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*Original Citation:*

*Availability:*

This version is available <http://hdl.handle.net/2318/1635955> since 2017-05-19T11:18:35Z

*Published version:*

DOI:10.1016/j.biomaterials.2016.01.055

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1     **The AGMA1 poly(amidoamine) inhibits the infectivity of herpes simplex virus in cell lines, in**  
2                     **human cervicovaginal histocultures, and in vaginally infected mice**

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17  
18    Running title: “Pre-clinical development of AGMA1 as anti-HSV microbicide”

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31 **Abstract**

32 The development of topical microbicides is a valid approach to protect the genital mucosa from  
33 sexually transmitted infections that cannot be contained with effective vaccination, like HSV and HIV  
34 infections. A suitable target of microbicides is the interaction between viral proteins and cell surface  
35 heparan sulfate proteoglycans (HSPGs). AGMA1 is a prevalingly cationic agmatine-containing  
36 polyamidoamine polymer previously shown to inhibit HSPGs dependent viruses, including HSV-1,  
37 HSV-2, and HPV-16. The aim of this study was to elucidate the mechanism of action of AGMA1  
38 against HSV infection and assess its antiviral efficacy and biocompatibility in preclinical models. The  
39 results show AGMA1 to be a non-toxic inhibitor of HSV infectivity in cell cultures and human  
40 cervicovaginal histocultures. Moreover, it significantly reduced the burden of infection of HSV-2  
41 genital infection in mice. The investigation of the mechanism of action revealed that AGMA1 reduces  
42 cells susceptibility to virus infection by binding to cell surface HSPGs thereby preventing HSV  
43 attachment. This study indicates that AGMA1 is a promising candidate for the development of a topical  
44 microbicide to prevent sexually transmitted HSV infections.

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50 **Keywords:**

51 Antiviral activity; herpes simplex virus; poly(amidoamine); attachment inhibitor; microbicide; sexually  
52 transmitted infections

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56 **1. Introduction**

57 Herpes Simplex Viruses type 1 and 2 (HSV-1 and HSV-2) are closely related pathogens belonging to  
58 the *Herpesviridae* family of DNA viruses that cause a wide variety of clinical manifestations in  
59 humans: HSV-1 is more frequently associated with oral and labial lesions, whereas HSV-2 typically  
60 infects genital mucosa. However, both viruses can infect both oral and genital regions, and the  
61 incidence of genital infections, particularly those caused by HSV-1, are on the increase [1]. Following  
62 primary infection, HSVs establish life-long latency in the neurons of the sensory ganglia proximal to  
63 the site of entry. Then, triggered by several viral and host factors, they periodically reactivate, descend  
64 into the primary site of infection, and replicate; leading to asymptomatic or symptomatic viral shedding  
65 [2]. Occasionally, HSV reactivation may result in life-threatening infections of the central nervous  
66 system [3, 4]. Both HSV-1 and HSV-2 infections are efficiently transmitted by sexual route and genital  
67 herpes is one of the most prevalent sexually transmitted infections (STIs) worldwide. Of note, genital  
68 ulcer disease, primarily associated with HSV-2 infection, increases the risk of HIV acquisition by  
69 damaging the genital mucosa; it induces local inflammation and the production of cytokines and  
70 chemokines that activate and recruit CD4<sup>+</sup> HIV target cells [5,6,7].

71 Indeed, in resource-limited countries where both viruses are highly prevalent, a high proportion of HIV  
72 infections can be ascribed to a pre-existing HSV-2 infection [8,9]. Strategies that prevent or treat HSV  
73 infections are expected to reduce rates of the sexual transmission of HIV and should therefore be part  
74 of HIV-1 prevention programs [7,10-12]. At present, there are a number of antiviral medications with  
75 activity against HSV-1 and HSV-2 and all are nucleoside analogues. These include acyclovir,  
76 penciclovir and their derivatives, valacyclovir, and famciclovir. However the effectiveness of antiviral  
77 therapy sometimes is limited by the development of antiviral resistance and relative high toxicity [13].

78 There are no vaccines currently available to prevent and treat HSV infection, but the pipeline is rich  
79 with candidates in various phases of development (for a comprehensive and update review see

80 reference 14) and studies directed at developing alternative approaches are underway; for instance,  
81 through the development of topical microbicides able to protect the genital mucosa from HSV (and  
82 HIV) acquisition and transmission. Easy-to-use microbicides, able to prevent most common sexually  
83 transmitted viruses, should be associated to PrEP strategy, that is mainly directed against HIV-1 and  
84 not able to prevent totally HSV infections [15].

