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ABSTRACT

The agmatine-containing poly(amidoamine) polymer AGMA1 was recently shown to inhibit the infectivity of several viruses, including human papillomavirus type 16 (HPV-16), that exploit cell surface heparan sulfate proteoglycans (HSPGs) as attachment receptors. The aim of this work was to assess the antiviral potency of AGMA1 and its spectrum of activity against a panel of low-risk and high-risk HPVs and to elucidate its mechanism of action. AGMA1 was found to be a potent inhibitor of mucosal HPV types (i.e., types 16, 31, 45, and 6) in pseudovirus-based neutralization assays. The 50% inhibitory concentration was between 0.34 μg/ml and 0.73 μg/ml and no evidence of cytotoxicity was observed. AGMA1 interacts with immobilized heparin and with cellular heparan sulfates, exerting its antiviral action by preventing virus attachment to the cell surface. The findings from this study indicate AGMA1 to be a leading candidate compound for further development as an active ingredient of a topical microbicide against HPV and other sexually transmitted viral infections.

INTRODUCTION

Human papillomaviruses (HPVs) are members of the *Papillomaviridae* family of double-stranded DNA, non-enveloped viruses (1). The 8-kb HPV genome is enclosed in a capsid shell comprising major (L1) and minor (L2) structural proteins. Most of the HPVs belonging to the *alpha* genus are sexually-transmitted and infect the anogenital mucosa. In the great majority of immunocompetent individuals, HPV infection is transient causing asymptomatic epithelial infections or benign epithelial hyperplasia. The genital warts are the most common lesions, caused mainly by HPV-6 and HPV-11. A small proportion of men and women fail to control viral infection and develop HPV-related malignancies, including carcinoma of the cervix, vulva, vagina, penis, anus and oropharynx. Several HPV types belonging to HPV species 7 (HPV-18, -39, -45, -59, -68) and species 9 (HPV-16, -31, -33, -35, -52, -58, -67) can confer a high oncogenic risk. HPV-16 and HPV-18 cause about 70% of all cases of invasive cervical cancer worldwide (followed by HPV 31, 33 and 45) (2).

It has been estimated that more than 528,000 new cases occur every year, and in 2012 it caused 266,000 deaths worldwide. (3, 4). Eighty-five percent of cervical cancer cases occur in women living in low socio-economic settings, primarily due to a lack of access to effective cervical cancer screening programs. No direct anti-HPV drugs are available to cure HPV lesions, therefore the current treatments are ablative and directed at the abnormal cells associated with HPV, rather than at the virus itself. The development of new ways to prevent genital infections is therefore essential in order to reduce the burden of HPV diseases. Two prophylactic vaccines are currently available: Gardasil and Cervarix. The first, is designed to protect against oncogenic HPV types 16 and 18 and low-risk HPV types 6 and 11, and is therefore is preventive against both cancer and genital warts (5), the latter is designed to protect against HPV types 16 and 18 only (5). Although the protective activity of these vaccines is undeniable, they also come with a number of limitations, such as the lack of protection against other oncogenic HPV types, the need for a cold chain distribution and storage, and low worldwide vaccine coverage, partly due to the very high cost of their

administration. Additional prevention tools for HPV infections are thus required, particularly in low-resource settings where the burden of HPV infection is highest. In this context, topical antiviral microbicides that can prevent the attachment of the full spectrum of mucosal HPV to the epithelial cells lining the anogenital tract would be extremely useful to complement the distribution of prophylactic vaccines.

Primary attachment of papillomavirus particles to the cell surface is mediated through the binding of HPV capsid proteins to the cellular heparan sulfate proteoglycans (HSPGs) (6, 7) – polyanionic structures widely expressed on eukaryotic cells that act as receptors for many other viruses (8, 9, 10). They consist of a core protein with glycosaminoglycan (GAG) chains of unbranched sulfated polysaccharides known as heparan sulfates (HS), which are structurally related to heparin. Consequently, heparin and other polyanionic compounds have been reported to act as HSPG-antagonists, binding and sequestering HPV in the extracellular environment, thus hampering its attachment to the cell surface and hence infections (11, 12 and references therein). The *in vivo* effectiveness of this anti-HPV strategy was recently demonstrated using the polyanionic sugar carrageenan (13, 14).

In addition to the virus-binding polyanionic compounds are the polycationic compounds, which instead bind to and mask HSPGs, in turn, preventing virus attachment. We have recently shown that AGMA1, a poly(amidoamine) (shown in Fig. 1) displays antiviral activity against a panel of viruses that utilize HSPG as attachment receptors including HPV (11). Its prevailing cationic nature (15) and its spectrum of antiviral activity suggest that it could prevent virus infectivity by binding to HSPG. The aim of the present work was to investigate the spectrum of AGMA1's antiviral activity against several low-risk and high-risk HPV types and to elucidate its mechanism of action. AGMA1 emerged as a broad-spectrum inhibitor of HPV infectivity that prevents HPV attachment by binding to and masking cell surface HSPGs.

MATERIALS AND METHODS

Materials. All solvents and reagents, unless otherwise indicated, were analytical-grade commercial products and used as received. 2,2-Bis(acrylamido)acetic acid (BAC) was prepared as reported in the literature and its purity (99.7%) was determined by NMR and titration (16). Phosphate buffer solution (PBS) 10 mM was prepared using Sigma Aldrich tablets according to the manufacturer's instructions. D_2O (99.9%) was purchased from Aldrich and used as received. Conventional heparin (13.6 kDa) was from Laboratori Derivati Organici S.p.A. (Milan, Italy). Heparinase II, a glycosidase that digests the glycosaminoglycan (GAG) moiety of HSPGs (17) was from Sigma-Aldrich (St Louis, MO).

Synthesis of AGMA1. AGMA1 (Fig 1) was prepared as previously reported (18). Briefly, Agmatine sulfate (2.000 g, 8.5 mmol) and lithium hydroxide monohydrate (0.360, 8.5 mmol) were added to a solution of 2,2-bisacrylamidoacetic acid (1.689 g, 8.5 mmol) and lithium hydroxide monohydrate (0.360 g, 8.5 mmol) in distilled water (2.8 mL). This mixture was maintained under a nitrogen atmosphere and occasionally stirred for 78 h. At the end of this period, it was diluted with water (100 mL), acidified with hydrochloric acid to pH 4-4.5, and then ultrafiltered through membranes with a nominal cutoff of 5000. The fractions retained in each case were freeze-dried and the product obtained as a white powder. Yields: 1.9 g.

AGMA1, $\overline{M}_n$ = 7800, $\overline{M}_w$ = 10100, and PD = 1.29.

Since AGMA1 is available in polydisperse preparations with average molecular mass not unequivocally determinable, we will quantitatively refer to the compound in µg/ml (11), with the exception of the calculation of the Kd (dissociation constant) value by Scatchard's analysis of the SPR data.

Preparation of biotinylated AGMA1

Biotinylated AGMA1 was prepared in two steps, (a) and (b), by reacting biotin N-hydroxysuccinimide ester (biotinNHS) with modified AGMA1 carrying approximately 8% 2-aminoethyl substituted units, in turn prepared by substituting in part agmatine with mono-*tert*-boc ethylenediamine in the polymerization recipe and then cleaving the protective group.

Step (a): 2,2-bisacrylamidoacetic acid (5.0005 g), lithium hydroxide (1.0644 g) and mono-*tert*-boc ethylenediamine (0.285 mL) were dissolved in distilled water (20mL), stirred until clear and then allowed to stand 24 hrs at room temperature (20°C) in the dark. After this time, agmatine sulphate (5.500 g) and lithium hydroxide (0.9936 g) were added under stirring, the resultant mixture was let standing as above for further 120 hrs, then diluted with water, acidified to pH 5 with hydrochloric acid and ultrafiltered through a membrane of nominal cut-off 3000. The product was retrieved by lyophilizing, dissolving in 2M hydrochloric acid (50 mL) and stirring 2 hrs at r.t. under a slow stream of nitrogen to favor eliminating the reaction by-product. The resultant aminated AGMA1 was then isolated as above. Yield 4.735 g.

