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Novel β -Cyclodextrin-Based Heptavalent Glycyrrhetic Acid Conjugates: Synthesis, Characterization, and Anti-Influenza Activity

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19 **Abstract**

20 In our continuing efforts toward the design of novel pentacyclic triterpene derivatives as
21 potential anti-influenza virus entry inhibitors, a series of homogeneous heptavalent glycyrrhetic
22 acid derivatives based on β -cyclodextrin scaffold were designed and synthesized by click
23 chemistry. The structure was unambiguously characterized by NMR, IR, and MALDI-TOF-MS
24 measurements. Seven conjugates showed sufficient inhibitory activity against influenza virus
25 infection based on cytopathic effect reduction assay with IC_{50} values in the micromolar range. The
26 interactions of conjugate **37**, the most potent compound ($IC_{50} = 2.86 \mu M$, $CC_{50} > 100 \mu M$), with
27 influenza virus were investigated using hemagglutination inhibition assay. Moreover, surface
28 plasmon resonance assay further confirmed that compound **37** bound to influenza HA protein
29 specifically with a dissociation constant of $5.15 \times 10^{-7} M$. Our results suggest the promising role of
30 β -cyclodextrin as scaffold for preparing a variety of multivalent compounds as influenza entry
31 inhibitors.

32

33 **Keywords:** Glycyrrhetic acid; β -cyclodextrin; influenza virus; multivalency; hemagglutinin

34

35 1. INTRODUCTION

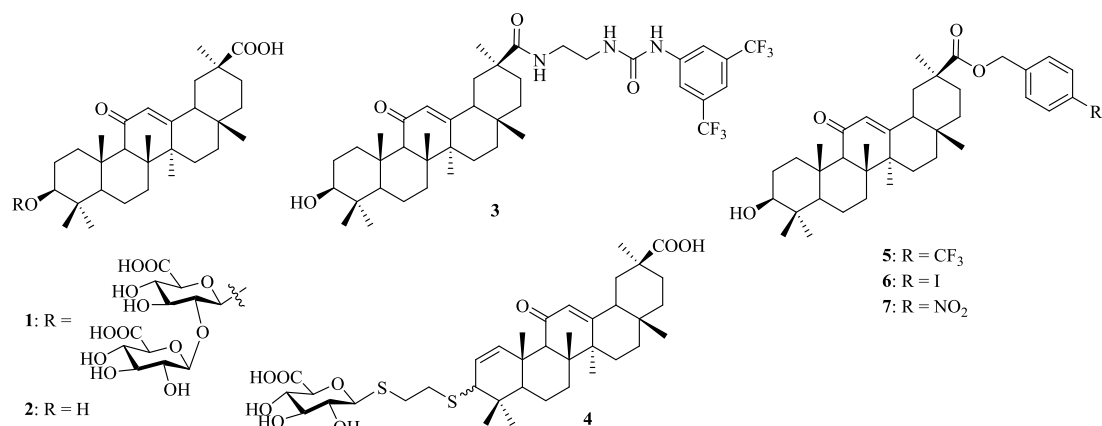
36 Influenza A virus is a highly contagious respiratory pathogen that can cause seasonal
37 epidemics and irregular pandemics due to its rapid transmission and frequent genetic alteration
38 (Neumann et al., 2009; Lagacé-Wiens et al., 2010). The structure of the viruses consists of a lipid
39 envelope that is generated from the host cell, to which two dominant membrane proteins
40 hemagglutinin (HA) (80%, ~300 copies of trimer) and neuraminidase (NA) (17%, ~50 copies of
41 tetramer) are anchored (Leser et al., 2005). Based on the antigenicity of HA and NA, they can be
42 classified into different subtypes. To date, 18 major antigenic variants of HA and 11 antigenic
43 variants of NA have been recognized, which are found in numerous combinations. In addition,
44 newly mutated forms of the influenza virus appear every year. The current available options to
45 combat influenza A viral infections include the prophylactic, yearly reformulated vaccines and
46 two classes of anti-influenza drugs (the M2 ion-channel inhibitors and the NA inhibitors) (Das,
47 2012). Since the vaccines that are available now offer a very limited breadth of protection (van
48 Dongen et al., 2019), the antiviral drugs are still the first line of protection to treat acute influenza
49 infection. Unfortunately, all circulating influenza A viral strains appear to be resistant to the M2
50 ion-channel inhibitors. Recently, the continually emerging of resistant virus against NA inhibitors
51 has been also reported (Hurt et al., 2006; van der Vries et al., 2010; Hurt, 2014). For these reasons,
52 novel anti-influenza viral agents with new targets and mechanisms of action are urgently required
53 to overcome the rapid emergence of drug resistance (Heo, 2018; van Dongen et al., 2019).

54 In the first step of infection, the virion adheres to the host cell surface through the binding of
55 HA to sialic acid-terminated carbohydrates present on the cell membranes (Mammen et al., 1998;
56 Skehel et al., 2000). Therefore, HA plays a pivotal role in virus entry, making it a potential target
57 for antiviral intervention. HA is a homotrimeric integral membrane glycoprotein, which has ca.
58 300-400 copies on the viral surface (Mammen et al., 1998). The monovalent interaction between
59 HA and sialic acid is mediated essentially by hydrogen bonds, and the dissociation constant of
60 them shows a relative weak binding ($K_D \sim 2$ mM) (Sauter et al., 1989). Therefore, a multitude of
61 HA is involved in order to increase the overall strength of the interaction between the virus and
62 the host cell. The binding between multiple pairs of HA trimer and sialic acid ligand increases
63 substantially as characterized by a multivalent affinity constant of estimated 10^{13} M^{-1} (Mammen et
64 al., 1998). This process provides important clues for the design of efficient multivalent ligands to
65 mimic the binding of HA to sialic acid to block the viral protein-host receptor interactions (Lu et
66 al., 2019). Over the past few years, many efforts have been made for the design and synthesis of
67 carbohydrate-based multivalent HA binders, including polymers, dendrimers, nanoparticles and so
68 on, serving as potent influenza A virus inhibitors (Chaudhary et al., 2019; Lu et al., 2019).

