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Running title: "Anti-HPV activity of AGMA1"

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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.

41 **INTRODUCTION**

42 Human papillomaviruses (HPVs) are members of the *Papillomaviridae* family of double-stranded
43 DNA, non-enveloped viruses (1). The 8-kb HPV genome is enclosed in a capsid shell comprising
44 major (L1) and minor (L2) structural proteins. Most of the HPVs belonging to the *alpha* genus are
45 sexually-transmitted and infect the anogenital mucosa. In the great majority of immunocompetent
46 individuals, HPV infection is transient causing asymptomatic epithelial infections or benign
47 epithelial hyperplasia. The genital warts are the most common lesions, caused mainly by HPV-6
48 and HPV-11. A small proportion of men and women fail to control viral infection and develop
49 HPV-related malignancies, including carcinoma of the cervix, vulva, vagina, penis, anus and
50 oropharynx. Several HPV types belonging to HPV species 7 (HPV-18, -39, -45, -59, -68) and
51 species 9 (HPV-16, -31, -33, -35, -52, -58, -67) can confer a high oncogenic risk. HPV-16 and
52 HPV-18 cause about 70% of all cases of invasive cervical cancer worldwide (followed by HPV 31,
53 33 and 45) (2).

54 It has been estimated that more than 528,000 new cases occur every year, and in 2012 it caused
55 266,000 deaths worldwide. (3, 4). Eighty-five percent of cervical cancer cases occur in women
56 living in low socio-economic settings, primarily due to a lack of access to effective cervical cancer
57 screening programs. No direct anti-HPV drugs are available to cure HPV lesions, therefore the
58 current treatments are ablative and directed at the abnormal cells associated with HPV, rather than
59 at the virus itself. The development of new ways to prevent genital infections is therefore essential
60 in order to reduce the burden of HPV diseases. Two prophylactic vaccines are currently available:
61 Gardasil and Cervarix. The first, is designed to protect against oncogenic HPV types 16 and 18 and
62 low-risk HPV types 6 and 11, and is therefore is preventive against both cancer and genital warts
63 (5), the latter is designed to protect against HPV types 16 and 18 only (5). Although the protective
64 activity of these vaccines is undeniable, they also come with a number of limitations, such as the
65 lack of protection against other oncogenic HPV types, the need for a cold chain distribution and
66 storage, and low worldwide vaccine coverage, partly due to the very high cost of their

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

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

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

91

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.

115 **Preparation of biotinylated AGMA1**

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

120 Step (a): 2,2-bisacrylamidoacetic acid (5.0005 g), lithium hydroxide (1.0644 g) and mono-*tert*-boc
121 ethylenediamine (0.285 mL) were dissolved in distilled water (20mL), stirred until clear and then
122 allowed to stand 24 hrs at room temperature (20°C) in the dark. After this time, agmatine sulphate
123 (5.500 g) and lithium hydroxide (0.9936 g) were added under stirring, the resultant mixture was let
124 standing as above for further 120 hrs, then diluted with water, acidified to pH 5 with hydrochloric
125 acid and ultrafiltered through a membrane of nominal cut-off 3000. The product was retrieved by
126 lyophilizing, dissolving in 2M hydrochloric acid (50 mL) and stirring 2 hrs at r.t. under a slow
127 stream of nitrogen to favor eliminating the reaction by-product. The resultant aminated AGMA1
128 was then isolated as above. Yield 4.735 g.

129 Step (b): aminated AGMA1 (0.500 g) was dissolved in water (25 mL), the solution brought to pH
130 9.0 with dilute sodium hydroxide, dropwise added with a solution of biotinNHS (0.035 g) in DMSO
131 (2 mL) and stirred 5 hrs at r.t. The reaction mixture was then acidified to pH 4.5, and the product
132 isolated as in the previous cases by diluting with distilled water, ultrafiltering and lyophilizing.
133 Yield 330 mg.

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

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

139 Tris buffer pH 8.00 ±0.05 with 0.2 M sodium chloride. The flow rate was 1 mL/min and sample
140 concentration 1% w/w.
141
142 **HPV PsV production.** Plasmids and 293TT cells, used for pseudovirus (PsV) production, were
143 kindly provided by John Schiller (National Cancer Institute, Bethesda, MD) or bought from
144 Addgene (Cambridge, MA). Detailed protocols and plasmid maps for this study can be obtained
145 from http://home.ccr.cancer.gov/lco/protocols.asp. HPV-16, HPV-31, HPV-45, HPV-6, and bovine
146 papillomavirus type 1 (BPV-1) PsVs were produced according to previously described methods
147 (19). Briefly, 293TT cells were transfected with plasmids expressing the papillomavirus major and
148 minor capsid proteins (L1 and L2, respectively), together with a reporter plasmid expressing the
149 secreted alkaline phosphatase (SEAP) or green fluorescent protein (GFP), named pYSEAP or pfwB,
150 respectively. HPV-16, HPV-6, and BPV-1 PsVs were produced using bicistronic L1/L2 expression
151 plasmids (p16sheLL, p6sheLL, and pSheLL, respectively). Capsids were allowed to mature
152 overnight in cell lysate; the clarified supernatant was then loaded on top of an Optiprep density
153 gradient of 27 to 33 to 39% (Sigma-Aldrich, St. Louis, MO) at room temperature for 3 h. The
154 material was centrifuged at 28,000 rpm for 18h at room temperature in an SW41.1 rotor (Beckman
155 Coulter, Inc., Fullerton, CA) and then collected by bottom puncture of the tubes.
156 Fractions were inspected for purity in 10% sodium dodecyl sulfate (SDS)–Tris–glycine gels, titrated
157 on 293TT cells to test for infectivity by SEAP or GFP detection, and then pooled and frozen at -
158 80°C until needed. The L1 protein content of PsV stocks was determined by comparison with
159 bovine serum albumin standards in Coomassie-stained SDS-polyacrylamide gels.
160
161 **Cell culture.** The human cervical carcinoma cell lines SiHa, HeLa, and C33A were grown as
162 monolayers in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL, Gaithersburg, MD)
163 supplemented with heat-inactivated 10% fetal calf serum (FCS; Gibco- BRL) and Glutamax-I
164 (Invitrogen, Carlsbad, CA). The 293TT cell line, derived from human embryonic kidney cells

