesis approach. Recent studies have shown that activation of BLT2 regulates cell motility functions, such as chemotaxis.[27] Measuring the biological activities with chemotaxis logically validates how the agonists trigger the activity. We performed a competitive binding experiment of CAY10583 with LTB_4 to confirm that it does bind in the same pocket (Figure 4). To check if Tyr271$^{6.51}$ triggers BLT2 activation, we produced a BLT2 Y271A mutant and examined its ability to elicit chemotaxis in transiently transfected CHO-K1 cells. In accordance with our prediction, the potency of the four agonists of BLT2 to induce chemotaxis decreased in the BLT2 Y271A mutant when compared with the wild-type BLT2 (Figure 3B).

However, the chemotactic motility of the cells transfected with the Y271A mutant was not completely blocked in response to LTB_4 or 12(*S*)-HETE. According to previous GRCR activation mechanistic studies,[4,28] GPCR undergoes helical rearrangement of TM5, TM6, and TM7 while transforming from the inactivation form to the activation form. The bound agonist stabilizes the active state by interacting with the key residues. A crystal structure of the A_{2A} complexed with agonist NECA offers structural features of agonist binding.[12] In this case, NECA has hydrophobic contacts with residues of TM2 and TM3 and hydrogen

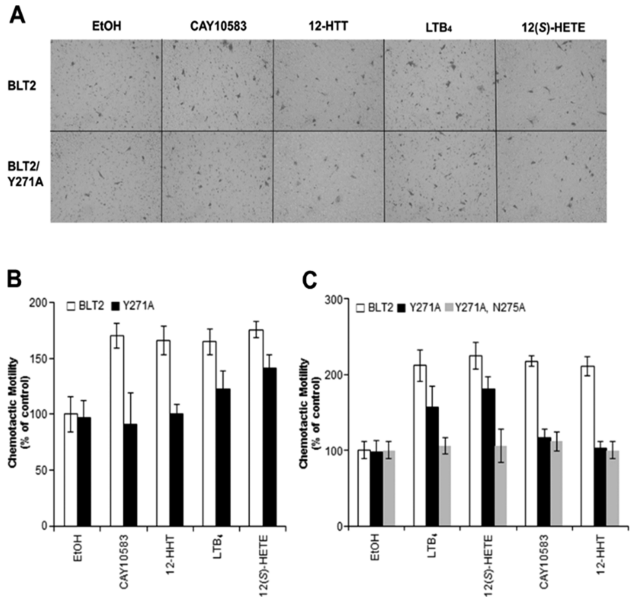

Figure 3. (A) Migrating cells were fixed and stained with hematoxylin/eosin. (B) Agonists-induced chemotactic motility was determined in wild-type and mutant BLT2 (Y271A) expressed CHO cells. (C) Agonists-induced chemotactic motility was determined in wild-type and mutant BLT2 (Y271A/N275A) expressed CHO cells.

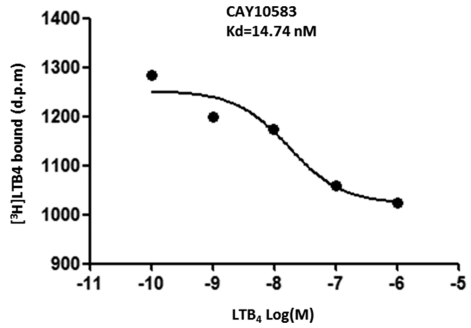

Figure 4. Competitive binding for CAY10583 against LTB_4.

bonds with residues of TM6. In our modeling results, all agonists form hydrogen bonds with Tyr271 of TM6 and van der Waals contacts with binding site residues of TM2 and TM3, as shown in Figure 2. In our mutagenesis experiments, however, LTB_4 and 12(*S*)-HETE did not lose their activity completely at BLT2 Y271A mutant, as shown in Figure 3. This phenomenon can be attributed to the observation that the two agonists have longer hydrophobic chains than 12-HTT, which maintain van der Waals contacts with TM2 and TM3 (Figure 2) and form additional hydrogen bonds with Asn275$^{6.55}$ located at a higher position than Tyr271$^{6.51}$ in TM6. Therefore, we formulated an additional hypothesis that for LTB_4 and 12(*S*)-HETE, there exists a second agonist-specific interaction, namely the hydrogen bond with Asn275, which leads to partial agonism.