85 On this regard, significant progresses to the development of effective microbicides against STI have  
86 been achieved with negatively charged polyanions and dendrimers of different formulations [16, 17-  
87 20]. Unfortunately, most of these compounds did not pass phase III clinical trials and one, a dendrimer  
88 with highly anionic charged branches developed by Starpharma Pty Ltd (Melbourne, Australia) has  
89 proved active against bacterial vaginosis in humans and is currently under testing for efficacy against  
90 STIs in Phase 3 trials [20,21].

91 Poly(amidoamine)s (PAAs) are a family of synthetic and highly biocompatible polymers with a highly  
92 versatile structure [22]. They are degradable polymers obtained by Michael-type polyaddition of  
93 primary or bis secondary amines to bisacrylamides. Many PAAs exhibit a combination of properties  
94 imparting them a considerable potential in the biomedical field. They are usually degradable in water at  
95 a rate depending on their structure. Therefore, if injected, they are bioeliminable [23]. Most PAAs are  
96 only moderately toxic despite their polycationic nature. According to a number of tests, the toxicity of  
97 most PAAs is significantly lower than that of poly-L-lysine (PLL) or polyethyleneimine (PEI) [24].  
98 Amphoteric PAAs carrying side carboxyl groups switch from a prevailingly anionic to a prevailingly  
99 cationic state in a relatively small pH interval. Those that at pH 7.4 are prevailingly anionic proved  
100 nontoxic and nonhemolytic. By contrast, those that at the same pH are prevailingly cationic showed  
101 significant toxicity and hemolytic activity. An interesting exception is the PAA named AGMA1,  
102 prepared by polyaddition of monoprotonated (4-aminobutyl)guanidine (agmatine) to BAC. The  
103 repeating unit of AGMA1 contains three ionizable groups, a strong acid (pKa 2.3), a medium-strength

104 base (pKa 7.4), and a strong base (pKa 12.1). AGMA1, an amphoteric, but prevailingly cationic  
105 polymer, proved nontoxic and nonhemolytic in vitro within the entire pH range tested (4.0-7.4). [25-  
106 27].

107 In a previous work, we screened a minilibrary of PAAs against a panel of DNA and RNA viruses to  
108 search for new antiviral chemical entities. AGMA1 selectively inhibited a panel of viruses, including  
109 HSV-1, HSV-2, and human papillomavirus-16 (HPV-16) [28], which exploit cell surface heparan  
110 sulfate proteoglycans (HSPGs) as attachment receptors. HSPGs consist of a protein core and  
111 glycosaminoglycan (GAG) side chains of unbranched sulfated polysaccharides, known as heparan  
112 sulfates, which are structurally related to heparin. The interaction between positively charged basic  
113 amino acids in HSV envelope and HPV capsid proteins and negatively charged sulfated/carboxyl  
114 groups of cellular HSPGs has been described [29-30] and is considered an attractive target for the  
115 development of microbicides able to block infection by sexually transmitted viruses [16].

116 Polycationic dendrimers have been so far developed mainly for the transfection of genetic material into  
117 eukaryotic cells for gene therapy, an approach that has been found however to be burden by the  
118 tendency of these dendrimers to bind to glycosaminoglycans of the cells surface [31]. With these  
119 premises, some HSPG-targeting polycationic dendrimers have been developed and assayed in vitro  
120 against different viruses, showing promising features: the peptide dendrimer SB105-A10, containing  
121 clusters of basic amino acids, proved to be a potent inhibitor of cytomegalovirus [32], HSV-1, HSV-2,  
122 a broad spectrum of genital HPV types, R5, and X4 HIV-1 and was found to exert its action mainly by  
123 binding to HS exposed on the cell surface [33-35]. Accordingly, we have recently demonstrated that  
124 AGMA1 interacts with immobilized heparin and cellular heparan sulfates, and that this, in turn, is able  
125 to prevent HPV attachment to the cell surface [36].