165 transformed with the simian virus 40 (SV40) large T antigen, was cultured in the medium described
166 above supplemented with nonessential amino acids. 293TT cells allow high levels of protein to be
167 expressed from vectors containing the SV40 origin due to over-replication of the expression
168 plasmid (20). Wild-type Chinese hamster ovary cells (CHO)-K1 cells and GAG-deficient A745
169 CHO cells (21) were kindly provided by J.D. Esko (University of California, La Jolla, CA) and
170 grown in Ham's F-12 medium supplemented with 10% FCS.
171
172 **SEAP-based PsV neutralization assays.** 293TT cells were seeded in 96-well tissue culture-treated
173 flat-bottom plates at a density of 25,000 cells/well in 100 μl of DMEM without phenol red (Life
174 Technologies, Inc., Gaithersburg, MD) and with 10% heat-inactivated FBS, 1% glutamate, 1%
175 nonessential amino acids, 1 % antibiotic-antimycotic solution (Zell Shield, Minerva Biolabs GmbH,
176 Berlin, Germany) and 10 mM HEPES (neutralization buffer). The following day, to generate dose-
177 response curves, diluted PsV stocks (80 μl/well) were combined with 20 μl of serially diluted
178 compound. The 100-μl PsV-compound mixture was transferred to the cell monolayers and
179 incubated for 72 h at 37°C at a final concentration of PsV equal to approximately 1 ng/ml L1 (about
180 750 capsid equivalents of L1/cell) (22). Following incubation, 50-μl aliquots of supernatant were
181 collected and the SEAP content in the clarified supernatant determined using a Great Escape SEAP
182 chemiluminescence kit 2.0 (BD Clontech, Mountain View, CA) as directed by the manufacturer.
183 Thirty minutes after the addition of the substrate, samples were read using a Wallac 1420 Victor
184 luminometer (PerkinElmer Life and Analytical Sciences, Inc., Wellesley, MA).
185 The 50% inhibitory concentration (IC50) values and the 95% confidence intervals (CIs) were
186 determined using the Prism program (GraphPad Software, San Diego, CA).
187 **GFP-based assays.** Cells were seeded in 96-well plates at a density of 25,000 cells/well in 100 μl
188 of DMEM supplemented with 10% FBS. The next day, serial dilutions of AGMA1 were added to
189 pre-plated cells together with dilutions of PsV stock. After 72 h of incubation at 37°C fluorescent
190 cells were counted on an inverted Zeiss LSM510 fluorescence microscope.

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

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

199 **Pre-attachment assays.** 293TT cell monolayers in 96-well plates were incubated with serial
200 dilutions of AGMA1 for 2 h at 4°C. After removal of the compound and a gentle wash, HPV-16–
201 SEAP PsVs (1 ng/ml L1) were added to the cells for 2 h at 4°C. After two gentle washes, the cells
202 were shifted to 37°C, and SEAP activity was measured in the cell culture supernatants 72 h after
203 PsV inoculation. Alternatively HeLa cells were incubated with a fixed dose of AGMA1 for 1 h at
204 37°C. After removal of the compound and a gentle wash, cells were overlaid with medium for
205 different times (23, 5, 3 or 1 h(s)) and then infected with 16–GFP PsV (1 ng/ml L1). Fluorescence
206 was evaluated in the cell culture 72 h after PsV inoculation.

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

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

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

217 **Post-entry assay.** HeLa cell monolayers in 96-well plates were incubated with HPV-16–GFP PsV
218 (1 ng/ml L1) for 2 h at 4°C, followed by two gentle washes to remove unbound virus. The cells
219 were then shifted to 37°C for 5 h to allow viral entry. After this incubation, cells were washed with
220 PBS at pH 10.5 (23) to remove un-entered virus and then washed twice with normal medium to
221 restore the physiological pH. Serial dilutions of AGMA1 were then added to the cells. Fluorescence
222 was evaluated in the cells 72 h after PsV inoculation.

223 **Cell viability assay.** Cells were seeded at a density of 25,000/well in 96-well plates; the next day,
224 they were treated with serially diluted peptide compounds to generate dose-response curves. After
225 72 h of incubation, cell viability was determined using the CellTiter 96 Proliferation Assay Kit
226 (Promega, Madison, WI, USA), according to the manufacturer's instructions. Absorbances were
227 measured using a Microplate Reader (Model 680, BIORAD) at 490 nm. 50% cytotoxic
228 concentration (CC_{50}) values and 95% confidence intervals (CIs) were determined using Prism
229 software (GraphPad Software, San Diego, CA).

