Small-Molecule Inhibition of TLR8 through Stabilization of its Resting State

Toll-like receptors (TLRs) play critical roles in the innate immune system by recognizing microbial lipids, carbohydrates, nucleic acids, and proteins. However, excessive TLR activation disrupts the immune homeostasis and consequently contributes to the development of many inflammatory and autoimmune diseases, such as systemic lupus erythematosus, infection-associated sepsis and so on. Therefore, inhibition of TLR signaling pathways has been predicted to be an effective therapeutic strategy for the pathogenesis of a variety of autoimmune diseases. TLR8 senses a single-stranded RNA and has been suggested to play pivotal roles in various inflammatory disorders and autoimmune diseases, but there have been no reports of small molecule inhibitors of TLR8 in the literature. We identified the first-of-its-kind human TLR8-specific small molecule antagonist with high potency and selectivity. We also solved the crystal structure of TLR8-antagonist complex. The antagonist selectively bound to the unliganded form of TLR8 and stabilized the inactive dimer conformation of TLR8. These results not only suggest a novel strategy for TLR inhibitor design, but also shed critical mechanistic insight into these clinically important immune receptors.

Toll-like receptors (TLRs) play a key role in the innate immune system by recognizing structurally conserved pathogen-associated molecular patterns and danger-associated molecular patterns. TLR is a type I transmembrane receptor consisting of extracellular leucine-rich repeat (LRR) domain, transmembrane domain, and intracellular Toll/IL-1 receptor (TIR) domain. The LRR and TIR domains are responsible for the ligand recognition and signaling, respectively. Among 10 TLRs in humans, TLR3, 7, 8 and 9 localize in endosomal compartments and detect viral and bacterial signature molecules, including double-stranded RNA (TLR3), singled-stranded RNA (TLR7/8), or unmethylated CpG sequences in DNA (TLR9). There is a considerable amount of evidence indicating that excessive activation of endosomal TLRs including TLR8 significantly contributes to the pathogenesis of a variety of autoimmune diseases, such as systemic lupus erythematosus and rheumatoid arthritis. However, few small molecules that specifically target TLR8 have been identified, which drove us to pursue TLR8 inhibitors in view of their significant potential. The development of potent and selective small-molecule inhibitors targeting TLR8 could provide useful tools to further the mechanistic understanding of TLR8 inhibition and regulation, and help the future development of anti-autoimmune disease therapeutics.

To discover specific TLR8 signaling inhibitors, we first developed a cell-based, high-throughput screening assay with an engineering HEK293 cells overexpressing human TLR8 and identified pyrazolo[1,5-a]pyrimidine derivatives as potent TLR8 inhibitors with a K_d value of 220 nM measured by ITC. With hit-to-lead SAR efforts, we successfully identified a highly potent TLR8 inhibitor, CU-CPT9b, with a K_d value of 21 nM. The derivatives of compound CU-CPT8m effectively inhibited TLR8-mediated proinflammatory signaling in a variety of

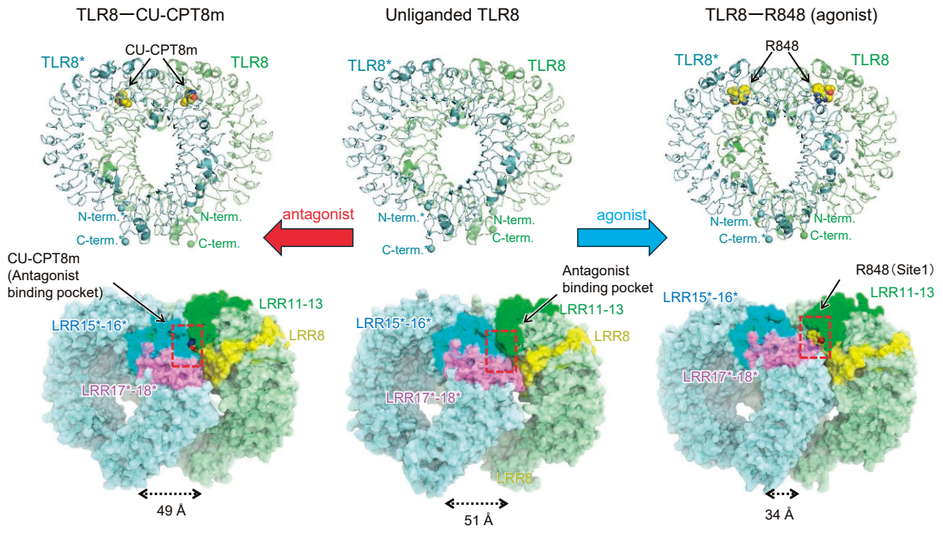

Figure 1: Crystal structures of antagonist bound, unliganded and agonist bound forms of TLR8.