126 The aim of the present study was to elucidate the mechanism of action of AGMA1 against HSV  
127 infection and assess its antiviral potency and biocompatibility in preclinical models. The results show

128 AGMA1 to be a non-toxic inhibitor of HSV infectivity in cell cultures and human-derived vaginal  
129 epithelium. Moreover, it significantly reduced the burden of infection of HSV-2 genital infection in  
130 mice.

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## 133 **2. Materials and Methods**

### 134 *2.1. Cells and viruses*

135 African green monkey kidney cells (Vero) (ATCC CCL-81) were purchased from American Type  
136 Culture Collection (ATCC; Manassas, VA). The culture medium was Eagle's minimal essential  
137 medium (E-MEM) (Gibco/BRL, Gaithersburg, MD) supplemented with heat-inactivated 10% fetal calf  
138 serum (FCS) (Gibco/BRL) and 1% antibiotic-antimycotic solution (Zell Shield, Minerva Biolabs  
139 GmbH, Berlin, Germany). The neurovirulent strains LV [37] and MS (ATCC VR-540) of HSV-1 and  
140 HSV-2, respectively, were used for most *in vitro* studies and all *in vivo* experiments. Both strains were  
141 sensitive to Acyclovir (ACV). Two laboratory HSV-2 strains (ACV-r1 and ACV-r2) with phenotypic  
142 resistance to ACV were generated by serial passage of the reference strain in the presence of increasing  
143 ACV concentrations. The fluorescence virus, HSV-1(GFP), encoding GFP fused to the gH envelope  
144 glycoprotein was kindly provided by Dr. E. Caselli, University of Ferrara, Italy. To generate viral  
145 stocks, semiconfluent T175 flasks of Vero cells were propagated in complete E-MEM and inoculated  
146 with 1 PFU/cell of virus. After 6 h, cells were fed with fresh E-MEM and cultured until cell lysis.  
147 Culture fluids were spun at 1200-g, and the pellets frozen-thawed three times to release intracellular  
148 virions. Supernatants were then clarified, pooled, and ultracentrifuged to concentrate the virus as  
149 previously described [38]. Pelleted virus was resuspended in 1/100 of the initial volume in saline and  
150 and stored in small aliquots at -80°C until use. Viral titer of randomly picked frozen aliquots was  
151 determined *in vitro* by plaque assay on Vero cells.



152 2.2. *EpiVaginal<sup>TM</sup> tissues*

153 The EpiVaginal Tissue Model (VEC-100/VEC-100-FT) was purchased from MatTek Corporation  
154 (Ashland, MA, USA) and consists of Human 3-D Vaginal-Ectocervical Tissues cultured to form a  
155 multilayered and highly differentiated tissue closely resembling the epithelial architecture found *in*  
156 *vivo*. According to the supplier's instructions, EpiVaginal cultures were seeded with the apical surface  
157 exposed to air in 6-well plates containing 0.9 ml MatTek assay medium (VEC-100-ASY) per well.  
158 Plates were incubated overnight at 37°C in 5% CO<sub>2</sub>.

159 2.3. *Animals*

160 Inbred C57Bl/6 mice were purchased from Harlan Italy (Correzzana, Milan, Italy) and housed and bred  
161 in a Biosafety Level 3 animal facility approved for mice detention and reproduction. Mice were  
162 maintained on a 12/12 hour dark/light cycle and handled according to European (2010/63/EU) and  
163 Italian (26/2014) guidelines. Since age and estrous cycle influence susceptibility to genital herpes and  
164 disease course [39], all *in vivo* experiments were carried out in mice of 11 weeks of age and with their  
165 estrous cycle synchronized with 2 mg depot medroxyprogesterone acetate (Depo-Provera) inoculated  
166 subcutaneously 5 days before infection. All manipulations were performed under deep anesthesia with  
167 2 ml/hg 2,2,2-tribromoethanol inoculated intraperitoneally. The project was approved by the University  
168 of Pisa Ethical Committee for Animal Research.

