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# Synthesis and Immunostimulatory Activity of Sugar-conjugated TLR7 ligands

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**Abstract**

Toll-like receptors (TLRs) are a type of pattern recognition receptors (PRRs), which are activated by recognizing pathogen-associated molecular patterns (PAMPs). The activation of TLRs initiates innate immune responses and subsequently leads to adaptive immune responses. TLR agonists are effective immuomodulators in vaccine adjuvants for infectious diseases and cancer immunotherapy. In exploring hydrophilic small molecules of TLR7 ligands using the cell-targeted property of a vaccine adjuvant, we conjugated 1V209, a small TLR7 ligand molecule, with various low or middle molecular weight sugar molecules that work as carriers. The sugar-conjugated 1V209 derivatives showed increased water solubility and higher immunostimulatory activity in both mouse and human cells compared to unmodified 1V209. The improved immunostimulatory potency of sugar-conjugates was attenuated by an inhibitor of endocytic process, cytochalasin D, suggesting that conjugation of sugar moieties may enhance the uptake of TLR7 ligand into the endosomal compartment. Collectively our results support that sugar-conjugated TLR7 ligands are applicable to novel drugs for cancer and vaccine therapy.

KEYWORDS:

TLR7; ligand; agonist; sugar conjugate

## Introduction

Toll-like receptors (TLRs), which are membrane type 1 proteins, are classified as pattern recognition receptors (PRRs), and play significant roles in the innate and adaptive immune responses.[1-4] In mammals, 13 kinds of TLRs (TLR1 to TLR13) have been found to date. Each TLR recognizes specifically microbial components called pathogen-associated molecular patterns (PAMPs); for example, lipoproteins and lipopeptides interact with TLR1, TLR2, and TLR6,[5] lipopolysaccharide with TLR4,[6] or nucleic acids with TLR3, TLR7, TLR8, and TLR9[7]. Although most PAMPs are macromolecules, small molecule TLR ligands have been identified among natural or synthetic compounds.[8-11] These compounds have attracted much attention as new candidates for cancer immunotherapy and as vaccine adjuvants because they modulate host immune responses.

Several TLR7 ligands based on scaffolds of imidazoquinoline,[12,13] purine,[14-19] or guanine analogs[20] have been studied intensively, and some of them have already been used in clinical treatment. Imiquimod (R837) is approved by the FDA for treatment of external genital warts, superficial basal cell carcinoma, and actinic keratosis.[21,22] However, its clinical use is limited to only topical administration due to undesired side effects such as cytokine release syndrome following systemic administration.[23-25] To improve bioavailability and pharmacokinetic properties of

small TLR7 ligand molecules, conjugates to a variety of accessory molecules such as proteins, lipids, PEG, or polysaccharides have been performed and showed higher immunostimulatory activity *in vitro*.[18,19,26,27] *In vivo* analysis of these conjugates in mice indicated that immune response varied depending on accessory molecules. For example, the lipid-PEG conjugate induced Th-2 immune responses in mice,[19] and the dextran conjugate induced a strong Th-1 biased immune response.[27] It was suggested that these differences were derived from the uptake mechanism and the location of TLR7 conjugates, and different pathway for the activation of TLR7 signals. Thus, the accessory molecules may contribute to potency and characteristics of immune response *in vivo*. In this study, we focused on sugar molecules, which are involved in cell-selective transport in living system,[28] and prepared several sugar-conjugated TLR7 ligands and evaluated their immunostimulatory activities.

## Result and discussion

### Synthesis of sugar-conjugated TLR7 ligands

We prepared 13 types of sugar molecules containing amino groups, which are commercially available or readily synthesized by amination from commercially available sugar molecules (Figure 1). Monosaccharide (glucosamine,

galactosamine, glucose, galactose, and mannose) and disaccharide (maltose and lactose) components were chosen since they are widely distributed as biomolecules. Cyclodextrines (CDs) were chosen since their derivatives are often utilized as additives for food and drug.