69 β -Cyclodextrin (β -CD) is a torus shaped cyclic oligosaccharide consisting of seven
70 (α -1,4)-linked D-glucopyranose units and it is well-known for their ability to form inclusion
71 complexes with a broad variety of organic and inorganic compounds (Crini, 2014). It is widely

72 used in pharmaceutical formulations to enhance the solubility of poorly soluble drugs, increase
73 drugs permeability through biological membranes and improve the bioavailability of drugs
74 (Jansook et al., 2018). The unique steric accessibility and acidity of the three types of hydroxyls in
75 β -CD have been taken into account to conceive efficient position-selective and face-selective
76 chemical functionalization methodologies (Khan et al., 1998). It has been shown to serve as a
77 multivalent scaffold in the design of glycoconjugates (Martinez et al., 2013), glycoclusters
78 (Gomez-Garcia et al., 2005; Gomez-Garcia et al., 2012), glycodendrimers (Ortega-Caballero et al.,
79 2001; Vargas-Berenguel et al., 2002) and star polymers (Zhang et al., 2012; Gonzalez-Gaitano et
80 al., 2017). In addition, target-specific multivalent drug delivery systems have shown significant
81 improvement of anti-tumor drugs delivery and hence antitumor efficacy and prevention of clinical
82 multi-drug resistance (Kim et al., 2017; Zhou et al., 2019).

83 In spite of the progress, the design of multivalent ligands with optimized biological properties
84 still remains a challenge considering the complexity of the multivalent ligand-receptor interactions
85 (Lepage et al., 2015). Glycyrrhizic acid (also known as glycyrrhizin, **1**) (Fig. 1), an oleanane-type
86 pentacyclic triterpene saponin, is the primary bioactive constituent of the roots and rhizomes of
87 *Glycyrrhiza glabra* (licorice) which is employed as an herbal medicine in both Western and
88 Eastern countries (Fiore et al., 2005; Asl et al., 2008). Compound **1** and its aglycone,
89 glycyrrhetic acid (GA, **2**), have been reported to possess antitumor, anti-inflammatory and other
90 pharmacological activities (Yang et al., 2015; Hussain et al., 2018). In a notable example,
91 Lallemand *et al.* have reported that compound **3**, with a
92 2-(3-(3,5-bis(trifluoromethyl)phenyl)ureido)ethyl group at C-30 of **2**, shows strong antitumor
93 activity with IC₅₀ in signal-digit micromolarity in a panel of eight cancer cell lines (Lallemand et
94 al., 2011). Recently, attention to GAs has been greatly increased due to their broad spectrum of
95 antiviral activities, such as anti-hepatitis B virus (HBV) (Wang et al., 2012), anti-Epstein-Barr
96 virus (EBV) (Lin et al., 2008), anti-rotavirus (Hardy et al., 2012), anti-influenza virus (Stanetty et
97 al., 2012), anti-herpes simplex virus (HSV) (Zigolo et al., 2018), etc. 3-Thioglucuronide derivative
98 **4** and several C-30 ester derivatives, such as 4-(trifluoromethyl) benzyl ester (**5**), 4-iodobenzyl
99 ester (**6**) and 4-nitrobenzyl ester (**7**), show promising antiviral activity (Stanetty et al., 2012; Wang
100 et al., 2012). In our previous study, a series of monovalent β -CD-GA conjugates have been
101 synthesized and several conjugates show weak antiviral activity at high concentration (~50 μ M)
102 (Liang et al., 2019). As a natural step in our efforts towards the design of new pentacyclic
103 triterpene conjugates with antiviral activity (Yu et al., 2014; Xiao et al., 2016; Si et al., 2018), we
104 described herein the design and synthesis of a series of heptavalent GA functionalized β -CD
105 conjugates and evaluated their *in vitro* anti-influenza activity.



106

107 **Figure 1.** Chemical structures of glycyrrhizic acid (1), GA (2) and its derivatives (3-7).

108

2. MATERIALS AND METHODS

2.1 Materials

110 GA was kindly supplied by Nanjing Zelang Medical Technology Co., Ltd. (China). β -CD,
 111 *O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluroniumtetrafluoroborate (TBTU),
 112 *N,N*-Diisopropylethylamine (DIPEA), iodine, triphenylphosphine, sodium azide,
 113 4-dimethylaminopyridine (DMAP), cupric sulfate were purchased from Shanghai Aladdin
 114 Bio-ChemCo. Ltd. (China). Sodium ascorbate, sodium methoxide, 5-hexyn-1-amine and
 115 4-ethynylaniline were supplied by Shanghai Energy Chemicals (China). Silica gel 60 (200-300
 116 mesh) was provided by Qingdao Haiyang Chemical Co., Ltd. (China). Sodium carbonate,
 117 *N,N*-dimethylformamide, tetrahydrofuran, methanol, pyridine, acetic anhydride as well as all other
 118 chemicals and reagents used were of analytical grade and purchased from Sinopharm Chemical
 119 Reagent Co., Ltd. (China) and used throughout this research without further purification.

120 Thin-layer chromatography (TLC) was conducted on a pre-coated silica gel 60 F₂₅₄ plate (E.
 121 Merck, Darmstadt, Germany) and eluted with a mixture of methanol/dichloromethane (15:1~10:1)
 122 for per-*O*-acetyl- β -CD-GA conjugates or methanol for the deacetylated β -CD-GA conjugates. For
 123 spot detection, the plates were immersed in a yellow solution of Ce(NH₄)₂(NO₃)₆ (0.5
 124 g)/(NH₄)₆Mo₇O₂₄·4H₂O (24.0 g)/6% H₂SO₄ (500 mL). The plate was heated by hot gun in order to
 125 visualize the spots. FT-IR spectra were obtained using a Nicolet Nexus 470 FTIR spectrometer
 126 (Thermo Electron Scientific Instruments LLC, USA). MALDI-TOF mass spectra were obtained
 127 on an AB Sciex TOF/TOFTM 72115 spectrometer using methanol or chloroform as solvents and
 128 α -cyano-4-hydroxy-cinnamic acid (HCCA) as matrix. NMR spectra were recorded on a Bruker
 129 DRX 400 or DRX 600 spectrometer at ambient temperature.