169 2.4. *Reagents*

170 AGMA1 and biotinylated AGMA1(b-AGMA1) were prepared as reported elsewhere [28,36]. AGMA1  
171 has an average molecular weight of 10100 and a polydispersity of 1.25. It is very soluble in water at all  
172 pH values, but hardly soluble in most organic solvents. It is amphoteric with isoelectric point 10.2. The  
173 pKa values of the carboxyl-, guanidine- and ter-amine- groups present in AGMA1 repeating unit are  
174 2.25, 7.4 and >12, respectively. Therefore, in the pH interval 5-10 each unit carries both one positive  
175 and one negative charge, whereas the tert-amine group is >90% protonated, that is, cationic at pH 6 and

176 approximately 50% protonated at pH 7.4 [25-27,36]. Since AGMA1 is available in polydisperse  
177 preparations with an average molecular mass not unequivocally determinable, we quantitatively refer to  
178 the compound in  $\mu\text{g/ml}$ . Acyclovir (ACV), 2,2,2-tribromoethanol, gelatin, horseradish peroxidase-  
179 labeled streptavidin, methylcellulose, crystal violet, sodium dodecyl sulfate (SDS), NP-40, sodium  
180 deoxycholate, a cocktail of protease inhibitors, Tween 20, glycine and Triton X-100 were purchased  
181 from Sigma-Aldrich (Milan, Italy). Conventional heparin (13.6 kDa) was from Laboratori Derivati  
182 Organici S.p.A. (Milan, Italy). Heparinase II, a glycosidase that digests the GAG moiety of HSPGs  
183 [40] was from Sigma-Aldrich (St Louis, MO). Depot medroxyprogesterone acetate (Depo-Provera) was  
184 purchased from Pfizer Italia (Latina, Italy). Chromogenic substrate ABTS was from Kierkegaard &  
185 Perry Laboratories (Gaithersburg, MD). The anti-HSV-1/2 ICP27 MAb (8.F.137B) and the anti-HSV-  
186 1 ICP8 MAb (clone 10A3) were from Abcam (Cambridge, UK). The anti-HSV-1/2 gD MAb (clone  
187 2C10) was from Virusys Corporation (Taneytown, MD). The anti-actin MAb was from Chemicon  
188 International (Billerica, MA). The anti-mouse Ab conjugated to horseradish peroxidase, used in  
189 immunoblotting, was from Amersham Italia (Milan, Italy). The rabbit polyclonal anti-HSV-2 antibody  
190 and the biotin-free polymer-conjugated secondary antibody, used in immunohistochemistry, were from  
191 Dako (Glostrup, Denmark). Cyclophosphamide was purchased from Baxter (Rome, Italy). All the other  
192 reagents and solvents are commercially available and used as received.

### 193 *2. 5. Preparation and characterization of AGMA1 solution*

194 To prepare the polymer solution, a weighed amount of AGMA1 was added to a 2.4 % glycerol  
195 aqueous solution in water. The pH of the solution was corrected to 5.0 using a 0.1 M NaOH. AGMA1  
196 solution was characterized measuring osmolarity and viscosity values using a semi-micro osmometer  
197 K-7400 (Knauer) and a capillary viscosimeter (Ubeholde) respectively. These parameters were  
198 determined just prepared and after three months.

### 199 *2.6. Cell viability assay*

200 Confluent Vero cell cultures in 96-well plates were incubated with MTS [3-(4,5-dimethylthiazol-2-yl)-  
201 5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] at different concentrations and in  
202 triplicate. Cells were cultured as for the *in vitro* antiviral assays and viability was determined using the  
203 CellTiter 96 Proliferation Assay Kit (Promega, Madison, WI, USA) according to the manufacturer's  
204 instructions. Absorbances were measured using a Microplate Reader (Model 680, BIORAD) at 490 nm.  
205 The effect on cell viability of AGMA1 tested at different concentrations was expressed as a percentage,  
206 by comparing the absorbances of treated cells with those of cells incubated with culture medium alone.  
207 The 50% cytotoxic concentrations (CC<sub>50</sub>s) and 95% confidence intervals (CIs) were determined using  
208 Prism software (Graph-Pad Software, San Diego, CA).

### 209 *2.7. AGMA1 binding to Vero cells assays*

210 Monolayers of Vero cells in 96-well plates were incubated for 2 hours at 4°C in phosphate-buffered  
211 saline (PBS) containing 0.1 mg/ml CaCl<sub>2</sub>, 0.1 mg/ml MgCl<sub>2</sub>, and 0.1% gelatin, with sub-saturating  
212 concentrations of b-AGMA1 (0.01 µg/mL or 0.1 µg/ml) in the absence or presence of heparin (10  
213 µg/ml). At the end of incubation, cells were washed with PBS, and the amount of cell-associated b-  
214 AGMA1 was determined with horseradish peroxidase-labeled streptavidin (1/5,000) and the  
215 chromogenic substrate ABTS. In some experiments, cell monolayers were washed with PBS containing  
216 2 M NaCl, a treatment known to remove cationic polypeptides from cell surface HSPGs [41].  
217 Alternatively, cells were incubated with heparinase II (15 mU/ml) for 1 hour at 37°C (an experimental  
218 condition demonstrated to efficiently remove HSPGs from the epithelial cells surface [36]) or left  
219 untreated before the binding assay.