230 **Electron microscopy.** An aliquot of diluted HPV-PsV preparation was allowed to adsorb for about
231 3 min on carbon and formvar-coated grids and then rinsed several times with water. Grids were
232 negatively stained with 0.5% uranyl acetate and excess fluid removed with filter paper.
233 Observations and photographs were made using a CM 10 electron microscope (Philips, Eindhoven,
234 The Netherlands).

235 **Attachment and pre-treatment followed by Western blot.** HeLa cells were seeded at a density of
236 300,000/well in 6-well plates; the next day they were treated with a fixed dose of AGMA1 or
237 heparin (i.e. 100 µg/ml) 2 h before or during the 4 h infection period at 4°C. Following incubation,
238 cells were washed with cold medium to ensure the removal of unbound virus; cells were then

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

244 **AGMA1/cell-associated HSPG binding assays.** Monolayers of CHO-K1 cells, GAG-deficient
245 A745 CHO-K1 cells or HeLa cells in 96-well plates were incubated for 2 h at 4°C in phosphate-
246 buffered saline (PBS) containing 0.1 mg/ml $CaCl_2$, 0.1 mg/ml MgCl2, and 0.1% gelatin, with sub-
247 saturating concentrations of biotinylated AGMA1 (b-AGMA1) (0.01 μg/mL or 0.1 μg/ml) in the
248 absence or presence of heparin (10 μg/ml). At the end of incubation, cells were washed with PBS,
249 and the amount of cell-associated b-AGMA1 determined with horseradish peroxidase-labeled
250 streptavidin (1/5,000) and the chromogenic substrate ABTS (Kierkegaard & Perry Laboratories,
251 Gaithersburg, MD). In some experiments, cell monolayers were washed with PBS containing 2 M
252 NaCl, a treatment known to remove cationic polypeptides from cell surface HSPGs (Urbinati,
253 2004). Alternatively, cells were incubated with heparinase II (15mU/ml) for 1 h at 37°C, or left
254 untreated, before the binding assay.

255 **SPR assay.** Surface plasmon resonance (SPR) measurements were performed on a BIAcore X
256 instrument (GE Healthcare, Milwaukee, WI), using a research grade CM3 sensorchip. The reagents
257 1-ethyl-3-(3-diaminopropyl)-carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS)
258 were purchased from GE Healthcare and used according to recommended protocols.

259 To study the interaction of AGMA1 with heparin, the latter was immobilized on a BIAcore
260 sensorchip as described previously (24). Briefly, a CM3 sensorchip (GE Healthcare) previously
261 activated with 50 μl of a mixture containing 0.4 M EDC and 0.1 M NHS was coated with
262 streptavidin. Heparin was biotinylated at its reducing end and immobilized onto the streptavidin-
263 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

273 **RESULTS**

274 **Characterization of purified HPV-16 PsV.**

275 HPV-16 was chosen as a pivotal model virus because it is the most frequent genotype identified in
276 cervical carcinomas (25). First of all, we evaluated the quality of the HPV-16–SEAP PsV
277 preparations by SDS-PAGE and electron microscopy analysis. As shown in Fig. 2A, a major band
278 migrating at 55 kDa was detected by Coomassie brilliant blue staining (lane 1) and was confirmed
279 to be the L1 major capsid protein by Western blot analysis with anti-L1 antibody (lane 2). No L1-
280 reactive proteolytic degradation products were observed at molecular masses below 55 kDa. Figure
281 2B shows an electron micrograph of the same PsV stock. The PsV particles exhibited an average
282 diameter of 50 to 60 nm, similar to that of an authentic HPV capsid, and appeared as individual,
283 well-defined particles with no aggregation. When observed at a higher magnification, the particles
284 appeared to be well-assembled, icosahedral capsids (Fig.2B, inset). Similar results were obtained
285 with the other PsV types used in this study (data not shown).

286

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

288 The ability of AGMA1 to block HPV-16 PsV infection was tested on several cell lines. 293TT cells
289 are preferred for PsV inhibition assays based on SEAP expression because high levels of the SV40
290 large T antigen in these cells allow for the over-replication of the SEAP reporter plasmid.
291 Moreover, the analysis was extended to cell lines derived from the uterine cervix (i.e. SiHa, HeLa,
292 and C33A), the major anatomical target for high-risk HPV infection. Unlike 293TT, these cell lines
293 do not express the SV40 large T antigen, resulting in very low levels of SEAP protein expression.
294 Therefore, we employed GFP as a reporter gene because it allows reliable analyses of cell types in
295 which the reporter plasmid does not over-replicate. GFP-expressing PsV were also tested in 293TT
296 and the IC_{50} values compared to those obtained with SEAP-expressing PsV. As reported in Table 1,
297 AGMA1 inhibited the infectivity of HPV-16 PsV in all cell lines tested with IC_{50} values between
298 0.38 μg/ml - 0.53 μg/ml. Of note, the results show that the IC_{50} values obtained from cells infected

