Cryo-EM and Crystallographic Analyses Reveal the Inhibition Mechanism of TLR7

Toll-like receptor 7 (TLR7) is an innate immune receptor for RNA viruses such as HIV-1 and coronaviruses. TLR7 boosts the immune response upon activation by ssRNAs, guanosine nucleosides and certain low-molecular ligands. However, unwanted activation of TLR7 by endogenous ssRNAs mediates the pathogenesis of certain autoimmune diseases. A series of TLR7-specific antagonists has been developed via modification of the TLR7 agonistic ligand. Using a combination of crystallographic and cryo-EM studies, we revealed the detailed inhibition mechanism of TLR7 by its antagonists. These antagonists bound to a newly observed ligand pocket inside the TLR7 dimerization interface and induced the conformational dynamics of dimeric TLR7, and thus inhibited TLR7 activation.

Toll-like receptors (TLRs) are crucial innate immune receptors for the surveillance of a broad spectrum of pathogenic molecules [1]. Upon activation by pathogenic stimulators, TLRs dimerize to activate downstream immune responses [1]. Among ten human TLRs, TLR7 is identified as a receptor for RNA viruses [2]. In recent years, our research group reported a number of crystal structures of TLR7/agonist complexes in the activated form and disclosed the activation mechanism of TLR7 [3, 4]. TLR7 contains two ligand binding sites (site 1 and site 2). Site 1 binds to small-molecule ligands, such as guanosine, 2',3'-cGMP and imidazoquinoline derivatives. Site 1 ligands directly mediate the dimerization of TLR7. Uridine-containing ssRNAs bind to site 2 and enhance the binding of site 1 ligands. TLR7 can also be accidentally activated by endogenous ssRNAs which leads to the pathogenesis of certain autoimmune diseases such as systemic lupus erythematosus (SLE) [5]. Thus, TLR7 is considered as a promising drug target for the treatment of related autoimmune diseases. For this purpose, a series of TLR7 antagonists (such as Cpd-6 and Cpd-7) have been developed through a strategy of modifying a potent TLR7 agonist (Cpd-3) (**Fig. 1A**) [6]. Cpd-6 and Cpd-7 showed potent TLR7-specific inhibitory activity and could ameliorate deadly symptoms in an autoimmune mouse model [6]. Nonetheless, the detailed inhibitory mechanisms of Cpd-6 and Cpd-7 have not been demonstrated.

We prepared recombinant *Macaca mulatta* TLR7 extracellular domain using *Drosophila* S2 cell lines. To obtain functional protein and facilitate crystallization, the Z-loop of TLR7 (a conserved autoinhibition structural motif of the TLR7 family) was cleaved using thrombin and *N*-glycans were artificially trimmed. We conducted crystallization of TLR7/Cpd-3, TLR7/Cpd-6 and TLR7/Cpd-7 complexes. As a result, the crystal structures of TLR7/Cpd-3 and TLR7/Cpd-6 complexes were successfully determined. Unfortunately, we did not obtain good-quality crystals of the TLR7/Cpd-7 complex. As an agonist, Cpd-3 bound to TLR7 site 1 and the crystal structure of TLR7/Cpd-3 forms a typical activated dimeric structure which is highly similar to other reported TLR7/agonist complex structures (**Fig. 1B**). Surprisingly, the crystal structure of TLR7/Cpd-6 complex also

