The Inhibitory Properties of Acidic Functionalised Calix[4]arenes on Human Papillomavirus Pentamer Formation

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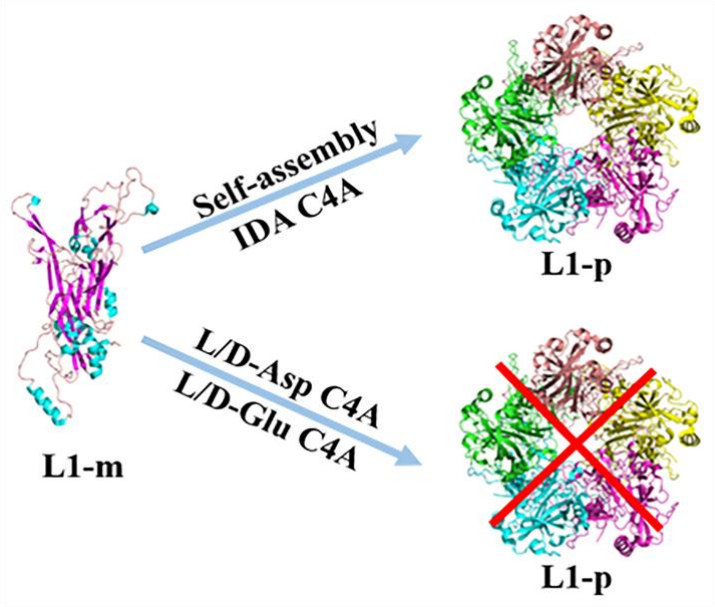
Abstract

Human Papillomavirus (HPV) is the leading cause of cervical cancer, with only a portion of HPV types prevented with vaccines and no treatments for the viral infection itself. Inhibiting the formation of the viral capsid is one way to target the viral infection. This can be done by inhibiting the assembly of the L1 monomer into a pentamer, which forms the viral capsid. Four calix[4]arene compounds functionalised with D- and L-aspartic and glutamic acid were synthesised and tested for L1 pentamer formation inhibition, in addition to an iminodiacetic functionalised calix[4]arene. The amino acid functionalised calix[4]arene derivatives showed millimolar inhibition (IC_{50} = 0.72 to 2.67 mM) of L1 pentamer formation, with a small difference between the inhibitory concentrations of the stereoisomers. The iminodiacetic acid calix[4]arene derivative showed no inhibitory properties, despite sharing structural similarities with the four other calix[4]arenes, suggesting the flexibility of the amino acid side chain and/or the hydrogen of the secondary amide were important for the compounds to bind to the L1 protein. Confirmation of binding the negatively charged compounds to the positive residues of the L1 protein was achieved with a trypsin digestion. This study will be helpful to develop cost-effective inhibitor to prevent HPV assembly.

Mandatory graphical abstract (525 pixels wide)(jpeg, png, gif)



Key words: 3 to 5

Calixarene, human papillomavirus, assembly inhibitor, L1 pentamer.

Introduction

Protein-protein interactions are a fundamental part of functional biological systems. The specificity of the intermolecular interactions between proteins is crucial for good, stable interactions. These stable interactions are essential for biological processes to occur (*1*), and include hydrogen-bonding, dipole interactions, salt bridges, dispersion forces and electrostatic attractions, in addition to covalent bonding in the form of disulfide bridges. It is possible to exploit these interactions to manipulate or inhibit the processes facilitated by protein-protein interactions (*1*). This is of particular interest for medicinal purposes due to the wide range of processes proteins regulate. The specificity of protein interactions also means there is the potential to develop highly specific inhibitors that could reduce the toxicity of drugs that arise due to unwanted interactions with other biological processes (*2*).

In the last three decades, interruption of protein-protein interfaces has been investigated, generally by targeting the intermolecular forces that are crucial to the stabilisation of the protein-protein interface (*1*). A large portion of this research has been focused on developing therapeutics that work to inhibit these interactions as treatment options (*2*). Biological drugs were the primary choice for targeting protein-protein interactions due to their large size and ability to effectively bind to proteins and inhibit their interactions. Small-molecule drugs are a more recent tool used to target the interactions of proteins. One of the most notable examples

of small-molecule drugs inhibiting protein-protein interactions is Tirofiban, which is used to reduce blood coagulation. Tirofiban inhibits platelet aggregation by inhibiting the interaction between platelets and glycoproteins IIb/IIIa (*3*). Vinblastine, a current antitumor drug used in the treatment of various tumours, inhibits cell replication by binding to tubulin and affecting the assembly of microtubules (*4*). While biological and small-molecule drugs have been used to inhibit the interactions between proteins, there has been little interest in the mid-range molecular mass compounds until fairly recently. In 1997 a mid-range molecular weight protein interaction inhibitor was shown to interact with cytochrome *c* (*5*). This compound, a calix[4]arene functionalised with various amino acids at the wider rim, has a molecular mass upwards of 2700 Da (*5*).

Protein-protein interactions have been targeted to inhibit the formation of the viral capsid in the human papillomavirus (HPV) (*6, 7*). HPV is the leading cause of cervical cancer, the cause of 5% of all cancer and the most commonly sexually transmitted infection. Consequently, there is a need for a treatment option for the HPV infections that does not just target the symptoms (*8, 9*). There are over 200 types of HPV, including high-risk cancer-causing types and others that cause minor effects, such as warts and lesions (*10*). Nine of these HPV types can be vaccinated against, with three vaccines available, however there are still over a hundred types whose infections can be harmful that cannot be prevented (*11*).

For the virus to leave human cells, the viral capsid has to be formed to encapsulate the viral DNA (*12*). Once the virus has left the cells, it is free to infect nearby cells and the infection propagates (*12*). By interrupting the formation of the viral capsid with compounds that do not specifically inhibit one type of HPV, the spread any HPV infection can be prevented. This would greatly reduce the rate of HPV infection related side-effects, such as cancer, warts and lesions, due to there being a higher rate of HPV clearance by the human immune system.