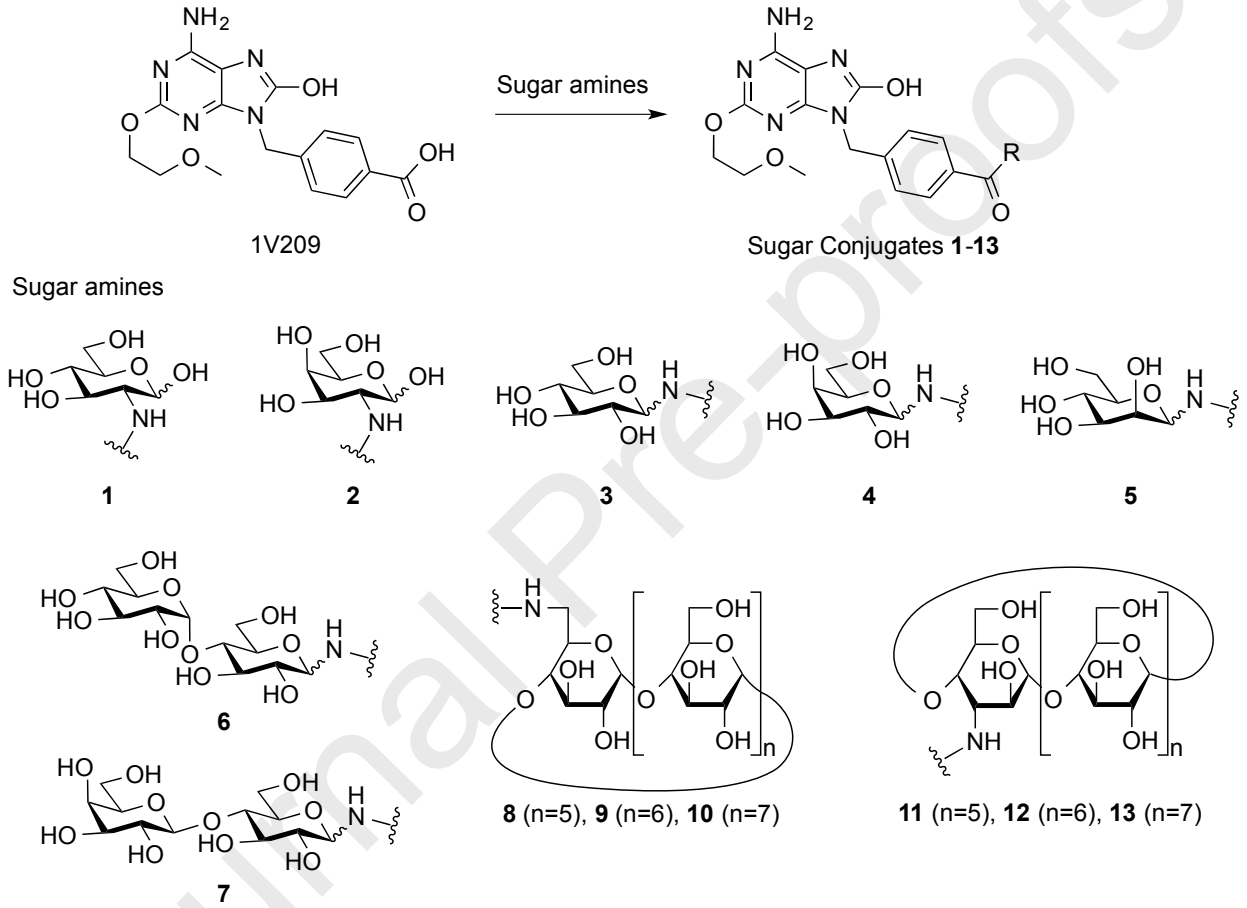


**Figure 1.** Sugar-conjugated TLR7 ligands.

The carboxylic acid functionalized TLR7 ligand, 1V209 (2-methoxyethoxy-8-oxo-9-(4-carboxybenzyl)adenine), is easily coupled to the amino containing molecules with the coupling reagent, HATU