299 with GFP- or SEAP-expressing PsV are comparable. Cell viability assays performed under identical
300 culture conditions for antiviral assays (i.e. cell density and time of incubation with the compound)
301 demonstrated that AGMA1 did not affect cell viability at any concentration tested (i.e. up to 300
302 μg/ml).
303
304 **The inhibitory activity of AGMA1 in not papillomavirus type restricted.**
305 To assess whether the inhibitory activity of AGMA1 was papillomavirus type-specific, the assays
306 were repeated in 293TT cells using two additional high-risk HPV types (i.e. HPV-31 and HPV-45),
307 one low-risk type (HPV-6) and the bovine papillomavirus type 1 (BPV-1 PsV). The results shown
308 in Table 2 demonstrate that AGMA1 inhibits the infection of all the papillomaviruses tested with a
309 similar potency indicating that its inhibitory activity is not type-restricted.
310
311 **AGMA1 does not inactivate HPV PsV particles.**
312 To assess whether the inhibitory activity was a consequence of a direct inactivation of PsV particles
313 by AGMA1 we performed a viral inactivation assay. As shown in Fig. 3, the virus titers of samples
314 treated with AGMA1 did not significantly differ to those determined in untreated samples ($P>0.05$),
315 indicating that AGMA1 does not inactivate HPV particles.
316
317 **AGMA1 interacts with the cell surface via HSPGs.**
318 The polycationic nature of AGMA1 (15, 18) and its demonstrated capacity to selectively inhibit
319 HSPG dependent viruses (11) suggested that AGMA1 could inhibit HPV infection by interacting
320 with cell surface HSPGs. To investigate this hypothesis, we first investigated the effective capacity
321 of AGMA1 to bind to the cell surface via HSPGs. To this end, in a first set of experiments, we
322 exploited the CHO cell model. As shown in Fig. 4A, the binding of b-AGMA1 to A745 CHO-K1

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

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

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

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

349 Having demonstrated the interaction between AGMA1 and HSPGs, we wanted to examine whether
350 AGMA1 exerted its inhibitory activity by blocking HPV attachment. To this end, pre-attachment
351 and attachment assays were performed. As shown in Fig. 5A and 5B, under both these experimental
352 conditions AGMA1 strongly inhibited HPV-16 infection, with IC_{50} values of 2.21 μg/ml and 1.01
353 μg/ml, respectively. This result suggests that the antiviral activity depended on the AGMA1
354 capacity to prevent virus binding to the cell surface. To verify this hypothesis, a Western blot
355 analysis was carried out to detect the HPV particles bound to cells treated with AGMA1 before or
356 during the PsV inoculum. In the same assay, heparin was used as a reference compound, being a
357 known inhibitor of HPV attachment. As shown in Figure 5C, pre-treatment with AGMA1 totally
358 prevented the binding of HPV-16 PsV. By contrast, heparin was only slightly active when added
359 before virus inoculum. Instead, when the compounds were added during infection at 4°C (Figure
360 5D), they were both able to inhibit HPV binding. These results support the hypothesis that AGMA1
361 prevents HPV attachment through a direct interaction with cells, instead of binding to the virus
362 particle as heparin does.

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

370

371

372

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

387 **DISCUSSION**

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

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

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

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

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

441 Additional properties of AGMA1 make it appealing for further development as an active
442 pharmaceutical ingredient of topical microbicides. AGMA1 is water-soluble, biodegradable and
443 biocompatible. Its preparation process is simple, easily scalable and environmentally friendly,
444 taking place in water or alcohols, at room temperature and without the need for added catalysts (33).
445 Its activity is not papillomavirus type-restricted, since it extends across three HPV species
446 belonging to the *alpha* genus of the *Papillomaviridae* family. Indeed, AGMA1 has been found to be
447 active against three high-risk oncogenic types, namely HPV-16, HPV-31 (species 9), and HPV-45
448 (species 7), and one low-risk type (HPV-6) belonging to species 10. Of note, HPV-31 and HPV-45,
449 whose worldwide prevalence in cervical cancer is about 4% and 6%, respectively, (34) are not
450 included in the bivalent or quadrivalent vaccines. Interestingly, the fact that AGMA1 is active
451 against HPV-31, whose attachment does not appear to be dependent on HSPG (35), suggests that an
452 additional, as yet unidentified, mechanism of anti-HPV activity exists. The finding that AGMA1 is
453 even active against BPV-1, which is phylogenetically distant from *alpha*- papillomaviruses (36),
454 further supports its broad-spectrum activity. Since the existing prophylactic vaccines are HPV type
455 restricted, a broad-spectrum microbicide could be a useful adjuvant to vaccination programs,
456 especially in resource-limited settings were the burden of HPV infections is greatest.
457 Moreover, AGMA1 was recently reported to inhibit HSV-1 and HSV-2 infectivity (11). This
458 finding supports its use in conditions in which the concomitant infection of various sexually
459 transmitted viruses may occur, such as in the case of HSV-2 infection, which enhances the
460 transmission of HIV-1 infection (37). In turn, HIV infection-driven immunodeficiency causes a well
461 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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606

607

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.

632 **Fig 5. AGMA1 inhibits HPV binding.** In the pre-treatment assay, AGMA1 was added to cells for
633 2 h at 4°C, it was then washed out and HPV16 PsVs added. SEAP activity was evaluated 72 h later
634 (A). In the attachment assay (B), AGMA1 and HPV16 PsVs were co-incubated on cells at 4°C for 2
635 h, followed by a washout and 72 h incubation. The results show means and SEMs for triplicates.
636 Figure C presents a Western blot directed against L1 after 2 h pre-treatment with AGMA1 and
637 heparin at 100 μg/ml followed by a washout and addition of HPV16 PsVs for 4 h at 4°C and
638 subsequent lysis. Figure D presents a Western blot directed against L1 after an incubation on cells
639 of AGMA1 and heparin (100 μg/ml) with HPV16 PsVs for 4 h at 4°C with subsequent lysis.
640
641 **Fig 6. AGMA1 prevents HPV infection for extended periods following its removal.**
642 Cells were pre-treated with AGMA1 for 1h at 37°C at fixed doses of 100 μg/ml and 33 μg/ml,
643 followed by washout; at different time points post washout (23, 5, 3, or 1 h) cells were then infected
644 with HPV-16 PsVs. After 72 h incubation, infection was evaluated. The results show means and
645 SEMs for triplicates
646
647 **Fig 7. AGMA1 is able to detach HPV from the cell surface.**
648 In the entry assay, HPV-16–GFP PsVs were added to cells for 2 h at 4°C then washed out to
649 remove unbound virus. AGMA1 was then added and the cells incubated for 5 h at 37°C to allow
650 viral entry. The cells were then washed with PBS pH 10.5 to remove everything that remained
651 outside the cell; 72 h after viral inoculum, GFP expression was evaluated. In the post-entry assay,
652 AGMA1 was not added before but after the 5 h incubation at 37°C and the washout with PBS pH
653 10.5 (A). In the post-treatment assay (B), AGMA1 was added 0, 2 or 4 h after the removal of the
654 PsV inocula; GFP expression was evaluated 72 h later. The results show means and SEMs for
655 triplicates.

