AAC Journals.ASM.org

The Agmatine-Containing Poly(Amidoamine) Polymer AGMA1 Binds Cell Surface Heparan Sulfates and Prevents Attachment of Mucosal Human Papillomaviruses

Valeria Cagno,[a] Manuela Donalisio,[a] Antonella Bugatti,[b] Andrea Civra,[a] Roberta Cavalli,[c] Elisabetta Ranucci,[d] Paolo Ferruti,[d] Marco Rusnati,[b] David Lembo[a]

Department of Clinical and Biological Sciences, Università degli Studi di Torino, Orbassano, Italy[a]; Department of Molecular and Translational Medicine, University of Brescia, Brescia, Italy[b]; Dipartimento di Scienza e Tecnologia del Farmaco, Università degli Studi di Torino, Turin, Italy[c]; Dipartimento di Chimica, Università degli Studi di Milano, Milan, Italy[d]

The agmatine-containing poly(amidoamine) polymer AGMA1 was recently shown to inhibit the infectivity of several viruses, including human papillomavirus 16 (HPV-16), that exploit cell surface heparan sulfate proteoglycans (HSPGs) as attachment receptors. The aim of this work was to assess the antiviral activity 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 interacted 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 that AGMA1 is a leading candidate compound for further development as an active ingredient of a topical microbicide against HPV and other sexually transmitted viral infections.

Human papillomaviruses (HPVs) are members of the *Papillomaviridae* family of double-stranded DNA nonenveloped 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 *Alphapapillomavirus* genus are sexually transmitted and infect the anogenital mucosa. For the great majority of immunocompetent individuals, HPV infections are transient, causing asymptomatic epithelial infections or benign epithelial hyperplasia. Genital warts are the most common lesions, caused mainly by HPV-6 and HPV-11. Small proportions of men and women fail to control viral infections and develop HPV-related malignancies, including carcinoma of the cervix, vulva, vagina, penis, anus, or oropharynx. Several HPV types belonging to HPV species 7 (HPV-18, HPV-39, HPV-45, HPV-59, and HPV-68) or species 9 (HPV-16, HPV-31, HPV-33, HPV-35, HPV-52, HPV-58, and HPV-67) can confer high oncogenic risk. HPV-16 and HPV-18 cause about 70% of all cases of invasive cervical cancer worldwide (followed by HPV-31, HPV-33, and HPV-45) (2).

It has been estimated that more than 528,000 new cases of cervical cancer occur every year, and cervical cancer caused 266,000 deaths worldwide in 2012 (3, 4). Eighty-five percent of cervical cancer cases occur in women living in low-socioeconomic settings, primarily due to a lack of access to effective cervical cancer screening programs. No 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 essential in order to reduce the burden of HPV diseases. Two prophylactic vaccines, Gardasil and Cervarix, are currently available. The first is designed to protect against oncogenic HPV types 16 and 18 and low-risk HPV types 6 and 11 and 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, the vaccines also have a number of limitations, such as the lack of protection against other oncogenic HPV types, the need for cold chain distribution and storage, and low worldwide vaccine coverage, partly due to the very high costs of their administration. Additional preventive tools for HPV infections are thus required, particularly in low-resource settings where the burden of HPV infections is greatest. In this context, topical antiviral microbicides that could prevent the attachment of the full spectrum of mucosal HPVs 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), which are polyanionic structures that are widely expressed on eukaryotic cells and act as receptors for many other viruses (8–10). They consist of a core protein with glycosaminoglycan (GAG) chains of unbranched sulfated polysaccharides known as heparan sulfates, 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 extracel-

Received 20 February 2015 **Returned for modification** 10 April 2015
Accepted 8 June 2015

Accepted manuscript posted online 15 June 2015

Citation Cagno V, Donalisio M, Bugatti A, Civra A, Cavalli R, Ranucci E, Ferruti P, Rusnati M, Lembo D. 2015. The agmatine-containing poly(amidoamine) polymer AGMA1 binds cell surface heparan sulfates and prevents attachment of mucosal human papillomaviruses. Antimicrob Agents Chemother 59:5250–5259. doi:10.1128/AAC.00443-15.

Address correspondence to David Lembo, david.lembo@unito.it.

doi:10.1128/AAC.00443-15

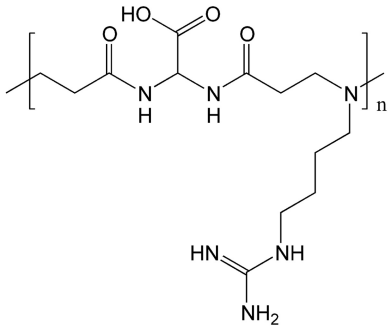


FIG 1 Chemical structure of AGMA1.

lular environment and thus hampering the cell surface attachment of HPV and hence infection (references 11 and 12 and references therein). The *in vivo* effectiveness of this anti-HPV strategy was demonstrated recently using the polyanionic sugar carrageenan (13, 14).

In addition to the virus-binding polyanionic compounds are the polycationic compounds, which bind to and mask HSPGs, preventing virus attachment. We showed recently that AGMA1, a poly(amidoamine) (Fig. 1), displays antiviral activity against a panel of viruses that utilize HSPGs as attachment receptors, including HPV (11). The prevailing cationic nature of AGMA1 (15) and its spectrum of antiviral activity suggest that it might prevent virus infectivity by binding to HSPGs. The aim of the present work was to investigate the antiviral activity of AGMA1 against several low-risk and high-risk HPV types and to elucidate the mechanism of action of AGMA1. 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 analysis-grade commercial products and were 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 nuclear magnetic resonance (NMR) spectroscopy and titration (16). Phosphate-buffered saline (PBS) (10 mM) was prepared using Sigma-Aldrich tablets, according to the manufacturer's instructions. D_2O (99.9%) was purchased from Aldrich and was used as received. Conventional heparin (13.6 kDa) was obtained from Laboratori Derivati Organici S.p.A. (Milan, Italy). Heparinase II, a glycosidase that digests the glycosaminoglycan (GAG) moiety of HSPGs (17), was obtained from Sigma-Aldrich (St. Louis, MO). The heparan sulfate-specific monoclonal antibody 10E4 was kindly provided by G. David (Leuven University, Leuven, Belgium).

Synthesis of AGMA1. AGMA1 (Fig. 1) was prepared as reported previously (18). Briefly, agmatine sulfate (2.000 g, 8.5 mmol) and lithium hydroxide monohydrate (0.360 g, 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 stirred occasionally for 78 h. At the end of this period, it was diluted with water (100 ml), acidified with hydrochloric acid to pH 4 to 4.5, and then ultrafiltered through membranes with a nominal molecular weight cutoff value of 5,000. The fractions retained in each case were freeze-dried, and the product was obtained as a white powder (yield, 1.9 g; AGMA1 number average molecular weight, 7,800; weight average molecular weight, 10,100; polydispersity, 1.29). Since AGMA1 is available in polydisperse preparations with average molecular masses that are not unequivocally determinable, we refer quantitatively to the compound in micrograms per milliliter (11), with the exception of calculation of the K_d (dissociation constant) value by Scatchard analysis of the surface plasmon resonance (SPR) data.