130 Madin-Darby canine kidney (MDCK) cells were donated by Crown Bioscience Inc. (USA).
 131 Dulbecco's Modified Eagle Medium (DMEM) was purchased from Gibco BRL, Inc. (USA).
 132 CellTiter-Glo[®] reagent was purchased from Promega Corp Inc. (USA). Recombinant influenza
 133 HA was purchased from Sino Biological Inc. (China). Deionized double-distilled water was used

134 throughout the biological study. Surface plasmon resonance (SPR) assay was analyzed using the
135 Biacore 8k system (GE Healthcare, Uppsala, Sweden).

136 2.2 Synthesis of heptavalent β -CD-GA conjugates

137 Heptakis (2,3-di-*O*-acetyl-6-deoxy-6-azide-)- β -CD **11** was prepared in a three-step procedure
138 as previously reported (Gadelle et al., 1991). The GA intermediate **12** was synthesized based on
139 the method of Schwarz *et al.* with minor modifications (Schwarz et al., 2014). The terminal
140 alkynyl substituted amines **13-16** were prepared as previously described (Murelli et al., 2009;
141 Zhang et al., 2010; Tran et al., 2013; Sanhueza et al., 2017). Alkynyl-functionalized GA
142 intermediates **17-20** and **30-32** were synthesized as previously described (Liang et al., 2019).
143 Heptavalent GA functionalized β -CD conjugates were synthesized through a two-step process
144 involving the copper-catalyzed alkyne-azide cycloaddition reaction (CuAAC) under microwaves
145 (step 1) and de-*O*-acetylation reaction under Zemblén conditions (step 2).

146 2.2.1 General procedure A for the click reaction (step 1)

147 To a solution of heptakis (2,3-di-*O*-acetyl-6-deoxy-6-azide-)- β -CD **11** (189.8 mg, 0.10 mmol)
148 and alkynyl-functionalized GA derivatives (0.84 mmol) in 50% of THF and H₂O (5 mL) was
149 added CuSO₄ (15.7 mg, 0.10 mmol) and sodium ascorbate (30.7 mg, 0.15 mmol). The resulting
150 solution was heated in a microwave reactor at 100°C until the azide was completely consumed
151 (typically about 1 h) as determined by TLC. Then the reaction mixture was extracted with CH₂Cl₂
152 (10 mL \times 3). The CH₂Cl₂ fraction was dried over Na₂SO₄, filtered, evaporated, and purified by
153 silica gel column chromatography using CH₂Cl₂/CH₃OH (15:1~10:1 v/v) as eluent to give the
154 acetylated intermediates as a white foam.

155 2.2.2 General procedure B for the deacetylation reaction (step 2)

156 The multivalent GA functionalized per-*O*-acetylated β -CD conjugates were dissolved in dry
157 methanol (5 mL per 100 mg of compound), and a solution of sodium methoxide (30% in methanol,
158 0.1 eq per mol of acetate) was added. The solution was stirred (180 rpm) at room temperature for
159 4~6 h in a N₂ atmosphere. After completion (TLC), the reaction mixture was neutralized with
160 Amberlite IR-120 (H⁺) ion exchange resin, then filtered and concentrated. The crude product was
161 purified by RP column chromatography using CH₃OH as eluent to give the final products in 73-95%
162 yields.

163 The ¹H and ¹³C NMR and ESI-HRMS or MALDI-TOF MS data of the synthesized new
164 compounds **21-28** and **33-38** are available in supporting information ([Supplementary Data, S6-S14](#)).
165

166 2.3 FT-IR analysis

167 FT-IR spectra of the heptavalent β -CD-GA conjugates as well as their parent compounds **11**
168 and **17** were obtained using a Nicolet Nexus 470 FTIR spectrometer (Thermo Electron Scientific
169 Instruments LLC, USA) over a scanning range 500-4,000 cm⁻¹. In addition, 1.0 mg of different
170 samples was mixed with KBr pellets.

171 2.4 NMR study

172 ¹H and ¹³C NMR spectra of GA derivatives **17-20** and **29-32** were acquired on a Bruker DRX
173 400 MHz spectrometer. ¹H NMR, ¹³C NMR, ¹H-¹H COSY NMR and ¹H-¹³C HSQC NMR spectra
174 of β-CD-GA conjugates **21-28** and **33-38** were acquired on a Bruker DRX 600 MHz spectrometer.
175 Sample solutions were prepared with CDCl₃ or CDCl₃/CD₃OD (2:1) as solvent. CDCl₃ or CD₃OD
176 were also employed as reference: proton (δ 7.26 ppm) and carbon (δ 77.00 ppm) for CDCl₃,
177 proton (δ 3.31 ppm) and carbon (δ 49.00 ppm) for CD₃OD. Detection temperature was set at
178 25 °C.

179 2.5 Cell culture and viruses

180 MDCK cells were donated by Crown Bioscience Inc. (USA) and grown in DMEM
181 supplemented with 10% (v/v) fetal bovine serum (FBS) (PAA Laboratories, Linz Austria) at 37 °C
182 under 5% CO₂. The A/WSN/33 (H1N1) influenza virus used in this study was artificially
183 generated from the 12-plasmid influenza virus rescue system.

184 2.6 Cytotoxicity assay

185 Cell viability assays were performed using CellTiter-Glo[®] reagent following the
186 manufacturer's instructions. Briefly, the cells (1×10⁴ per well) were seeded into 96-well tissue
187 culture plates and incubated for 16 h at 37 °C with 5 % CO₂ to allow the cells to adhere to the
188 surface of the wells. The culture medium was then replaced with fresh medium containing
189 compound or paclitaxel (PTX) at 10 and 25 μM in triplicate, and the negative control wells
190 contained the equivalent volume of medium with 1% DMSO, after which they were incubated for
191 48 h at 37 °C with 5% CO₂. CellTiter-Glo[®] reagent was added, and the plates were read using a
192 Tecan Infinite M2000 PRO[™] plate reader.

193 2.7 Cytopathic effect (CPE) reduction assay

194 MDCK cells (1 × 10⁴ per well) were seeded into 96-well plates, incubated at 37 °C with 5 %
195 CO₂ for 24 h and infected with influenza virus (MOI = 0.1). Cells were suspended in DMEM
196 supplemented with 1% FBS, containing different concentrations test compound or Oseltamivir
197 (OSV) and 2 μg/mL TPCK-treated trypsin, and a final DMSO concentration of 1% was added in
198 each well. After 48 h of incubation at 37 °C with 5 % CO₂, CellTiter-Glo[®] reagent (Promega
199 Corp., Madison, WI, USA) was added, and the cells were assessed with the CellTiter-Glo[®] assay
200 according to the manufacturer's instructions.