This research focused on the inhibition of the viral pentamer formation by using the functionalized calix[4]arenes (**Figure 1**). The L1 pentamers are composed of five L1 protein monomers that undergo molecular self-assembly to form the pentamer through various intermolecular interactions, including disulfide bonds, hydrogen bonding, ionic interactions and hydrophobic interactions (*13*). These L1 pentamers then assemble to form virus like particles or, with the inclusion of viral DNA, the infective virus. These L1-L1 protein interactions include entwined loops between the proteins and a buried surface area of

approximately one third of the total surface area (*14*). Calix[4]arenes functionalised with acidic functional groups have been shown to successfully inhibit HPV pentamer formation by interacting with the basic residues of the L1 protein, including lysine and arginine (*6*). One of the compounds tested was a *p*-sulfonatocalix[4]arene derivative (*6*). This compound has also been demonstrated to bind with cytochrome *c*, predominantly through strong interactions with lysine residues of the protein within the cavity of the calix[4]arene (*15*). Calix[4]arenes functionalised with L- and D-proline were used to initially investigate the impact the stereochemistry has on the inhibitory properties, with the L- derivative (half maximal inhibitory concentration (IC_{50}) of 2.21 mM) inhibiting slightly better than the D- derivative (IC_{50} of 3.06 mM) (*7*). The impact of stereochemistry is further investigated here.

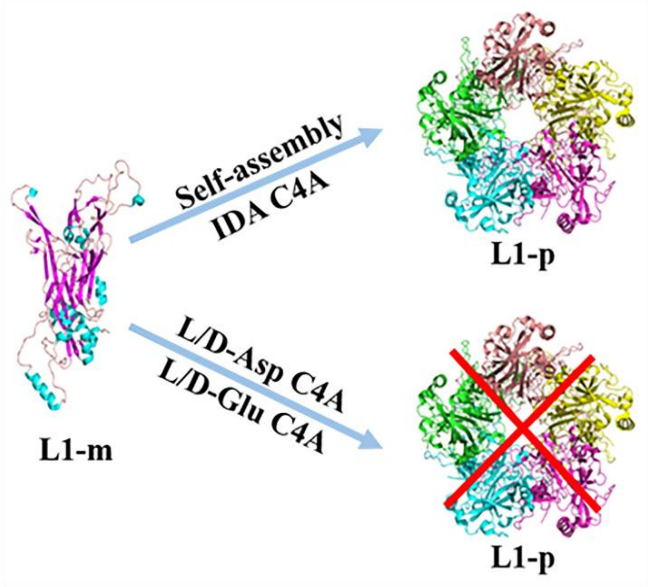


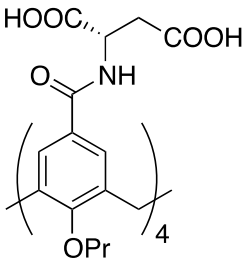
Figure 1: The assembly of the viral capsid of HPV L-1 pentamer from the L1 monomer. The crystal structure of L1-monomer and L1-pentamer are from PDB ID: 1DZL, 2R5H.

Results and Discussion

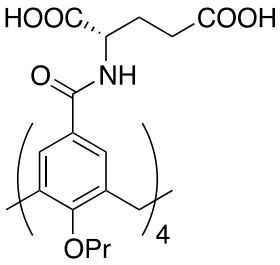
Synthesis

Five compounds were synthesised for testing against HPV L1 pentamer formation (**Figure 2**). The synthesis of **L-Asp C4A**, **L-Glu C4A** and the iminodiacetic acid calix[4]arene (**IDA C4A**) have been previously described (*16*). Briefly, the calix[4]arene was locked into the cone conformation by propylating the narrow rim, followed by formylation and oxidation at the wider rim to give the tetracarboxylic acid calix[4]arene. The amino acids were attached to the calix[4]arene with a peptide bond, both via an acyl chloride and activation of the carboxylic acid with two peptide coupling reagents, dicyclohexylcarbodiimide (DCC) with *N*-

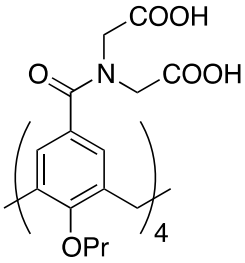
hydroxybenzotriazole (HOBt) and *O*-(benzotriazole-1-yl)-*N*,*N*,*N'*,*N'*-tetramethyluronium tetrafluoroborate (TBTU) (*16*).



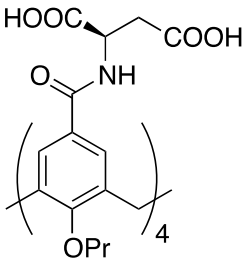
L-Asp C4A



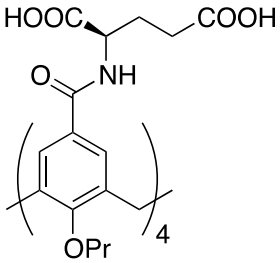
L-Glu C4A



IDA C4A



D-Asp C4A

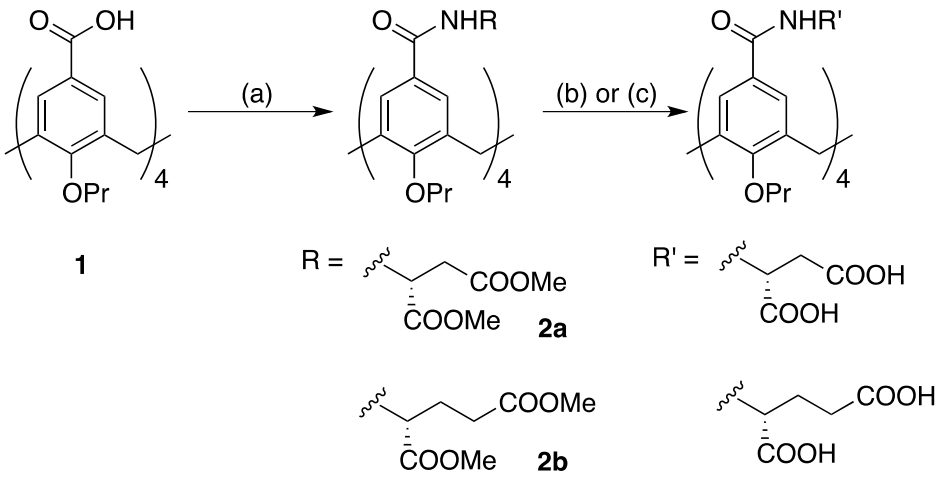


D-Glu C4A

Figure 2: The calix[4]arene structures synthesised for testing against HPV pentamer formation.