Step (b): aminated AGMA1 (0.500 g) was dissolved in water (25 mL), the solution brought to pH 9.0 with dilute sodium hydroxide, dropwise added with a solution of biotinNHS (0.035 g) in DMSO (2 mL) and stirred 5 hrs at r.t. The reaction mixture was then acidified to pH 4.5, and the product isolated as in the previous cases by diluting with distilled water, ultrafiltering and lyophilizing. Yield 330 mg.

AGMA1, $\overline{M}_n$ = 8400, $\overline{M}_w$ = 11900, and PD = 1.42.

Size exclusion chromatography (SEC) traces were obtained with a Knauer Pump 1000 equipped with a Knauer Autosampler 3800, TSKgel G4000 PW and G3000 PW TosoHaas columns connected in series, a light scattering (LS) Viscotek 270 Dual Detector, a Waters 486 UV detector operating at 230 nm, and a Waters 2410 differential refractometer. The mobile phase was a 0.1 M

Tris buffer pH 8.00 ±0.05 with 0.2 M sodium chloride. The flow rate was 1 mL/min and sample concentration 1% w/w.

HPV PsV production. Plasmids and 293TT cells, used for pseudovirus (PsV) production, were kindly provided by John Schiller (National Cancer Institute, Bethesda, MD) or bought from Addgene (Cambridge, MA). Detailed protocols and plasmid maps for this study can be obtained from http://home.ccr.cancer.gov/lco/protocols.asp. HPV-16, HPV-31, HPV-45, HPV-6, and bovine papillomavirus type 1 (BPV-1) PsVs were produced according to previously described methods (19). Briefly, 293TT cells were transfected with plasmids expressing the papillomavirus major and minor capsid proteins (L1 and L2, respectively), together with a reporter plasmid expressing the secreted alkaline phosphatase (SEAP) or green fluorescent protein (GFP), named pYSEAP or pfwB, respectively. HPV-16, HPV-6, and BPV-1 PsVs were produced using bicistronic L1/L2 expression plasmids (p16sheLL, p6sheLL, and pSheLL, respectively). Capsids were allowed to mature overnight in cell lysate; the clarified supernatant was then loaded on top of an Optiprep density gradient of 27 to 33 to 39% (Sigma-Aldrich, St. Louis, MO) at room temperature for 3 h. The material was centrifuged at 28,000 rpm for 18h at room temperature in an SW41.1 rotor (Beckman Coulter, Inc., Fullerton, CA) and then collected by bottom puncture of the tubes.
Fractions were inspected for purity in 10% sodium dodecyl sulfate (SDS)–Tris–glycine gels, titrated on 293TT cells to test for infectivity by SEAP or GFP detection, and then pooled and frozen at -80°C until needed. The L1 protein content of PsV stocks was determined by comparison with bovine serum albumin standards in Coomassie-stained SDS-polyacrylamide gels.

Cell culture. The human cervical carcinoma cell lines SiHa, HeLa, and C33A were grown as monolayers in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL, Gaithersburg, MD) supplemented with heat-inactivated 10% fetal calf serum (FCS; Gibco- BRL) and Glutamax-I (Invitrogen, Carlsbad, CA). The 293TT cell line, derived from human embryonic kidney cells

transformed with the simian virus 40 (SV40) large T antigen, was cultured in the medium described above supplemented with nonessential amino acids. 293TT cells allow high levels of protein to be expressed from vectors containing the SV40 origin due to over-replication of the expression plasmid (20). Wild-type Chinese hamster ovary cells (CHO)-K1 cells and GAG-deficient A745 CHO cells (21) were kindly provided by J.D. Esko (University of California, La Jolla, CA) and grown in Ham's F-12 medium supplemented with 10% FCS.

SEAP-based PsV neutralization assays. 293TT cells were seeded in 96-well tissue culture-treated flat-bottom plates at a density of 25,000 cells/well in 100 μl of DMEM without phenol red (Life Technologies, Inc., Gaithersburg, MD) and with 10% heat-inactivated FBS, 1% glutamate, 1% nonessential amino acids, 1 % antibiotic-antimycotic solution (Zell Shield, Minerva Biolabs GmbH, Berlin, Germany) and 10 mM HEPES (neutralization buffer). The following day, to generate dose-response curves, diluted PsV stocks (80 μl/well) were combined with 20 μl of serially diluted compound. The 100-μl PsV-compound mixture was transferred to the cell monolayers and incubated for 72 h at 37°C at a final concentration of PsV equal to approximately 1 ng/ml L1 (about 750 capsid equivalents of L1/cell) (22). Following incubation, 50-μl aliquots of supernatant were collected and the SEAP content in the clarified supernatant determined using a Great Escape SEAP chemiluminescence kit 2.0 (BD Clontech, Mountain View, CA) as directed by the manufacturer. Thirty minutes after the addition of the substrate, samples were read using a Wallac 1420 Victor luminometer (PerkinElmer Life and Analytical Sciences, Inc., Wellesley, MA).

The 50% inhibitory concentration (IC50) values and the 95% confidence intervals (CIs) were determined using the Prism program (GraphPad Software, San Diego, CA).

GFP-based assays. Cells were seeded in 96-well plates at a density of 25,000 cells/well in 100 μl of DMEM supplemented with 10% FBS. The next day, serial dilutions of AGMA1 were added to pre-plated cells together with dilutions of PsV stock. After 72 h of incubation at 37°C fluorescent cells were counted on an inverted Zeiss LSM510 fluorescence microscope.

Virus inactivation assay. Diluted PsV containing GFP stock and the test compounds at a concentration of 3.6 μg/ml were added to MEM and mixed in a total volume of 100 μl. The virus compound mixtures were incubated for 2 h at 37°C or 4°C then serially diluted to the non-inhibitory concentration of test compound, and the residual viral infectivity was determined.

Attachment assay. Serial dilutions of AGMA1 were mixed with HPV-16–SEAP PsV (1 ng/ml L1) and then added to cooled 293TT cells in 96-well plates and incubated for 2 h at 4°C to ensure PsV attachment but not entry. After two gentle washes, cells were shifted to 37°C, and SEAP activity was measured in the cell culture supernatants 72 h after PsV inoculation.

Pre-attachment assays. 293TT cell monolayers in 96-well plates were incubated with serial dilutions of AGMA1 for 2 h at 4°C. After removal of the compound and a gentle wash, HPV-16–SEAP PsVs (1 ng/ml L1) were added to the cells for 2 h at 4°C. After two gentle washes, the cells were shifted to 37°C, and SEAP activity was measured in the cell culture supernatants 72 h after PsV inoculation. Alternatively HeLa cells were incubated with a fixed dose of AGMA1 for 1 h at 37°C. After removal of the compound and a gentle wash, cells were overlaid with medium for different times (23, 5, 3 or 1 h(s)) and then infected with 16–GFP PsV (1 ng/ml L1). Fluorescence was evaluated in the cell culture 72 h after PsV inoculation.

Post-attachment assay. HeLa cell monolayers in 96-well plates were incubated with HPV-16–GFP PsV (1 ng/ml L1) for 2 h at 37°C, followed by two gentle washes to remove unbound virus. Serial dilutions of AGMA1 were added to cultures after washout of the inoculums or after 2 or 4 h. Fluorescence was evaluated in the cell culture 72 h after PsV inoculation.

Entry assay. HeLa cell monolayers in 96-well plates were incubated with HPV-16–GFP PsV (1 ng/ml L1) for 2 h at 4°C, followed by two gentle washes to remove unbound virus. Serial dilutions of AGMA1 were then added to the cultures, which were shifted to 37C and incubated for 5 h to allow viral entry. After this incubation, cells were washed with PBS at pH 10.5 (23) to remove the

un-entered virus and two washes with normal medium to restore the physiological pH. Fluorescence was evaluated in the cells 72 h after PsV inoculation.