201 2.8 Hemagglutination inhibition (HI) assay

202 HI assay was performed as described previously (Yu et al., 2014). Briefly, compound from a
203 2-fold serial dilution in saline was mixed with an equal volume of influenza virus (2 HA units) in
204 the V-bottomed 96-well microplates. Subsequently, 50 μL of freshly prepared chicken red blood
205 cells (cRBC) (1% v/v in saline) was added to each well. The mixture was incubated at room
206 temperature for 30 min before observing cRBC aggregation on the plate.

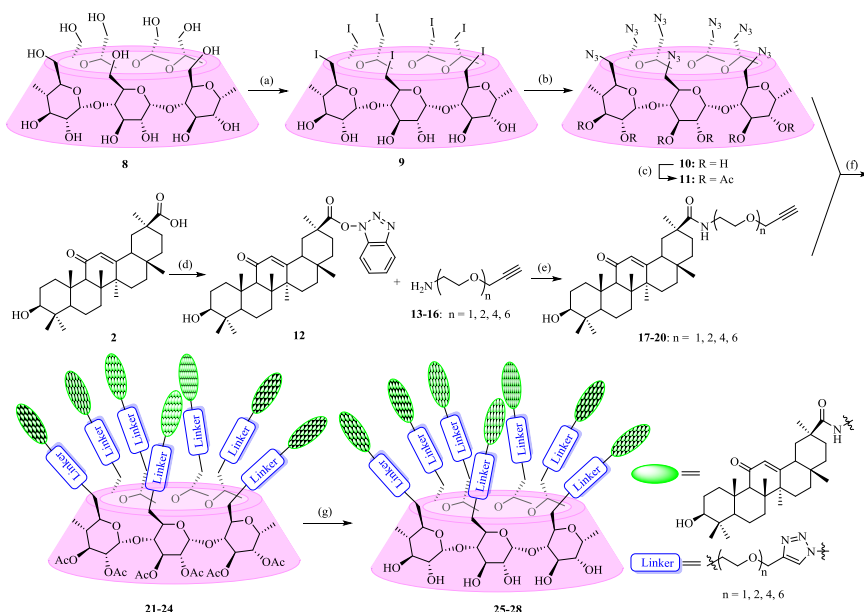
207 2.9 SPR assay

208 Interactions between influenza HA and the compounds were analyzed using the Biacore 8k
209 system (GE Healthcare, Uppsala, Sweden) at 25 °C. Recombinant influenza HA (Sino Biological
210 Inc., Beijing, China) was immobilized on a sensor chip (CM5) using EDC and NHS for activation.
211 Final HA immobilized levels were typically ~9,000 RU. Subsequently, compounds were injected
212 as analytes at various concentrations, and PBS-T (10 mM phosphate buffer with 5% DMSO, 0.05%
213 Tween, pH 7.4) was used as the running buffer. For binding studies, analytes were applied at
214 corresponding concentrations in running buffer at a flow rate of 30 μ L/min with a contact time of
215 60 s and a dissociation time of 180 s. Chip platforms were washed with running buffer and 50%
216 DMSO. Data were analyzed with the Biacore insight evaluation software (version 1.05) by curve
217 fitting using a binding model of 1:1.

218 **3. RESULTS AND DISCUSSION**

219 *3.1 Synthesis of heptavalent β -CD-GA conjugates*

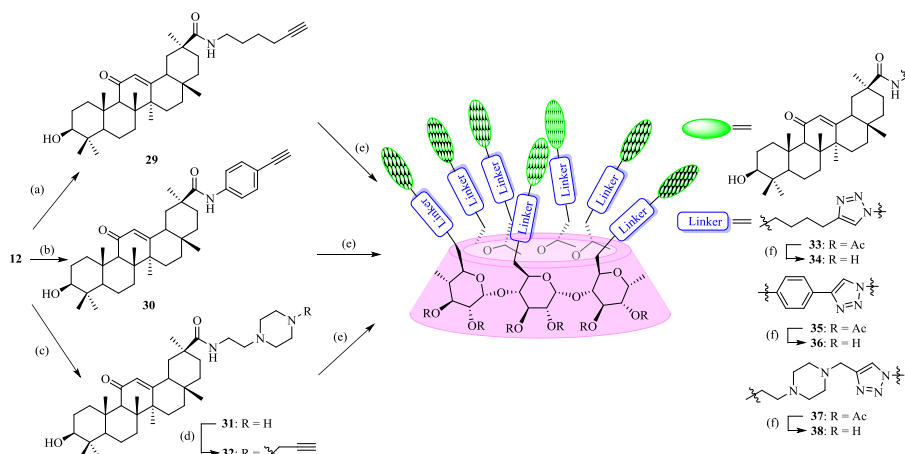
220 As shown in [Scheme 1](#), the synthetic strategy was based on coupling of alkynyl-functionalized
221 Gas with heptaazide substituted β -CD via click chemistry. At first, oligo (ethylene glycols) (OEGs)
222 were chosen as linkers to enhance and optimize the properties of conjugates. Various
223 heterobifunctional OEGs with amine and alkynyl terminals **13-16** were synthesized as described
224 previously (Murelli et al., 2009; Zhang et al., 2010; Tran et al., 2013; Sanhueza et al., 2017). The
225 coupling of OEGs **13-16** with compound **12**, which obtained from commercially available GA **2**
226 by activation of the carboxylic acid at C-30, was performed to provide the alkynyl-functionalized
227 GAs **17-20**. Then **17-20** underwent a “click chemistry” reaction with heptakis
228 (2,3-di-*O*-acetyl-6-deoxy-6-azide)- β -CD **11**, which was prepared from β -CD **8** in three steps using
229 the conventional method as previously described (Gadelle et al., 1991; Ashton et al., 1996;
230 Karginov et al., 2006) in the presence of copper sulfate and sodium ascorbate as reducing agent to
231 give **21-24** with yields ranging from 54% to 88%. At last, the acetyl groups of multivalent
232 conjugates **21-24** were removed under Zemblén conditions, followed by neutralization with H⁺
233 resin to afford **25-28** in 73-86% yields.