A similar synthetic route was used to synthesise the **D-Asp C4A** and **D-Glu C4A** derivatives (**Scheme 1**), however, TBTU was used as it showed increased yield previously (*16*). Additionally, the reaction using peptide coupling reagents is less water-sensitive than using acyl chlorides, resulting in a more robust reaction. In order to attach the amino acid to the calix[4]arene scaffold, the free amine of D-Asp or D-Glu methyl ester was coupled to the carboxylic acid calix[4]arene **1** using TBTU and triethylamine. Purification by silica gel column chromatography afforded the D-Asp C4A methyl ester **2a** and D-Glu C4A methyl ester **2b**. Both **2a** and **2b** were fully characterised by ^{1}H and ^{13}C NMR spectroscopy and high resolution mass spectrometry confirmed the presence of the tetra substituted derivatives. Deprotection of the carboxylic acids of **2a** was achieved with potassium carbonate in methanol at 50 °C to give **D-Asp C4A** in 30% yield. The final compound appeared to be quite water soluble and so only a fraction was extracted out of the aqueous phase with ethyl acetate. The deprotection of D-Glu C4A methyl ester **2b** was carried out using potassium hydroxide in

methanol at room temperature, affording **D-Glu C4A** in 36% yield, with similar solubility issues as the **D-Asp C4A** derivative. The successful hydrolysis to give **D-Asp C4A** and **D-Glu C4A** was verified by the absence of signals of the methyl ester in both the ^{1}H and ^{13}C NMR spectrum. This was consistent with the data of the previously published L- isomers (*16*).



Scheme 1: (a) D-Asp methyl ester HCl or D-Glu methyl ester HCl, triethylamine, TBTU, dichloromethane, room temperature, overnight; (b) to produce D-Asp C4A, potassium carbonate, water, methanol, 50°C, 2 hours; (c) to produce D-Glu C4A, potassium hydroxide, water, methanol, room temperature, 6 hours.

Biology

All four calix[4]arenes functionalised with amino acids showed inhibitory properties against HPV pentamer formation (Figure 3). The **L-Asp C4A** and **L-Glu C4A** derivatives had the best inhibitory properties, with IC_{50} values of 0.72 mM and 0.76 mM, respectively. The inhibitory properties of the D-amino acid derivatives appear to be less than the L-amino acid counterparts, which is in agreeance with the previously tested proline derivatives (*7*). However, it appears there is no significant difference in the inhibitory properties between the two L-amino acid derivatives, suggesting the extra carbon in the glutamic acid has little impact on the compound interaction with the L1 protein. There appears to be a larger difference between the IC_{50} values determined for the **D-Asp C4A** (1.87 mM) and **D-Glu C4A** (2.67 mM) derivatives than the L-amino acid counterparts. This difference is difficult to explain given the added flexibility of the glutamic acid moiety.

The **IDA C4A** derivative showed no inhibition of HPV pentamer formation when analysed by size-exclusion chromatography at a ratio of 300:1 (**IDA C4A**: GST L1) (Figure S2). All other compounds tested showed obvious pentamer formation inhibitory properties at concentrations

lower than this, despite the fact that all compounds tested contained the same number of carboxylic acid groups. Initially this result was somewhat unanticipated as **IDA C4A** is an isomer of the glutamic acid derivatives. However, when considering the planar nature of the amide group and the restricted rotation around the amide linkage, only one of the two carboxylic acid groups on each ring is directed towards the protein surface. This lack of conformational flexibility effectively restricts the capacity of the acidic groups to orientate themselves to achieve good binding to the positively charges sites on the protein surface. A contributing factor may also be that the **IDA C4A** is a tertiary amide and lacks a hydrogen atom for hydrogen bonding. This suggests that the secondary amide of the amino acid functionalised calix[4]arenes tested may be important for hydrogen bonding between the tested compounds and the L1 protein.