(1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxide hexafluorophosphate). Coupling of commercially available hexosamines (glucosamine: GlcN, galactosamine: GalN) and 3A-aminocyclodextrins (3A-$NH_2$-α-CD, 3A-$NH_2$-β-CD, and 3A-$NH_2$-γ-CD) with 1V209 afforded sugar-conjugates **1**, **2**, **11**, **12**, and **13**, respectively. The additional sugar amine derivatives were prepared according to two routes (Figure 2, Route A or B) and then conjugated to 1V209. In Route A, sugars were treated with 7 M methanolic ammonia in autoclave reactor according to the method reported by Zhang *et al.*[29] with modifications, and the resulting sugar amines were reacted with 1V209 to produce conjugates. In Route B, the transformation via azide groups was applied;[30] sugar molecules were peracetylated, brominated at the one position using HBr in AcOH, and azidated using $NaN_3$. Reduction of the azide group, subsequent coupling with 1V209 using HATU, and then the successive deacetylation afforded the sugar conjugates. The conjugates **3**, **5**, and **6** were synthesized by Route A, whereas **4** and **7** were synthesized by Route B to avoid Amadori byproducts[31] and other side products produced in Route A. The monosaccharide and disaccharide conjugates were used without isolation of anomers for the immunomodulating experiments. On the other hand, cyclodextrin conjugates were prepared via 6A-azido-cyclodextrins using a method previously reported[32] (Route C), and were

purified by reversed phase (ODS) column chromatography with MeOH-$H_2O$ eluents. As expected, the water solubility of the obtained sugar conjugates was higher than that of 1V209 (see supporting information, Table 1S).