To test this additional hypothesis, we examined the ability of each ligand to elicit chemotaxis from the BLT2 Y271A/N275A double mutant. As shown in Figure 3C, now LTB_4-elicited chemotaxis was completely blocked in cells transfected with the BLT2 Y271A/N275A double mutant along with all the other ones elicited by other agonists. Our experiments verified that Tyr271$^{6.51}$ is the preferential residue that leads to BLT2 activation and Asn275$^{6.55}$ plays an assisting role.

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Our prediction for the role of Tyr271$^{6.51}$ was also verified through chemical variation. We synthesized a new compound, dubbed AC-1657, in which a methyl group replaces the carboxyl group of CAY10583 (Figure 5). As the carboxyl group

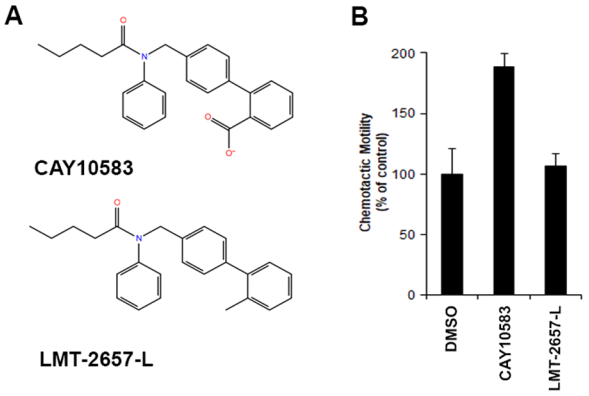

Figure 5. Synthetic compound AC-1657 could not induce chemotactic motility.

of CAY10583 formed a hydrogen bond with Tyr271$^{6.51}$, we could verify the role of Tyr271$^{6.51}$ through a chemotactic motility test using LMT-2657-L. The cells transfected with wild-type BLT2 exhibited significant chemotactic motility in response to CAY10583 but not to LMT-2657-L as shown in Figure 5. Furthermore, we tested the criticality of the hydrogen bond between Tyr271$^{6.51}$ and the carboxyl group of CAY10583 via binding affinity measurement. By measuring the ability of LMT-2657-L to compete for ^{3}H-LTB$_4$ binding to the membranes of cells expressing BLT2, we observed that the AC-1657 compound has no ligand binding affinity. Tyr271$^{6.51}$ plays important roles not only as an activation trigger for agonists but also as an important residue in the ligand binding of them. The major interaction force between Tyr271$^{6.51}$ and the agonists is revealed to be the hydrogen bond.

Our simulation results showed that the agonists form hydrogen bonds with Tyr271$^{6.51}$ and this interaction stabilizes the active state of the receptor. Thus, we hypothesized that a compound that has a weak hydrogen bond with Tyr271$^{6.51}$ could act as a partial agonist. To test this hypothesis, we synthesized two compounds called LMT-1886-L and LMT-1887-L in which the carboxyl group at the ortho- position of CAY10583 was shifted to the meta- and para-positions, respectively (Figure 6). The chemical modifications increased the distances of the hydrogen bond between the carboxyl group and Tyr271$^{6.51}$, and the strength of the two hydrogen bonds would be weakened. Indeed, the chemotactic motilities of the cells transfected with the wild-type BLT2 partially decreased in response to LMT-1886-L and LMT-1887-L. Our results strongly suggest that the hydrogen bond between Tyr271$^{6.51}$ and the agonist is critical in stabilizing the active state of the receptor.

We also designed selective BLT2 antagonists based on the revealed activation mechanism of BLT2 thereby proving our hypotheses even further. Using the LMT compound as a template scaffold, we designed a series of derivatives and predicted their ligand efficacies and binding energies using the molecular modeling approaches. LMT-2074-L, the full structure of which we cannot reveal in this paper, emerged in the end. LMT-2074-L does not form any hydrogen bond with Tyr271$^{6.51}$ and Asn275$^{6.55}$ but a hydrogen bond and an

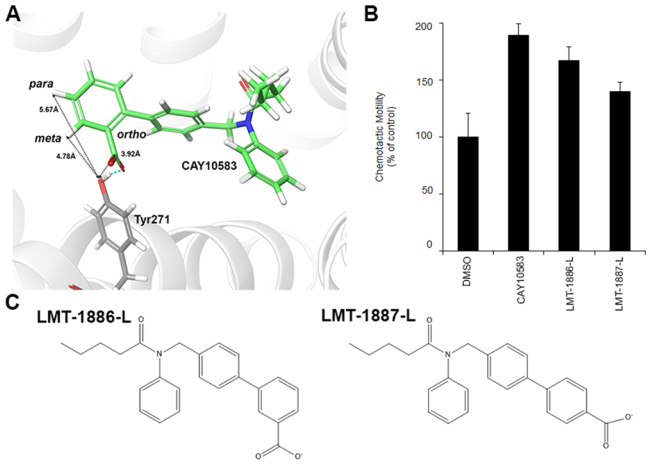

Figure 6. Chemotactic motility decreased with increasing distance of hydrogen bond between Tyr271$^{6.51}$ and carboxyl group. (A) Distance measurements between Tyr271 and carbon atoms of terminal aromatic ring. (B) Chemotactic motility results. (C) Chemical structures of LMT-1886-L and LMT-1887-L.