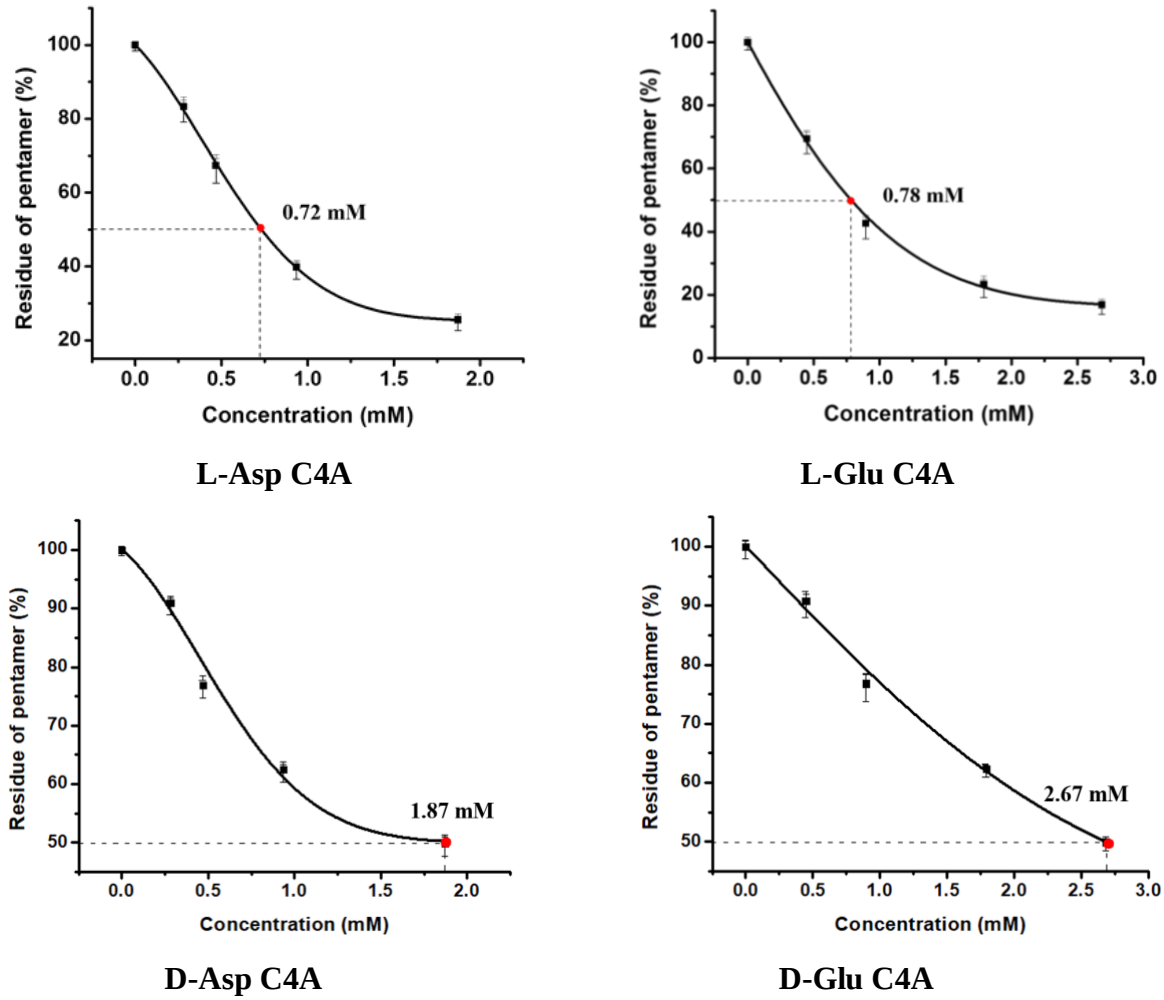


Figure 3: The percentage inhibition of HPV L1 pentamer formation with the varying concentration of the functionalised calix[4]arenes, determined using size-exclusion

chromatography. The determined IC_{50} of the calix[4]arene on L1 pentamer formation is also given.

Trypsin digestion

An enzymatic digestion experiment was also carried out using trypsin to further confirm whether **Asp C4A**/**Glu C4A** was truly bound at the positive residues of full-length GST-L1. It is well known that trypsin exclusively cleaves the arginine (Arg) and lysine (Lys) residues in proteins (*7*, *17*). Taking GST-L1 as an example, if the sites for enzyme digestion and **Asp C4A**/**Glu C4A** binding were the same, the pre-incubation of GST-L1 with **Asp C4A**/**Glu C4A** would prevent the digestion of GST-L1 by trypsin; otherwise, there would be no interference on the digestion. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was used as an assay to verify this hypothesis. The results showed that without C4A participation, the GST-L1 was almost degraded completely by trypsin in seven minutes after addition (Figure 4, panel 1). It was seen in the presence of **Asp C4A**/**Glu C4A**, the degree of hydrolysis was clearly reduced (Figure 4, panel 2 and 3). These results confirm that C4A indeed binds to the positively charged residues of Lys and Arg and, therefore, protects it from trypsin degradation.

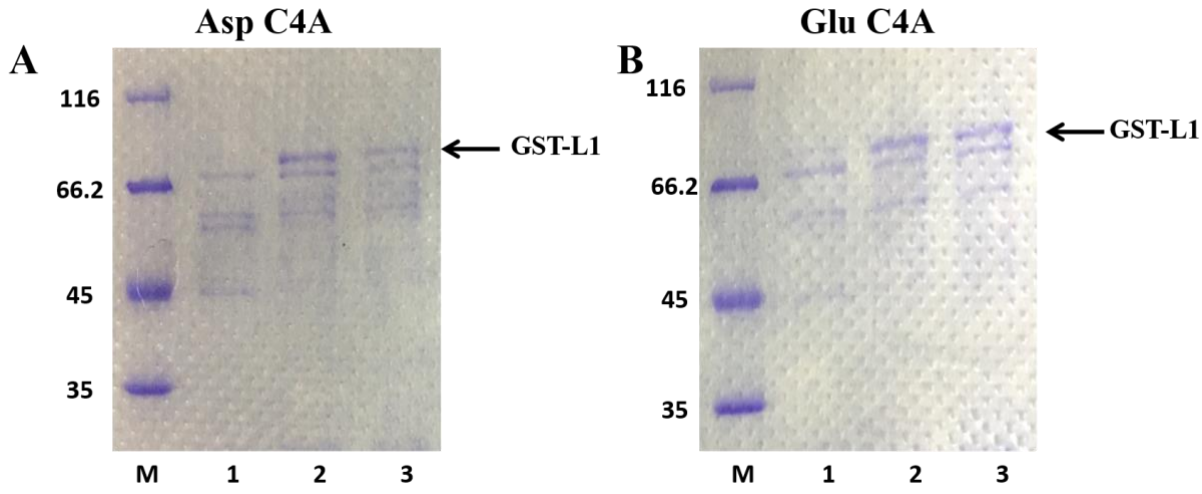


Figure 4: SDS-PAGE analysis shows the effect of (A) Asp C4A (B) Glu C4A on GST-L1 digestion by trypsin. Panel 1: GST-L1+Trypsin, Panel 2: (GST-L1+L-Asp C4A/L-Glu C4A)+Trypsin, Panel 3: (GST-L1+D-Asp C4A/D-Glu C4A)+Trypsin, respectively, after incubation for seven minutes.