Preparation of biotinylated AGMA1. Biotinylated AGMA1 (b-AGMA1) was prepared in two steps, steps a and b, by reacting biotin *N*-hydroxysuccinimide (NHS) ester (biotin-NHS) with modified AGMA1 carrying approximately 8% 2-aminoethyl-substituted units, which was prepared by substituting in part agmatine with mono-*tert*-butoxycarbonyl (Boc)-ethylenediamine in the polymerization recipe and then cleaving the protective group. In 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 (20 ml); the mixture was stirred until clear and then allowed to stand in the dark at room temperature (20°C) for 24 h. After that time, agmatine sulfate (5.500 g) and lithium hydroxide (0.9936 g) were added with stirring, and the resultant mixture was left standing for another 120 h, as described above, and then diluted with water, acidified to pH 5 with hydrochloric acid, and ultrafiltered through a membrane with a nominal molecular weight cutoff value of 3,000. The product was retrieved by lyophilization, dissolution in 2 M hydrochloric acid (50 ml), and stirring for 2 h at room temperature under a slow stream of nitrogen, to favor elimination of the reaction byproduct. The resultant aminated AGMA1 was then isolated as described above (yield, 4.735 g).

In step b, aminated AGMA1 (0.500 g) was dissolved in water (25 ml), the solution was brought to pH 9.0 with dilute sodium hydroxide, a solution of biotin-NHS (0.035 g) in dimethyl sulfoxide (DMSO) (2 ml) was added dropwise, and the mixture was stirred for 5 h at room temperature. The reaction mixture was then acidified to pH 4.5, and the product was isolated as in the previous cases, by dilution with distilled water, ultrafiltration, and lyophilization (yield, 330 mg; AGMA1 mean mass number, 8,400; mean molecular weight, 11,900; positive distribution, 1.42).

Size exclusion chromatography (SEC) traces were obtained with a Knauer pump 1000 system equipped with a Knauer autosampler 3800, TSKgel G4000 PW and G3000 PW columns (Tosoh) 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 0.1 M Tris buffer (pH 8.00 ± 0.05) with 0.2 M sodium chloride. The flow rate was 1 ml/min, and the sample concentration was 1% (wt/wt).

HPV PsV production. Plasmids and 293TT cells used for pseudovirus (PsV) production were kindly provided by John Schiller (National Cancer Institute, Bethesda, MD) or were purchased from Addgene (Cambridge, MA). Detailed protocols and plasmid maps for this study are available online (http://home.ccr.cancer.gov/lco/pseudovirusproduction.htm). HPV-16, HPV-31, HPV-45, HPV-6, and bovine papillomavirus 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 secreted alkaline phosphatase (SEAP) or green fluorescent protein (GFP) (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, to separate PsVs from cellular debris and empty capsids. The material was centrifuged at 28,000 rpm for 18 h 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 contents of PsV stocks were 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. The 293TT cell line allows high levels of protein to be expressed from vectors containing the SV40 origin, due to overreplication of the expression plasmid (20). Wild-type Chinese hamster ovary (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.

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% FCS. The next day, serial dilutions of AGMA1 were added to preplated cells together with dilutions of PsV stocks at a multiplicity of infection (MOI) of 0.05 (determined for each PsV genotype tested by calculation of the fraction of cells positive for reporter protein expression in untreated cells, as reported in reference 22). After 72 h of incubation at 37°C, fluorescent cells were counted on an inverted Zeiss LSM510 fluorescence microscope.

SEAP-based PsV neutralization assays. The 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 FCS, 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 an MOI of 0.05 (calculated by comparing Coomassie-stained SDS-polyacrylamide gels for SEAP and GFP PsVs, after having verified a linear correlation between PsV amounts and relative light unit [RLU] values). Following incubation, 50-μl aliquots of supernatant were collected, and the SEAP contents in the clarified supernatant were determined by 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 assessed using a Wallac 1420 Victor luminometer (PerkinElmer Life and Analytical Sciences, Inc., Wellesley, MA). The 50% inhibitory concentration (IC_{50}) values and the 95% confidence intervals (CIs) were determined using the Prism program (GraphPad Software, San Diego, CA).

Virus inactivation assay. Diluted stocks of PsVs containing GFP (10^5 focus-forming units) and the test compounds at concentrations of 3.6 μg/ml were added to minimal essential medium (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 and then serially diluted to a noninhibitory concentration of test compound, and the residual viral infectivity was determined.

Attachment assay. Serial dilutions of AGMA1 were mixed with HPV-16–SEAP PsV (MOI, 0.05) and added to cooled 293TT cells in 96-well plates, and the cells were incubated for 2 h at 4°C to ensure PsV attachment but not entry. 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.

Preattachment assays. Monolayers of 293TT cells in 96-well plates were incubated with serial dilutions of AGMA1 for 2 h at 4°C. After removal of the compound and gentle washing, HPV-16–SEAP PsV (MOI, 0.05) was 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 gentle washing, cells were overlaid with medium for different times (23, 5, 3, or 1 h) and then infected with HPV-16–GFP PsV (1 ng/ml L1). Fluorescence was evaluated in the cell cultures 72 h after PsV inoculation.

Postattachment assay. HeLa cell monolayers in 96-well plates were incubated with HPV-16–GFP PsV (MOI, 0.05) 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 inocula or after 2 or 4 h. Fluorescence was evaluated in the cell cultures 72 h after PsV inoculation.

Entry assay. HeLa cell monolayers in 96-well plates were incubated with HPV-16–GFP PsV (MOI, 0.05) 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 37°C and incubated for 5 h to allow virus entry. After this incubation, cells were washed with medium or PBS at pH 10.5 (23) to remove virus that had not entered the cells, followed by two washes with normal medium to restore the physiological pH. Fluorescence was evaluated in the cells 72 h after PsV inoculation.

Postentry assay. HeLa cell monolayers in 96-well plates were incubated with HPV-16–GFP PsV (MOI, 0.05) 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 virus entry. After this incubation, cells were washed with PBS at pH 10.5 (23) to remove virus that had not entered the cells, followed by two washes 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 cells/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), according to the manufacturer's instructions. Absorbance values were measured at 490 nm using a microplate reader (model 680; Bio-Rad), and 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 the grids were rinsed several times with water. Grids were negatively stained with 0.5% uranyl acetate, and excess fluid was removed with filter paper. Observations and photographs were made using a CM 10 electron microscope (Philips, Eindhoven, The Netherlands).

Attachment and pretreatment followed by Western blotting. HeLa cells were seeded at a density of 300,000 cells/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 (MOI, 0.5), at 4°C. Following incubation, cells were washed with cold medium to ensure the removal of unbound virus; the 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 a mouse monoclonal antibody (Ab30908; Abcam, Cambridge, United Kingdom) at a 1:2,000 dilution, followed by horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Santa Cruz Biotechnology, Inc.). Actin was detected using a mouse monoclonal antibody (anti-actin MAB1501R; Millipore), followed by HRP-conjugated anti-mouse IgG (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 $MgCl_2$, and 0.1% gelatin, with subsaturating 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 the incubation, cells were washed with PBS, and the amounts of cell-associated b-AGMA1 were determined with HRP-labeled streptavidin (1:5,000) and the chromogenic substrate ABTS [2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid)] (Kierkegaard & Perry Laborato-

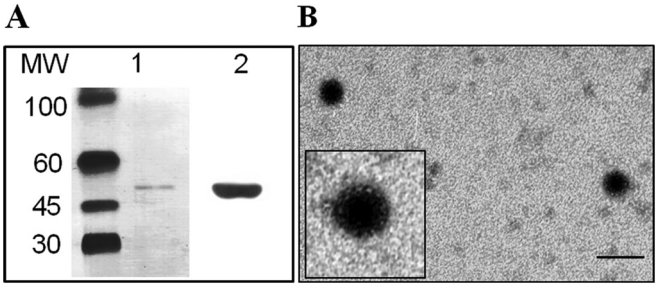


FIG 2 Characterization of purified HPV-16–SEAP PsV. (A) An aliquot of purified PsV preparation was analyzed by SDS-PAGE with Coomassie brilliant blue staining (lane 1) or immunoblotted with an anti-L1 antibody (lane 2). MW, molecular weight (in thousands). (B) Electron micrograph of a purified PsV preparation (bar, 100 nm). Inset, pseudovirus at ×2 magnification.

