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Pentacyclic triterpenes grafted on CD cores to interfere with influenza virus entry: A dramatic multivalent effect

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ABSTRACT

Multivalent effect plays an important role in biological processes, particularly in the specific recognition of virus with its host cell during the first step of infection. Here we report the synthesis of multivalent pentacyclic triterpene grafted on cyclodextrin core and potency of against influenza entry activity. Nine star-shaped compounds containing six, seven and eight pentacyclic triterpene pharmacophore on cyclodextrin scaffold were prepared by way of copper-catalyzed azide-alkyl cycloaddition reaction under microwave activation. Some of the multimers exhibited much potent antiviral activity against H1N1 virus (A/WSN/33), even equivalent or superior to oseltamivir. The most active compound **31**, a heptavalent oleanolic acid-β-cyclodextrin conjugate, shows an up to 125-fold potency enhancement by its IC_{50} value over the corresponding monovalent conjugate and oleanolic acid, disclosing a clear multivalent effect. Further studies show that three compounds **31**–**33** exhibited broad spectrum inhibitory activity against other two human influenza A/JX/312 (H3N2) and A/HN/1222 (H3N2) viruses with the IC_{50} values at 2.47 −14.90 μM. Most importantly, we found that compound **31,** one of the best representative conjugate, binds tightly to the viral envelope hemagglutinin with a dissociation constant of K_D = 2.08 μM, disrupting the interaction of hemagglutinin with the sialic acid receptor and thus the attachment of viruses to host cells. Our study might establish a strategy for the design of new pharmaceutical agents based on multivalency so as to block influenza virus entry into host cells.

1. Introduction

Influenza A viruses are responsible for causing annual epidemics and occasional pandemics with significant morbidity and mortality [1]. These viruses are usually structurally similar, making up of a viral envelope containing two main types of proteins wrapped around a central core, as rough spherical particles with 80–120 nm in diameter [2]. The influenza virus infection occurs by an attachment of its surface protein hemagglutinin (HA) to the host cell. HA is a homotrimeric integral membrane glycoprotein [3], shaped like a cylinder in approximately 13.5 nm length, and appears at high concentrations on the surface of the virus, with 600–1200 molecules per virus particle [4]. The three identical HA monomers are constructed into a central α helix coil with their spherical heads (HA1) contain the SA binding sites and the long, helical chains (HA2) anchored in the viral membrane. The function of HA1 is to cause the viral particles stick to the cell's surface via binding to the monosaccharide SA (SA)-containing receptors on host membrane. Once bound, HA2 facilitates the entry of the viral genome into the host by fusing host endosomal membrane with the viral membrane [5]. A plenty of distinct antigenic mappings have found that mutations in the HA1, except the SA receptor-binding domain, are tolerated without affecting the overall functionality of HA [6].

Since HA is the major envelope protein essential for virus entry and this region is known to be highly conserved in all strains of the virus, specific binders to HA are of interest for influenza therapy as well as for diagnosis. The association constant for the single SA molecule to a single HA receptor is relatively weak with 10^3 M^{-1},

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but the binding between viral receptor and cellular SA increases substantially as characterized by a multivalent affinity constant of estimated 10^{13} M^{-1} [4]. Viral HA is a homotrimeric receptor, tri- or multivalent ligands should have a high binding affinity. Therefore, a lot of multivalent molecules, built on various templates including small molecule scaffolds, polypeptides, polysaccharides, polyacrylamides, and nanoparticles, have been previously reported as inhibitors for competing the binding between SA and HA [4,7–10]. Despite these progresses, there is still an urgent need for improved design to address issues of the relatively low activity observed in some classes of the multivalent ligands, the cytotoxicity of polyacrylamide-based inhibitors and the unwanted immunogenicity of protein-based ligands [4,11–13].

We have recently identified certain pentacyclic triterpenes displayed modest anti-influenza virus activity by competing with SA moiety to HA and thus disrupting the attachment of viruses to host cells [14]. However, given the presence of multivalent HA and SA moieties on respective virus and cell surface, a marked tendency may be conferred to form virus-cell aggregations rather than triterpene-virus complexes. It is thus anticipated that simultaneous conjugation of multivalent ligands using a proper scaffold may be an ideal approach to reverse the tendency. Cyclodextrins (CDs) are the cyclic oligomers with six, seven and eight α-1,4-linked glucopyranose units. Typically, CDs are topologically represented as toroids with their larger and the smaller openings exposing to the solvent secondary and primary hydroxyl groups, respectively [15–17]. Because of this arrangement, the interior of CDs is considerably less hydrophilic than the aqueous environment and thus able to host hydrophobic molecules. The exterior is sufficiently hydrophilic to impart CD-conjugates water solubility and a rigid backbone for the construction of multivalent ligands with unique properties. Therefore CDs have become an attractive building block for the development of new biocompatible materials with many potential applications in drug delivery [18]. We report herein the design and synthesis of CD-based multivalent conjugates in which a variety of pentacyclic triterpene pharmacophore was tethered onto CD using the copper-catalyzed alkyne–azide cycloaddition (CuAAC) reaction between an alkyne-labeled pentacyclic triterpene and multi-azide-modified CD scaffold (Fig. 1). A bioassay conducted by the cytopathic effect (CPE) reduction assay and the CellTiter-Glo assay indicated that the multivalent pentacyclic triterpene-CD conjugates exhibited intriguing anti-influenza activity with IC_{50} values in the μM scale. Mechanistic studies indicated that these conjugates bind tightly to HA protein, thus blocking the interaction of HA with SA receptor.