Conclusion

Synthesis of stereoisomers of calix[4]arenes functionalised with aspartic and glutamic acid was achieved through a simple peptide coupling reaction using TBTU. The four amino acid functionalised calix[4]arene compounds showed half-maximal inhibition concentrations (IC_{50}) on the L1 pentamer formation of the human papillomavirus in concentrations ranging from 0.72 to 2.67 mM, with only a small difference between the enantiomers. Binding of the negatively charged compounds to the positive residues of the L1 protein was demonstrated with a trypsin digestion and subsequent analysis. A fifth compound, an iminodiacetic acid functionalised calix[4]arene, showed no inhibition of L1 pentamer formation, suggesting the flexibility of the amino acid side chain and/or the hydrogen of the secondary amide were important for the compounds to bind to the L1 protein. These findings lay the foundation for the development of cost-effective inhibitor to prevent HPV L1 pentamer and consequent virus like particle (VLP) assembly.

Experimental

Synthesis

General Methods

NMR spectra were recorded on a Bruker UltraShield Avance 400 spectrometer (400 MHz for ^{1}H, 100 MHz for ^{13}C) and were calibrated to their respective solvent signal. Optical rotations were measured on a Rudolph Research Analytical Autopol I automatic polarimeter. A Thermo Scientific Q-Exactive Orbitrap mass spectrometer was employed for high accuracy mass determination. Thin layer chromatography was performed using 200 µm silica gel F-254 aluminium-backed sheets. Preparative TLC was performed using 20 × 20 cm, 1000 µm thick silica gel F-254 glass-backed plates. Column chromatography was performed using 40-63 µm silica gel.

The carboxylic acid calix[4]arene **1** was synthesised from $1_2,3_2,5_2,7_2$-tetrapropoxycalix[4]arene using literature methods (*18-20*). The synthesis of **L-Asp C4A**, **L-Glu C4A** and **IDA C4A** was previously reported (*16*).

*$1_2,3_2,5_2,7_2$-tetrapropoxycalix[4]arene-$1_5,3_5,5_5,7_5$-tetrakis-(((2R)-2-[(carbonyl)amino]))butanedioic acid dimethyl ester (**2a**)*

The carboxylic acid **(1)** (0.337 g, 0.438 mmol), D-aspartic acid dimethyl ester hydrochloride (0.673 g, 3.41 mmol) and triethylamine (0.43 mL) were combined in dichloromethane (30 mL) and stirred at room temperature (5 min). TBTU (0.852 g, 2.65 mmol) and triethylamine

(0.4 mL) were then added and the mixture stirred at room temperature (4 h). Further triethylamine (1 mL) was added in the first 30 minutes to maintain the pH between 8.0 to 9.0. The reaction was quenched by the addition of hydrochloric acid (1 M, 30 mL). The organic phase was washed with sodium bicarbonate solution (5%, 30 mL) and water (30 mL) before concentrating *in vacuo*. The residue was diluted with ethyl acetate, washed with water (3 x 30 mL) and brine (3 x 30 mL). The organic phase was dried ($MgSO_4$), filtered, and concentrated *in vacuo* resulting in an orange-brown oil. The oil was purified via column chromatography [EtOAc/CH_2Cl_2/MeOH (80:19:1, v/v)] to afford a yellow glass (92 mg, 16%): $[\alpha]_D^{23}$ -33.8° (chloroform); HRMS (ESI): $[M+H]^+$ $C_{68}H_{85}O_{24}N_4$ requires: 1341.5554; found: 1341.5537; ^1H NMR ($CDCl_3$) δ 7.16 (4H, d, *J* = 2.0 Hz, Ar*H*), 7.03 (4H, d, *J* = 2.0 Hz, Ar*H*), 6.98 (4H, d, *J* = 8.0 Hz, N*H*), 4.90 (4H, m, NHC*H*R_2), 4.46 (4H, d, *J* = 13.6 Hz, ArC*H*$_2$Ar), 3.88 (8H, t, *J* = 7.2 Hz, OC*H*$_2$$CH_2$), 3.74 (12H, s, COOC*H*$_3$), 3.71 (12H, s, COOC*H*$_3$), 3.27 (4H, d, *J* = 13.6 Hz, ArC*H*$_2$Ar), 3.07-2.94 (8H, m, CHC*H*$_2$C), 1.94-1.85 (8H, apparent sextet, CH_2C*H*$_2$$CH_3$), 1.00 (12H, t, *J* = 7.6 Hz, CH_2C*H*$_3$); ^{13}C NMR ($CDCl_3$) δ 171.9 (*C*OOCH_3), 171.7 (*C*OOCH_3), 167.0 (Ar*C*ON), 159.6 (*C*O, Ar), 135.0 (*C*CH_2, Ar), 134.8 (*C*CH_2, Ar), 128.1 (*C*H, Ar), 128.0 (*C*CO, Ar), 127.3 (*C*H, Ar), 77.2 (overlapping with $CDCl_3$, OCH_2), 52.8 (COO*C*H_3), 52.2 (COO*C*H_3), 49.1 (NH*C*HR_2), 36.1 (CH*C*H_2C), 31.1 (Ar*C*H_2Ar), 23.3 (CH_2*C*$H_2$$CH_3$), 10.4 ($CH_2$*C*$H_3$).