ries, Gaithersburg, MD). In some experiments, cell monolayers were washed with PBS alone or PBS containing 2 M NaCl, a treatment known to remove cationic polypeptides from cell surface HSPGs (24). Alternatively, cells were incubated for 1 h at 37°C with culture medium alone or medium containing heparinase II (15 mU/ml), before the binding assay. In previous experiments, these experimental conditions have been demonstrated to inhibit almost completely HSPG-dependent binding and biological activities of different heparin-binding proteins in various cell types (25, 26), including epithelial cells (27, 28). To evaluate the efficiency of the heparinase treatment, monolayers of HeLa cells on glass coverslips were incubated for 1 h at 37°C with culture medium alone or medium containing heparinase II (15 mU/ml), fixed with 3% paraformaldehyde and 2% sucrose in PBS, and saturated with PBS containing 3% bovine serum albumin. Then cells were incubated with the heparan sulfate-specific monoclonal antibody 10E4 (1:200), followed by a 45-min incubation with fluorescein isothiocyanate (FITC)-conjugated goat IgM (1:200) directed against mouse IgM. Cells were photographed using an Axioplan 2 microscope equipped for epifluorescence (Carl Zeiss, Gottingen, Germany) (original magnification, ×630).

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

To study the interaction of AGMA1 with heparin, the latter was immobilized on a BIAcore sensor chip as described previously (24). Briefly, a CM3 sensor chip (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 sensor chip. These experimental conditions allowed the immobilization of 80 resonance units (RU), equal to 5.8 fmol/mm^2 of heparin. A sensor chip coated with streptavidin alone was used for evaluation of the nonspecific binding of AGMA1 to the sensor chip and for blank subtraction. The compound was resuspended in 10 mM HBS-EP buffer (HEPES buffer [pH 7.4] containing 150 mM NaCl, 3 mM EDTA, and 0.005% surfactant P20) and injected over the heparin or streptavidin surfaces for 4 min (to allow association with immobilized heparin), and then the sensor chip was washed until dissociation was observed. After every run, the sensor chip was regenerated by injection of 2 M NaCl. The K_d (dissociation constant) was calculated by using the k_{off}/k_{on} ratio or by Scatchard analysis of the SPR RU values at equilibrium (directly proportional to the moles of bound ligand) as a function of the ligand concentration in solution.

RESULTS

Characterization of purified HPV-16 PsV. HPV-16 was chosen as a model virus because it is the most frequent genotype identified in cervical carcinomas (29). First, we evaluated the quality of the HPV-16–SEAP PsV preparations by SDS-PAGE and electron microscopic analysis. As shown in Fig. 2A, a major band migrating at 55 kDa was detected by Coomassie brilliant blue staining (Fig. 2A, lane 1) and was confirmed to be the L1 major capsid protein by Western blotting with anti-L1 antibody (Fig. 2A, 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 well-defined individual 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 with 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 overreplication of the SEAP reporter plasmid (20). 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 infections. Unlike 293TT cells, 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 is not overreplicated. GFP-expressing PsVs were also tested in 293TT cells and the IC_{50}s were compared to those obtained with SEAP-expressing PsVs. As reported in Table 1, AGMA1 inhibited the infectivity of HPV-16 PsV in all cell lines tested, with IC_{50}s between 0.38 μg/ml and 0.53 μg/ml. Of note, the results showed that the IC_{50}s obtained from cells infected with GFP-expressing versus SEAP-expressing PsVs were comparable. Cell viability assays performed under culture conditions identical to those used 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).

Inhibitory activity of AGMA1 is 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 bovine papillomavirus 1 (BPV-1). The results shown in Table 2 demonstrate that AGMA1 inhibited infection with all of the papillomaviruses tested

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

Cell line	IC_{50} (95% CI) (μg/ml)[a]	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

[a] Values are means and CIs for three separate determinations. IC_{50}, 50% inhibitory concentration; CI, confidence interval; CC_{50}, 50% cytotoxic concentration; SI, selectivity index.

TABLE 2 AGMA1 antiviral activity against different types of papillomavirus PsVs

Genotype	IC_{50} (95% CI) (μg/ml)[a]	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

[a] Values are means and CIs for three separate determinations. IC_{50}, 50% inhibitory concentration; CI, confidence interval; CC_{50}, 50% cytotoxic concentration; SI, selectivity index.

with similar potencies, indicating that the inhibitory activity of AGMA1 is not type restricted.

AGMA1 does not inactivate HPV PsV particles. To assess whether the inhibitory activity was a consequence of 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 differ significantly from those determined for 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. In the first set of experiments, we exploited the CHO cell model. Biotinylated AGMA1 bound CHO-K1 cells (which express HSPGs) in a dose-dependent manner, with an ED_{50} equal to 0.004 μg/ml and saturation binding being reached at 0.1 μg/ml (data not shown). The binding of b-AGMA1 to A745 CHO-K1 cells (mutant cells with defective HSPG synthesis) was significantly reduced with respect to wild-type CHO-K1 cells (Fig. 4B). Moreover, the binding of AGMA1 to wild-type CHO-K1 cells could be reduced to a level comparable to or even lower than those measured in A745 CHO-K1 cells with a 2 M NaCl wash, a treatment known to disrupt the binding of cationic molecules to HSPGs (24), and in the presence of a molar excess of heparin, a structurally related HSPG antagonist. Also, cell treatment with heparinase, an enzyme that specifically removes the heparan sulfate chains from cell surface-associated HSPGs, partially inhibited the cell surface association of AGMA1 (Fig. 4B).

We wondered whether the HSPG dependence of AGMA1 binding to cell surfaces also applied to cervical adenocarcinoma epithelial cells. Therefore, we evaluated the binding of b-AGMA1 to HeLa cells. As shown in Fig. 4C, b-AGMA1 bound to the surface of HeLa cells in a dose-dependent manner, with an ED_{50} equal to 0.04 μg/ml and saturation binding being reached at 0.5 μg/ml. Also, as seen for CHO-K1 cells, the binding could be efficiently inhibited by a 2 M NaCl wash and by heparin. At variance was the finding that heparinase treatment, which removed HSPGs almost completely from HeLa cell surfaces (Fig. 4E), exerted only partial inhibition (Fig. 4D). Taken together, these data suggest that the binding of AGMA1 to the cell surface depends on HSPGs but also on other (as yet unidentified) receptors.

To confirm further the interaction of AGMA1 with HSPGs, we evaluated the capacity of AGMA1 to bind to heparin (a structurally similar molecule) immobilized on a BIAcore sensor chip, a cell-free model that resembles the interactions of cationic proteins with cell surface HSPGs (28). In a typical experiment, increasing concentrations of AGMA1 were injected over the heparin surface, and a set of sensograms were obtained (Fig. 4E). An association rate constant (k_{on}) equal to 5.3×10^4 M^{-1} s^{-1} and a low dissociation rate constant (k_{off}) equal to 1.2×10^{-3} s^{-1} characterized the interaction of AGMA1 with immobilized heparin. Thus, the AGMA1-heparin interaction occurred with relatively high affinity (dissociation constant [K_d] calculated independently of AGMA1 concentrations as k_{off}/k_{on} equal to 22.6 nM). Finally, equilibrium binding data from Fig. 4E were used to generate the saturation curve shown in Fig. 4F, which in turn was used to calculate a K_d value independent of kinetic parameters; a K_d value equal to 17 nM was obtained, very similar to that calculated above.