AGMA1

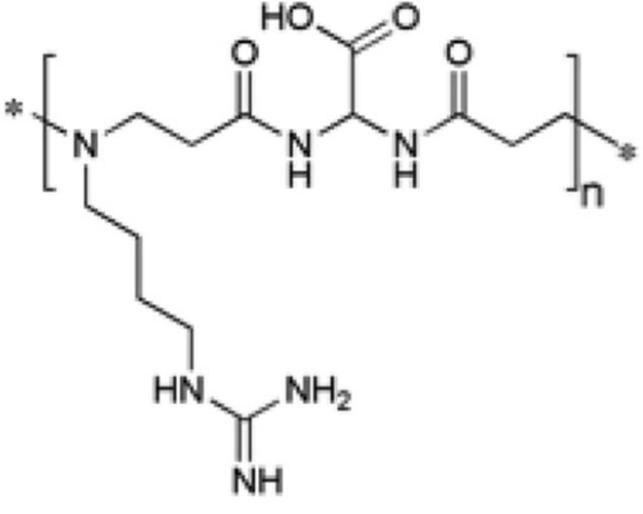


Table 1. AGMA1 antiviral activity against HPV-16 in different cell lines.

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

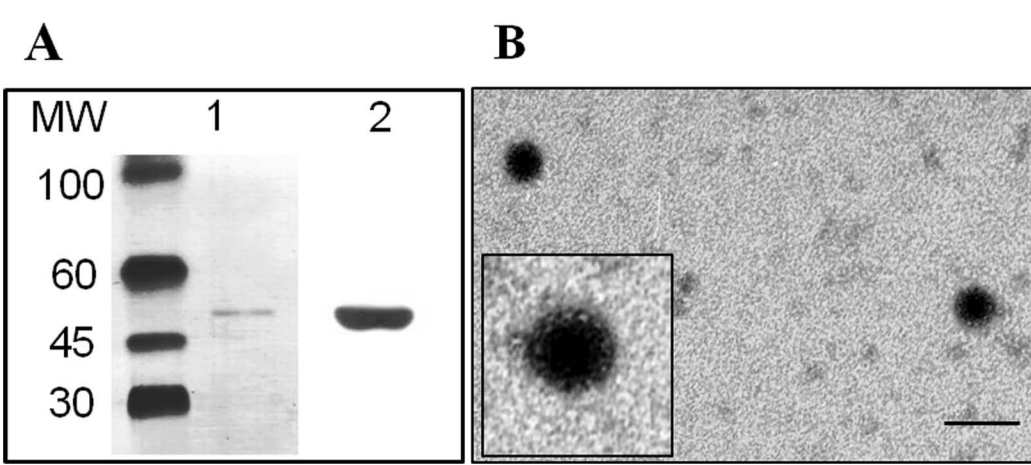
Cell line	**IC_{50} * (µg/ml)**	**95% CI****	**CC_{50}*** (µg/ml)**	**SI**
HPV-16	0.53	0.51-0.55	>300	>566
HPV-31	0.36	0.28-0.46	>300	>836
HPV-45	0.74	0.70-1.80	>300	>407
HPV-6	0.54	0.36-0.81	>300	>553
BPV-1	0.34	0.23-0.50	>300	>875