Post-entry assay. HeLa cell monolayers in 96-well plates were incubated with HPV-16–GFP PsV (1 ng/ml L1) for 2 h at 4°C, followed by two gentle washes to remove unbound virus. The cells were then shifted to 37°C for 5 h to allow viral entry. After this incubation, cells were washed with PBS at pH 10.5 (23) to remove un-entered virus and then washed twice with normal medium to restore the physiological pH. Serial dilutions of AGMA1 were then added to the cells. Fluorescence was evaluated in the cells 72 h after PsV inoculation.

Cell viability assay. Cells were seeded at a density of 25,000/well in 96-well plates; the next day, they were treated with serially diluted peptide compounds to generate dose-response curves. After 72 h of incubation, cell viability was determined using the CellTiter 96 Proliferation Assay Kit (Promega, Madison, WI, USA), according to the manufacturer's instructions. Absorbances were measured using a Microplate Reader (Model 680, BIORAD) at 490 nm. 50% cytotoxic concentration (CC_{50}) values and 95% confidence intervals (CIs) were determined using Prism software (GraphPad Software, San Diego, CA).

Electron microscopy. An aliquot of diluted HPV-PsV preparation was allowed to adsorb for about 3 min on carbon and formvar-coated grids and then rinsed several times with water. Grids were negatively stained with 0.5% uranyl acetate and excess fluid removed with filter paper. Observations and photographs were made using a CM 10 electron microscope (Philips, Eindhoven, The Netherlands).

Attachment and pre-treatment followed by Western blot. HeLa cells were seeded at a density of 300,000/well in 6-well plates; the next day they were treated with a fixed dose of AGMA1 or heparin (i.e. 100 µg/ml) 2 h before or during the 4 h infection period at 4°C. Following incubation, cells were washed with cold medium to ensure the removal of unbound virus; cells were then

collected and lysed. The lysate proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. L1 was detected using mouse monoclonal antibody (Ab30908, Abcam, Cambridge, UK) at a 1:2000 dilution, followed by anti-mouse IgG-HRP (Santa Cruz Biotechnology Inc.). Actin was detected using mouse monoclonal antibody (Anti-actin MAB1501R, Millipore), followed by anti-mouse IgG-HRP (Santa Cruz Biotechnology Inc.).

AGMA1/cell-associated HSPG binding assays. Monolayers of CHO-K1 cells, GAG-deficient A745 CHO-K1 cells or HeLa cells in 96-well plates were incubated for 2 h at 4°C in phosphate-buffered saline (PBS) containing 0.1 mg/ml $CaCl_2$, 0.1 mg/ml MgCl2, and 0.1% gelatin, with sub-saturating concentrations of biotinylated AGMA1 (b-AGMA1) (0.01 μg/mL or 0.1 μg/ml) in the absence or presence of heparin (10 μg/ml). At the end of incubation, cells were washed with PBS, and the amount of cell-associated b-AGMA1 determined with horseradish peroxidase-labeled streptavidin (1/5,000) and the chromogenic substrate ABTS (Kierkegaard & Perry Laboratories, Gaithersburg, MD). In some experiments, cell monolayers were washed with PBS containing 2 M NaCl, a treatment known to remove cationic polypeptides from cell surface HSPGs (Urbinati, 2004). Alternatively, cells were incubated with heparinase II (15mU/ml) for 1 h at 37°C, or left untreated, before the binding assay.

SPR assay. Surface plasmon resonance (SPR) measurements were performed on a BIAcore X instrument (GE Healthcare, Milwaukee, WI), using a research grade CM3 sensorchip. The reagents 1-ethyl-3-(3-diaminopropyl)-carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) were purchased from GE Healthcare and used according to recommended protocols.

To study the interaction of AGMA1 with heparin, the latter was immobilized on a BIAcore sensorchip as described previously (24). Briefly, a CM3 sensorchip (GE Healthcare) previously activated with 50 μl of a mixture containing 0.4 M EDC and 0.1 M NHS was coated with streptavidin. Heparin was biotinylated at its reducing end and immobilized onto the streptavidin-coated sensorchip. These experimental conditions allowed the immobilization of 80 resonance

264 units (RU), equal to 5.8 fmol/mm^2 of heparin. A sensorchip coated with streptavidin alone was used
265 to evaluate the nonspecific binding of AGMA1 to the sensorchip and for blank subtraction. The
266 compound was resuspended in 10 mM HBS-EP buffer (HEPES buffer, pH 7.4, containing 150 mM
267 NaCl, 3 mM EDTA, 0.005% surfactant P20) and injected over the heparin or streptavidin surfaces
268 for 4 min (to allow its association with immobilized heparin) and then washed until dissociation
269 was observed. After every run, the sensorchip was regenerated by injection of 2 M NaCl. The Kd
270 (dissociation constant) was calculated using the Koff/Kon ratio or by Scatchard's analysis of the
271 SPR values of RU at equilibrium (directly proportional to the moles of bound ligand) as a function
272 of the ligand concentration in solution.
273

RESULTS

Characterization of purified HPV-16 PsV.

HPV-16 was chosen as a pivotal model virus because it is the most frequent genotype identified in cervical carcinomas (25). First of all, we evaluated the quality of the HPV-16–SEAP PsV preparations by SDS-PAGE and electron microscopy analysis. As shown in Fig. 2A, a major band migrating at 55 kDa was detected by Coomassie brilliant blue staining (lane 1) and was confirmed to be the L1 major capsid protein by Western blot analysis with anti-L1 antibody (lane 2). No L1-reactive proteolytic degradation products were observed at molecular masses below 55 kDa. Figure 2B shows an electron micrograph of the same PsV stock. The PsV particles exhibited an average diameter of 50 to 60 nm, similar to that of an authentic HPV capsid, and appeared as individual, well-defined particles with no aggregation. When observed at a higher magnification, the particles appeared to be well-assembled, icosahedral capsids (Fig.2B, inset). Similar results were obtained with the other PsV types used in this study (data not shown).

Inhibition of HPV-16 PsV infectivity in different cell lines by AGMA1.

The ability of AGMA1 to block HPV-16 PsV infection was tested on several cell lines. 293TT cells are preferred for PsV inhibition assays based on SEAP expression because high levels of the SV40 large T antigen in these cells allow for the over-replication of the SEAP reporter plasmid. Moreover, the analysis was extended to cell lines derived from the uterine cervix (i.e. SiHa, HeLa, and C33A), the major anatomical target for high-risk HPV infection. Unlike 293TT, these cell lines do not express the SV40 large T antigen, resulting in very low levels of SEAP protein expression. Therefore, we employed GFP as a reporter gene because it allows reliable analyses of cell types in which the reporter plasmid does not over-replicate. GFP-expressing PsV were also tested in 293TT and the IC_{50} values compared to those obtained with SEAP-expressing PsV. As reported in Table 1, AGMA1 inhibited the infectivity of HPV-16 PsV in all cell lines tested with IC_{50} values between 0.38 μg/ml - 0.53 μg/ml. Of note, the results show that the IC_{50} values obtained from cells infected

with GFP- or SEAP-expressing PsV are comparable. Cell viability assays performed under identical culture conditions for antiviral assays (i.e. cell density and time of incubation with the compound) demonstrated that AGMA1 did not affect cell viability at any concentration tested (i.e. up to 300 μg/ml).

The inhibitory activity of AGMA1 in not papillomavirus type restricted.

To assess whether the inhibitory activity of AGMA1 was papillomavirus type-specific, the assays were repeated in 293TT cells using two additional high-risk HPV types (i.e. HPV-31 and HPV-45), one low-risk type (HPV-6) and the bovine papillomavirus type 1 (BPV-1 PsV). The results shown in Table 2 demonstrate that AGMA1 inhibits the infection of all the papillomaviruses tested with a similar potency indicating that its inhibitory activity is not type-restricted.