AGMA1 blocks HPV binding to host cells through direct interaction with cells. Having demonstrated the interaction between AGMA1 and the cell surface, we wanted to examine

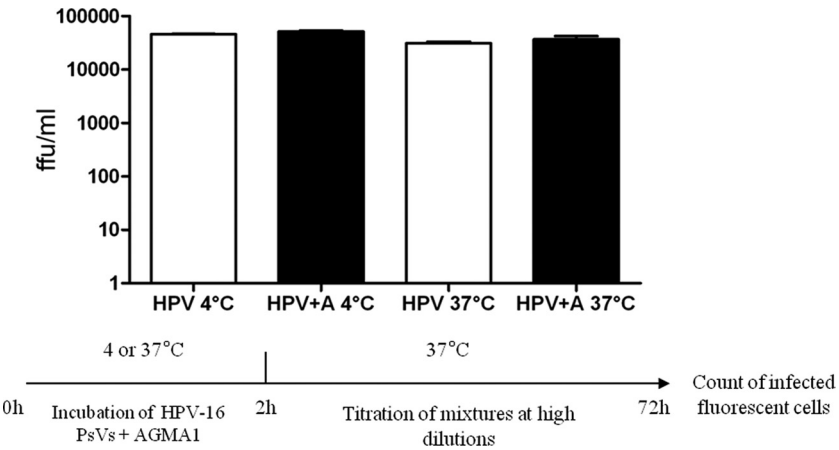
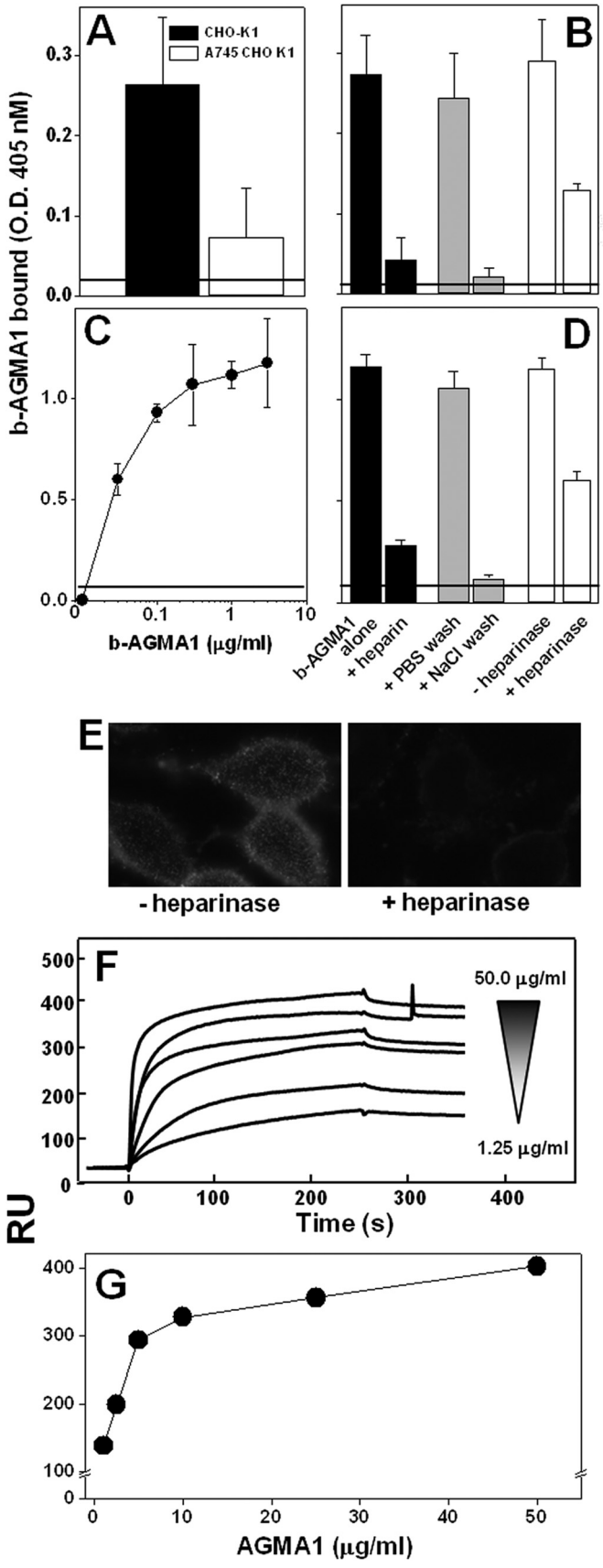


FIG 3 AGMA1 does not inactivate HPV PsV particles. HPV PsVs were incubated with 3.6 μg/ml AGMA1 for 2 h at 4°C or 37°C. Mixtures were then titrated on HeLa cells at high dilutions, such that the concentration of compound was not active. The titers, expressed in focus-forming units (ffu) per milliliter, are means and standard errors of the means (SEMs) for triplicates.



whether AGMA1 exerted its inhibitory activity by blocking HPV attachment. To this end, preattachment and attachment assays were performed. As shown in Fig. 5A and B, AGMA1 strongly inhibited HPV-16 infection under both experimental conditions, with IC_{50}s of 2.21 μg/ml and 1.01 μg/ml, respectively. This result suggested that the antiviral activity depended on the capacity of AGMA1 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 inoculation. In the same assay, heparin was used as a reference compound, being a known inhibitor of HPV attachment. As shown in Fig. 5C, pretreatment with AGMA1 totally prevented the binding of HPV-16 PsV. In contrast, heparin was only slightly active when added before the virus inoculum. When the compounds were added during infection at 4°C (Fig. 5D), they were both able to inhibit HPV binding. These results support the hypothesis that AGMA1 prevents HPV attachment through direct interactions 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 pretreatment assay in which the virus inoculum was added 23, 5, 3, or 1 h after the cells were exposed to 100 μg/ml or 33 μg/ml AGMA1 (two doses higher than IC_{90}) for 1 h and then washed. As shown in Fig. 6, addition of the virus inoculum 5, 3, or 1 h after AGMA1 pretreatment resulted in almost complete suppression of infection (>97%) for all doses of AGMA1; at 23 h posttreatment, 76.7% inhibition or 45.2% inhibition was observed in cells treated with 100 μg/ml or 33 μg/ml, respectively.

AGMA1 displaces HPV-16 bound to cells. It was reported previously that HPV exhibits slow entry kinetics, with an average half-time of 12 h for HPV-16 (30). Therefore, we used postattachment assays to investigate whether AGMA1 could displace bound HPV PsVs. We first performed an entry assay in which the virus was incubated with cells for 2 h at 4°C, a condition that allows virus attachment but not entry. Immediately after removal of the virus inoculum, AGMA1 was added to the cells and the temperature was shifted to 37°C to allow virus entry. Five hours later, a time sufficient to allow detectable amounts of PsVs to enter the cells, the virus particles that had bound but not entered were detached by washing with PBS at pH 10.5 (23) or with medium. The IC_{50} determined for AGMA1 in the entry assay with PBS (pH 10.5) washing was 2.07 μg/ml, while the value in the assay with medium washing was 2.37 μg/ml, demonstrating the ability of AGMA1 to

FIG 4 (A to D) Binding of AGMA1 to heparin and HSPGs. (A) Wild-type CHO-K1 cells and HSPG-deficient A745 CHO-K1 cells were incubated with b-AGMA1 (0.01 μg/ml) and washed with PBS. (B) In parallel experiments, wild-type CHO-K1 cells were (i) incubated with b-AGMA1 alone or in the presence of a molar excess (10 μg/ml) of heparin, (ii) washed with PBS alone or PBS containing 2 M NaCl, or (iii) left untreated or pretreated with heparinase before b-AGMA1 incubation. (C and D) HeLa cells were incubated with increasing concentrations of b-AGMA1 (C) or were incubated with 0.1 μg/ml b-AGMA1 and subjected to the three different treatments described for CHO-K1 cells (D). The amounts of cell-associated b-AGMA1 were then measured. O.D., optical density. Horizontal lines, absorbance values measured for the negative controls (cells not incubated with b-AGMA1). (E) Immunofluorescence analysis showing HSPG expression on the surface of HeLa cells and the efficiency of heparinase II treatment. (F) Overlay of blank-subtracted sensorgrams generated by the injection of AGMA1 onto sensor-chip-immobilized heparin. (G) Saturation curves of the binding of AGMA1 to sensor-chip-immobilized heparin. The saturation curves were obtained using the bound RU values at equilibrium, calculated from the sensorgrams presented in panel E.