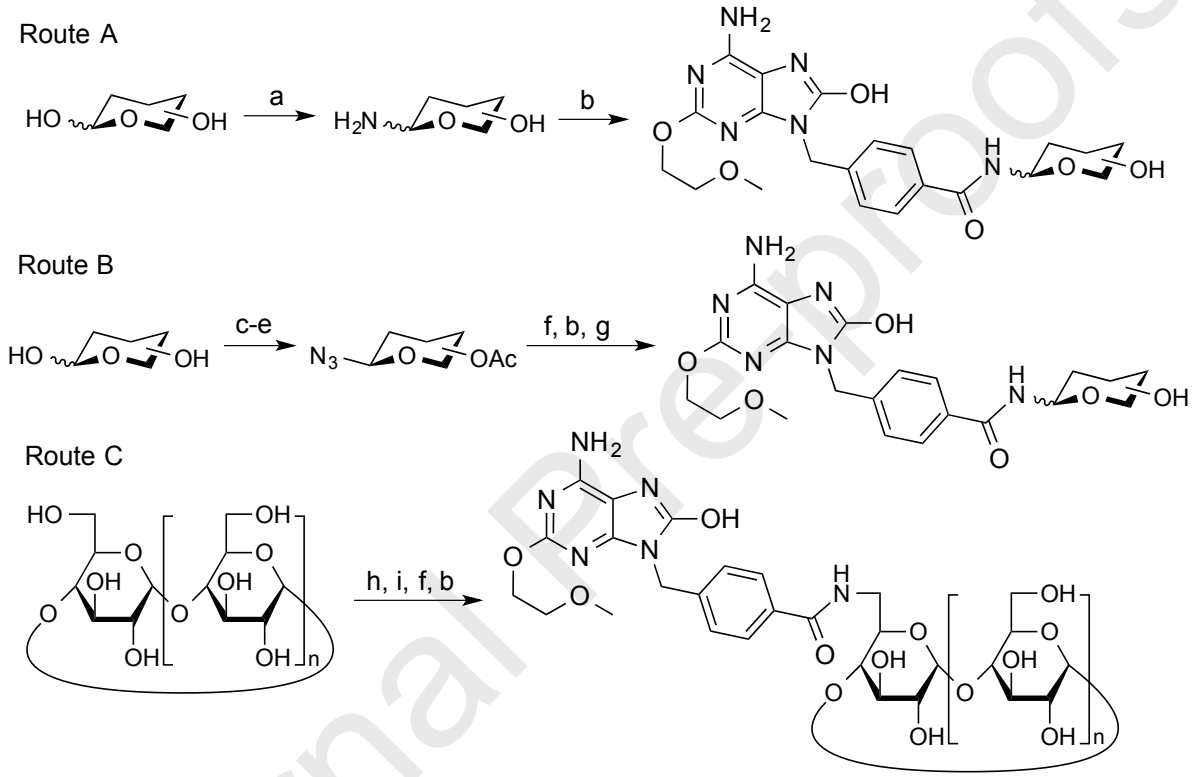


**Figure 2.** Synthesis of glycosyl amine (Route A or B) and 6A-amino-CD (Route C) derivatives. a) 7 M $NH_3$ in MeOH, 60 ˚C, b) HATU in DMF, c) $Ac_2O$, Pyr., d) HBr in AcOH, e) $NaN_3$ in DMF, 60 ˚C, f) $H_2$, Pd/C in MeOH, g) $K_2CO_3$ in MeOH, h) TsCl, Pyr. in DMF, i) $NaN_3$ in DMF.

**Immunostimulatory activity of sugar-conjugated TLR7 ligands**

Immunostimulatory activity of the sugar-conjugated TLR7 ligands was evaluated in two types of murine cells: macrophage cell line (RAW264.7) and primary bone marrow-derived dendritic cells (mBMDC). The levels of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) from those cells into the culture supernatants were evaluated by ELISA. The results are shown in Figure 3 (A, B, C, D, E, and F) and summary of half maximal effective concentrations ($EC_{50}$) and the concentration of maximum induction ($E_{max}$) is shown in Table 1. Sugar-conjugated TLR7 ligands showed higher or equal cytokine release potency compared to unconjugated TLR7 ligand, 1V209. The potency of IL-6 production in mBMDC with sugar conjugates varied with size and structure of sugar moiety, and tended to be higher as the size of sugar molecule increased. Cyclodextrin conjugates linked at 3A position showed 10-fold higher potency than 1V209 in mBMDC.

The conjugates showing favorable potencies in murine cells (sugar-conjugates **1**, **6**, and **11)** were also tested using human peripheral blood mononuclear cells (PBMC) to assess whether the conjugates with different sugar size have activity in human cells. It was found that the sugar-conjugates showed higher potencies compared to 1V209 (conjugates **1** and **6**: 2-fold, conjugate **11**: 3-fold, Figure 3G).

Immune cells contain receptors or transporters that can facilitate uptake of sugar molecules. On antigen presenting cells (APC), various types of lectins are

expressed as scavenger receptors such as C-type lectins.[33,34] Thus, we hypothesized that enhancement of the immunostimulatory potencies of sugar-conjugated TLR7 ligands may be attributed to receptor-mediated cellular uptake facilitated by the receptors and/or transporters on the cell surface. To test this hypothesis, we treated RAW264.7 cells with the sugar-conjugated TLR7 ligands (**3**, **6**, and **11**) in the presence or absence of cytochalasin D, an inhibitor of actin polymerization preventing the endocytic process (Table 2, Figure 3H, I). In the presence of cytochalasin D, the sugar conjugates showed decreased potencies. Especially, the potency of the conjugate **11** with cyclodextrin was significantly decreased by cytochalasin D. On the other hand, the unconjugated TLR7 ligand, 1V209, were not inhibited by cytochalasin D, suggesting that 1V209 was incorporated by passive diffusion. These results indicate that enhanced immunostimulatory potencies of sugar conjugates are due to enhanced endocytic uptake via sugar transporter and/or lectins on the cell surface.