AGMA1 does not inactivate HPV PsV particles.

To assess whether the inhibitory activity was a consequence of a direct inactivation of PsV particles by AGMA1 we performed a viral inactivation assay. As shown in Fig. 3, the virus titers of samples treated with AGMA1 did not significantly differ to those determined in untreated samples ($P>0.05$), indicating that AGMA1 does not inactivate HPV particles.

AGMA1 interacts with the cell surface via HSPGs.

The polycationic nature of AGMA1 (15, 18) and its demonstrated capacity to selectively inhibit HSPG dependent viruses (11) suggested that AGMA1 could inhibit HPV infection by interacting with cell surface HSPGs. To investigate this hypothesis, we first investigated the effective capacity of AGMA1 to bind to the cell surface via HSPGs. To this end, in a first set of experiments, we exploited the CHO cell model. As shown in Fig. 4A, the binding of b-AGMA1 to A745 CHO-K1

(cell mutants with defective HSPG synthesis) is significantly reduced with respect to wild type CHO-K1 cells. Moreover, the binding of AGMA1 to wild type CHO-K1 cells could be reduced to a level comparable or even lower than those measured in A745 CHO-K1 cells by: i) a 2 M NaCl wash, a treatment known to disrupt the binding of cationic molecules to HSPGs (24); ii) the presence of a molar excess of heparin, a structurally related HSPG antagonist; or iii) cell treatment with heparinase, an enzyme that removes the heparan sulfate chains from cell surface-associated HSPGs (Fig. 4B).

We thus wondered whether the HSPG-dependence of AGMA1 binding to cell surfaces also held in relation to cervix adenocarcinoma epithelial cells. To this end, we evaluated the binding of b-AGMA1 to HeLa cells. As shown in Fig. 4C, b-AGMA1 binds to the surface of HeLa cells in a dose-dependent and saturable manner. Also, the binding could be inhibited by a 2M NaCl wash, by heparin, or by heparinase treatment, thus confirming its dependence on surface-associated HSPGs (Fig. 4D).

To confirm further the interaction of AGMA1 with HSPGs, we evaluated its capacity to bind to heparin (a structurally similar molecule) immobilized on a BIAcore sensorchip – a "cell-free" model that resembles the interaction of cationic proteins with cell surface HSPGs (26). In a typical experiment, increasing concentrations of AGMA1 were injected over the heparin surface, and a set of sensograms obtained (Fig. 4E). An association rate constant (Kon) equal to 5.3×10^4 M-1 s-1 and a slow dissociation rate constant (Koff) equal to 1.2×10^{-3} s-1 characterized the interaction of AGMA1 with immobilized heparin. Thus, the AGMA1-heparin interaction occurs with a relatively high affinity [dissociation constant (Kd) calculated independently of AGMA1 concentration as Koff/Kon equal to 22.6 nM]. Finally, equilibrium binding data from Fig. 4E were used to generate the saturation curve shown in Fig. 4F, which was in turn used to calculate a Kd value independent from kinetic parameters; a Kd equal to 17 nM was obtained, thus very similar to that calculated above.

AGMA1 blocks HPV binding to host cells through a direct interaction with cells.

Having demonstrated the interaction between AGMA1 and HSPGs, we wanted to examine whether AGMA1 exerted its inhibitory activity by blocking HPV attachment. To this end, pre-attachment and attachment assays were performed. As shown in Fig. 5A and 5B, under both these experimental conditions AGMA1 strongly inhibited HPV-16 infection, with IC_{50} values of 2.21 μg/ml and 1.01 μg/ml, respectively. This result suggests that the antiviral activity depended on the AGMA1 capacity to prevent virus binding to the cell surface. To verify this hypothesis, a Western blot analysis was carried out to detect the HPV particles bound to cells treated with AGMA1 before or during the PsV inoculum. In the same assay, heparin was used as a reference compound, being a known inhibitor of HPV attachment. As shown in Figure 5C, pre-treatment with AGMA1 totally prevented the binding of HPV-16 PsV. By contrast, heparin was only slightly active when added before virus inoculum. Instead, when the compounds were added during infection at 4°C (Figure 5D), they were both able to inhibit HPV binding. These results support the hypothesis that AGMA1 prevents HPV attachment through a direct interaction with cells, instead of binding to the virus particle as heparin does.

To explore further the inhibitory activity of AGMA1 when added to the cells before infection, we performed a pre-treatment assay in which the virus inoculum was added 23, 5, 3, or 1 hour(s) after exposure of the cells to 100 μg/ml or 33 μg/ml of AGMA1 for 1 hour and then washed. As shown in Fig 6, addition of the virus inoculum 5, 3, or 1 h after AGMA1 pretreatment resulted in an almost complete suppression of infection (>97%) for all doses of AGMA1, whereas at 23 h post-treatment a 76.7% inhibition and a 45.2% inhibition was observed in cells treated with 100 μg/ml or 33 μg/ml, respectively.

373 **AGMA1 displaces HPV-16 bound to cells.**

374 It has been previously reported that HPV exhibits slow entry kinetics, with an average half-time of
375 12 h for HPV16 (27). We therefore used post-attachment assays to investigate whether AGMA1
376 could displace bound HPV PsV. We first performed an entry assay in which the virus was incubated
377 with cells for 2 h at 4°C, a condition that allows viral attachment but not entry. Immediately after
378 the removal of the virus inoculum, AGMA1 was added to the cells and the temperature shifted to
379 37°C to allow viral entry. Five hours later – a time sufficient to let a detectable amount of PsVs
380 enter the cells – the bound but not entered viruses were detached by washing with PBS at pH 10.5
381 (23). The IC_{50} determined for AGMA1 in the entry assay was 2.07 μg/ml, demonstrating its ability
382 to displace PsV particles already bound to cells. By contrast, when AGMA1 was added after the
383 washout with PBS pH 10.5 (post-entry assay) no reduction of reporter gene expression (Fig 7A)
384 could be observed. Moreover, we tested the inhibitory activity of AGMA1 when it was added to the
385 cells 2 or 4 h after the removal of the PsV inoculum (Fig 7B) and demonstrated that a 60%
386 inhibitory activity was still present at 4 h post-infection at the highest dose tested (i.e. 100 μg/ml).

387

DISCUSSION

The wide distribution of HSPGs on eukaryotic cells and their strong interactive capacity has made them attractive adhesion molecules for viruses, such as HPV, HSV, and HIV (10, 12, 28, 29). On the molecular level, cationic viral proteins, determinants of infectivity, interact with the negatively charged sulfate groups present on the GAG chains of HSPGs (8). In the case of HPV, the basic domains on the L1 and L2 capsid proteins mediate the initial interaction between the virus and the HSPGs (30, 31). This interaction has therefore been put forward as being a suitable molecular target for virus attachment inhibitors with the scope of developing novel topical microbicides for the prevention of sexually transmitted HPV infections. The present study shows the prevailingly cationic polymer AGMA1 to be a broad spectrum inhibitor of HPV attachment and demonstrates that its inhibitory activity depends on its capacity to bind to cellular HSPGs. The latter feature is supported by biochemical, genetic, pharmacological and enzymatic evidence herein presented. We observed that the binding of AGMA1 to HSPG-deficient A745 CHO-K1 cells is reduced with respect to wild type CHO-K1 cells. Moreover, washing with 2M NaCl, known to disrupt the electrostatic bonds between various proteins and heparin/HSPGs (24), displaced AGMA1 from the cell surface. Finally, heparin, a structural analog of HSPG GAG chains, competed with cell surface HSPGs for AGMA1 binding. Importantly, both of these treatments have previously been demonstrated to act selectively on HSPG binding events, leaving the interactions of other proteins with their receptors unaffected (24, 32). Finally, the direct removal of HSPG GAG chains using the enzyme heparinase significantly reduced the binding of AGMA1 to cell surfaces. However, heparinase treatment did not completely abolish the binding of AGMA1 to the cell, suggesting that other surface receptors, as yet unidentified, may exist able to interact with the polymer.