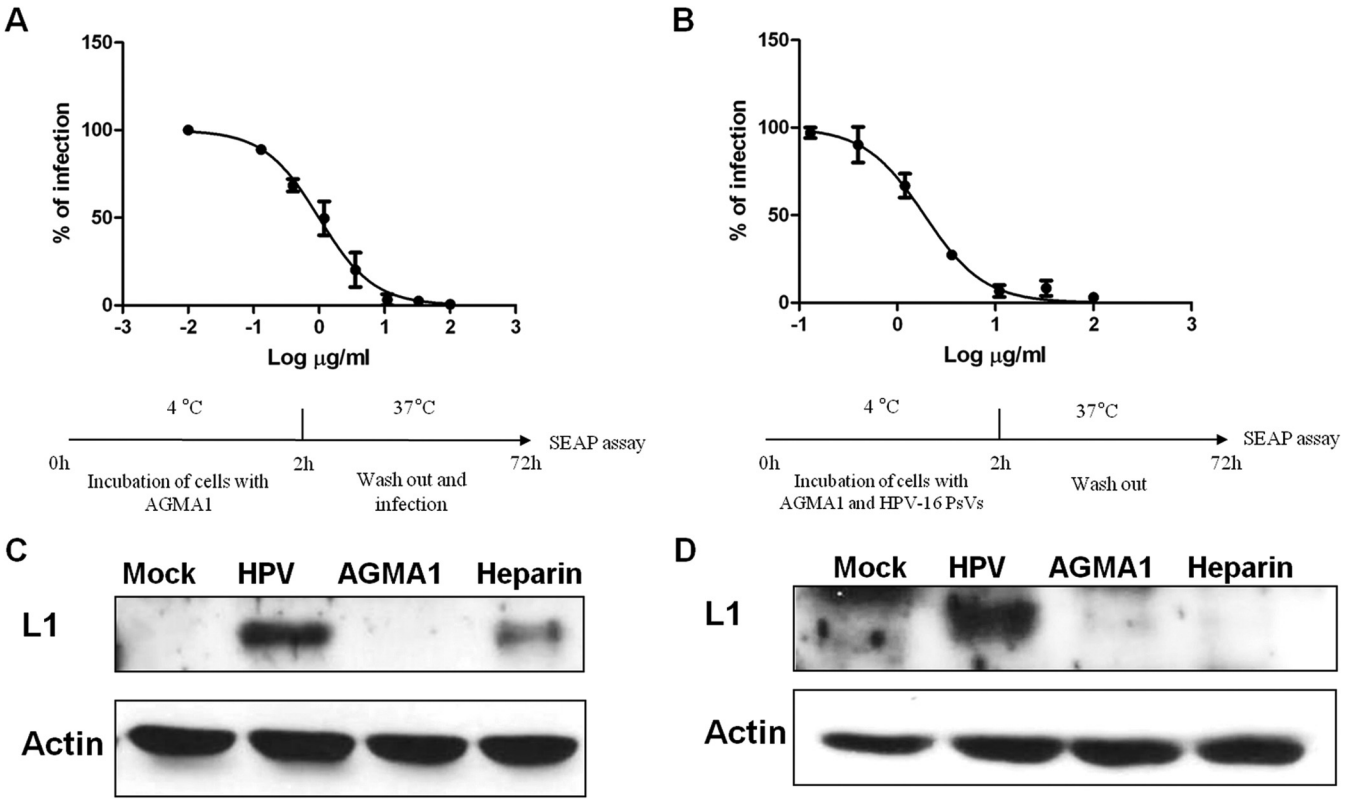


FIG 5 AGMA1 inhibition of HPV binding. (A) In the pretreatment assay, AGMA1 was added to cells for 2 h at 4°C and then washed out, and HPV-16 PsVs were added. SEAP activity was evaluated 72 h later. (B) In the attachment assay, AGMA1 and HPV-16 PsVs were coincubated on cells for 2 h at 4°C, followed by washout and 72 h of incubation. The results show means and SEMs for triplicates. (C) Western blotting directed against L1 was performed after a 2-h pretreatment with AGMA1 and heparin (100 μg/ml), followed by washout, addition of HPV-16 PsVs for 4 h at 4°C, and subsequent lysis. (D) Western blotting directed against L1 was performed after incubation on cells of AGMA1 and heparin (100 μg/ml) with HPV-16 PsVs for 4 h at 4°C, with subsequent lysis.

displace PsV particles already bound to cells. In contrast, when AGMA1 was added after the washout with PBS (pH 10.5) (postentry assay), no reduction of reporter gene expression could be observed (Fig. 7A). Moreover, we tested the inhibitory activity of AGMA1 when it was added to the cells 2 or 4 h after removal of the PsV inoculum (Fig. 7B), and we observed that 60% inhibitory activity was still present 4 h postinfection at the highest dose tested (i.e., 100 μg/ml).

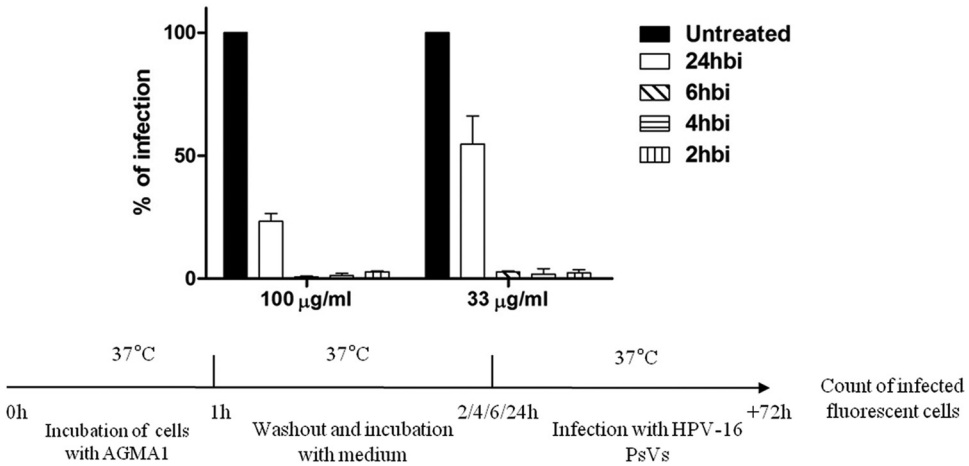


FIG 6 Prevention of HPV infection by AGMA1 for extended periods following its removal. Cells were pretreated with AGMA1 for 1 h at 37°C at fixed doses of 100 μg/ml or 33 μg/ml, followed by washout; at different time points postwashout (23, 5, 3, or 1 h), cells were infected with HPV-16 PsVs. After 72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