1$_2$*,3*$_2$*,5*$_2$*,7*$_2$*-tetrapropoxycalix[4]arene-1*$_5$*,3*$_5$*,5*$_5$*,7*$_5$*-tetrakis-(((2R)-2-[(carbonyl)amino]))pentanedioic acid dimethyl ester (**2b**)*

The carboxylic acid **(1)** (0.249 g, 0.324 mmol), D-glutamic acid dimethyl ester hydrochloride (0.491 g, 2.32 mmol), triethylamine (3 portions: 0.43 mL; 0.40 mL, 3.0 mL) and TBTU (0.64 g, 2.0 mmol) were combined in dichloromethane (30 mL) as described above and the reaction mixture stirred overnight. Workup, column chromatography [ethyl acetate/dichloromethane/methanol (80:19:1, v/v)] followed by preparatory TLC [ethyl acetate/dichloromethane/methanol (80:19:1, v/v)] gave an orange glass (71 mg, 16%): $[\alpha]_D^{23}$ -7.47° (chloroform); HRMS (ESI): $[M+H]^+$ $C_{72}H_{93}O_{24}N_4$ requires: 1397.6180; found: 1397.6161; ^1H NMR ($CDCl_3$) δ 7.18 (8H, dd, *J* = 8.4, 2.0 Hz Ar*H*), 6.89 (4H, d, *J* = 7.6 Hz, N*H*), 4.66-4.61 (4H, m, NHC*H*R_2), 4.47 (4H, d, *J* = 13.6 Hz, ArC*H*$_2$Ar), 3.89 (8H, t, *J* = 7.6 Hz, OC*H*$_2$$CH_2$), 3.74 (12H, s, COOC*H*$_3$), 3.51 (12H, s, COOC*H*$_3$), 3.28 (4H, d, *J* = 13.6 Hz, ArC*H*$_2$Ar), 2.44 (8H, apparent t, CH_2C*H*$_2$C), 2.29-2.20 (4H, m, CHC*H*$_2$$CH_2$), 2.11-2.02 (4H, m, CHC*H*$_2$$CH_2$), 1.97-1.88 (8H, apparent sextet, CH_2C*H*$_2$$CH_3$), 1.00 (12H, t, *J* = 7.2 Hz,

CH_2CH_3); ^{13}C NMR (Chloroform-*d*) δ 174.0 (*C*OOCH$_3$), 172.6 (*C*OOCH$_3$), 167.5 (Ar*C*ON), 159.4 (*C*O, Ar), 135.0 (*C*CH$_2$, Ar), 134.9 (*C*CH$_2$, Ar), 128.5 (*C*H, Ar), 128.0 (*C*CO, Ar), 127.8 (*C*H, Ar), 77.3 (O*C*H$_2$), 52.5 (COO*C*H$_3$), 52.5 (COO*C*H$_3$), 51.9 (NH*C*HR$_2$), 31.2 (CH_2CH_2C), 30.6 (Ar*C*H$_2$Ar), 27.3 (CH*C*H$_2$CH$_2$), 23.3 ($CH_2CH_2CH_3$), 10.4 (CH_2CH_3).

*1$_2$,3$_2$,5$_2$,7$_2$-tetrapropoxycalix[4]arene-1$_5$,3$_5$,5$_5$,7$_5$-tetrakis-(((2R)-2-[(carbonyl)amino]))butanedioic acid (**D-Asp C4A**)*

Potassium carbonate (158 mg, 1.14 mmol) in water (2 mL) was added to a solution of the aspartic octaester **(2a)** (95 mg, 0.071 mmol) in methanol (5 mL) and the reaction mixture stirred at 50˚ C (2 h). The reaction mixture was cooled to room temperature and concentrated *in vacuo*. The residue was diluted with water (10 mL), acidified to pH 1 with hydrochloric acid (1 M) and extracted with ethyl acetate (4 x 10 mL). The organic extracts were combined, washed with brine (30 mL), dried ($MgSO_4$), filtered, and concentrated *in vacuo* to afford a yellow glass (26 mg, 30%): ^1H NMR (DMSO-*d*$_6$) δ 8.46 (4H, d, *J* = 7.8 Hz, N*H*), 7.41 (s, 8H, Ar*H*), 4.60-4.66 (, NC*H*R$_2$), 4.45 (4H, d, *J* = 13.0 Hz, ArC*H*$_2$Ar), 3.93 (8H, t, *J* = 7.6 Hz, OC*H*$_2$CH$_2$), 3.37 (4H, d, *J* = 13.0 Hz, ArC*H*$_2$Ar), 2.82 (4H, dd, *J* = 16.4, 6.6 Hz, CHC*H*$_2$C), 2.64 (4H, dd, *J* = 16.4, 6.6 Hz, CHC*H*$_2$C), 2.08 – 1.80 (8H, apparent sextet, $CH_2CH_2CH_3$), 0.98 (12H, t, *J* = 7.4 Hz, CH_2CH_3); ^{13}C NMR (DMSO-*d*$_6$) δ 173.0 (*C*OOH), 172.4 (*C*OOH), 166.2 (Ar*C*ON), 159.2 (*C*O, Ar), 134.4 (*C*CH$_2$, Ar), 128.5 (*C*H and *C*CO, Ar), 77.1 (O*C*H$_2$), 49.7 (NH*C*HR$_2$), 36.3 (CH*C*H$_2$C), 29.5 (Ar*C*H$_2$Ar), 23.1 ($CH_2CH_2CH_3$), 10.6 (CH_2CH_3).

*1$_2$,3$_2$,5$_2$,7$_2$-tetrapropoxycalix[4]arene-1$_5$,3$_5$,5$_5$,7$_5$-tetrakis-(((2R)-2-[(carbonyl)amino]))pentanedioic acid (**D-Glu C4A**)*

Potassium hydroxide (320 mg, 5.70 mmol) in water (2 mL) was added to a solution of the glutamic octaester **(2b)** (37 mg, 0.026 mmol) in methanol (5 mL) and the reaction mixture stirred at room temperature (6 h). The reaction mixture was acidified to pH 1 with hydrochloric acid (1 M) and then concentrated *in vacuo*. The residue was diluted with water (10 mL) and the aqueous layer was extracted with ethyl acetate (3 x 15 mL). The organic extracts were combined, washed with brine (30 mL), dried ($MgSO_4$), filtered, and concentrated *in vacuo* to afford a yellow glass (12 mg, 36%): ^1H NMR (DMSO-*d*$_6$) δ 12.31 (8H, br s, COO*H*), 8.31 (4H, d, *J* = 7.2 Hz, N*H*), 7.44 (8H, d, *J* = 9.2 Hz, Ar*H*), 4.45 (4H, d, *J* = 12.8 Hz, ArC*H*$_2$Ar), 4.25-4.20 (4H, apparent q, NHC*H*R$_2$), 3.92 (8H, t, *J* = 7.6 Hz, OC*H*$_2$CH$_2$), 3.38 (4H, d, *J* = 12.8 Hz, ArC*H*$_2$Ar), 2.33 (8H, apparent t, CH$_2$C*H*$_2$C), 2.04-1.87 (16H, m, CH$_2$C*H*$_2$CH$_3$, CH$_2$C*H*$_2$CH), 0.99 (12H, t, *J* = 7.6 Hz, CH_2CH_3); ^{13}C NMR (DMSO-*d*$_6$) δ 174.4 (*C*OOH), 173.9 (*C*OOH),