AGMA1's interaction with the cell surface is further supported by the observation that AGMA1 prevents virus binding even when administered before virus inoculum (pre-treatment assays). By contrast, heparin, a known attachment inhibitor that interacts directly with the virus particle rather

than with the cells, only prevents viral binding when in the presence of the virus (Fig 5). Interestingly, AGMA1 suppresses infection even when it is added to cell cultures after the virus attachment has already occurred (Fig 7), indicating that AGMA1 may be able to displace HPV particles that are bound to cells but not yet internalized (due to their slow entry kinetics). Taken together, these results identify valuable properties of AGMA1 as a topical microbicide that could potentially prevent HPV infections if applied before or immediately after sexual intercourse.

Joyce and coworkers reported that virus-like particles composed of HPV L1 protein bind to heparin with an affinity that is comparable to those of other heparin-binding proteins (30). Interestingly, the SPR binding assays performed here also showed that AGMA1 binds to heparin with an affinity (Kd: 17.0-22.6 nM) that is comparable to those of many other heparin-binding viral proteins (10). Taken together, these data suggest that the binding of AGMA1 to HSPGs *in vivo* might occur with an affinity that is comparable to that of HPV itself, resulting in efficient competition between AGMA1 and the virus for cell interaction. In turn, this results in an equally efficient inhibition of HPV infection, as shown by the very low value of IC50, calculated for the inhibitory activity of AGMA1 (0.34 - 0.74 μg/ml) (Table 2). Besides affinity, another interesting binding feature displayed by the AGMA1/heparin interaction is its slow dissociation rate (Koff), which identifies the formation of very stable complexes between the polymer and heparin. Again, a similar, slow Koff has been calculated for the HPV/heparin interaction (30). These similarities may be tentatively explained by the multimeric nature shared by the polymer and HPV, both exposing multiple binding domains on their surface for the HSPG GAG chains (themselves presenting multiple binding sites for their ligands). This kind of situation very often leads to the establishment of cooperative interactions. Briefly, cooperativity is a form of allostery in which a macromolecule (AGMA1 or HPV) has more than one binding site, and interaction with a receptor (HSPGs) at one site increases its affinity at the contiguous site, stabilizing the complex (10). *In vivo*, the formation of stable complexes between AGMA1 and HSPGs may result in extended inhibitory activity; i.e.

once the polymer is bound to HSPGs it may be able to keep them masked and prevent virus interaction for prolonged periods of time. Those considerations nicely correlate to the observation that, once bound to the cell surface, AGMA1 retains its inhibitory activity when cells are challenged with HPV infection 3, 5 and even 23 hours after initial exposure to the polymer (Fig 6).

Additional properties of AGMA1 make it appealing for further development as an active pharmaceutical ingredient of topical microbicides. AGMA1 is water-soluble, biodegradable and biocompatible. Its preparation process is simple, easily scalable and environmentally friendly, taking place in water or alcohols, at room temperature and without the need for added catalysts (33). Its activity is not papillomavirus type-restricted, since it extends across three HPV species belonging to the *alpha* genus of the *Papillomaviridae* family. Indeed, AGMA1 has been found to be active against three high-risk oncogenic types, namely HPV-16, HPV-31 (species 9), and HPV-45 (species 7), and one low-risk type (HPV-6) belonging to species 10. Of note, HPV-31 and HPV-45, whose worldwide prevalence in cervical cancer is about 4% and 6%, respectively, (34) are not included in the bivalent or quadrivalent vaccines. Interestingly, the fact that AGMA1 is active against HPV-31, whose attachment does not appear to be dependent on HSPG (35), suggests that an additional, as yet unidentified, mechanism of anti-HPV activity exists. The finding that AGMA1 is even active against BPV-1, which is phylogenetically distant from *alpha*- papillomaviruses (36), further supports its broad-spectrum activity. Since the existing prophylactic vaccines are HPV type restricted, a broad-spectrum microbicide could be a useful adjuvant to vaccination programs, especially in resource-limited settings were the burden of HPV infections is greatest.

Moreover, AGMA1 was recently reported to inhibit HSV-1 and HSV-2 infectivity (11). This finding supports its use in conditions in which the concomitant infection of various sexually transmitted viruses may occur, such as in the case of HSV-2 infection, which enhances the transmission of HIV-1 infection (37). In turn, HIV infection-driven immunodeficiency causes a well documented increase in HPV and HSV infections (38, 39). Of note, HIV is also a HSPG-dependent

462 virus (9), and may also, therefore, be sensitive to AGMA1.

463 In conclusion, our results identify AGMA1 as a lead compound for further development as an active
464 pharmaceutical ingredient of a topical microbicide against HPV and other sexually transmitted viral
465 infections. Preclinical efficacy and toxicology studies are ongoing to assess the clinical potential of
466 this inhibitor.

467

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607 **FIGURE LEGENDS**

608 **Fig 1. Chemical structure of AGMA1.**

609

610 **Fig 2. Characterization of purified HPV-16–SEAP PsV.** (A) An aliquot of purified PsV
611 preparation was analyzed by SDS-PAGE with Coomassie brilliant blue staining (lane 1) or
612 immunoblotted with an anti-L1 antibody (lane 2). (B) Electron micrograph of a purified PV
613 preparation (bar 100 nm); inset shows a pseudovirus at higher magnification.

614

615 **Fig 3. AGMA1 does not inactivate HPV PsV particles.** HPV PsVs were incubated with 3.6 μg/ml
616 of AGMA1 for 2 h at 4°C or 37°C. Mixtures were then titrated on HeLa cells at high dilutions such
617 that the concentration of compound was not active. The titers, expressed as ffu/ml, show means and
618 SEMs for triplicates.

619

620 **Fig 4. Binding of AGMA-1 to heparin and HSPGs. A)** Wild type CHO-K1 cells and HSPGs-
621 deficient A745 CHO-K1 cells were incubated with b-AGMA1 (0.01 μg/ml) and washed with PBS.
622 **B)** In parallel experiments, wild type CHO-K1 cells were: i) incubated with b-AGMA1 alone and
623 then washed with PBS containing 2 M NaCl; ii) incubated with b-AGMA1 in the presence of a
624 molar excess (10 μg/ml) of heparin; or iii) pre-treated with heparinase before b-AGMA1 incubation.
625 HeLa cells were incubated with increasing concentrations of b-AGMA1 (panel **C**) or with 0.1 μg/ml
626 b-AGMA1 and subjected to the three different treatments described above (panel **D**). Then, the
627 amount of cell-associated b-AGMA was measured. In panel **B** and **D**, data are expressed as percent
628 of b-AGMA1 bound to control cells. **E**) Overlay of blank-subtracted sensorgrams generated by the
629 injection of AGMA1 onto sensorchip-immobilized heparin. **F**) Saturation curves of the binding of
630 AGMA1 to sensorchip-immobilized heparin. The saturation curves were obtained using the values
631 of RU bound at equilibrium, calculated from the sensorgrams reported in panel E.

Fig 5. AGMA1 inhibits HPV binding. In the pre-treatment assay, AGMA1 was added to cells for 2 h at 4°C, it was then washed out and HPV16 PsVs added. SEAP activity was evaluated 72 h later (A). In the attachment assay (B), AGMA1 and HPV16 PsVs were co-incubated on cells at 4°C for 2 h, followed by a washout and 72 h incubation. The results show means and SEMs for triplicates. Figure C presents a Western blot directed against L1 after 2 h pre-treatment with AGMA1 and heparin at 100 μg/ml followed by a washout and addition of HPV16 PsVs for 4 h at 4°C and subsequent lysis. Figure D presents a Western blot directed against L1 after an incubation on cells of AGMA1 and heparin (100 μg/ml) with HPV16 PsVs for 4 h at 4°C with subsequent lysis.