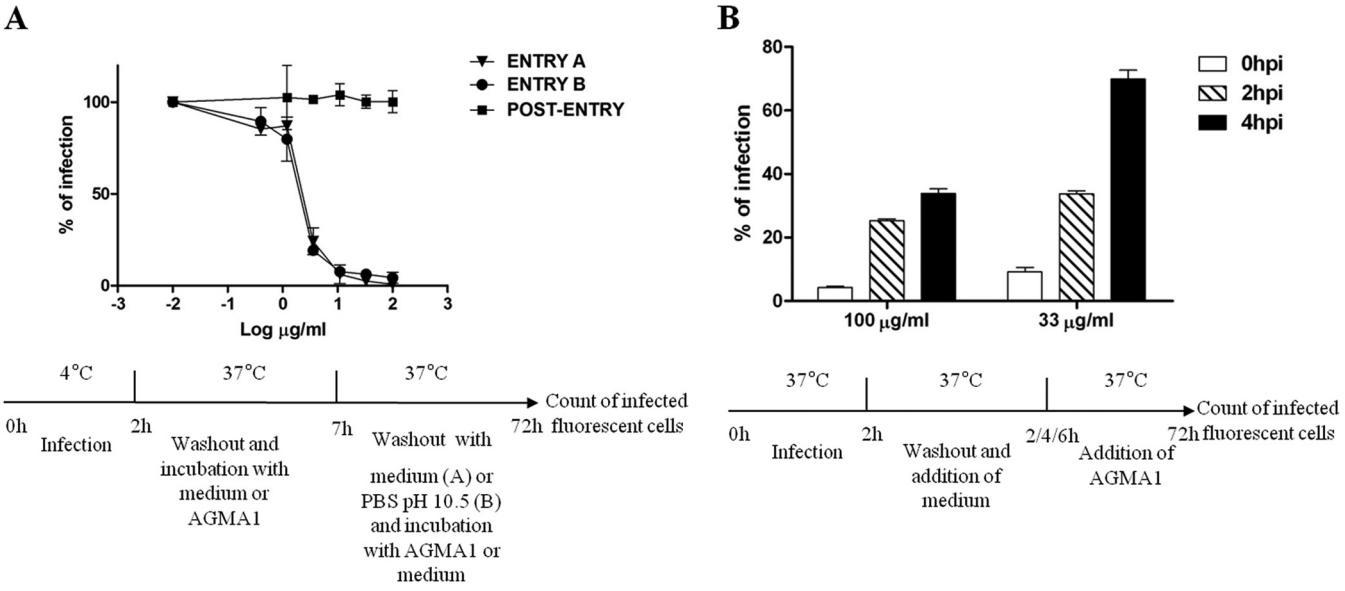


FIG 7 AGMA1 detachment of HPV from the cell surface. In the entry assay, HPV-16–GFP PsVs were added to cells for 2 h at 4°C and unbound PsVs were washed out. AGMA1 was then added, and the cells were incubated for 5 h at 37°C to allow virus entry. The cells were washed with medium (entry A) or with PBS (pH 10.5) (entry B) to remove viruses that remained outside the cell; 72 h after virus inoculation, GFP expression was evaluated. (A) In the postentry assay, AGMA1 was added not before but after the 5-h incubation at 37°C and the washout with PBS (pH 10.5). (B) In the posttreatment assay, AGMA1 was added 0, 2, or 4 h after removal of the PsV inoculum; GFP expression was evaluated 72 h later. hpi, hours postinfection. The results show means and SEMs for triplicates.

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, herpes simplex virus (HSV), and HIV (10, 12, 31, 32). 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 (33, 34). Therefore, this interaction has been put forward as being a suitable molecular target for virus attachment inhibitors, with the goal 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 it demonstrates that its inhibitory activity depends, at least in part, on its capacity to bind to cellular HSPGs. The latter feature is supported by biochemical, genetic, pharmacological, and enzymatic evidence presented herein. We observed that the binding of AGMA1 to HSPG-deficient A745 CHO-K1 cells was reduced with respect to wild-type CHO-K1 cells. Moreover, washing with 2 M NaCl, which is 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. Although both of these treatments have been demonstrated to act mainly on HSPG binding events (24, 35), the possibility that they also affect interactions of AGMA1 with other (as yet undefined) receptors cannot be ruled out. The treatment of cells with heparinase, which specifically and efficiently removes GAG chains from HSPGs (Fig. 4E), did not completely abolish the binding of AGMA1 to the cells (leaving 50% of binding unaffected), suggesting that other surface receptors, as yet unidentified, may be able to interact with the polymer. Of note, beside HSPGs, other cell receptors have been identified that contribute to HPV infection, including α6β4 integrin (36) and annexin A2 (37), and some reports even suggest the possibility that HPV infection occurs in a HSPG-independent manner (38, 39). Interestingly, the levels of cell surface expression of HSPGs (40) and of heparinase (41) can vary during inflammation, which is normally triggered by viral infections, suggesting the possibility that, depending on the pathological setting, HSPGs or other (as yet unidentified) HPV receptors may contribute differently to HPV infection.

Whatever receptors are bound, the interaction of AGMA1 with the cell surface is supported by the observation that AGMA1 prevents virus binding even when AGMA1 is administered before the virus inoculum (pretreatment assays). In contrast, heparin, a known attachment inhibitor that interacts directly with the virus particles rather than with the cells, completely prevents virus binding only in the presence of the virus (Fig. 5). The modest activity of heparin observed in Fig. 5C may be explained by interaction with an unidentified receptor on the cell surface. Annexin A2, which has been reported to interact with heparin (42) and to be involved in HPV-16 entry (37), could be a candidate. Interestingly, AGMA1 suppresses infection even when it is added to cell cultures after 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 bound to heparin with an affinity that was comparable to those of other heparin-binding proteins (33). Interestingly, the SPR binding assays performed here also showed

that AGMA1 bound to heparin with an affinity (K_d of 17.0 to 22.6 nM) 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 interactions. This would result in equally efficient inhibition of HPV infection, as shown by the very low IC_{50} calculated for the inhibitory activity of AGMA1 (0.34 to 0.74 μg/ml) (Table 2). Besides affinity, another interesting binding feature displayed by the AGMA1-heparin interaction is its low dissociation rate constant (k_{off}), which identifies the formation of very stable complexes between the polymer and heparin. A similar low k_{off} has been calculated for the HPV-heparin interaction (33). These similarities may be tentatively explained by the multimeric nature shared by the polymer and HPV, with both exposing multiple binding domains on their surfaces 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 the 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 interactions for prolonged periods. Those considerations correlate well with the observation that, once bound to the cell surface, AGMA1 retains its inhibitory activity when cells are challenged with HPV 3, 5, and even 23 h after initial exposure to the polymer (Fig. 6).

The results reported here also point to the possibility that, beside HSPGs, AGMA1 binds other (as yet unidentified) receptors on the cell surface. It is tentatively hypothesized that these receptors bound (and masked) by AGMA1 may be involved in HPV infection as well, pointing to the compound as a multitarget inhibitor that is able to interfere with multiple HPV interactions at the cell surface, which explains its high efficiency in inhibiting HPV infections under different experimental conditions.

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 (43). Its activity is not papillomavirus type restricted, since it extends across three HPV species belonging to the *Alphapapillomavirus* 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 rates in cervical cancer are about 3% and 7%, respectively (44), 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 (39), suggests that an additional, as yet unidentified, mechanism of anti-HPV activity exists. The finding that AGMA1 is active even against BPV-1, which is phylogenetically distant from *Alphapapillomavirus* species (45), supports the broad-spectrum activity of AGMA1. Since the existing prophylactic vaccines are HPV type restricted, a broad-spectrum microbicide could be a useful addition to vaccination programs, especially in resource-limited settings where the burdens of HPV infections are greatest.

Moreover, AGMA1 was recently reported to inhibit HSV-1 and HSV-2 infectivity (11). This finding supports its use under conditions in which concomitant infections with various sexually transmitted viruses may occur, such as in the case of HSV-2 infection, which enhances the transmission of HIV-1 (46). In turn, HIV infection-driven immunodeficiency causes well-documented increases in HPV and HSV infections (47, 48). Of note, HIV is a HSPG-dependent virus (9) and therefore may also be sensitive to AGMA1.

