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Hexagonal-shaped chondroitin sulfate self-assemblies have exalted anti-HSV-2 activity

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ABSTRACT

The initial step in mucosal infection by the herpes simplex virus type 2 (HSV-2) requires its binding to certain glycosaminoglycans naturally present on host cell membranes. We took advantage of this interaction to design biomimetic supramolecular hexagonal-shaped nanoassemblies composed of chondroitin sulfate having exalted anti-HSV-2 activity in comparison with native chondroitin sulfate. Nanoassemblies were formed by mixing hydrophobically-modified chondroitin sulfate with α-cyclodextrin in water. Optimization of alkyl chain length grafted on chondroitin sulfate and the ratio between hydrophobically-modified chondroitin sulfate and α-cyclodextrin showed that more cohesive and well-structured nanoassemblies were obtained using higher α-cyclodextrin concentration and longer alkyl chain lengths. A structure-activity relationship was found between anti-HSV-2 activity and the amphiphilic nature of hydrophobically-modified chondroitin sulfate. Also, antiviral activity of hexagonal nanoassemblies against HSV-2 was further improved in comparison with hydrophobically-modified chondroitin sulfate. This work suggests a new biomimetic formulation approach that can be extended to other heparan-sulfate-dependent viruses.

1. Introduction

The herpes simplex viruses (HSVs) cause various forms of diseases such as lesions on the lips, eyes, or genitalia, encephalitis and even disseminated disease in immunocompromised individuals (Kleymann, 2005). Herpes simplex viral diseases are caused by two members of the *Herpesviridae* family of viruses namely the herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2). Genital herpes is a sexually transmitted infection caused by HSV-2 and to a lesser extent HSV-1. According to the last estimation, 536 million people worldwide aged 15–49 were living with HSV-2 with an annual incidence of 23.6 million (Looker, Garnett, & Schmid, 2008). HSV-2 is the most frequent cause of genital ulcer disease (Corey et al., 2004). The lesions are often very painful, can lead to substantial psychological morbidity and even mortality and an increased incidence of HIV-1 transmission. Since genital herpes may increase the risk of HIV acquisition, interventions against HSV-2 may also have a key role in HIV prevention (Freeman et al., 2007).

The first step in the infection of mucosal surfaces by viruses involves interaction between the viral glycoproteins and epithelial cell receptors of the mucosa. Cell surface heparan sulfate proteoglycans (HSPGs) mediate the initial attachment of many viruses including herpesviruses to the target cell and the subsequent cellular entry and infection (WuDunn & Spear, 1989; Tiwari, Manus, Sigar, Ramsey, & Shukla, 2012). HSPGs are associated with the surfaces of many cell types and consist of a core protein with glycosaminoglycan (GAG) chains of unbranched sulfated polysaccharides. In the case of HSV-1 and HSV-2, attachment to heparan sulfate (HS) seems to be primarily mediated through glycoprotein C (gC) although glycoprotein B (gB) may contribute to this function (Herold, WuDunn, Soltys, & Spear, 1991; Cheshenko & Herold, 2002). Bergefall et al. (2005) postulated that HSV-1 glycoprotein C (gC) binds to chondroitin sulfate (characterized by E disaccharide units) and that the chondroitin sulfate-E unit is an essential component to function as a HSV-1 receptor. The same article reported that the concentrations of CS-E that reduced the number of viral plaques

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by 50% (IC50) for HSV-1 and for HSV-2 are substantially exceeding the antiviral potency of heparin (Bergefall et al., 2005). More recently, this group showed that chondroitin 4-*O*-sulfotransferase-1 regulates the E disaccharide expression of chondroitin sulfate required for HSV-1 infection (Uyama et al., 2006). Chondroitin sulfate-A, B, C and D do not interfere with (or very little) viral glycoproteins.

Even if sulfated polysaccharides showed their ability to inhibit virus attachment to cells by competitive inhibition, there is a clear gap between fundamental data and the design of efficient locally-administrated formulations. The aim of this work is to "learn from nature" to design new formulation strategies for the prevention of vaginal infections caused by HSV-2. Our formulation strategy is based on the design of GAG nanoassemblies able to interact with HSV-2 envelop protein, better than native GAG. The proof of the concept of the formation of GAG-based nanoassemblies was already demonstrated using heparin (Lembo et al., 2014). This GAG biomimetic barrier could act like a "trap" able to specifically catch up the viruses and avoid their attachment to cells. Heparin nanoassemblies were formed by non-covalent interaction between hydrophobically-modified heparin and α-cyclodextrin. Hydrophobically-modified heparin was obtained by the esterification of heparin using palmitoyl chloride (Lembo et al., 2014).

Although heparin exhibits significant anti-herpetic activity, its use could be limited because of its anticoagulant activity, which can cause undesirable side effects. Furthermore, we have previously shown that heparin esterification using acyl chloride results in acidification of the medium and decrease of sulfate group content and consequently, antiviral activity (Lembo et al., 2014). Thus, the development of nanoassemblies from other GAGs and using other chemical reactions allowing sulfate groups to be preserved was considered in this work. Our attention was oriented toward the design of nanoassemblies composed of chondroitin sulfate-E. Chondroitin sulfate is commercially available in the form of heterogeneous mixture of different component types, mainly chondroitin sulfate types A and C derived from bovine cartilage, porcine or chicken (Volpi, 2004). Here, chondroitin sulfate from shark cartilage is used. Indeed, unlike other cartilages, it has a relatively high percentage of chondroitin sulfate-E (≈13%) and is therefore more likely to have a significant anti-herpetic activity (Ji, 2007).

