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Efficient inhibition of human papillomavirus 16 L1 pentamer formation by a carboxylatopillarene and a *p*-sulfonatocalixarene†

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Dong-Dong Zheng,[a] Ding-Yi Fu,[a] Yuqing Wu,*[a] Yu-Long Sun,[a] Li-Li Tan,[a] Ting Zhou,[a] Shi-Qi Ma,[b] Xiao Zha[b] and Ying-Wei Yang*[a]

Pillarenes and calixarenes showed obvious inhibition of HPV16 L1 pentamer formation *via* their selective binding to Arg and Lys residues at the monomer interface, which was reversible after the release of cyclic arenes. Pillarenes are more effective than calixarenes in terms of the inhibition efficiency, attributing to the different kinetics and binding affinity.

Human papillomavirus (HPV) is the causative agent of cervical cancer and other anogenital and oropharyngeal cancers.[1] The major capsid protein L1 and minor capsid protein L2 assemble into virus particles essentially and mediate the primary attachment of viral particles to cells[2] and the extracellular matrix,[3] emphasizing their significance in viral infections. Current HPV vaccines target the major capsid protein L1 of the most common cancer-causing types and protect humans against HPV infection and the development of neoplasias[4] successfully, but they are type-specific, expensive and require cold chain transportation.[5] Therefore, there is an urgent need for the development of cost-effective and/or more broad-spectrum alternatives.

Creating disturbances in virus assembly is a particularly attractive method for antiviral intervention because viral structures are formed by multiple, relatively weak noncovalent interactions,[6] and essentially the viral infectivity is critically dependent on capsid formation and its stability.[7] The cleavage of the glutathione-S-transferase (GST) fusion L1, *i.e.*, GST-L1, by PreScission™ Protease (PPase) liberates the capsid proteins for self-assembly into pentamers and further virus-like particles (VLPs).[6a,8] Structural determination of L1 pentamers[6a] indicated that the L1 monomer represents individual subdomains within pentamers. Image reconstruction of *in vitro* assembled VLPs shows that VLPs are organized by interaction between helices 2 and 4 of L1 and linked to each other *via* the monomer interface of L1. Therefore, novel small-molecule inhibitors of HPV, targeting specific sites of proteins to stop further virus assembly, are needed to complement or replace current treatments. Although inhibitors have been widely used for controlling the assembly process of other health-related viruses,[9] few/no small-molecular inhibitors have been identified for HPV assembly mainly due to the insufficient target sites and unavailability of agents. Small molecules that recognize protein surfaces specifically are important tools for modifying protein interactions. Molecules that target the cationic side chains of lysine and arginine have great potential as generic surface binders.[10] Pillarenes,[11] as a new class of synthetic macrocycles, have exhibited intriguing and peculiar host–guest properties and many potential applications in chemistry, biology and materials science.[12] Pillarene-based molecular recognition in organic solvents has been extensively investigated, but few examples have been shown in aqueous solutions,[12d–g] particularly the complexation with biologically important entities such as proteins has rarely been reported yet.[12d]

A negatively charged pillarene derivative, *i.e.*, a carboxylatopillar[5]arene sodium salt (CP5A), possessing a three-dimensional, rigid and π-rich cavity, as well as two anionic portals, has shown high water solubility and good selective binding towards basic amino acids (AAs), such as L-lysine (Lys), L-arginine (Arg) and L-histidine (His).[12d] These results inspired us to employ CP5A to bind to the arginine/lysine-rich HPV major capsid protein, L1, and further inhibit the formation of L1 pentamers and VLPs. The controlled assembly of L1 pentamers by CP5As has been examined by static light scattering (SLS) and fast protein liquid chromatography (FPLC). In the presence of CP5A, the L1 pentamer formation was efficiently suppressed. The comparison of CP5A with a water-soluble sulfonatocalix[4]arene (SC4A) by monitoring the L1 pentamer formation was also performed, and big differences in the intrinsic binding mechanisms and kinetics were also explored. So far, CP5A and SC4A are the first family of small molecular inhibitors directed against pentamer formation of HPV L1.