ionic interaction network with Arg301 of TM7, Gln278 of TM6, and His203 of ECl2. AC-1074 not only acted as an antagonist in cell-based chemotaxis test but also displayed IC$_{50}$ value of 132 nM from competitive ligand binding affinity measurement using radiolabeled LTB$_4$ (Figure 7), which is

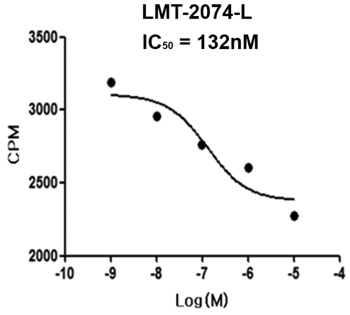

Figure 7. Competitive binding for LMT-2074-L against LTB$_4$.

lower than that of a known BLT2 antagonist LY255283 (150 nM). Our calculation of the binding affinities indeed conforms to this result giving −6.15 kcal/mol for LMT-2074-L and −5.985 kcal/mol for LY255283.

In conclusion, we identified the binding site residues, which determine ligand efficacy at BLT2. In GPCRs, ligand efficacy is related to the structural rearrangement of the receptor; however, gaining structural insights by exclusively using experimental methods is difficult. In this work, we overcame such difficulty by employing molecular modeling techniques. We constructed a 3D structure of BLT2 and predicted binding poses of agonists and antagonists using our own GPCR-specific structure modeling protocol and docking method. Utilizing molecular modeling, we predicted that Tyr271$^{6.51}$ and Asn275$^{6.55}$ act as activation triggers of BLT2. Then we verified the modeling results through chemotactic motility tests using BLT2 mutants. The chemical variation test further confirmed that the hydrogen bond between the agonist and Tyr271$^{6.51}$ is an important interaction for agonist binding and BLT2 activity. Finally, we designed two partial agonists based on the BLT2 activation mechanism we constructed herein.

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Several site-directed mutagenesis experiments have stated that residues at position 6.51 are associated with GPCR activation.[29] However, to the best of our knowledge, this is the first study that identifies residues related to the activation of leukotriene receptor BLT2. Furthermore, because of the variety of residues expressed at the 6.51 position of GPCRs, investigating the distinct interactions between the 6.51 residue and agonists using only site-directed mutagenesis experiments is difficult. However, we have successfully shown that the hydrogen bond between $Tyr271^{6.51}$ and agonists is an important interaction to stabilize the active state of BLT2 using docking and LMT-2657-L chemical variation studies.

The discovery of activation triggers would help develop selective BLT2 agonists and antagonists. We were able to design an antagonist with significant binding affinity to BLT2, but as is evident in comparison of its binding with that of a known agonist (Figures 4 and 7), missing the interaction to the trigger residues weakens the binding. Therefore, to design an effective antagonist, how to strengthen the binding without interacting with the two trigger residues must be investigated. We are continuing our research effort along this line to design more potent antagonists.

Additionally, our strategy of combining computational prediction with site-directed mutagenesis can be used to elucidate different biases of other agonists by highlighting involved residues. Overall, the cooperation of computational and experimental approaches reported herein provides a promising strategy for investigating ligand efficacy at GPCRs.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmedchemlett.0c00065.

Experimental details for molecular modeling, cell chemotaxis assay and compound synthesis. NMR data for newly synthesized compounds. Molecular modeling figures. (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

GPCRs, G protein-coupled receptors; BLT2, leukotriene B_4 receptor 2; LTB_4, leukotriene B_4; MD simulation, molecular dynamics simulation; ECL2, extra cellular loop 2; QM/MM, quantum mechanics/molecular mechanics; ARDS, acute respiratory distress syndrome; ALI, acute lung injury; COPD, chronic obstructive pulmonary disease; HA, hemeagglutinin; MM/GBSA, molecular mechanics/generalized Born surface area; PCR, polymerase chain reaction; BAC, bacterial artificial chromosome

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