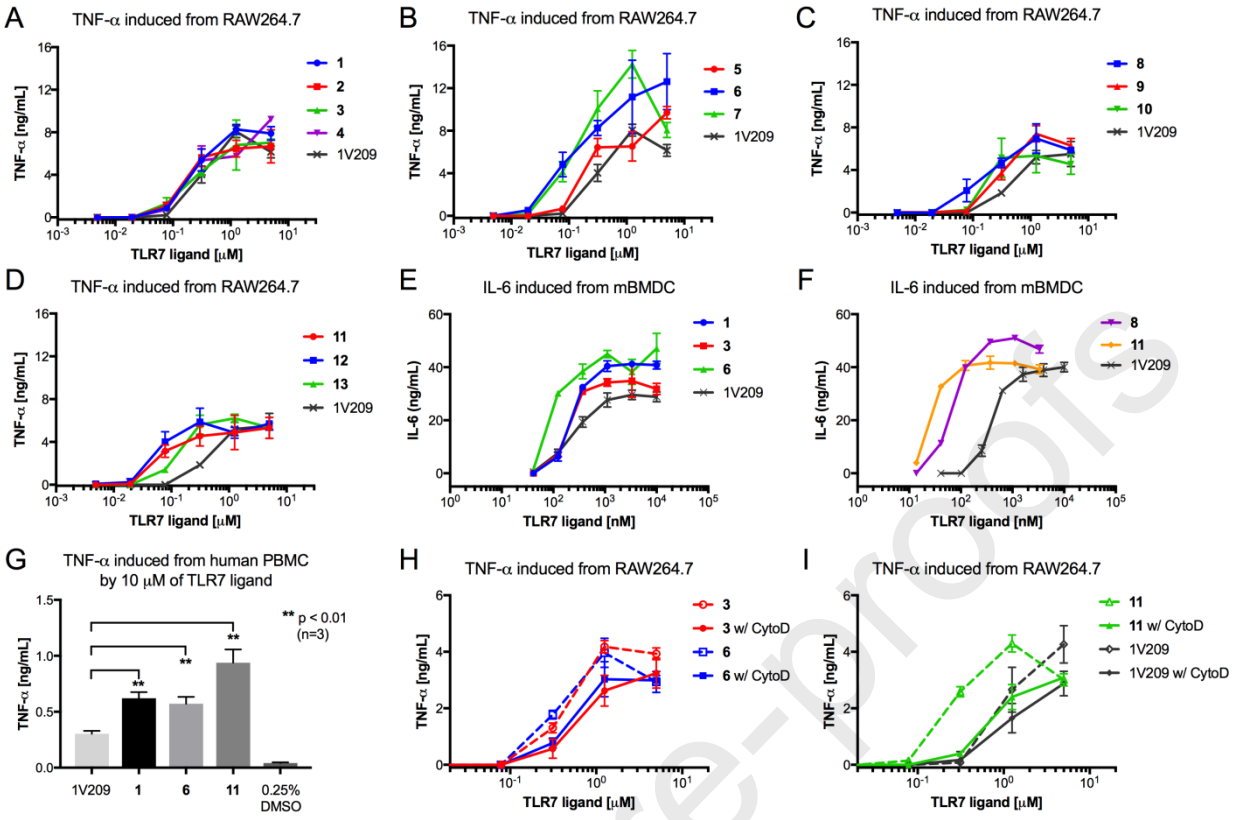


**Figure 3.** *In vitro* cytokine induction by sugar-conjugated TLR7 ligands in murine and human cells. (A−D) RAW264.7 cells ($1 \times 10^4$ cells/well) were plated and incubated with serially diluted sugar-conjugated TLR7 ligands (**1**−**13**) or unconjugated TLR7 ligand (1V209) for 18 h. The levels of TNF-α were measured by ELISA. (E, F) The compounds were also tested in mBMDC ($1 \times 10^5$ cell/well). IL-6 was measured by ELISA. The potencies of representative compounds (**1**, **3**, **6**, **8**, and **11**) are presented. Vehicle (0.25% DMSO)-stimulation was not observed, respectively. (G) Human PBMC were plated at $2 \times 10^5$ cells/well and incubated with 10 μM glycan-conjugated TLR7 ligands (1, 6, and 11) or 1V209 for 18 h. Control cells were treated with 0.25% DMSO. (H, I) RAW264.7 cells ($1.5 \times 10^4$