Here, drug-free chondroitin sulfate based nanoassemblies were designed by a self-association in water of hydrophobically-modified chondroitin sulfate and α-cyclodextrin. The hydrophobization of chondroitin sulfate was conducted on carboxylic groups through amide linkage. A structure-activity relationship was established between the length of hydrophobic chain grafted on chondroitin sulfate-E and anti-HSV-2 activity.

2. Materials

Chondroitin sulfate from shark cartilage ($M_w \approx 40$–50 kDa evaluated by Polyacrylamide Gel Electrophoresis) was from TCI (Zwijndrecht, Belgium). Three amines: dodecylamine ($CH_3(CH_2)_{11}NH_2$), hexadecylamine ($CH_3(CH_2)_{15}NH_2$) and octadecylamine ($CH_3(CH_2)_{17}NH_2$), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), α-cyclodextrin (α-CD), methylcellulose, dry hydranal formamide, ethanol and chloroform were from Sigma-Aldrich (St. Quentin Fallavier, France). Dimethylformamide (DMF), methanol, isopropanol and acetone were from Carlo Erba Reagents (Val de Reuil, France).

2.1. Cells

African green monkey fibroblastoid kidney cells (Vero) (ATCC CCL-81), human epithelial cells Hep-2 (ATCC CCL-23), A549 (ATCC CCL-185) and African green monkey kidney epithelial (MA-104) cells (ATCC CRL-2378.1) were grown as monolayers in Eagle's minimal essential medium (MEM) (Gibco/BRL, Gaithersburg, MD) supplemented with 10% heat inactivated fetal calf serum and 1% antibiotic-antimycotic solution (Zell Shield, Minerva Biolabs GmbH, Berlin, Germany). The 293TT cell line, derived from human embryonic kidney cells transformed with the simian virus 40 (SV40) large T antigen, was cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL, Gaithersburg, MD) supplemented with heat-inactivated 10% fetal calf serum (FCS; Gibco-BRL), Glutamax-I 1% (Invitrogen, Carlsbad, CA) and nonessential amino acids 1% (Sigma Aldrich, Steinheim, Germany). 293TT cells allow high levels of protein to be expressed from vectors containing the SV40 origin due to overreplication of the expression plasmid.

2.2. Viruses

Clinical isolates of HSV-2 were kindly provided by Prof. M. Pistello, University of Pisa, Italy. HSV-2 strains were propagated and titrated by plaque assay on Vero cells. Virus stocks were maintained frozen (−80 °C).

3. Methods

3.1. Chemical preparation of hydrophobically-modified chondroitin sulfate

The impact of hydrophobic chain length grafted on chondroitin sulfate on the ability to form nanoassemblies was investigated using three different amines: dodecylamine (DDAm, C_{12}), hexadecylamine (HDAm, C_{16}) and octadecylamine (ODAm, C_{18}). Amines were grafted on carboxylic acids functions of chondroitin sulfate using EDC as coupling agent to activate carboxyl functions of chondroitin sulfate, as shown in Fig. 1 of Supporting information. Briefly, 2.0 g of chondroitin sulfate were dissolved in 30 mL of formamide at 60 °C under magnetic stirring. Once fully dissolved, the mixture was cooled at 25 °C and EDC (715 mg) was added. Amount of EDC was calculated to provide a molar ratio of 1:1 between the EDC and the carboxylic acid groups of chondroitin sulfate.

Then, the amines were dispersed into 15 mL of DMF and added to the mixture. Amount of each amine was 3 equivalents calculated for chondroitin sulfate disaccharide unit (corresponding to 2.07 g of DDAm, 2.70 g of HDAm, 3.02 g of ODAm).

The resulting mixture was magnetically stirred for 24 h at 25 °C. Hydrophobically-modified chondroitin sulfate formed were then precipitated in excess of acetone (about 200 mL) and allowed to stir 30 min for grafting by the dodecylamine and 1 h30 for grafting by the two other amines. These conditions were optimized according to the solubility of the amines allowing complete dissolution of eventual free unreacted amines. Finally, the precipitate was filtrated under vacuum and washed 3 times with acetone. Powder was dried under vacuum for 24 h, redispersed in distilled water and lyophilized for 24 h (Alpha 1–2 freeze-dryer. Fisher Scientific Bioblock, Illkirch, France).

3.2. Chemical characterization of hydrophobically-modified chondroitin sulfate

Hydrophobically-modified chondroitin sulfate derivatives were characterized by Attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy. Infrared spectra were obtained with an ATR-FTIR spectrometer (FT/IR-4100, JASCO) operating at 4 cm^{-1} resolution. Fifty scans were accumulated in each run and referred to air. The ATR sampling device utilized a diamond internal reflection element embedded into a ZnSe support/focusing element in a single reflection configuration. The resultant spectra over the range of 4000–400 cm^{-1} were analyzed using the IR Protein