The HPV L1, with the GST fusion, was coded, expressed, and purified using the previously reported protocols.[6a] After removing the GST-tag by PPase cleavage, the liberated L1 can assemble into pentamers with structural features that resemble "donuts".[6a,8] The pentamer formation leads to an increase in sample turbidity, as monitored by fluorescence scattering intensity,[8] which was further used to probe the potential inhibitory effects of CP5A on L1 pentamer formation.

[a] *State Key Laboratory of Supramolecular Structure and Materials, College of Chemistry, Jilin University, 2699 Qianjin Street, Changchun, 130012, China. E-mail: yqwu@jlu.edu.cn, ywyang@jlu.edu.cn*

[b] *Sichuan Tumor Hospital & Institute, Chengdu, 610041, China*

† Electronic supplementary information (ESI) available: Experimental procedures, SLS, NMR, IC_{50}, TEM, DLS, FPLC data and structural analysis of the target protein interaction. See DOI: 10.1039/c3cc49789e

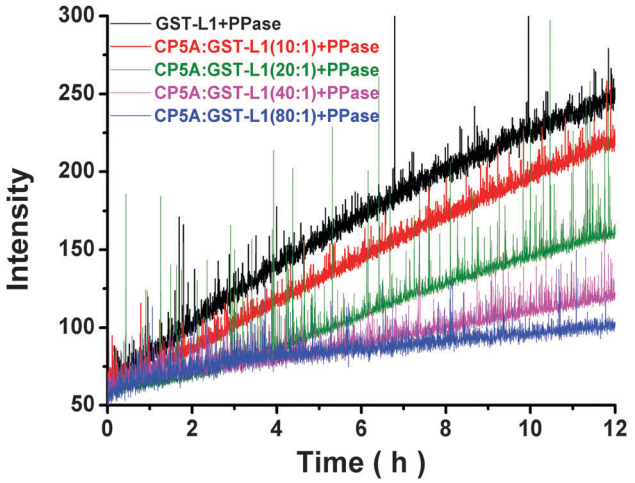

Fig. 1 Monitoring of the pentamer formation from GST-L1 monomers (0.1 mg mL^{-1}) by time-dependent static light scattering (SLS) after addition of the PPase for GST cleavage. The protein was incubated with CP5A in buffer L (50 mM Tris-HCl, pH = 8.0, 0.2 M NaCl, 1 mM DTT and 1 mM EDTA) at different concentrations for 30 min before PPase addition and measurement. The curves in different colours represent the molar ratios of CP5A to GST-L1 at 0 : 1, 10 : 1, 20 : 1, 40 : 1 and 80 : 1, from top to bottom, respectively.

In the absence of CP5A, the scattering intensity increases gradually with time after addition of PPase for GST cleavage (Fig. 1), suggesting that the particle size increases with L1 pentamer formation. The increase in rate was restrained in the presence of CP5A and the tendency was enhanced with the addition of more CP5As, which was demonstrated more clearly by the corresponding curve fitting plots (Fig. S1, ESI†). The pronounced inhibitory effects of CP5A on L1-pentamer formation suggest that tight binding exists between CP5A and L1, where the exposed residues of basic AAs might be the main binding sites.

Based on the standard methods,[13] ^{1}H NMR spectra of a peptide of HPV L1 helix 5 (DLDQFPLGRKFLLQ), a representative one of L1, and the peptide with CP5A/SC4A were obtained, and the proton peaks were assigned (Tables S1–S3, ESI†). The NH regions of the ^{1}H NMR spectra of 1 mM peptide with CP5A/SC4A at different molar ratios are shown in Fig. S3 and S4 (ESI†). The differences between the chemical shifts of the amide NH protons of the peptide backbone in the absence and presence of CP5A are pronounced (Fig. S5, ESI†). In other words, the chemical shifts of NH protons in the backbone and side chains of AAs, especially Arg and Lys, are markedly affected by CP5A/SC4A (Fig. S6, ESI†). Obviously, the results demonstrate that CP5A/SC4A can bind strongly to the basic residues of AAs in L1.