2. Materials and methods

2.1. General experimental details

2.1.1. Materials and methods

α-, β- and γ-CD were purchased from Aladdin Chemistry Co. Ltd (Shanghai, China). Oleanolic acid (OA) and ursolic acid (UA) were kindly supplied by Nanjing Zelang Medical Technology Co., Ltd (Nanjing, China) and Anboruila Biotechnology Co. Ltd (Nanjing, China), respectively. Echinocystic acid (EA) was separated from the ethanol/H_2O crude extract of *Gleditsia sinensis Lam*, a traditional Chinese herbal medicine. Mouse anti-NP monoclonal antibody (Cat No. sc-101352, 1 μg/μL) and rabbit anti-GAPDH polyclonal antibody (Cat No. sc-25778, 200 μg/mL) were purchased from Santa Cruz Biotechnology Inc. (USA). Rabbit monoclonal antibodies to influenza virus HA (H1N1) (Cat No. 11684-rp107-100, 1 μg/μL) were purchased from Sino Biological Inc. (Beijing, China). The goat anti-mouse IgG conjugated to FITC secondary antibody (Cat No. 115-095-003, 2 μg/μL) was purchased from Zhongshan Golden Bridge Biotechnology (Beijing, China). The anti-rabbit IgG (Cat No. ZB-2301, 1 μg/μL) and anti-mouse IgG (Cat No. ZB-2305, 1 μg/μL) coupled the horseradish peroxidase (HRP) were purchased from Sigma-Aldrich (USA). CellTiter-Glo reagent (Cat No. G7572) was purchased from Promega Corp. (USA). MDCK cells, HEK293T cells, A549 cells and HeLa cells were donated by Crown Bioscience Inc. (USA) and cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco BRL, Inc., Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS) (PAA Laboratories, Linz Austria) at 37 °C under 5% CO_2. α-2,6 glycans (6′-S-Di-LN: Neu5Aca2-6[Galb1-4GlcNAcb1-3]2b-SpNH-LC-LC-Biotin) was kindly provided by Scripps Research Institute (USA). Deionized double-distilled water was used throughout the biological study. All the other reagents and solvents are commercially available and used as received.

The results of cytopathic effect (CPE) assay were read using a Tecan Infinite M2000 PRO™ plate reader (Switzerland). The surface plasmon resonance (SPR) experiments were performed by a Biacore T200 system and analyzed with Biacore evaluation software (T200 Version 1.0) (Sweden). Microwave-assisted CuAAC reactions were performed using a CEM Discover SP microwaver reactor (USA). ^1H and ^{13}C NMR spectra were recorded using a Brucker 400 or 600 MHz spectrometer; the residual solvent protons were used to reference the chemical shift in ppm. ESI-HRMS and MALDI-TOF-MS were obtained with an APEX IV FT_MS (7.0 T) spectrometer (Bruker) and an AB Sciex TOF/TOF™ 72115 spectrometer, respectively. Reactions were monitored by thin-layer chromatography (TLC) on a pre-coated silica gel 60 F_{254} plate (layer thickness 0.2 mm; E. Merck, Darmstadt, Germany) and detected by staining with a yellow solution containing $Ce(NH_4)_2(NO_3)_6$ (0.5 g) and $(NH_4)_6Mo_7O_{24}4H_2O$ (24.0 g) in 6% H_2SO_4 (500 mL) followed by heating. Flash column chromatography was performed using 200–300 μM mesh silica gel 60 (Qingdao Haiyang Chemical Co. Ltd, Qingdao, China). Compounds **13**–**18** were synthesized according to the literature (Scheme 1) [19–22].

2.1.2. General procedure for the click reaction and deacetylation reaction

General procedure A: To a solution of azide (0.1 mmol) and alkyne (0.66–0.88 mmol) in 50% of THF and H_2O (15 mL) was added $CuSO_4$ (9.5 mg, 0.06 mmol) and sodium ascorbate (24 mg, 0.12 mmol). The resulting solution was heated in a microwave reactor at 100 °C until the azide was completely consumed (typically about 1 h) as determined by thin-layer chromatography (TLC). Then the reaction mixture was extracted with CH_2Cl_2 (10 mL × 3). The CH_2Cl_2 fraction was dried over Na_2SO_4, filtered, evaporated, and purified by silica gel column chromatography using DCM/CH_3OH (15:1–12:1 v/v) as eluent to give the purified white product.

General procedure B: The per-*O*-acetylated CD-triterpene conjugate was then dissolved in dry methanol (5 mL per 100 mg of compound) and a solution of sodium methoxide (30% in methanol, 0.1 eq per mol of acetate) was added. The solution was stirred (180 rpm) at room temperature for 6 h under N_2 atmosphere. After completion (TLC) the reaction mixture was neutralized with Amberlite IR-120 (H^+) ion exchange resin, then filtered, concentrated. The crude product was purified by RP column chromatography. The ^1H and ^{13}C NMR data, NMR spectra and mass spectra of compound **4**–**15** and **19**–**36** are available in supporting information.

2.2. Cytopathic effect (CPE) reduction assay

The assay was performed as described by Noah et al. [23] with some modifications. MDCK cells at a density of 1 × 10^4 cells per well were seeded into 96-well plates, incubated overnight and