In conclusion, our results identify AGMA1 as a lead compound for further development as an active pharmaceutical ingredient of a topical microbicide against HPV and other sexually transmitted viral infections. Several issues remain to be investigated, including the ability of AGMA1 to interact with the basal cells of wounded squamous stratified epithelia, which are the targets of HPV infection. Preclinical efficacy and toxicology studies are ongoing, to assess the clinical potential of this inhibitor.

ACKNOWLEDGMENTS

This work was supported by a grant from Ricerca Finanziata dall'Università degli Studi di Torino (grant ex60% 2012) to D.L.

We thank Emanuela Noris and Manuela Vecchiati for technical support.

REFERENCES

1. **Bernard HU, Burk RD, Chen Z, van Doorslaer K, zur Hausen H, de Villiers EM.** 2010. Classification of papillomaviruses (PVs) based on 189 PV types and proposal of taxonomic amendments. Virology **401:**70–79. http://dx.doi.org/10.1016/j.virol.2010.02.002.
2. **Tommasino M.** 2014. The human papillomavirus family and its role in carcinogenesis. Semin Cancer Biol **26:**13–21. http://dx.doi.org/10.1016/j.semcancer.2013.11.002.
3. **IARC.** 2015. GLOBOCAN 2012: estimated cancer incidence, mortality and prevalence worldwide in 2012. IARC, Lyon, France. http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx.
4. **Centers for Disease Control and Prevention.** 2014. Sexually transmitted disease surveillance 2012. Centers for Disease Control and Prevention, Atlanta, GA. http://www.cdc.gov/std/stats12/surv2012.pdf.
5. **Schiller JT, Lowy DR.** 2012. Understanding and learning from the success of prophylactic human papillomavirus vaccines. Nat Rev Microbiol **10:** 681–692. http://dx.doi.org/10.1038/nrmicro2872.
6. **Giroglou T, Florin L, Schafer F, Streeck RE, Sapp M.** 2001. Human papillomavirus infection requires cell surface heparan sulfate. J Virol **75:** 1565–1570. http://dx.doi.org/10.1128/JVI.75.3.1565-1570.2001.
7. **Shafti-Keramat S, Handisurya A, Kriehuber E, Meneguzzi G, Slupetzky K, Kirnbauer R.** 2003. Different heparan sulfate proteoglycans serve as cellular receptors for human papillomaviruses. J Virol **77:**13125–13135. http://dx.doi.org/10.1128/JVI.77.24.13125-13135.2003.
8. **Spillmann D.** 2001. Heparan sulfate: anchor for viral intruders? Biochimie **83:**811–817. http://dx.doi.org/10.1016/S0300-9084(01)01290-1.
9. **Rusnati M, Vicenzi E, Donalisio M, Oreste P, Landolfo S, Lembo D.** 2009. Sulfated K5 *Escherichia coli* polysaccharide derivatives: a novel class of candidate antiviral microbicides. Pharmacol Ther **123:**310–322. http://dx.doi.org/10.1016/j.pharmthera.2009.05.001.
10. **Rusnati M, Chiodelli P, Bugatti A, Urbinati C.** 2013. Bridging the past and the future of virology: surface plasmon resonance as a powerful tool to investigate virus/host interactions. Crit Rev Microbiol **41:**238–260. http://dx.doi.org/10.3109/1040841X.2013.826177.
11. **Donalisio M, Ranucci E, Cagno V, Civra A, Manfredi A, Cavalli R, Ferruti P, Lembo D.** 2014. Agmatine-containing poly(amidoamine)s as a novel class of antiviral macromolecules: structural properties and *in vitro* evaluation of infectivity inhibition. Antimicrob Agents Chemother **58:** 6315–6319. http://dx.doi.org/10.1128/AAC.03420-14.