After treatment with different molar ratios of CP5A to GST-L1 and then digestion with PPase to remove the GST-tag, the sample was injected into a gel filtration column to obtain the FPLC elution profile (Fig. 2). Without CP5A treatment, the results showed an intense peak corresponding to ~284 kDa in the FPLC elution profile (peak1 in Fig. 2A), which was in a good agreement with the mass of the HPV L1 pentamer (L1-p). Also eluted was a peak corresponding to ~58 kDa (peak2 in Fig. 2A) that could be attributed to the L1 monomer (L1-m); both show a decreased ratio of the integrated peak area, Sp to Sm. Pre-incubation of GST-L1 with CP5A resulted in a decrease of the L1-p peak and a simultaneous increase of the L1-m peak. The intense

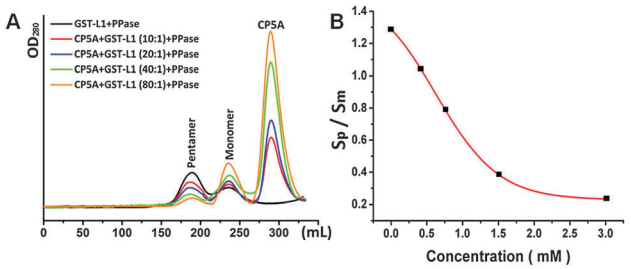

Fig. 2 Examination of the pentamer formation of L1 by size-exclusion chromatography. The GST-L1 (0.1 mg mL^{-1}) was treated with different molar ratios of CP5A : GST-L1 (0 : 1, 10 : 1, 20 : 1, 40 : 1 and 80 : 1). The graph on the right shows the decrease in the yield of L1-p to L1-m ratios upon addition of CP5A.

peaks that appeared to the right of the monomer peak correspond to the excess amount of CP5A used in the systems. The yield of L1-p to L1-m ratio was in positive correlation with that of the CP5A concentration in the samples (Fig. 2B). More precisely, the rate of pentamer formation was inhibited by CP5A in a dose-dependent manner, supplying a half-inhibition concentration (IC_{50}) of CP5A at 0.62 mM for 3 mg mL^{-1} GST-L1 (Fig. S7, ESI†). Once CP5A was released by dialysis, the cleaved L1 could further form L1 pentamers and VLPs, as revealed by dynamic light scattering (DLS, Fig. S9, ESI†) and transmission electron microscopy (TEM, Fig. 3). Similar inhibitory effects of SC4A on the L1-p formation and its reversibility were also observed for the SC4A–L1 system (Fig. S10, ESI†).

By using the same molar ratios of SC4A/GST-L1 as compared with CP5A/GST-L1, we observed that SC4A has a similar (Fig. S2, ESI†) but less pronounced efficacy in assembly inhibition (Fig. 4). That is, the inhibition rate of L1-p formation was enhanced in a dose-dependent manner by both CP5A and SC4A, while CP5A has a more distinct effect on it. The kinetics of L1-p formation under CP5A and SC4A are compared using *in situ* SLS plots (Fig. 4). At the same concentration, both CP5A and SC4A have similar effects on L1-p formation initially; however, with time especially several hours after addition of PPase, they exhibited obvious differences. The enhanced increase rate for SC4A indicates the formation of more L1-p, suggesting the less efficiency of SC4A to bind L1 when competing with the intermolecular binding between L1 monomers. In contrast, CP5A showed a more efficient inhibition with the increase of the L1particle size, suggesting a higher binding affinity of CP5A and L1. Thus, the binding of CP5A–L1 should be stronger than that of

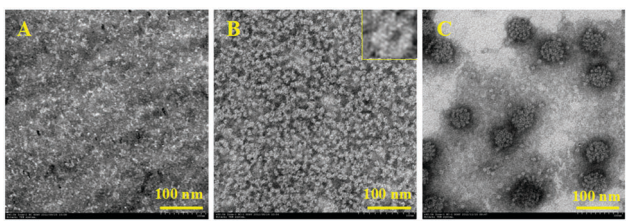

Fig. 3 Comparison of the electronic micrograms (EM) of HPV L1 in (A) monomer (L1-m, after incubated with CP5A at the molar ratios of 1 : 80 and GST cleavage); (B) pentamer (L1-p, after removal of CP5A by dialysis in buffer L) and (C) VLPs (after further assembly with L1-p in assembly buffer G). The concentration of initial GST-L1 was 0.1 mg mL^{-1}.