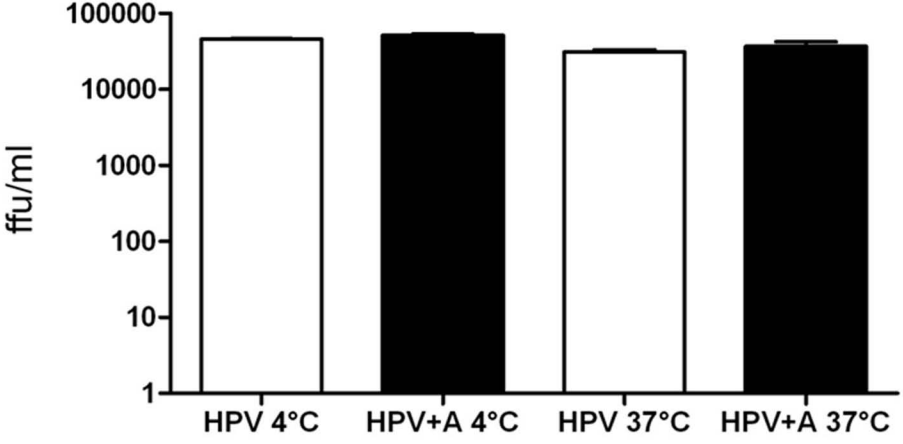
* IC_{50}: 50% inhibitory concentration

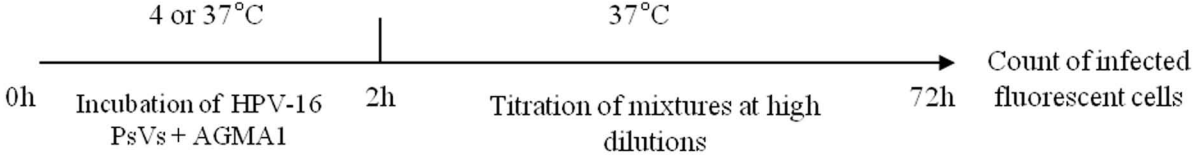
** 95% CI: 95% confidence interval