cell/well) were plated and pre-incubated with or without 1 μM of cytochalasin D, and incubated with serially diluted sugar-conjugated TLR7 ligands (**3**, **6**, and **11**) or unconjugated TLR7 ligand (1V209) for 6 h. TNF-α and IL-6 released in the culture supernatants were determined by ELISA. All data shown are means ± SD of triplicate and are representative of three independent experiments. **A value of $p < 0.01$ by one-way ANOVA with Dunnett's multiple comparisons test (G).

**Table 1.** Immunostimulatory activity of sugar conjugates.

| | TNF-α[a] | | IL-6[b] | |
|---|---|---|---|---|
| Compound | $EC_{50}$ (nM)[c] | $E_{max}$ (%)[d] | $EC_{50}$ (nM)[c] | $E_{max}$ (%)[d] |
| 1V209 | 252 | 100 | 434 | 100 |
| **1** | 234 | 103 | 239 | 139 |
| **2** | 153 | 83 | 210 | 139 |

| | | | | |
|---|---|---|---|---|
| **3** | 230 | 87 | 198 | 118 |
| **4** | 316 | 115 | 258 | 118 |
| **5** | 252 | 121 | 303 | 116 |
| **6** | 136 | 157 | 99 | 159 |
| **7** | 80 | 177 | 110 | 142 |
| **8** | 128 | 126 | 71 | 127 |
| **9** | 287 | 135 | 153 | 127 |
| **10** | 132 | 97 | 108 | 130 |
| **11** | 57 | 96 | 27 | 104 |
| **12** | 40 | 106 | 40 | 113 |
| **13** | 107 | 113 | 56 | 109 |

[a] RAW264.7 cells were used for production of TNF-α.

[b] mBMDC cells were used for production of IL-6.

[c] $EC_{50}$ was calculated using *Prism* software.

[d] $E_{max}$ values normalized to 1V209.

**Table 2.** Immunostimulatory activity of sugar conjugates in the presence or absence of cytochalasin D.

| Compound | TNF-α[a] ($EC_{50}$/μM)[b] | |
|---|---|---|
| | cytochalasin D (-) | cytochalasin D (+) |
| 1V209 | 2.15 | 2.11 |
| **3** | 0.490 | 0.909 |
| **6** | 0.277 | 0.585 |
| **11** | 0.186 | 1.06 |

[a] RAW264.7 cells were used for production of TNF-α.

[b] $EC_{50}$ was calculated using *Prism* software.

To evaluate the binding interaction of sugar conjugates to TLR7 ligand, computational modeling was further performed (Figure 4). Modeling of the monosaccharide conjugate **3** with monkey TLR7 (PDB: **5GMH**[35]) suggested that monosaccharide moiety of conjugate **3** is positioned outside of TLR7 dimer and no interaction between the sugar moiety and TLR7 dimer was observed. The disaccharide of **6** is also found to be positioned outside of the 1V209-TLR dimer complex (see supporting information, Figure 1S). The results suggest that sugar moiety is not responsible for dimerization of TLR7, and sugar moieties are important for water solubility, cellular uptake, and trafficking of ligands. Although

the detailed mechanism is still unclear, further analysis will clarify how sugar structures enhance the agonistic activity.