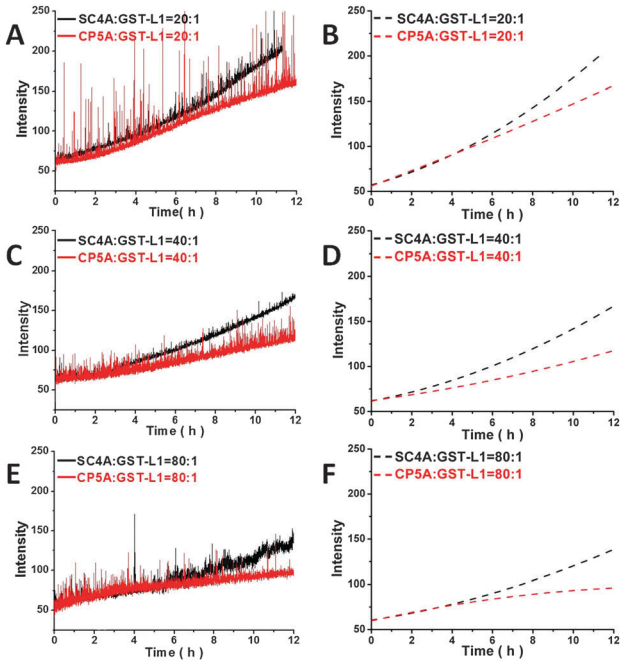

Fig. 4 The kinetics comparison of the inhibitory effects of SC4A and CP5A on L1 pentamer formation by SLS. The initial concentration of GST-L1 was 0.1 mg mL^{-1}.

L1–L1, while SC4A–L1 be weaker, as further confirmed by the different ratios of L1-p to L1-m in FPLC elution profiles for CP5A and SC4A (Fig. S8, ESI†).

The crystal structure of SC4A bound with lysine-rich cytochrome *c* showed that SC4A targeted Lys side chains at the surface of cytochrome *c*.[14] In addition to providing valuable information on protein recognition, the data also indicated that calixarenes are mediators of protein–protein interactions (PPIs), with potential applications in assemblies and promoting crystallization. On the other hand, the highly selective binding of basic AAs by CP5As indicated that the host shows strong binding abilities towards basic AAs such as Lys, Arg and His with the strongest K_a value of $(5.9 \pm 0.4) \times 10^3$ M^{-1} for Arg.[12d] Combined with the ^{1}H NMR results, we can deduce that CP5A and SC4A might explore different targets at the surface of HPV L1. Close inspection of the crystal structure of the HPV16 L1 pentamer reveals that 5 lateral Arg residues from one monomer interact with the specific sites on the neighbouring one by forming hydrogen bonds or other non-covalent interactions (Table S4, ESI†), while another two were observed in reverse (Fig. S11C and D, ESI†). The number of Lys at the monomer interface is much less than Arg; in total only 3 Lys participate in the interface interaction of monomers (Fig. S12, ESI†), explaining well the less efficiency of SC4A in inhibiting L1-p formation than CP5A. Besides the difference in the number of Lys and Arg at the monomer interface, their distributions also play crucial roles: two of three Lys are located at the loop, outer edge of the monomer interface (Fig. S12, ESI†), while most Arg residues are distributed well along the interface of two monomers (Fig. S11, ESI†).

In conclusion, CP5A and SC4A showed obvious inhibition of HPV L1 pentamer formation *via* binding to Arg and Lys at the monomer interface. The results show that CP5A is more efficient than SC4A in inhibiting the L1 pentamer formation, with different kinetics and binding affinity to L1. The GST-released L1 could form L1 pentamers and VLPs well after CP5A was removed by dialysis. These findings lay the groundwork for the development of assembly inhibitors as a new class of prophylactic and/or therapeutic agents for the treatment of HPV infections.

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