234

235 **Scheme 1.** Synthesis of heptavalent GA functionalized β -CD conjugates (**21-28**). Reagents and
 236 conditions: (a) I_2 , Ph_3P , DMF, $80^\circ C$, 44%; (b) NaN_3 , DMF, $60^\circ C$, 90% (c) Py, Ac_2O , DMAP, 92%;
 237 (d) TBTU, DIPEA, THF, 90%; (e) Na_2CO_3 , DMF, $60^\circ C$, 46-82%; (f) Sodium ascorbate, $CuSO_4$,
 238 THF- H_2O (1:1, v/v), 54-88%; (g) MeOH, MeONa, 73-86%.

239 To highlight the effect of the linkers on the anti-influenza virus activity, a parallel experiment
 240 was carried out to conjugate GA with β -CD moiety through an alkyl chain or a more rigidity
 241 chain, such as benzene ring or piperazine ring, rather than an OEG linker (**Scheme 2**). In a similar
 242 pathway, coupling of compounds **29**, **30** and **32** with heptaazide β -CD intermediate **11** was
 243 performed via click reaction, followed by de-*O*-acetylation under Zemplén conditions to give the
 244 corresponding conjugates **34**, **36** and **38**, respectively, as the final products.



245

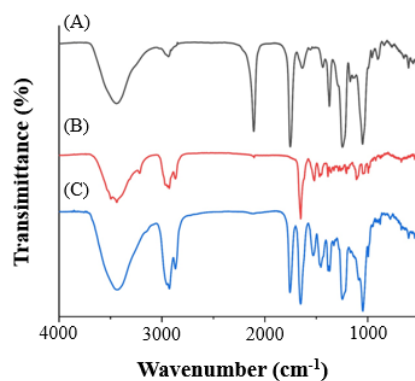
246 **Scheme 2.** Synthesis of heptavalent GA functionalized β -CD conjugates (**33-38**). Reagents and
 247 conditions: (a) 5-hexyn-1-amine, Na_2CO_3 , DMF, $60^\circ C$, 90%; (b) 4-ethynylaniline, Na_2CO_3 , DMF,
 248 $60^\circ C$, 30%; (c) 2-(piperazin-1-yl)ethan-1-amine, Na_2CO_3 , DMF, $60^\circ C$, 45%; (d) propargyl
 249 bromide, Na_2CO_3 , DMF, $60^\circ C$, 76%; (e) **11**, sodium ascorbate, $CuSO_4$, THF- H_2O (1:1, v/v),
 250 42-59%; (f) MeOH, MeONa, 90-95%.

251 3.2 Structure characterization of heptavalent β -CD-GA conjugates

252 The structures and symmetrical substitution of the heptavalent β -CD-GA conjugates **21-28** as
253 well as their precursors **33-38** were characterized by IR, NMR and MALDI-TOFMS
254 spectroscopy.

255 3.2.1 FT-IR analysis

256 The FTIR spectra of the all synthesized heptavalent β -CD-GA conjugates were recorded over
257 the range of 400-4,000 cm^{-1} in a Nicolet Nexus 470 FT-IR thermo-scientific spectrophotometer.
258 The spectrum of β -CD intermediate **11** contained the characteristic absorption band for the
259 spectrum with OH stretching at 3,386 cm^{-1} , CH stretching around 2,924 cm^{-1} and CO stretching
260 around 1,030 cm^{-1} (Supplementary data Fig. S1). For GA (**2**), several vibration characteristics of
261 the triterpenic moiety were present. The characteristic band attributed to the stretching vibration of
262 the OH group at C3 was found at 3,443 cm^{-1} . And the bands at 2,930 and 2,870 cm^{-1} were
263 assigned to the stretching vibration of the aliphatic CH (Supplementary data Fig. S2). The spectra
264 of these heptavalent β -CD-GA conjugates were very similar. In a typical example, Fig. 2 shows
265 the IR spectra of the two intermediates **11**, **17** and their conjugate **21**. It was noteworthy that the
266 strong absorption band located at 2,107 cm^{-1} in Fig. 2A and the weak absorption band located at
267 2,120 cm^{-1} in Fig. 2B, which should be assigned to the stretching vibration of azide and terminal
268 alkyne, respectively, were diminished in the spectrum of **21** (Fig. 2C). In addition, a few
269 representative absorption bands ascribable to 1,2,3-triazole units were shown around 1,529, 1,455
270 and 1,047 cm^{-1} (Rao et al., 1964).

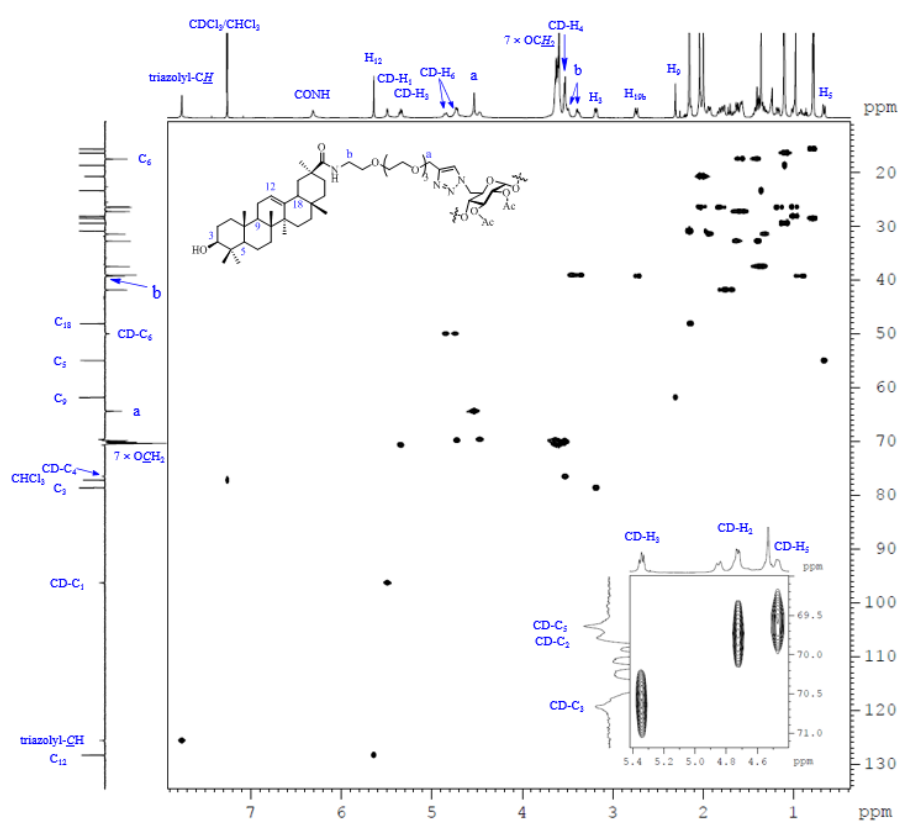


271
272 **Figure 2.** FTIR spectra of compounds **11** (A, black), **17** (B, red) and their conjugate **21** (C, blue).
273 Spectra were acquired between 500 and 4,000 cm^{-1} .