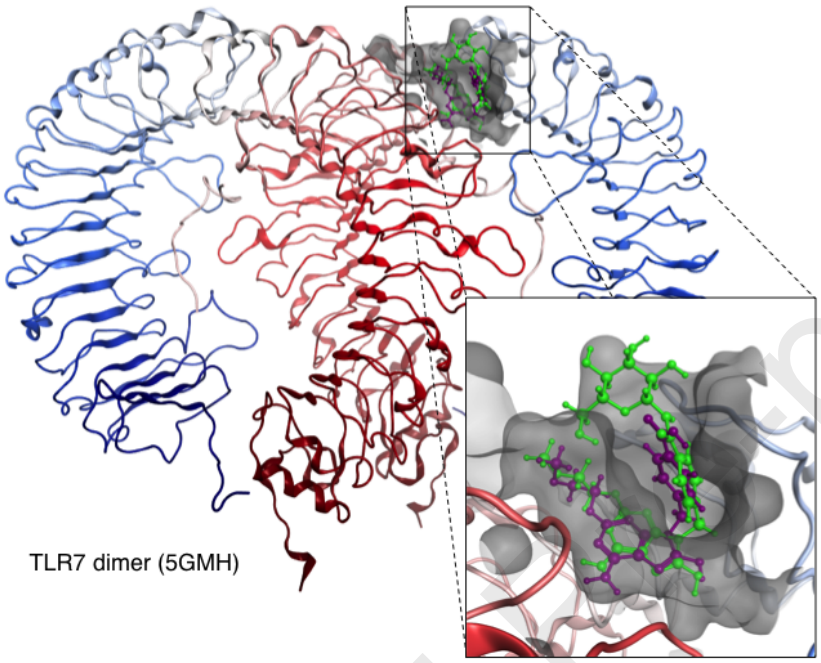


**Figure 4.** Computational docking studies of 1V209 and conjugate **3** to TLR7 dimer. Molecular docking of 1V209 (purple) and conjugate **3** (green) in the resiquimod (R848) binding pocket of monkey TLR7 dimer complex (PDB: **5GMH**). The van der Waals surface area of binding pocket is shown in gray. Overlay of binding geometry between 1V209 (purple) and conjugate **3** (green) is shown in the magnified view.

## Conclusion

We synthesized various sugar-conjugated TLR7 ligands and evaluated their immune stimulatory potency in mice and human cells. The conjugation of a TLR7 ligand with the amino group in sugar molecules was accomplished by simple amide condensation reaction using HATU. Synthesized sugar conjugates were water soluble and showed higher immunostimulatory potency compared to the unconjugated 1V209. Among them, the cyclodextrin conjugate **11** showed 10-fold higher potency in mBMDC and 3-fold higher activity in hPBMC relative to 1V209. We demonstrated that the higher potencies of sugar conjugates are due to incorporation into cells via the endocytic process. Molecular modeling of TLR7 with monosaccharide conjugates suggests that the sugar moiety is not involved in the interaction of TLR7 dimerization. Although the detailed analysis of the sugar structures relative to cellular trafficking property is needed, this study may lead to novel applications in cancer immunotherapy or infectious diseases aimed at selective activation of immune cells using sugar properties.