Fig 6. AGMA1 prevents HPV infection for extended periods following its removal.
Cells were pre-treated with AGMA1 for 1h at 37°C at fixed doses of 100 μg/ml and 33 μg/ml, followed by washout; at different time points post washout (23, 5, 3, or 1 h) cells were then infected with HPV-16 PsVs. After 72 h incubation, infection was evaluated. The results show means and SEMs for triplicates

Fig 7. AGMA1 is able to detach HPV from the cell surface.
In the entry assay, HPV-16–GFP PsVs were added to cells for 2 h at 4°C then washed out to remove unbound virus. AGMA1 was then added and the cells incubated for 5 h at 37°C to allow viral entry. The cells were then washed with PBS pH 10.5 to remove everything that remained outside the cell; 72 h after viral inoculum, GFP expression was evaluated. In the post-entry assay, AGMA1 was not added before but after the 5 h incubation at 37°C and the washout with PBS pH 10.5 (A). In the post-treatment assay (B), AGMA1 was added 0, 2 or 4 h after the removal of the PsV inocula; GFP expression was evaluated 72 h later. The results show means and SEMs for triplicates.

AGMA1

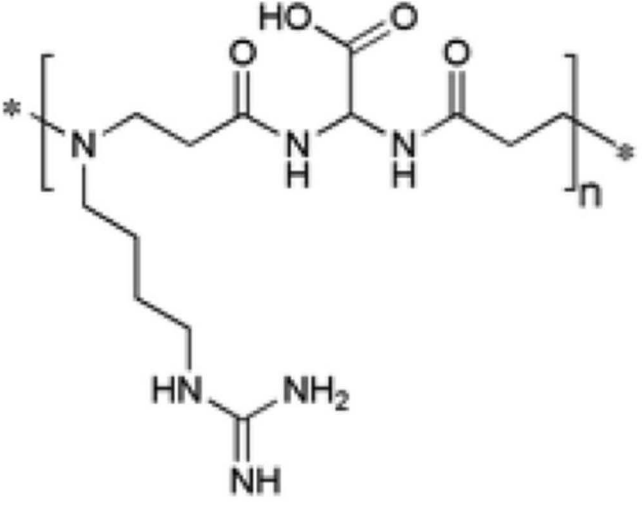


Table 1. AGMA1 antiviral activity against HPV-16 in different cell lines.

Cell line	IC_{50} * (µg/ml)	95% CI**	CC_{50}*** (µg/ml)	SI
293TT (SEAP)	0.53	0.51-0.54	>300	>566
293TT (GFP)	0.38	0.30-0.48	>300	>785
Hela	0.38	0.28-0.52	>300	>777
Siha	0.38	0.34-0.42	>300	>779
C33A	0.49	0.38-0.63	>300	>606

* IC_{50}: 50% inhibitory concentration

** 95% CI: 95% confidence interval

***CC_{50}: 50% cytotoxic concentration

Values are means and CIs for three separate determinations.

Table 2. AGMA1 antiviral activity against different types of Papillomaviruses

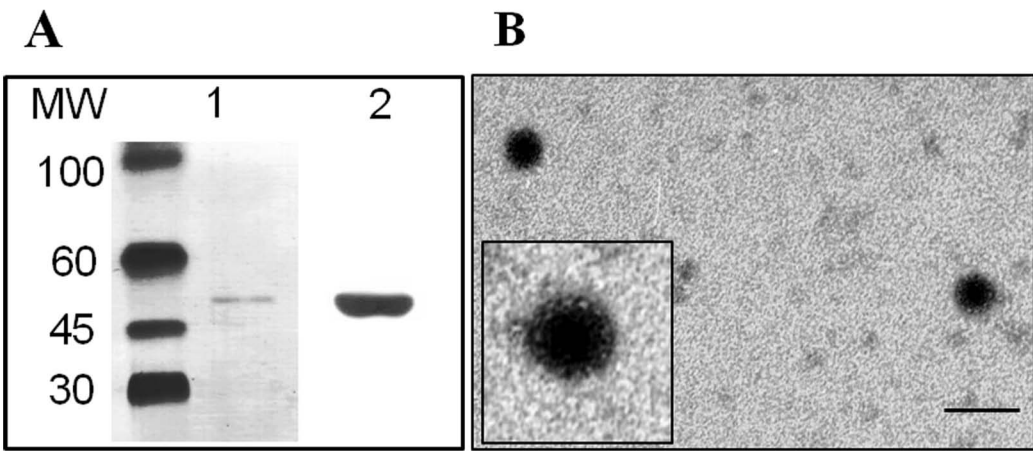
Cell line	IC_{50} * (µg/ml)	95% CI**	CC_{50}*** (µg/ml)	SI
HPV-16	0.53	0.51-0.55	>300	>566
HPV-31	0.36	0.28-0.46	>300	>836
HPV-45	0.74	0.70-1.80	>300	>407
HPV-6	0.54	0.36-0.81	>300	>553
BPV-1	0.34	0.23-0.50	>300	>875