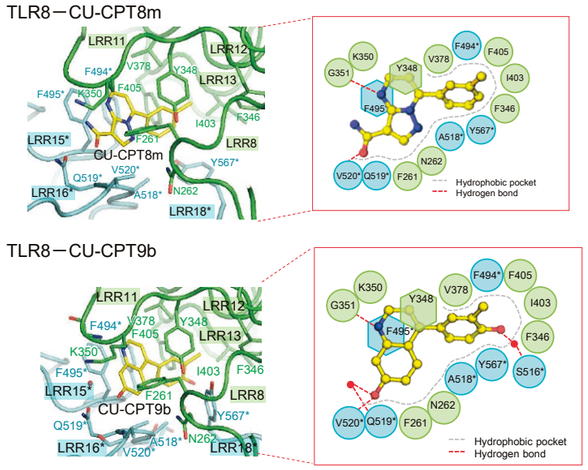

Figure 2: Close-up views of the antagonist binding site and schematic representation of interactions of TLR8–CU-CPT8m and TLR8–CU-CPT9b.

cultured cell lines (CU-CPT9b inhibited TLR8 signaling with a sub ~nM IC_{50} value in HEK293 cells) [1].

To obtain molecular insights into the inhibition mechanism, we solved the crystal structure of TLR8–CU-CPT8m complex [1]. A diffraction dataset was collected on beamline AR-NE3A and BL-5A under cryogenic conditions at 100 K. To our surprise, it was found that CU-CPT8m selectively bound to the unliganded form of TLR8 and stabilized the inactive dimer conformation of TLR8 (**Fig. 1**).

Previously, two ligand-binding sites were identified for TLR8 and TLR7 in the agonist bound form [2, 3]. In TLR8, small chemical ligands, such as R848 and CL097, and uridine bind to Site 1, whereas Site 2 is bound by the oligoribonucleotide. Interestingly, CU-CPT8m is accommodated in a hydrophobic pocket on the protein-protein interface of TLR8 and TLR8*. This pocket is only formed in the preformed dimer in the resting state (unliganded form).

CU-CPT8m is mainly recognized by van der Waals interactions with hydrophobic residues (**Fig. 2**). The pocket is mainly created by LRR11-13 and LRR15*-16*. Note that TLR8 utilizes LRR11-13 for both agonist and antagonist binding on one side of the interface, while on the other side LRR17*-18* and LRR15*-16* are used for agonist and antagonist binding, respectively. Therefore, this new binding site is close to but distinct from Site 1, implying a unique inhibitory mechanism by CU-CPT8m. We showed that the ectodomains of TLR8 undergo conformational changes upon agonist-induced activation, resulting in less separation of their C-termini [4]. The distances between the C-termini of the two protomers of TLR8 dimer are 49 Å in TLR8–CU-CPT8m and 51 Å in unliganded TLR8, respectively (**Fig. 1**). These values are obviously larger than that of agonist-bound activated dimer (34 Å), in which the two C-termini come closer to allow dimerization of intracellular domains and downstream signaling.

We also determined the crystal structure of TLR8–CU-CPT9b complex. CU-CPT9b binds to the same site as CU-CPT8m and is recognized in a similar manner (**Fig. 2**).

Our results indicate that apo TLR8 exists as preformed dimer in its resting state and CU-CPT8m recognizes a novel binding site distinct from Site 1, whose occupation prevents TLR8 activation. More importantly, such a resting state could provide a novel target for TLR inhibitor design, which could be generally applicable to all TLR family proteins. This new mechanistic insight may be a crucial step towards solving the global-inflammation problem that has plagued existing TLR7/8 vaccine adjuvants.

REFERENCES

[1] S. Zhang, Z. Hu, H. Tanji, S. Jiang, N. Das, J. Li, K. Sakaniwa, J. Jin, Y. Bian, U. Ohto, T. Shimizu and H. Yin, *Nature Chem. Biol.* **14**, 58 (2018).
[2] H. Tanji, U. Ohto, T. Shibata, M. Taoka, Y. Yamauchi, T. Isobe, K. Miyake and T. Shimizu, *Nature Struc. Mol. Biol.* **22**, 109 (2015).
[3] Z. Zhang, U. Ohto, T. Shibata, E. Krayukhina, M. Taoka, Y. Yamauchi, H. Tanji, T. Isobe, S. Uchiyama, K. Miyake and T. Shimizu, *Immunity* **45**, 737 (2016).
[4] H. Tanji, U. Ohto, T. Shibata, K. Miyake and T. Shimizu, *Science* **339**, 1426 (2013).

BEAMLINES

AR-NE3A and BL-5A

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