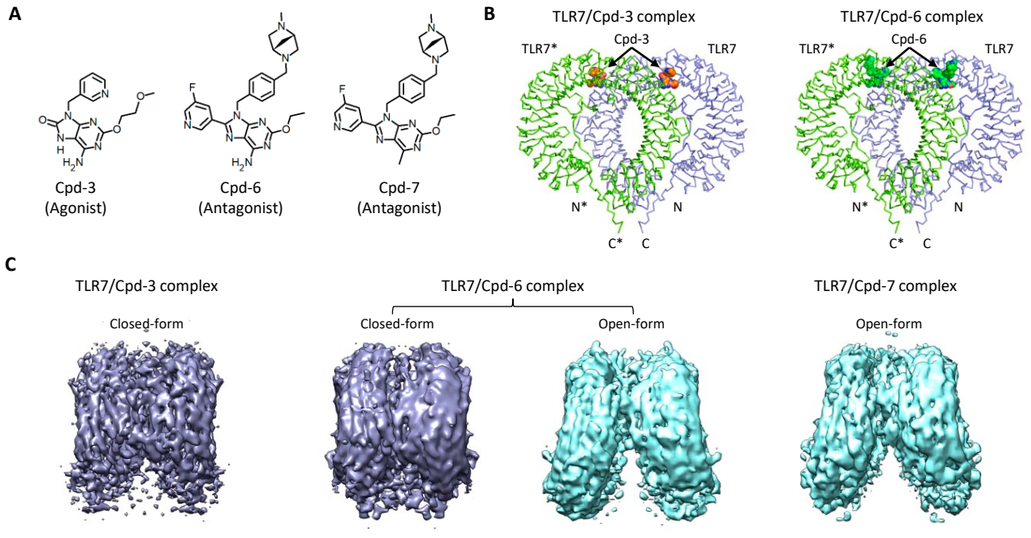

Figure 1: A Chemical structures of Cpd-3, Cpd-6 and Cpd-7. **B** Crystal structures of TLR7/Cpd-3 and TLR7/Cpd-6 complexes. **C** Cryo-EM reconstructions of TLR7/Cpd-3, TLR7/Cpd-6 and TLR7/Cpd-7 complexes.

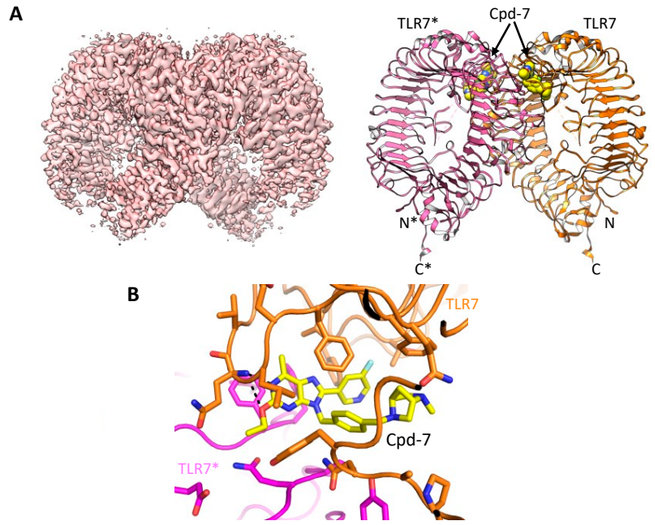

Figure 2: A High-resolution cryo-EM structure of TLR7/Cpd-7 complex. **B** Detailed view of Cpd-7 binding pocket.

adopts an activated dimeric conformation despite the fact that Cpd-6 is an antagonist (**Fig. 1B**). The binding mode of Cpd-6 also resembles that of Cpd-3 with the features commonly observed in other agonists. Therefore, it is difficult to explain its inhibitory mechanism by the activated dimer structure of TLR7 induced by Cpd-6.

Considering that a single crystal structure is static and might only represent one aspect of the TLR7 structure induced by Cpd-6, in the next stage, we shifted to cryo-EM analysis for visualizing possible conformational polymorphism of TLR7 induced by Cpd-6 and Cpd-7. We prepared glutaraldehyde-crosslinked TLR7/Cpd-3, TLR7/Cpd-6 and TLR7/Cpd-7 complexes for cryo-EM analysis. To our surprise, these three complexes output different conformations (**Fig. 1C**). For Cpd-3, TLR7 dimer predominantly adopts a closed-form conformation which is consistent with its crystal structure. In the case of Cpd-6, both the closed-form conformation and an open-form conformation, which was identified in this study for the first time, were observed. For Cpd-7, TLR7 dimer exclusively forms the open-form conformation. The C-termini of two TLR7 protomers in the open-form conformation are more separated from each other compared to those in the closed-form conformation. Therefore, the open-form conformation prevents the dimerization of the intracellular domain of TLR7 which is essential for signaling. Thus, the open-form conformation captured by cryo-EM represents an inhibited form of TLR7.

To further visualize the ligand binding mode inside the open-form conformation of TLR7, we successfully solved the cryo-EM structure of TLR/Cpd-7 complex at a 2.8-Å resolution using a Titan Krios microscope (**Fig. 2A**). A newly defined antagonist binding site was observed inside the TLR7 dimerization interface. Interestingly, this antagonist binding site is spatially close to site 1 in the closed form, although the binding mode is largely rearranged. Cpd-7 is mainly recognized via characteristic hydrophobic contacts and hydrogen bonds (**Fig. 2B**). Cpd-7 interacts with both TLR7 protomers and mediates TLR7 dimerization which stabilized the open-form conformation.

Based on our crystallographic and cryo-EM analyses, we can summarize the inhibitory mechanism of Cpd-6 and Cpd-7: these two antagonists induce TLR7 dynamics upon binding which inhibits the stable formation of the TLR7 activated dimer. Our work provides a structural basis for the development of therapies that target TLR7.

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BEAMLINES

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