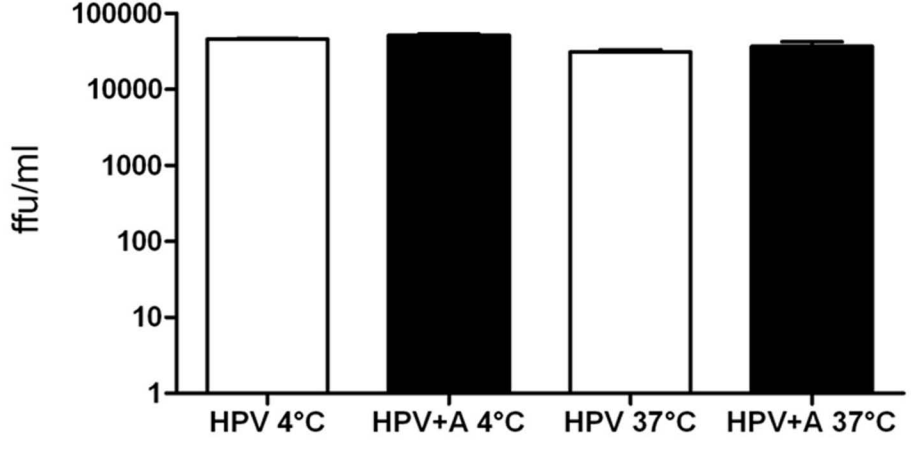
* IC_{50}: 50% inhibitory concentration

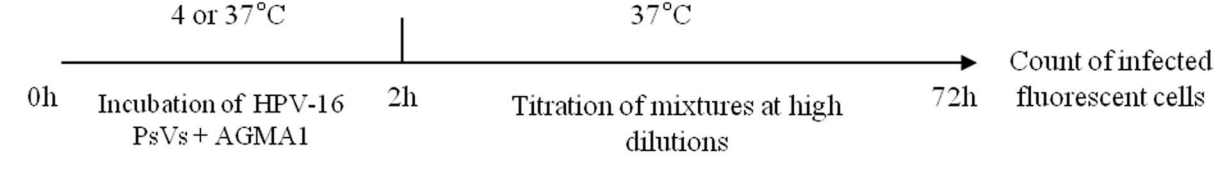
** 95% CI: 95% confidence interval

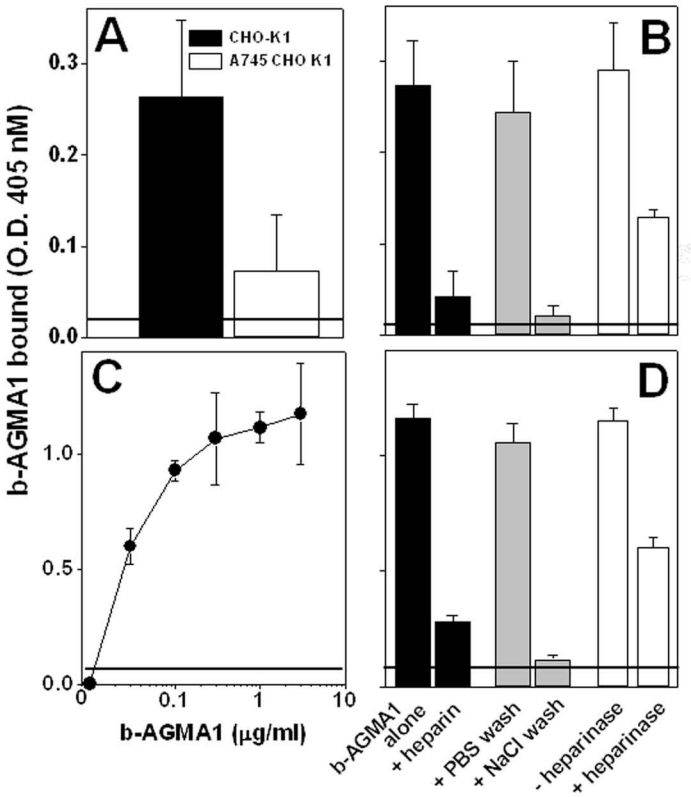
***CC_{50}: 50% cytotoxic concentration

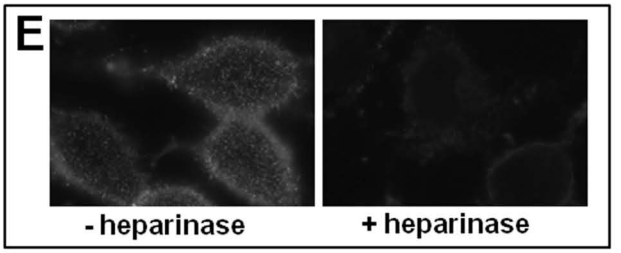
Values are means and CIs for three separate determinations.

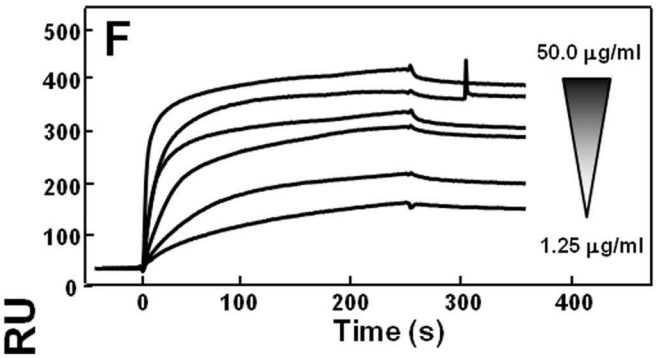

A
B
MW
1
2
100
60
45
30

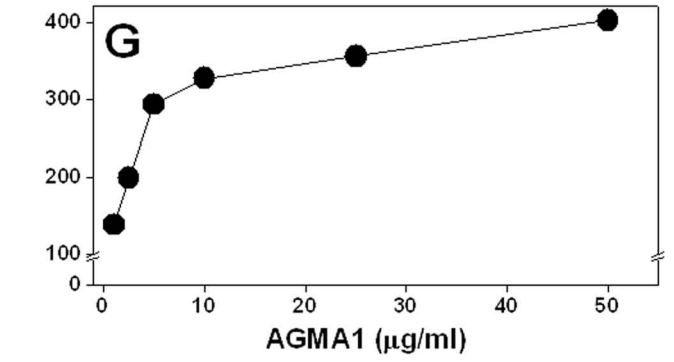

100000
10000
1000
100
10
1
ffu/ml
HPV 4°C
HPV+A 4°C
HPV 37°C
HPV+A 37°C

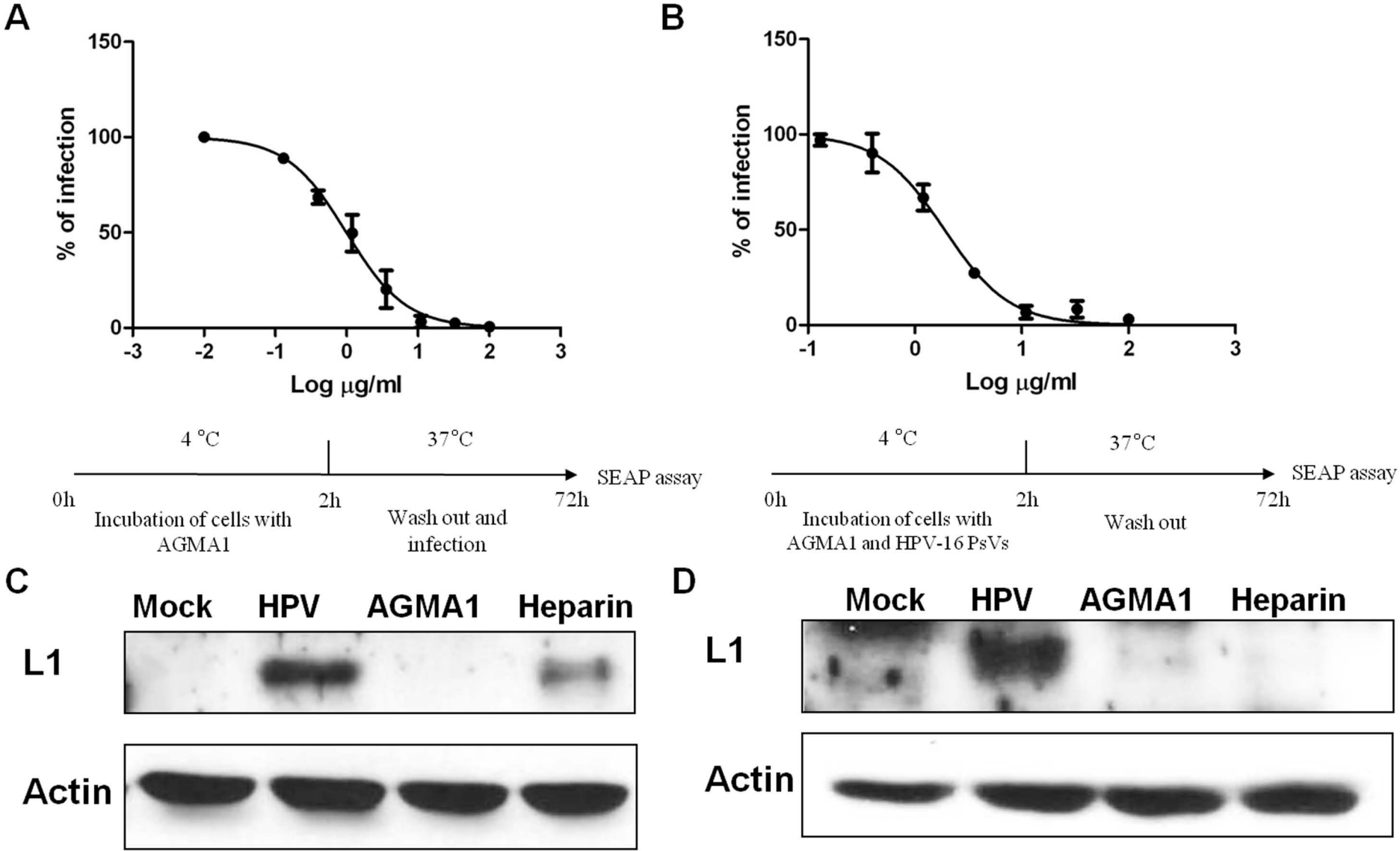

4 or 37°C
37°C
0h
Incubation of HPV-16 PsVs + AGMA1
2h
Titration of mixtures at high dilutions
72h
Count of infected fluorescent cells