166.9 (Ar*C*ON), 159.0 (*C*O, Ar), 134.4 (*C*CH_2, Ar), 128.8 (*C*H and *C*CH_2, Ar), 77.1 (O*C*H_2), 52.7 (NH*C*HR_2), 31.1 (CH_2*C*H_2C), 30.9 (Ar*C*H_2Ar), 26.4 (CH*C*H_2CH_2), 23.1 (CH_2*C*H_2CH_3),10.6 (CH_2*C*H_3).

Biology

Protein expression and purification

The HPV 16 L1 coding sequences, lacking four amino acids at the N-terminal and 29 amino acids at the C-terminal, were employed for better expression. Recombinant HPV 16 L1, which combines a GST tag at the N-terminal, was purified from *E. coli* strain BL21 containing the overexpression vector pGEX-6p-1. The protein expression and purification were carried out essentially as described previously (*21-23*). Briefly, cells from a one liter culture were re-suspended in buffer L (50 mM Tris-HCl, 0.2 M NaCl, 1 mM DTT, 1 mM EDTA, pH=8.0) and then were lysed by sonification for 30 min. The lysate was separated by centrifugation at 24000 *g* for 30 min. The obtained GST-L1 in the supernatant was first purified using the glutathione affinity column. Then the column was washed with 20 bed volumes of buffer L to remove the contaminants. After thorough washing, they were eluted from column by using 10 mM GSH. Further dialysis was applied to remove GSH and the obtained GST-L1 was then used to monitor the pentamer formation, both by fast protein liquid chromatography (FPLC) elution profile and static light scattering (SLS) measurements.

FPLC elution profile

The size-exclusion chromatography (SEC, Superdex-200, 26/60) was used to confirm the relative molecular masses of target protein and essentially the ratio of L1 pentamer to monomer. For this assay, all samples and solvents were filtered through a 0.22 μm filter unit before using. A flow rate of 3 mL/min was used in overall process and a 50 mL sample loop was employed for sample injection, all the steps of purification were conducted at 4 °C.

For the inhibition test of L1 pentamer formation by aspartic/glutamic acid modified calix[4]arenes, the protein (0.1 mg/mL) was initially treated for 3 h incubation with different ratios of **Asp-C4A**/**Glu-C4A** to GST-L1, and then digested by PPase overnight to remove GST-tag. The sample was subsequently injected into a gel-filtration column to obtain the FPLC elution profile, and finally to obtain the ratio of L1 pentamer to monomer.

In vitro *monitoring of pentamer formation from GST-L1 by static light scattering (SLS) measurement*

The purified GST-L1 diluted in buffer solution was used for the time dependent *in situ* SLS measurements on a fluorescence spectrophotometer, SHIMADZU (Japan) RF-5301 at 25 °C. GST-L1 fusion proteins (0.1 mg/mL, 2.0 mL) in buffer L was treated with **L-Asp C4A**, **D-Asp C4A**, **L-Glu C4A** or **D-Glu C4A**, respectively, at different concentrations for 30 min. The solutions were passed through a 0.2 μm filter unit to remove aggregates and dust particles before being added to a 10×10 mm quartz cuvette. Static fluorescence intensity was recorded with an excitation wavelength of 350 nm. Under constant stirring, PreScissionTM Protease (PPase, 200 IU/100 μL) was added into the cuvette and the moment was treated as the starting point (t=0) for the measurements continuously recording the SLS intensities by the spectrophotometer at 10 s intervals for 12 h.

Trypsin digestion on protein

A 7.5 nM GST-L1 was pre-incubated in buffer L in the absence and presence of **L-/D-Asp C4A** or **L-/D-Glu C4A** (in a molar ratio of 1:200 for GST-L1 to Asp C4A or Glu C4A) for 3 h at 4 °C, then it was subjected to proteolysis by trypsin under the identical conditions for 7 min. At the end of the reaction, all samples were supplemented with SDS-PAGE loading buffer, carefully heated at 95 °C for 10 min and then subjected to SDS-PAGE (*17*).

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Disclosure Statement

The authors declare no potential conflict of interest.

References

(1) Chen, J.; Sawyer, N.; Regan, L., Protein–protein interactions: General trends in the relationship between binding affinity and interfacial buried surface area. *Protein Science* **2013,** *22*, (4), 510-515.

(2) Laraia, L.; McKenzie, G.; Spring, D.R.; Venkitaraman, A.R.; Huggins, D.J., Overcoming Chemical, Biological, and Computational Challenges in the Development of Inhibitors Targeting Protein-Protein Interactions. *Chemistry & Biology* **2015,** *22*, (6), 689-703.