274 3.2.2 NMR and MALDI-TOF MS analysis

275 Except for those signals of the linkers, the heptavalent conjugates were similar in their NMR
276 spectra, and Fig. 3 represents the HSQC spectrum of conjugate **23** as an example. The low-field
277 regions of the conjugate were relatively well resolved and fully assigned. In the aromatic region,
278 the signal at δ 7.76 ppm was assigned to triazole-CH according to the ^1H - ^{13}C correlation spectra,
279 indicating that it was indeed connected by triazole linker. The assignment of NH proton at δ 6.31
280 ppm was confirmed as there was no correlation for NH by HSQC spectral analysis. The low-field

281 peak at δ 5.64 ppm, referring to 1H, was assigned to H₁₂, and one carbon appearing at δ 128.35
 282 ppm should be assigned to C₁₂ of GA. With a C₇-symmetry in the molecule, compound **23** showed
 283 only one set of CD-H₁ signal appearing at 5.49 ppm and one CD-C₁ signal appearing at 96.29 ppm.
 284 Similar observation was also made for other proton and carbon signals of β -CD unit, and the
 285 chemical shift data are summarized in Table 1. As C_b was connected with nitrogen rather than
 286 oxygen to which other carbons were bonded in tetraethylene glycol linker, the chemical shift of C_b
 287 in ¹³C NMR was at the highest field (NH₂CH₂ vs OCH₂). In addition, as C_a was connected with
 288 triazole, the chemical shift of H_a in ¹H NMR was at the lowest field among the eight -OCH₂-
 289 groups. The MALDI-TOF MS of compound **23** showed a sodium adduct ion [M + Na]⁺ at *m/z*
 290 6709.6 (Calcd. For C₃₅₇H₅₄₆N₂₈NaO₉₁, 6709.4), which also confirmed that it was a heptavalent
 291 conjugate.



292
 293 **Figure 3.** The 600 MHz HSQC spectrum of **23** recorded in CDCl₃ at 298 K, with the 1D ¹H and
 294 ¹³C NMR spectra along the top and the side, respectively.

295 **Table 1.**

296 Chemical shifts of ¹H and ¹³C NMR of β -CD unit in conjugate **23**.

¹ H NMR (ppm)		¹³ C NMR (ppm)	
CD-H ₁	5.64	CD-C ₁	96.29
CD-H ₂	4.73	CD-C ₂	69.81
CD-H ₃	5.34	CD-C ₃	70.67
CD-H ₄	3.53	CD-C ₄	76.51

CD-H ₅	4.47	CD-C ₅	69.64
CD-H ₆	4.85, 4.75	CD-C ₆	49.97

297 3.3 *In vitro* cytotoxicity of heptavalent β -CD-GA conjugates

298 It has been reported that GA **2** and some of its derivatives are cytotoxic in cancer cells (Satomi
 299 et al., 2005; Lallemand et al., 2011). In this study, the cytotoxicity of heptavalent β -CD-GA
 300 conjugates **21-28** and **33-38** was first determined in MDCK cells using the CellTiter-Glo[®]
 301 luminescent cell viability assay. Decrease in viability of MDCK cells was observed in a
 302 dose-dependent manner upon treatment of PTX (Supplementary data Fig. S3). Treatment of cells
 303 with **2** for 48 h resulted in decreased viability to 82.8% for the 25 μ M concentration and no
 304 viability for the 10 μ M concentration, indicating that **2** had weak cytotoxicity to cells and agreed
 305 with previously reported results that **2** shows weak cytotoxicity to eight cancer cell lines with IC₅₀
 306 values between 70.48-136.40 μ M (Gao et al., 2014). For all the tested conjugates, except that **25**
 307 and **28** showed weak cytotoxicity to uninfected MDCK cells with the reduced viability to 85.6%
 308 and 87.9%, respectively, at high concentration of 25 μ M, no significant cytotoxicity for the other
 309 conjugates was observed at concentrations of 10 μ M and 25 μ M.

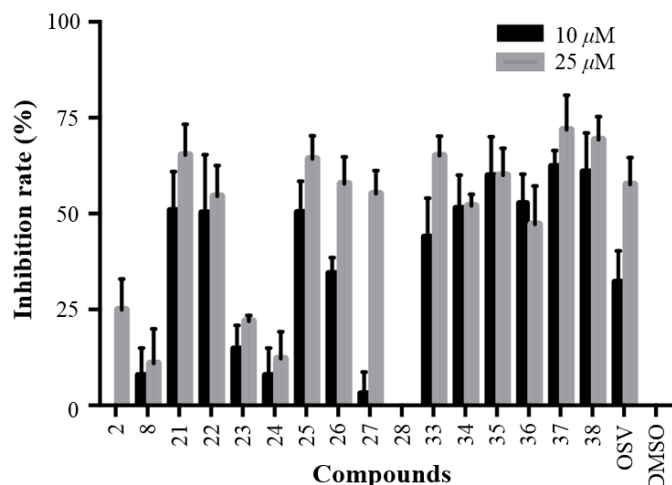
310 3.4 Structure-activity relationships (SAR) of heptavalent β -CD-GA conjugates

311 Concerning the fact that the use of multivalent sialopeptides as potential inhibitors of
 312 influenza infections has been proved to be a useful approach (Mammen et al., 1998; Marra et al.,
 313 2008), our interest was aimed at evaluating the inhibitory effect of the heptavalent GA conjugates
 314 **21-28** and **33-38** by microscopic examination of the viral CPE at 2 days post infection (Noah et al.,
 315 2007). Oseltamivir (OSV) and DMSO were used as positive and negative controls, respectively.