### 220 *2.8. In vitro antiviral activity assays*

#### 221 *2.8.1. HSV virus yield reduction assay*

222 The assay is finalized to quantify the antiviral effect of compound testing its effect on the production of  
223 infectious viruses. Vero cells were seeded in 24-well plates at a density of 10 x 10<sup>4</sup> cells/well and

224 infected in duplicate with HSV-1 or HSV-2 at a multiplicity of infection (MOI) of 0.01 plaque-forming  
225 units (PFU)/cell and in the presence of serial dilutions of the compound. Following adsorption at 37°C  
226 for 2 hours, the virus inoculum was removed and cultures were grown in the presence of AGMA1 until  
227 control cultures displayed extensive cytopathology. Supernatants were harvested and pooled as  
228 appropriate 48-72 hours after infection and cell-free virus infectivity titers were determined in duplicate  
229 by plaque assay in Vero cell monolayers. The end-point of the assay was the effective concentration of  
230 compound that reduced virus yield by 50% (EC<sub>50</sub>) compared to untreated virus controls.

### 231 2.8.2. *HSV plaque reduction assay*

232 The assay is finalized to quantify the antiviral effect of compound testing its ability to reduce the  
233 number of viral plaques. Vero cells were seeded in 24-well plates at a density of 10 x 10<sup>4</sup> cells/well  
234 and infected at 0.001 MOI in the presence of different concentrations of compound for 2 hours at 37°C,  
235 washed, and then overlaid with 1.2% methylcellulose. After 24 hours (HSV-2) or 48 hours (HSV-1) of  
236 incubation at 37°C, cells were fixed and stained with 0.1% crystal violet in 20% ethanol and viral  
237 plaques were counted. The concentration of compound that reduced plaque formation by 50% (EC<sub>50</sub>)  
238 was determined by comparing treated and untreated wells. PRISM 4 software (GraphPad Software, San  
239 Diego, California, U.S.A.) was used to fit a variable slope-sigmoidal dose-response curve and calculate  
240 EC<sub>50</sub> values. A selectivity index (SI) was calculated by dividing the CC<sub>50</sub> by the EC<sub>50</sub> value.

### 241 2.8.3. *Immunoblotting of viral proteins*

242 The assay is finalized to evaluate the ability of AGMA1 to inhibit the HSV-1 protein expression in  
243 treated-, infected- extracts of Vero cells. Whole-cell extracts were prepared by resuspending pelleted  
244 cells in lysis buffer containing 150 mM NaCl, 50 mM Tris-Cl (pH 8), 0.1% SDS, 1% NP-40, 0.5%  
245 sodium deoxycholate and a cocktail of protease inhibitors. Soluble proteins were collected by  
246 centrifugation at 15,000g. Supernatants were quantified and stored at -80°C as described [42]. For  
247 immunoblotting, proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and

248 transferred to Immobilon-P membranes (Millipore). Membranes were then incubated with blocking  
249 buffer consisting of 5% nonfat dry milk in 10 mM Tris-Cl (pH 7.5)–100 mM NaCl–0.1% Tween 20  
250 and immunostained with anti-HSV-1/2 MAbs against ICP27, ICP8 and gD proteins, and the anti-actin  
251 MAb. Immunocomplexes were detected using a sheep anti-mouse immunoglobulin Ab conjugated to  
252 horseradish peroxidase, and visualized using enhanced chemiluminescence (Super Signal; Pierce),  
253 according to the manufacturer's instructions.

#### 254 *2.8.4. Virus inactivation assay*

255 The assay evaluates the virucidal activity of compound. AGMA1 (33 µg/ml) was added to aliquots of  
256 10<sup>5</sup> PFU HSV-1 or HSV-2 and incubated at either 4 or 37°C for 2 hours. After incubation, samples  
257 were titrated on Vero cells at high dilutions, at which the compound was not active.