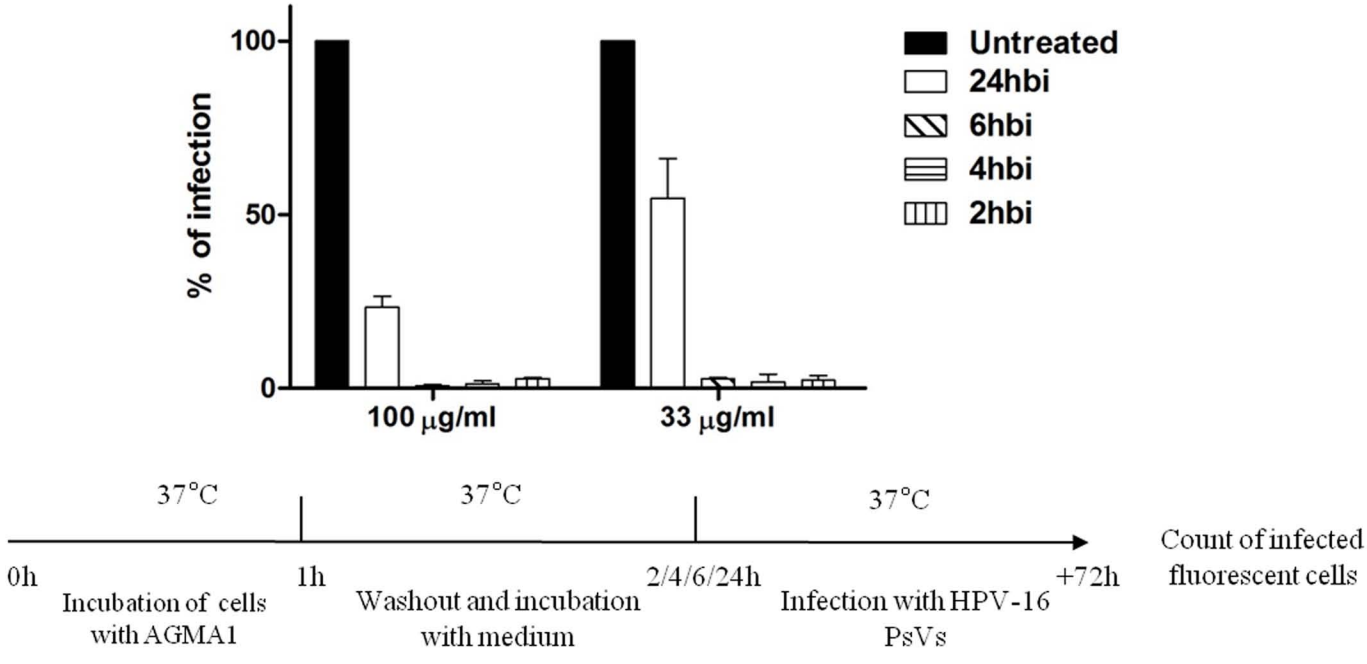

A
CHO-K1
A745 CHO K1
B
C
D
b-AGMA1 bound (O.D. 405 nM)
b-AGMA1 (μg/ml)
b-AGMA1 alone
+ heparin
+ PBS wash
+ NaCl wash
- heparinase
+ heparinase

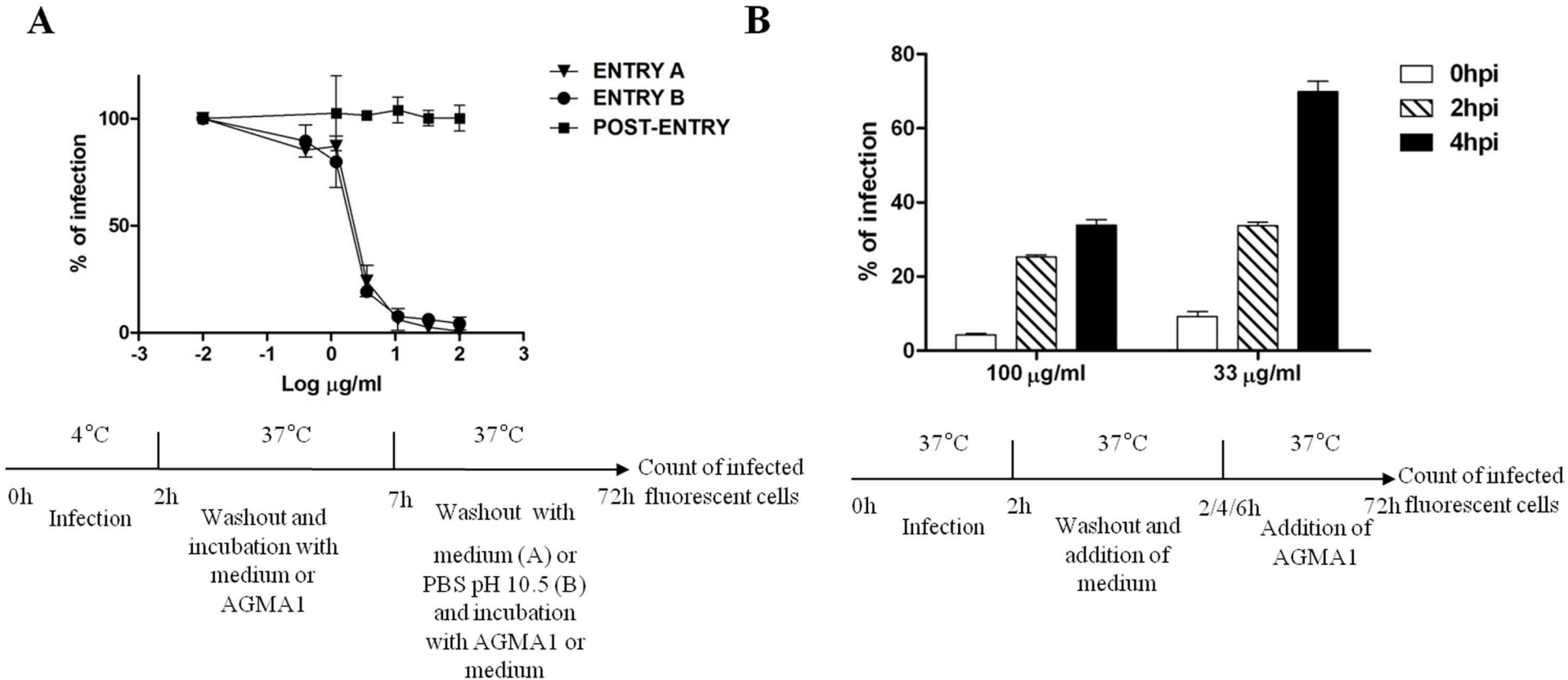

E
- heparinase
+ heparinase


F
50.0 μg/ml
1.25 μg/ml
RU
Time (s)


G
AGMA1 (μg/ml)


A
150
100
50
0
% of infection
-3
-2
-1
0
1
2
3
Log μg/ml
4 °C
37°C
SEAP assay
0h
2h
72h
Incubation of cells with AGMA1
Wash out and infection
B
150
100
50
0
% of infection
-1
0
1
2
3
Log μg/ml
4 °C
37°C
SEAP assay
0h
2h
72h
Incubation of cells with AGMA1 and HPV-16 PsVs
Wash out
C
Mock
HPV
AGMA1
Heparin
L1
Actin
D
Mock
HPV
AGMA1
Heparin
L1
Actin


% of infection
100
50
0
100 μg/ml
33 μg/ml
Untreated
24hbi
6hbi
4hbi
2hbi
37°C
37°C
37°C
0h
1h
2/4/6/24h
+72h
Incubation of cells with AGMA1
Washout and incubation with medium
Infection with HPV-16 PsVs
Count of infected fluorescent cells


A
ENTRY A
ENTRY B
POST-ENTRY
% of infection
100
50
0
-3
-2
-1
0
1
2
3
Log μg/ml
4°C
37°C
37°C
Count of infected
fluorescent cells
0h
Infection
2h
Washout and incubation with medium or AGMA1
7h
Washout with medium (A) or PBS pH 10.5 (B) and incubation with AGMA1 or medium
72h
B
0hpi
2hpi
4hpi
% of infection
80
60
40
20
0
100 μg/ml
33 μg/ml
37°C
37°C
37°C
Count of infected
fluorescent cells
0h
Infection
2h
Washout and addition of medium
2/4/6h
Addition of AGMA1
72h