316 The primarily screening results are shown in Fig. 4. A careful analysis and comparison of the
 317 data allowed us to reveal some interesting SAR trends. Regarding the OEG linkers between the
 318 triazole ring substituted GA and β -CD scaffold, we found that the anti-influenza activity of
 319 conjugates **21-24** was decreased in the order as follows: **21**>**22**>>**23**>**24**. Similar results were also
 320 observed for conjugates **25-28**, indicating that a length of four atoms was found to be optimal for
 321 the inhibitory activity (**21** and **25**). Especially for conjugate **28**, the inserting of two more ethylene
 322 glycols spacer in **26** resulted in the loss of activity under 25 μ M, suggesting the importance of the
 323 length of the linker between GA and β -CD for their anti-influenza activity. Concerning the nature
 324 of the linker's heteroatom, the replacement of the oxygen of ethylene glycol in conjugates **21** and
 325 **25** by methylene (**33** and **34**) showed slightly weaker or similar antiviral activity. Interesting, the
 326 increasing of the rigidity of the linker by further replacement of the four methylene with aromatic

327 benzene at positions at 1,4 (**35** and **36**) still retained reasonable anti-influenza activity.
 328 Surprisingly, the inserting of a piperazine space in the alkyl linker caused a substantial increase in
 329 activity of conjugates **37** and **38** (relative to **33** and **34**, respectively), which were proved to be the
 330 most two potent multivalent conjugates.

331



332

333 **Figure 4.** The CPE-based primarily screening of GA (**2**), β -CD (**8**) and their heptavalent
 334 β -CD-GA conjugates **21-28** and **33-38**. MDCK cells were utilized as the host cells to test
 335 A/WSN/33 virus infection; OSV and DMSO acted as positive and negative control, respectively.
 336 Error bars indicate standard deviations of triplicate experiments.

337 After the preliminary screening at two concentrations of 10 μ M and 25 μ M, 11 conjugates
 338 (**21-22**, **25-27** and **33-38**) with the inhibition rate over 50% at concentration of 25 μ M were
 339 selected to perform in dose response assays, and the concentrations required to inhibit viral
 340 replication by 50% (IC_{50}) are summarized in **Table 2**. In addition, no significant toxicity was
 341 observed for the 10 conjugates at concentrations up to 100 μ M. Based on these data, we
 342 investigated in detail the potential of a representative heptavalent conjugate **37**.

343 **Table 2.**

344 *In vitro* anti-influenza A virus activity of heptavalent conjugates **21-22**, **25-27** and **33-38**^a

Compd	IC_{50} , μ M ^b	CC_{50} , μ M	SI	Compd	IC_{50} , μ M	CC_{50} , μ M	SI
2	NA ^c	85±6.8	-	33	12.1±0.31	>100	>8.3
8	NA	ND ^d	-	34	9.03±0.50	>100	>11.1
21	6.64±0.31	>100	>15.1	35	20.7±1.02	>100	>4.8
22	7.70±0.74	>100	>13.0	36	11.0±0.87	>100	>9.1
25	6.96±0.39	>100	>14.4	37	2.86±0.22	>100	>35.0
26	8.14±0.11	>100	>12.3	38	4.89±0.15	>100	>20.4

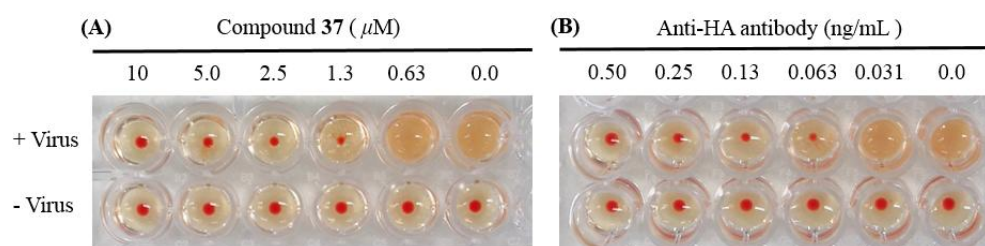
27 27.02±0.11 >100 >3.7

345 ^aUsing influenza A/WSN/33 (H1N1) strain. ^bThe concentration required for a test compound to
346 reduce the virus-induced CPE by 50% relative to the virus control was expressed as IC₅₀. ^cNo
347 activity. ^dNot detectable.

348 The CPE produced by influenza A/WSN/33 virus in MDCK cells and its abrogation by
349 treatment with **37** were further confirmed by direct observation under a microscope. No
350 cytotoxicity was observed for **37** when incubated with the MDCK cells for 48 h (Supplementary
351 data Fig. S4A vs. Fig. S4B), while **37** significantly reduced the CPE induced by influenza
352 A/WSN/33 virus infection (Supplementary Fig. S4C vs. S4D).