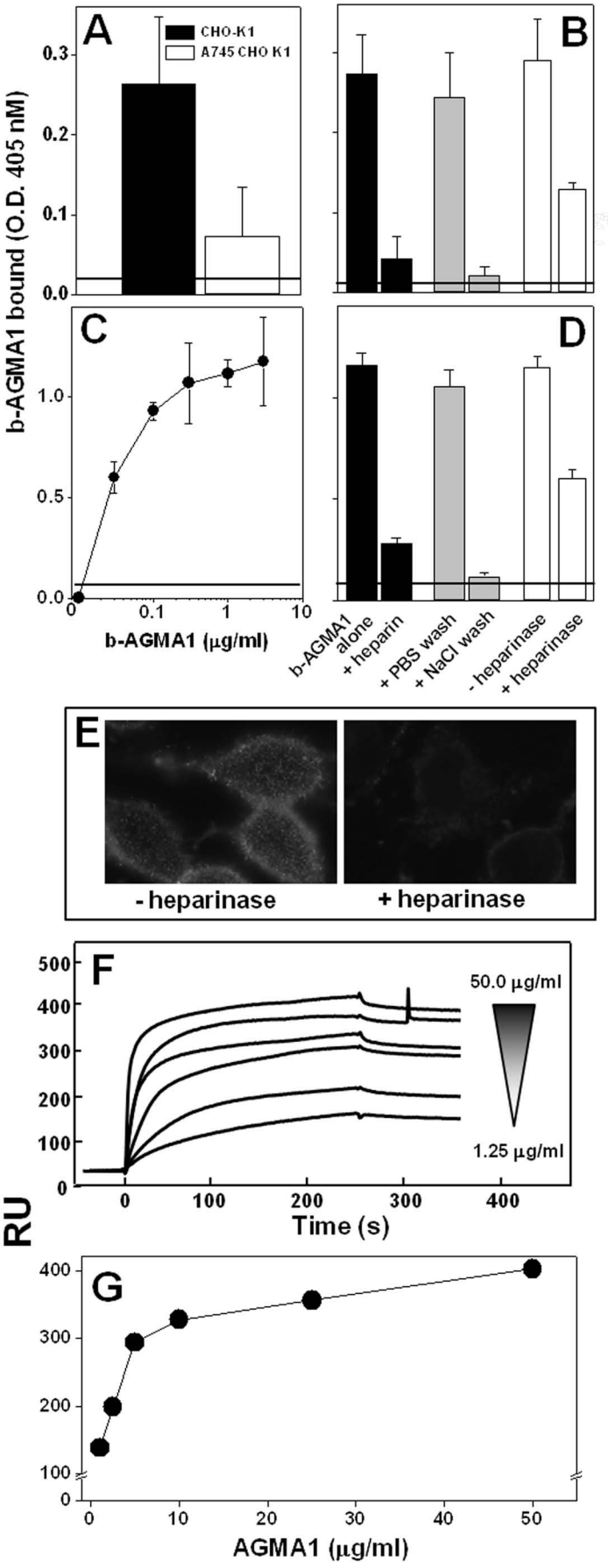
***CC_{50}: 50% cytotoxic concentration

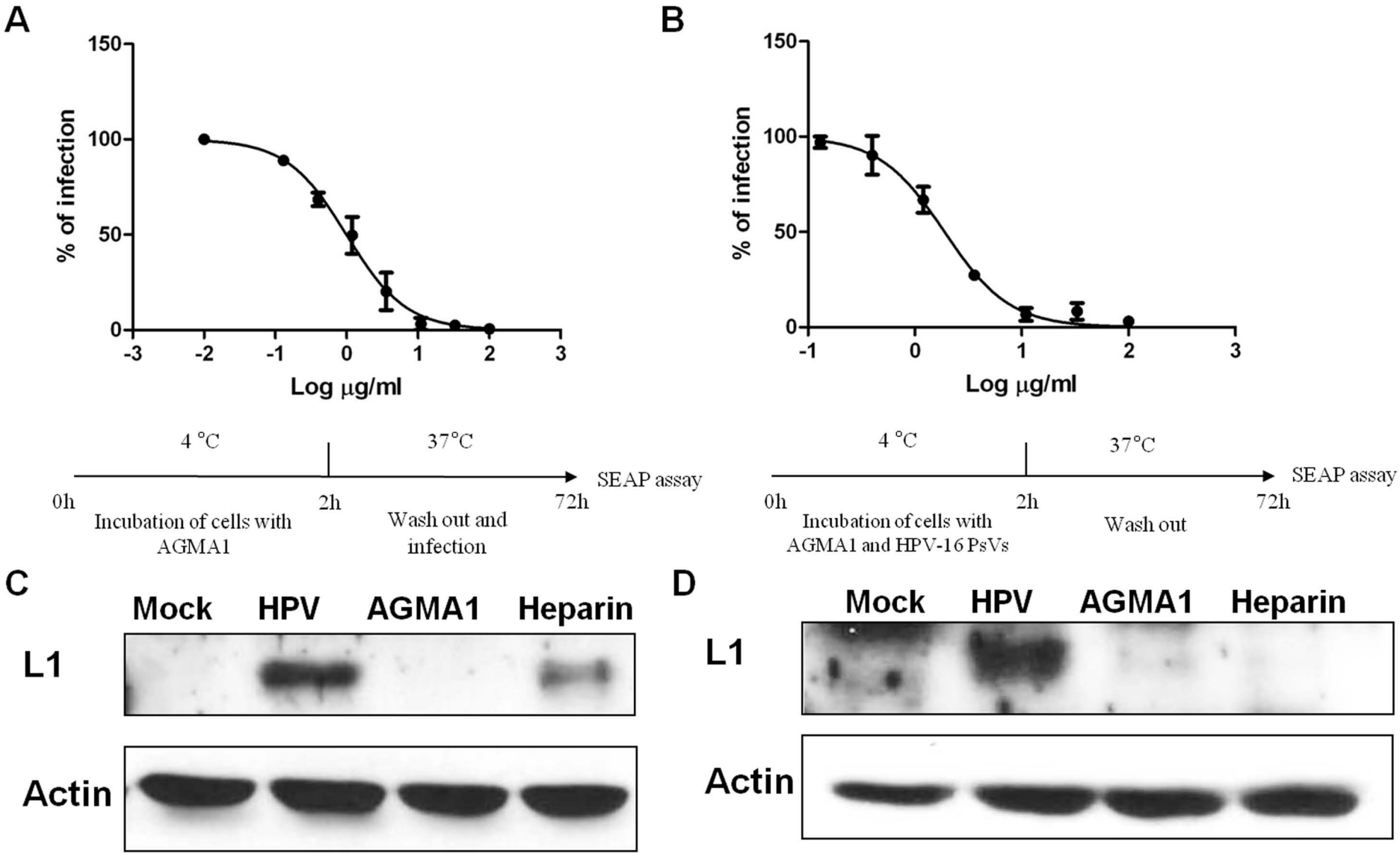
Values are means and CIs for three separate determinations.