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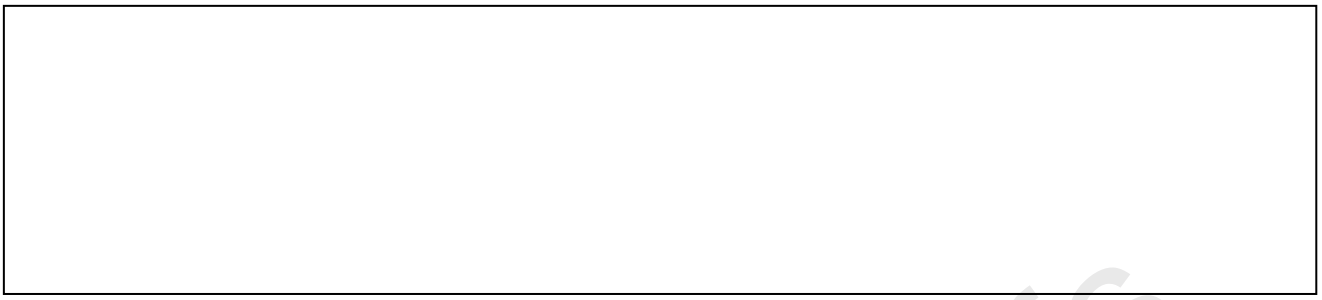
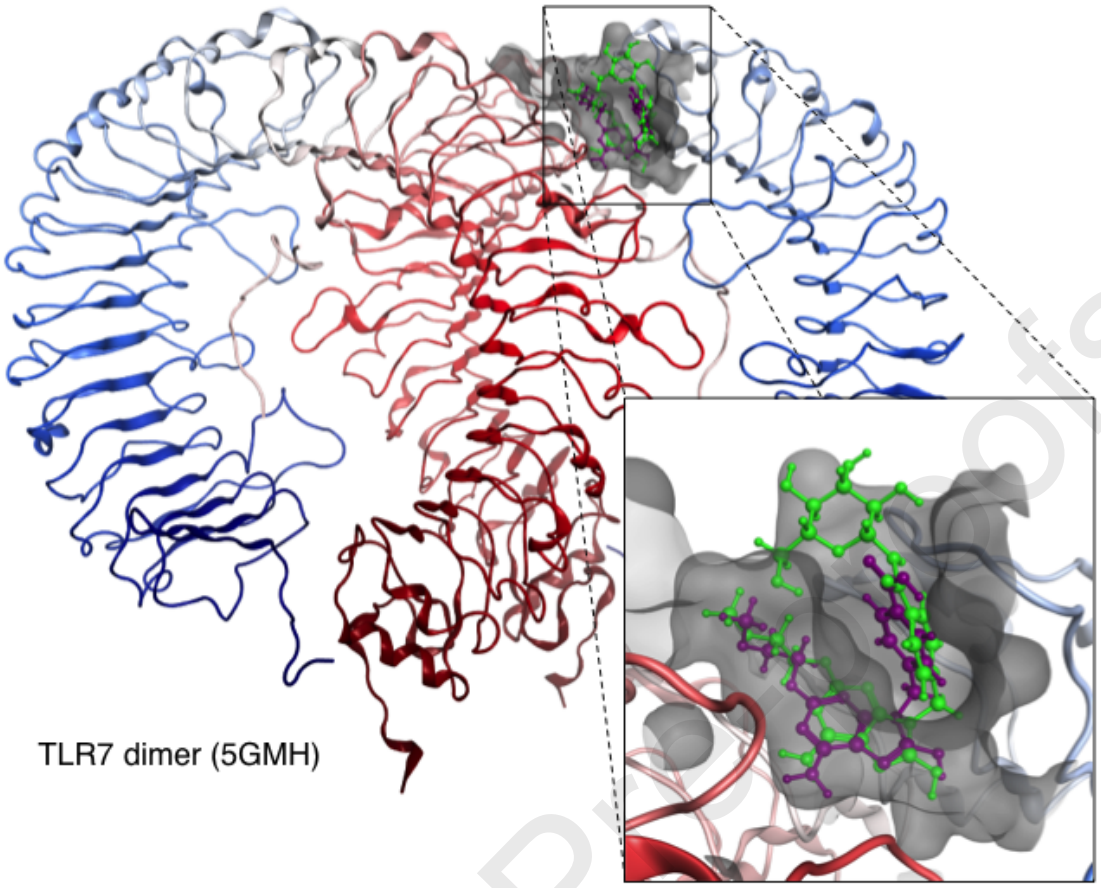
**Declaration of interests**

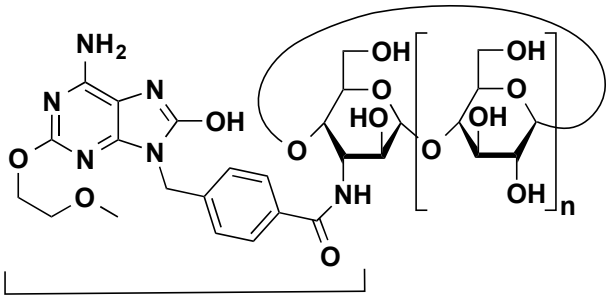
☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:





## Graphical Abstract




**Sugar conjugates with TLR7 ligand**