12. **Lembo D, Donalisio M, Rusnati M, Bugatti A, Cornaglia M, Cappello P, Giovarelli M, Oreste P, Landolfo S.** 2008. Sulfated K5 *Escherichia coli* polysaccharide derivatives as wide-range inhibitors of genital types of human papillomavirus. Antimicrob Agents Chemother **52:**1374–1381. http://dx.doi.org/10.1128/AAC.01467-07.
13. **Kizima L, Rodríguez A, Kenney J, Derby N, Mizenina O, Menon R, Seidor S, Zhang S, Levendosky K, Jean-Pierre N, Pugach P, Villegas G, Ford BE, Gettie A, Blanchard J, Piatak M, Jr, Lifson JD, Paglini G, Teleshova N, Zydowsky TM, Robbiani M, Fernández-Romero JA.** 2014. A potent combination microbicide that targets SHIV-RT, HSV-2 and HPV. PLoS One **9**(4): e94547. http://dx.doi.org/10.1371/journal.pone.0094547.
14. **Rodríguez A, Kleinbeck K, Mizenina O, Kizima L, Levendosky K, Jean-Pierre N, Villegas G, Ford BE, Cooney ML, Teleshova N, Robbiani M, Herold BC, Zydowsky T, Fernández Romero JA.** 2014. In vitro and in vivo evaluation of two carrageenan-based formulations to prevent HPV acquisition. Antiviral Res **108:**88–93. http://dx.doi.org/10.1016/j.antiviral.2014.05.018.
15. **Ferruti P, Franchini J, Bencini M, Ranucci E, Zara GP, Serpe L, Primo L, Cavalli R.** 2007. Prevailingly cationic agmatine-based amphoteric polyamidoamine as a nontoxic, nonhemolytic, and "stealthlike" DNA complexing agent and transfection promoter. Biomacromolecules **8:**1498–1504. http://dx.doi.org/10.1021/bm061126c.
16. **Ferruti P, Manzoni S, Richardson SCW, Duncan R, Patrick NG, Mendichi R, Casolaro M.** 2000. Amphoteric linear poly(amido-amine)s as endosomolytic polymers: correlation between physicochemical and biological properties. Macromolecules **33:**7793–7800. http://dx.doi.org/10.1021/ma000378h.
17. **Ernst S, Langer R, Cooney CL, Sasisekharan R.** 1995. Enzymatic degradation of glycosaminoglycans. Crit Rev Biochem Mol Biol **30:**387–444. http://dx.doi.org/10.3109/10409239509083490.
18. **Cavalli R, Bisazza A, Sessa R, Primo L, Fenili F, Manfredi A, Ranucci E, Ferruti P.** 2010. Amphoteric agmatine containing polyamidoamines as carriers for plasmid DNA in vitro and in vivo delivery. Biomacromolecules **11:**2667–2674. http://dx.doi.org/10.1021/bm100685t.
19. **Buck CB, Pastrana DV, Lowy DR, Schiller JT.** 2005. Generation of HPV pseudovirions using transfection and their use in neutralization assays. Methods Mol Med **119:**445–462.
20. **Buck CB, Pastrana DV, Lowy DR, Schiller JT.** 2004. Efficient intracellular assembly of papilloma viral vectors. J Virol **78:**751–757. http://dx.doi.org/10.1128/JVI.78.2.751-757.2004.
21. **Esko JD.** 1991. Genetic analysis of proteoglycan structure, function and metabolism. Curr Opin Cell Biol **3:**805–816. http://dx.doi.org/10.1016/0955-0674(91)90054-3.
22. **Zhang W, Kazakov T, Popa A, DiMaio D.** 2014. Vesicular trafficking of incoming human papillomavirus 16 to the Golgi apparatus and endoplasmic reticulum requires γ-secretase activity. mBio **5**(5):e01777-14. http://dx.doi.org/10.1128/mBio.01777-14.
23. **Schelhaas M, Shah B, Holzer M, Blattmann P, Kühling L, Day PM, Schiller JT, Helenius A.** 2012. Entry of human papillomavirus type 16 by actin-dependent, clathrin- and lipid raft-independent endocytosis. PLoS Pathog **8**(4):e1002657. http://dx.doi.org/10.1371/journal.ppat.1002657.
24. **Urbinati C, Bugatti A, Oreste P, Zoppetti G, Waltenberger J, Mitola S, Ribatti D, Presta M, Rusnati M.** 2004. Chemically sulfated *Escherichia coli* K5 polysaccharide derivatives as extracellular HIV-1 Tat protein antagonists. FEBS Lett **568:**171–177. http://dx.doi.org/10.1016/j.febslet.2004.05.033.
25. **Urbinati C, Nicoli S, Giacca M, David G, Fiorentini S, Caruso A, Alfano M, Cassetta L, Presta M, Rusnati M.** 2009. HIV-1 Tat and heparan sulfate proteoglycan interaction: a novel mechanism of lymphocyte adhesion and migration across the endothelium. Blood **114:**3335–3542. http://dx.doi.org/10.1182/blood-2009-01-198945.
26. **Chiodelli P, Mitola S, Ravelli C, Oreste P, Rusnati M, Presta M.** 2011. Heparan sulfate proteoglycans mediate the angiogenic activity of the vascular endothelial growth factor receptor-2 agonist gremlin. Arterioscler Thromb Vasc Biol **31**(12):e116–e127. http://dx.doi.org/10.1161/ATVBAHA.111.235184.
27. **Tyagi M, Rusnati M, Presta M, Giacca M.** 2001. Internalization of HIV-1 Tat requires cell surface heparan sulfate proteoglycans. J Biol Chem **276:**3254–3261. http://dx.doi.org/10.1074/jbc.M006701200.
28. **Donalisio M, Rusnati M, Civra A, Bugatti A, Allemand D, Pirri G, Giuliani A, Landolfo S, Lembo D.** 2010. Identification of a dendrimeric heparan sulfate-binding peptide that inhibits infectivity of genital types of human papillomaviruses. Antimicrob Agents Chemother **54:**4290–4299. http://dx.doi.org/10.1128/AAC.00471-10.
29. **Hong D, Lu W, Ye F, Hu Y, Xie X.** 2009. Gene silencing of HPV16 E6/E7 induced by promoter-targeting siRNA in SiHa cells. Br J Cancer **101:**1798–1804. http://dx.doi.org/10.1038/sj.bjc.6605344.
30. **Raff AB, Woodham AW, Raff LM, Skeate JG, Yan L, Da Silva DM, Schelhaas M, Kast WM.** 2013. The evolving field of human papilloma virus receptor research: a review of binding and entry. J Virol **87:**6062–6072. http://dx.doi.org/10.1128/JVI.00330-13.
31. **Shukla D, Spear PG.** 2001. Herpesviruses and heparan sulfate: an intimate relationship in aid of viral entry. J Clin Invest **108:**503–510. http://dx.doi.org/10.1172/JCI13799.
32. **Patel M, Yanagishita M, Roderiquez G, Bou-Habib DC, Oravecz T, Hascall VC, Norcross MA.** 1993. Cell-surface heparan sulfate proteoglycan mediates HIV-1 infection of T-cell lines. AIDS Res Hum Retroviruses **9:**167–174. http://dx.doi.org/10.1089/aid.1993.9.167.
33. **Joyce JG, Tung JS, Przysiecki CT, Cook JC, Lehman ED, Sands JA, Jansen KU, Keller PM.** 1999. The L1 major capsid protein of human papillomavirus type 11 recombinant virus-like particles interacts with heparin and cell-surface glycosaminoglycans on human keratinocytes. J Biol Chem **274:**5810–5822. http://dx.doi.org/10.1074/jbc.274.9.5810.
34. **Bousarghin L, Touzé A, Combita-Rojas AL, Coursaget P.** 2003. Positively charged sequences of human papillomavirus type 16 capsid proteins are sufficient to mediate gene transfer into target cells via the heparan sulfate receptor. J Gen Virol **84:**157–164. http://dx.doi.org/10.1099/vir.0.18789-0.
35. **Coltrini D, Rusnati M, Zoppetti G, Oreste P, Grazioli G, Naggi A, Presta M.** 1994. Different effects of mucosal, bovine lung and chemically modified heparin on selected biological properties of basic fibroblast growth factor. Biochem J **303:**583–590.
36. **Brendle SA, Christensen ND.** 2015. HPV binding assay to laminin-332/integrin α6β4 on human keratinocytes. Methods Mol Biol **1249:**53–66.
37. **Woodham AW, Taylor JR, Jimenez AI, Skeate JG, Schmidt T, Brand HE, Da Silva DM, Kast WM.** 2015. Small molecule inhibitors of the annexin A2 heterotetramer prevent human papillomavirus type 16 infection. J Antimicrob Chemother **70:**1686–1690.
38. **Richards KF, Mukherjee S, Bienkowska-Haba M, Pang J, Sapp M.** 2014. Human papillomavirus species-specific interaction with the basement membrane-resident non-heparan sulfate receptor. Viruses **6:**4856–4879. http://dx.doi.org/10.3390/v6124856.
39. **Cruz L, Meyers C.** 2013. Differential dependence on host cell glycosaminoglycans for infection of epithelial cells by high-risk HPV types. PLoS One **8**(7):e68379. http://dx.doi.org/10.1371/journal.pone.0068379.
40. **Pruessmeyer J, Martin C, Hess FM, Schwarz N, Schmidt S, Kogel T, Hoettecke N, Schmidt B, Sechi A, Uhlig S, Ludwig A.** 2010. A disintegrin and metalloproteinase 17 (ADAM17) mediates inflammation-induced shedding of syndecan-1 and -4 by lung epithelial cells. J Biol Chem **285:**555–564. http://dx.doi.org/10.1074/jbc.M109.059394.
41. **Meirovitz A, Goldberg R, Binder A, Rubinstein AM, Hermano E, Elkin M.** 2013. Heparanase in inflammation and inflammation-associated cancer. FEBS J **280:**2307–2319. http://dx.doi.org/10.1111/febs.12184.
42. **Shao C, Zhang F, Kemp MM, Linhardt RJ, Waisman DM, Head JF, Seaton BA.** 2006. Crystallographic analysis of calcium-dependent heparin binding to annexin A2. J Biol Chem **281:**31689–31695. http://dx.doi.org/10.1074/jbc.M604502200.
43. **Ferruti P.** 2013. Poly(amidoamine)s: past, present, and perspectives. J Polym Sci A Polym Chem **51:**2319–2353. http://dx.doi.org/10.1002/pola.26632.
44. **World Health Organization.** 2011. The immunological basis for immunization series. Module 19: human papillomavirus infection. WHO, Geneva, Switzerland. http://apps.who.int/iris/bitstream/10665/44604/1/9789241501590_eng.pdf?ua=1.
45. **de Villiers EM, Fauquet C, Broker TR, Bernard HU, zur Hausen H.** 2004. Classification of papillomaviruses. Virology **324:**17–27. http://dx.doi.org/10.1016/j.virol.2004.03.033.
46. **Freeman EE, Weiss HA, Glynn JR, Cross PL, Whitworth JA, Hayes RJ.** 2006. Herpes simplex virus 2 infection increases HIV acquisition in men and women: systematic review and meta-analysis of longitudinal studies. AIDS **20:**73–83. http://dx.doi.org/10.1097/01.aids.0000198081.09337.a7.
47. **McGrath BJ, Newman CL.** 1994. Genital herpes simplex infections in patients with the acquired immunodeficiency syndrome. Pharmacotherapy **14:**529–542.
48. **Heard I, Palefsky JM, Kazatchkine MD.** 2004. The impact of HIV antiviral therapy on human papillomavirus (HPV) infections and HPV-related diseases. Antivir Ther **9:**13–22.