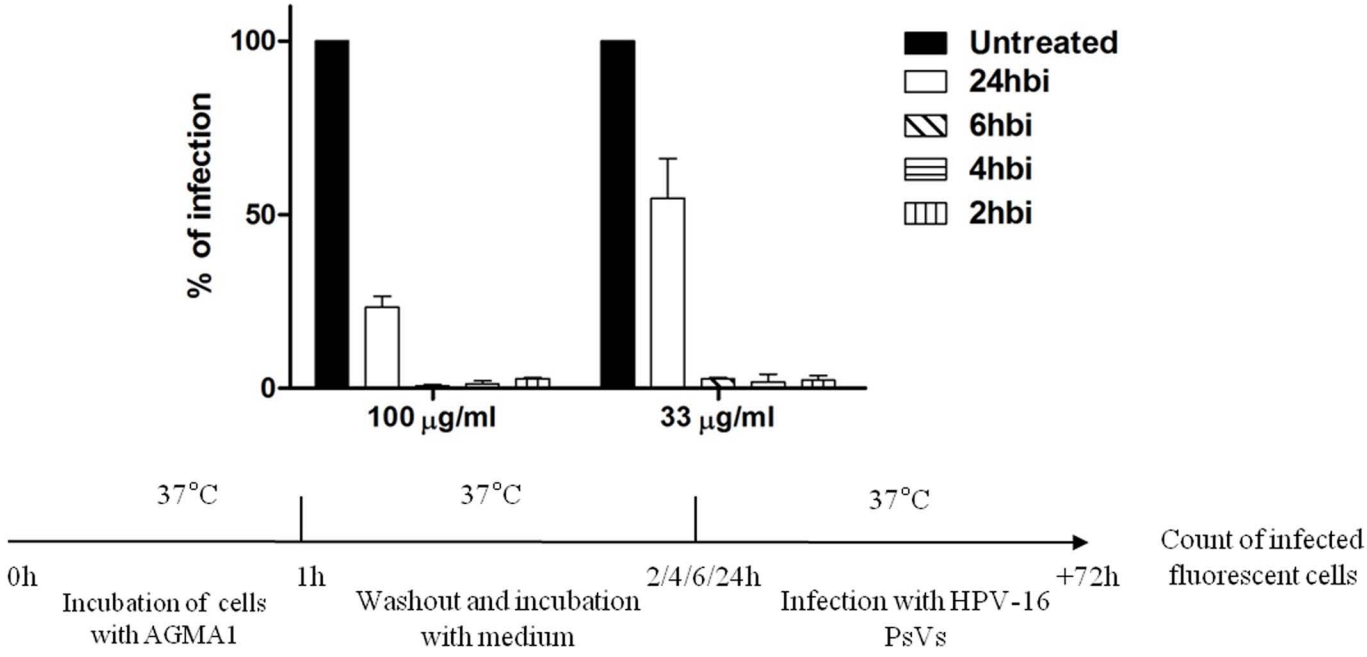

A
B
MW
1
2
100
60
45
30

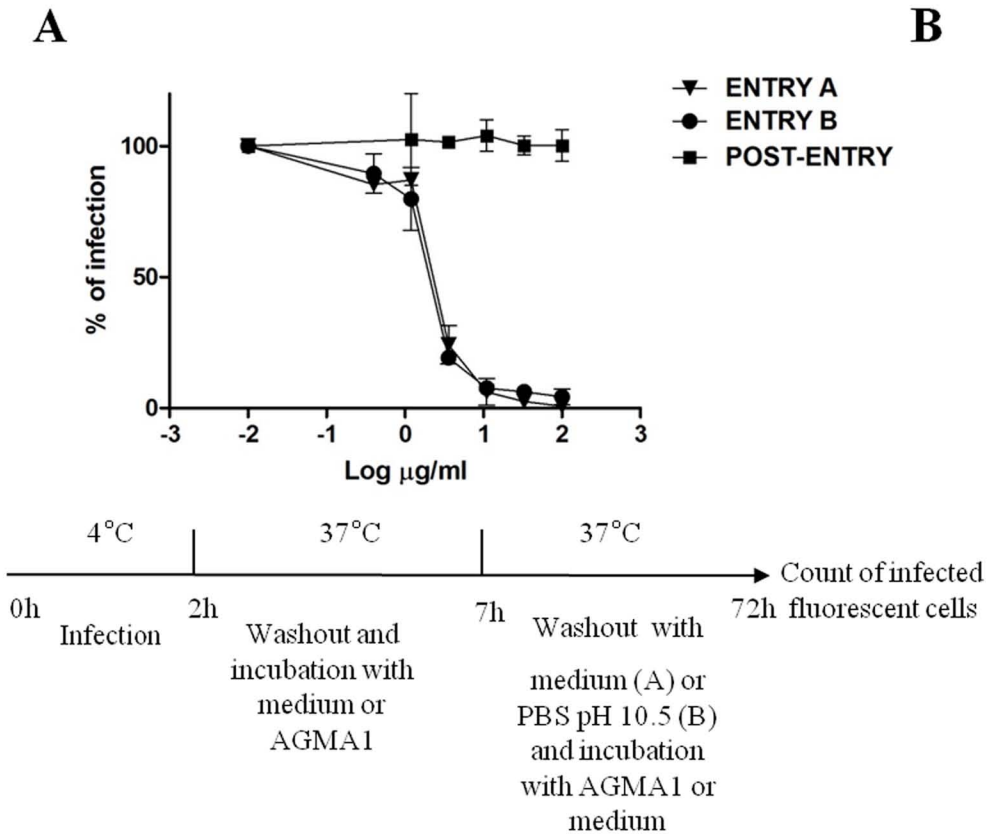

100000
10000
1000
100
10
1
ffu/ml
HPV 4°C
HPV+A 4°C
HPV 37°C
HPV+A 37°C

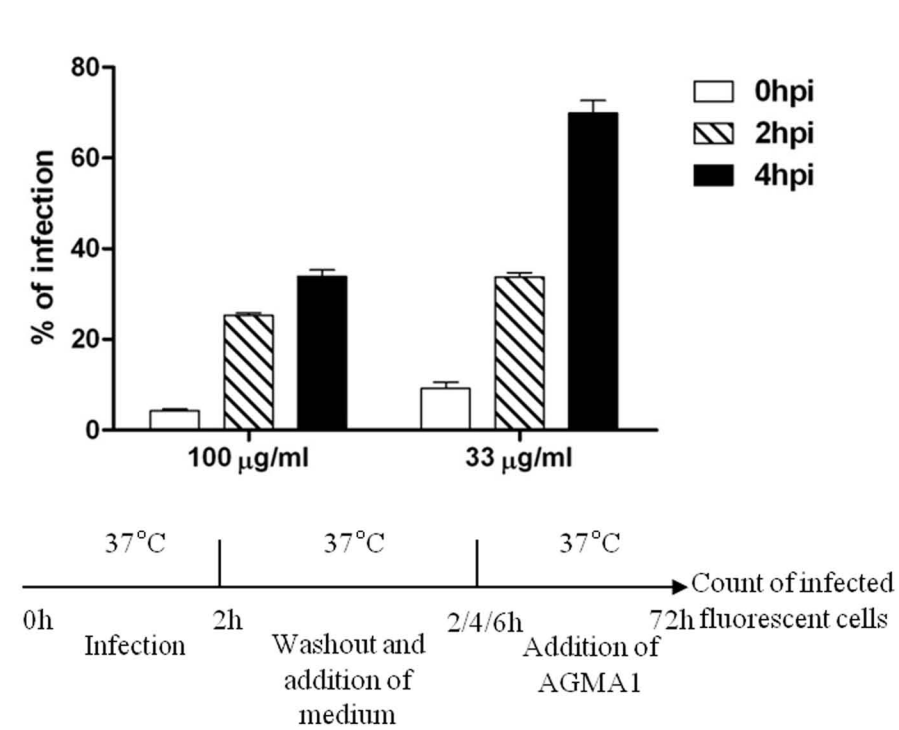

4 or 37°C
37°C
0h
Incubation of HPV-16 PsVs + AGMA1
2h
Titration of mixtures at high dilutions
72h
Count of infected fluorescent cells


A
CHO-K1
A745 CHO K1
B
C
D
b-AGMA1 bound (O.D. 405 nM)
0.0
0.1
0.2
0.3
0.5
1.0
0
0.1
1
10
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
0
100
200
300
400
500
Time (s)
G
AGMA1 (µg/ml)
0
10
20
30
40
50


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


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


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