353 3.5 HI assay

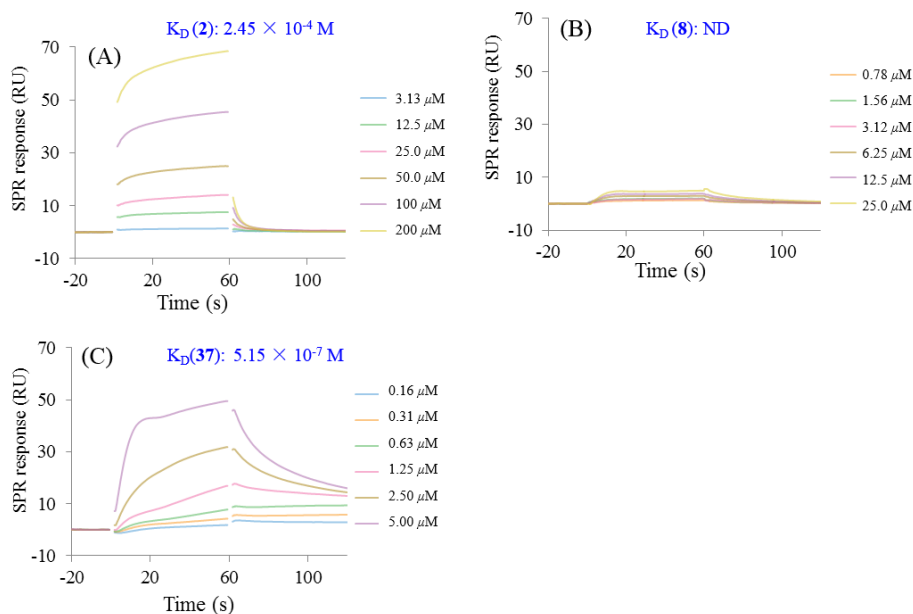
354 With the confirmation of the antiviral activity of the heptavalent conjugates, we were
355 interested in identifying the molecular target of them. It is well known that the viral envelop
356 glycoprotein HA plays a critical role in the initial step of influenza virus entry into host cells.
357 Therefore, HI assay was designed to detect whether the heptavalent conjugates could interfere
358 with the interaction between HA and its sialic acid receptors. Two fold dilutions of the heptavalent
359 conjugate **37** (from 10 μM) or anti-HA antibody (from 0.50 ng/mL) treated virus were incubated
360 with cRBC, and agglutination was observed (Fig. 5). In addition, **37** completely inhibited
361 influenza A/WSN/33 (H1N1) virus induced agglutination of cRBC in a dose-dependent manner at
362 concentrations of 1.3 μM or more. Similar results were observed for anti-HA antibody at
363 concentrations of 0.063 ng/mL or more. These results suggested that the heptavalent conjugates **37**
364 shared the same target of anti-HA antibody, thus interfering with virus-receptor interaction.



365
366 **Figure 5.** Effect of the heptavalent compound **37** (A) and anti-HA antibody (B) on HA by HI
367 assay. The chicken red blood cells (cRBC) (1% v/v in saline) were incubated with influenza
368 A/WSN/33 at 2 × HA with various concentrations of **37** (two-fold serial dilution from 10 μM) or
369 anti-HA antibody (two-fold serial dilution from 0.50 ng/mL).

370 3.6 Determination of the K_D for the interaction between the heptavalent conjugate **37** with HA
371 protein

372 To determine the binding affinity of conjugate **37** for the HA protein, SPR experiments were
373 performed on a Biacore 8K instrument. The SPR response was reported in the resonance units
374 (RU). Sensor grams were analyzed with a 1:1 (Langmuir) binding mode. As shown in Fig. 6, the
375 binding of recombinant influenza HA to GA (**2**) exhibited typical weak affinity binding with a
376 calculated equilibrium dissociation constant (K_D) value of 2.45×10^{-4} M, while β -CD (**8**) did not
377 bind to HA protein at the same concentration. However, the multivalent conjugate **37** showed
378 strong binding affinity to HA protein within the concentration range 0.16-5.00 μ M. The calculated
379 K_D value for conjugate **37** was 5.15×10^{-7} M, which seemed to be too large by about 470-fold of
380 magnitude to that of **2** (Fig. 6A vs. Fig. 6C). In addition, kinetic analysis revealed that changes in
381 multivalent conjugate and HA protein not only affected the association rate constant (K_a), but also
382 affected the dissociation rate constant (K_d) (Supplementary data Table S1).



383
384 **Figure 6.** SPR inhibition assay sensor grams showing response to influenza HA protein in the
385 presence of varying concentration of GA (**2**), β -CD (**8**) and their heptavalent conjugates **37**. The
386 binding kinetics was analyzed by BIA evaluation 1.0.5 software with 1:1 (Langmuir) binding
387 mode.

388 Compared with our previous study in which multiple pentacyclic triterpenes attached directly
389 on CD scaffold via 1,2,3-triazolyl group (Xiao et al., 2016), the insertion of a suitable linker
390 between them will help for the binding of multiple pentacyclic triterpenes with their target. To
391 test the feasibility of the strategy, various types of linkers including flexible linear alkyl/ether
392 chains combinations between 4 and 19 atoms, rigid aromatic space chain as well as semi-rigid
393 charged piperzine chain have been successfully introduced in monovalent β -CD-GA conjugates
394 in our recently study (Liang et al., 2019), which allowed us to further design and synthesis of the

395 second-generation multivalent pentacyclic triterpene- β -CD conjugates. This well-established
396 method will provide an alternative approach to the construction of novel multivalent derivatives
397 bearing different linkers based on natural or chemically modified CDs scaffolds. Combined with
398 our previous studies, we found that the pentacyclic triterpenes pharmacophore, the CD scaffold
399 and the linkers between the two parts were all affected their anti-influenza activity.

400 **4. CONCLUSIONS**

401 In this work, a series of multiple GA functionalized β -CD conjugates with different linkers
402 were synthesized and characterized. Except that two conjugates (**25** and **28**) showed weak
403 cytotoxicity to MDCK cells at concentration of 25 μ M, all other conjugates showed no significant
404 cytotoxicity based on an alamarBlue assay. When presented multivalently, seven β -CD-GA
405 conjugates (**21**, **22**, **25**, **26**, **34**, **37** and **38**) showed strong anti-influenza properties with IC₅₀ values
406 at a micromolar level (IC₅₀: 2.86-9.03 μ M). By using of HI and SPR assays, conjugate **37** showed
407 specific binding to HA protein with K_D value at 515 nM, which was about 470-fold potent to its
408 parent compound GA (**2**). Taken together, the results reported here for multivalent β -CD-GA
409 conjugates demonstrated that multivalent displays of anti-influenza entry agent afford
410 macromolecules with a remarkable activity and a strong binding to HA protein relative to their
411 parent compound alone and multivalency based on β -CD scaffold is a promising alternative to the
412 current available treatments for the management of viral infection by blocking virus entry into
413 host cells.

414 **DATA AVAILABILITY STATEMENT**

415 The raw data supporting the conclusions of this article will be made available by
416 the authors, without undue reservation.

417 **AUTHOR CONTRIBUTIONS**

418 SL and XM: design, synthesis and characterization of the multivalent conjugates;
419 ML and YC: contribution in anti-influenza viral assay, HI assay, cytotoxicity assay;
420 QG: SPR assay; YZ: discussion of all the experiments and assist in writing the
421 manuscript; and LZ, DZ and SX: designed the synthesis and biochemical
422 experiments, supervised the project, and wrote the manuscript.

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