(3) Hashemzadeh, M.; Furukawa, M.; Goldsberry, S.; Movahed, M.R., Chemical structures and mode of action of intravenous glycoprotein IIb/IIIa receptor blockers: A review. *Experimental and Clinical Cardiology* **2008,** *13*, (4), 192-197.
(4) Martino, E.; Casamassima, G.; Castiglione, S.; Cellupica, E.; Pantalone, S.; Papagni, F.; Rui, M.; Siciliano, A.M.; Collina, S., Vinca alkaloids and analogues as anti-cancer agents: Looking back, peering ahead. *Bioorganic & Medicinal Chemistry Letters* **2018,** *28*, (17), 2816-2826.
(5) Hamuro, Y.; Calama, M.C.; Park, H.S.; Hamilton, A.D., A Calixarene with Four Peptide Loops: An Antibody Mimic for Recognition of Protein Surfaces. *Angewandte Chemie International Edition in English* **1997,** *36*, (23), 2680-2683.
(6) Zheng, D.-D.; Fu, D.-Y.; Wu, Y.; Sun, Y.-L.; Tan, L.-L.; Zhou, T.; Ma, S.-Q.; Zha, X.; Yang, Y.-W., Efficient inhibition of human papillomavirus 16 L1 pentamer formation by a carboxylatopillarene and a p-sulfonatocalixarene. *Chemical Communications* **2014,** *50*, (24), 3201-3203.
(7) Fu, D.Y.; Lu, T.; Liu, Y.X.; Li, F.; Ogden, M.I.; Wang, Y.; Wu, Y.; Mocerino, M., Enantioselective Inhibition of Human Papillomavirus L1 Pentamer Formation by Chiral-Proline Modified Calix[4]arenes: Targeting the Protein Interface. *ChemistrySelect* **2016,** *1*, (19), 6243-6249.
(8) Stanley, M.A., Genital human papillomavirus infections: current and prospective therapies. *Journal of General Virology* **2012,** *93*, (4), 681-691.
(9) Mlynarczyk-Bonikowska, B.; Majewska, A.; Malejczyk, M.; Mlynarczyk, G.; Majewski, S., Antiviral medication in sexually transmitted diseases. Part I: HSV, HPV. *Mini-Reviews in Medicinal Chemistry* **2013,** *13*, (13), 1837-1845.
(10) Kocjan, B.J.; Bzhalava, D.; Forslund, O.; Dillner, J.; Poljak, M., Molecular methods for identification and characterization of novel papillomaviruses. *Clinical Microbiology and Infection* **2015,** *21*, (9), 808-816.
(11) Mohsen, M.O.; Zha, L.; Cabral-Miranda, G.; Bachmann, M.F., Major findings and recent advances in virus–like particle (VLP)-based vaccines. *Seminars in Immunology* **2017,** *34*, 123-132.
(12) Graham, S., The human papillomavirus replication cycle, and its links to cancer progression: A comprehensive review *Clinical Science* **2017,** *131*, 2201-2221.
(13) Baker, T.S.; Newcomb, W.W.; Olson, N.H.; Cowsert, L.M.; Olson, C.; Brown, J.C., Structures of bovine and human papillomaviruses. Analysis by cryoelectron microscopy and three-dimensional image reconstruction. *Biophysical Journal* **1991,** *60*, (6), 1445-1456.
(14) Li, Z.; Yan, X.; Yu, H.; Wang, D.; Song, S.; Li, Y.; He, M.; Hong, Q.; Zheng, Q.; Zhao, Q., et al., The C-Terminal Arm of the Human Papillomavirus Major Capsid Protein Is Immunogenic and Involved in Virus-Host Interaction. *Structure* **2016,** *24*, (6), 874-885.
(15) McGovern, R.E.; Fernandes, H.; Khan, A.R.; Power, N.P.; Crowley, P.B., Protein camouflage in cytochrome c–calixarene complexes. *Nature Chemistry* **2012,** *4*, 527.
(16) Goh, C.Y.; Baldini, L.; Casnati, A.; Jones, F.; Mocerino, M.; Ogden, M.I.; Sansone, F.; Ungaro, R., Upper-rim acidic peptidocalixarenes as crystal growth modifiers. *Supramolecular Chemistry* **2014,** *26*, (7-8), 488-499.
(17) Gao, N.; Sun, H.; Dong, K.; Ren, J.; Duan, T.; Xu, C.; Qu, X., Transition-metal-substituted polyoxometalate derivatives as functional anti-amyloid agents for Alzheimer's disease. *Nature Communications* **2014,** *5*, (1), 3422.
(18) Strobel, M.; Kita-Tokarczyk, K.; Taubert, A.; Vebert, C.; Heiney, P.A.; Chami, M.; Meier, W., Self-Assembly of Amphiphilic Calix[4]arenes in Aqueous Solution. *Advanced Functional Materials* **2006,** *16*, (2), 252-259.

(19) Dondoni, A.; Marra, A.; Scherrmann, M.C.; Casnati, A.; Sansone, F.; Ungaro, R., Synthesis and Properties of O-Glycosyl Calix[4]Arenes (Calixsugars). *Chemistry - A European Journal* **1997,** *3*, (11), 1774-1782.
(20) Sansone, F.; Barboso, S.; Casnati, A.; Fabbi, M.; Pochini, A.; Ugozzoli, F.; Ungaro, R., Synthesis and Structure of Chiral Cone Calix[4]arenes Functionalized at the Upper Rim with L-Alanine Units. *European Journal of Organic Chemistry* **1998,** *1998*, (5), 897-905.
(21) Fu, D.-Y.; Jin, S.; Zheng, D.-D.; Zha, X.; Wu, Y., Peptidic inhibitors for in vitro pentamer formation of human papillomavirus capsid protein l1. *ACS Medicinal Chemistry Letters* **2015,** *6*, (4), 381-385.
(22) Bishop, B.; Dasgupta, J.; Klein, M.; Garcea, R.L.; Christensen, N.D.; Zhao, R.; Chen, X.S., Crystal Structures of Four Types of Human Papillomavirus L1 Capsid Proteins. *The Journal of Biological Chemistry* **2007,** *282*, (43), 31803-31811.
(23) Fu, D.-Y.; Zhang, S.; Qu, Z.; Yu, X.; Wu, Y.; Wu, L., Hybrid Assembly toward Enhanced Thermal Stability of Virus-like Particles and Antibacterial Activity of Polyoxometalates. *ACS Applied Materials & Interfaces* **2018,** *10*, (7), 6137-6145.