#### 258 *2.8.5. Cell pre-treatment assay*

259 The assay evaluates the antiviral activity of compound when administered before infection. Cells were  
260 exposed to different concentrations of AGMA1 in a 24-well plate at 4°C or 37°C for two hours. After  
261 washing, cells were infected with HSV-1 or HSV-2 at 0.001 MOI for two hours, washed and treated as  
262 for plaque reduction assay.

#### 263 *2.8.6. Attachment assay*

264 The assay evaluates the ability of compound to inhibit the attachment of virus to cells. The assay was  
265 performed as described previously [43]. Prechilled Vero cells were treated with AGMA1 or heparin for  
266 30 minutes at 4°C and then infected with HSV-1 or HSV-2 at 0.004 MOI for 2 hours at 4°C in presence  
267 of the compound. After three washes with cold MEM to remove unbound virus, cells were overlaid  
268 with 1.2% methylcellulose and shifted to 37°C. After 24 hours (HSV-2) or 48 hours (HSV-1) of  
269 incubation, cells were stained and viral plaques counted. Cells infected in absence of compound were  
270 arbitrarily set at 100% of infection and served as positive control. To examine viral attachment without

271 entry, cells were incubated at 4°C and treated for two minutes with cold acidic glycine (100mM  
272 glycine, 150 mM NaCl, pH 3) to inactivate attached virus, resulting in 100% inhibition of infection.

#### 273 2.8.7. *Entry assay*

274 The assay evaluates the ability of compound to inhibit the entry of virus into cells. HSV-1 or HSV-2 at  
275 0.004 MOI was adsorbed for 2 hours at 4°C on prechilled confluent Vero cells. Cells were then washed  
276 with cold MEM three times to remove unbound virus, treated with different concentrations of AGMA1  
277 or Heparin, and incubated for three hours at 37°C. Outer virions were inactivated with acidic glycine  
278 for 2 minutes at room temperature as described [43]. Cells were washed with warm medium three  
279 times and treated as for plaque reduction assay.

#### 280 2.8.8. *Binding assay*

281 The assay evaluates the ability of compound to inhibit the binding of virus to cells. Cells were pre-  
282 incubated with AGMA1 or Heparin for 30 minutes or left untreated (control) at 4°C and then infected  
283 for 2 hours at 4°C with 5 MOI HSV-1 as described [44]. Cells were then washed four times with PBS  
284 and lysed as described below in Immunoblotting section. HSV was detected with a MAb against the  
285 Glycoprotein D. Actin was stained as input control.

#### 286 2.8.9. *Post-entry infection assay*

287 The assay evaluates the antiviral activity of compound when administered after infection. Vero cells  
288 monolayers in 96-well plate were infected with HSV- 1(GFP), HSV-1 or HSV-2 for two hours at 37°C,  
289 followed by two gentle washes to remove unbound virus. Increasing AGMA1 concentrations (at 0 hour  
290 post-infection) or 100 µg/ml (at 1, 2, 3, 6 hours post-infection) were then added to cultures in 1.2%  
291 methylcellulose medium. After incubation at 37°C for 24 hours (HSV-2) or 48 hours (HSV-1), cells  
292 were fixed and stained with 0.1% crystal violet in 20% ethanol to count the number and measure the  
293 size of viral plaques. Plaque size was measured with a Leica inverted microscope equipped with a  
294 Bresser MikroCam microscope camera and MikroCamLab software (Rhede, Germany). Plaques of

295 HSV-1(GFP) were analyzed with an inverted Zeiss LSM510 fluorescence microscope and measured  
296 using with ImageJ software. To assess the effect of AGMA1 added after infection, a virus yield  
297 reduction assay was performed and EC<sub>50</sub> determined by comparing drug-treated and untreated wells, as  
298 described above.

### 299 *2.9. Antiviral assay at acidic pHs*

300 To evaluate the stability of AGMA1 at different pHs [45], the compound was incubated in phosphate-  
301 buffered saline solutions of pH 3, pH 5, pH 7, for 2 hours at 37°C as previously described [45].  
302 Thereafter, different concentrations of pH-treated AGMA1 were incubated with confluent Vero cell  
303 monolayers for 1 hour at physiological pH. Cells were then infected at physiological pH with HSV-2 at  
304 an MOI of 0.001 for two hours, washed and treated as for plaque reduction assay.

### 305 *2.10. Assays on EpiVaginal™ tissues*

#### 306 *2.10.1. Viability assay*

307 EpiVaginal tissues were evaluated using the MTT ET-50 Tissue Viability Assay (MatTek  
308 Corporation), according to manufacturer's instructions. AGMA1 (100 µg/ml) was added to the cell  
309 culture insert placed on top of the EpiVaginal samples and incubated for 30 minutes, 1, 4, and 18 hours  
310 in duplicate. At the end of incubation, any liquid remaining on top of the tissue was decanted and  
311 inserts were washed with PBS to remove any residual material. Tissues were then processed according  
312 to the MTT protocol and read at 570 nm using an ELISA plate. Tissues were incubated with 1.0%  
313 Triton X-100 and ultrapure water as positive and negative controls, respectively. The ET-50 value  
314 refers to the time required to reduce tissue viability to 50% and was determined using Prism software.  
315 According to the manufacturer an ET<sub>50</sub> value > 18h indicates that a compound does not cause vaginal  
316 irritation and can be used for feminine hygiene products.

#### 317 *2.10.2. Cytotoxicity assay*

318 Any cytotoxic effect of AGMA1 (100 µg/ml) on EpiVaginal tissues was evaluated by analyzing the  
319 release of lactate dehydrogenase (LDH) into culture medium, which increases in a manner that is  
320 proportional to the number of dead cells. The LDH cytotoxicity assay was performed according to  
321 manufacturer's protocol (TAKARA bio inc, Japan).

### 322 *2.10.3. Analysis of inflammatory response*

323 This was evaluated by monitoring cytokine IL-1 $\alpha$  release into the culture medium of EpiVaginal  
324 tissues treated with AGMA1 (100 µg/ml) for 30 minutes, 1, 4, and 18 hours, as previously reported  
325 [46]. After incubation, the concentration of IL-1 $\alpha$  in the culture medium was measured using the IL-1  
326 alpha ELISA KIT, according to the manufacturer's instructions (Bender Medsystem). The  
327 concentration of IL-1 $\alpha$  was calculated by interpolation from a standard calibration curve.

### 328 *2.10.4. Antiviral assays*

329 EpiVaginal Tissue cultures were pre-incubated with 100 µl medium containing 100 µg/ml AGMA1.  
330 Medium was applied to the apical surface and cells were incubated at 37°C for 2 hours. After pre-  
331 incubation, the medium was removed and cultures were infected with 1000 pfu HSV-2 at 37°C for 2  
332 hours in the presence of AGMA1. Cultures were washed apically with 100 µl medium, incubated at  
333 37°C, and fed each day via the basolateral surface with 0.9 ml medium. Viruses were harvested at 24,  
334 48, 72 and 96 hpi by adding 100 µl medium per well to the EpiVaginal Tissue apical surface that was  
335 allowed to equilibrate for 30 minutes. Viral suspension was then collected and stored at -80°C until  
336 viral titers were determined by plaque assay in Vero cell monolayers. Harvesting was performed daily.

### 337 *2.10.5. Detection of HSV-2 by immunohistochemistry*

338 HSV-2 was detected on EpiVaginal cultures by immunohistochemistry using a polyclonal anti-HSV-2  
339 antibody. Briefly, EpiVaginal tissue cultures were fixed in buffered formalin, properly oriented, and  
340 embedded in paraffin together with adherent collagen membranes. Tissue sections were incubated with  
341 the anti-HSV-2 antibody or stained with hematoxylin and eosin. Tissues were processed for antigen



342 retrieval in citrate buffer using a dedicated pressure cooker (1 cycle for 5' at 125°C, followed by 10  
343 sec. at 90°C). After incubation with the primary antibody (1:500 dilution), the reaction was visualized  
344 using a biotin-free polymer-conjugated secondary antibody. In positive samples, the antibody showed  
345 cytoplasmic and nuclear immunoreactivity, mostly recognizable in cells of the superficial layers.  
346 Several sections were analyzed for each experimental condition.

347

## 348 *2.11. Analysis of antiviral activity in vivo*

### 349 *2.11.1. Titration of viral stocks in vivo*

350 All animals were treated in parallel and grouped at random. Eleven-week-old mice were infected via  
351 vagina following estrous cycle synchronization. To facilitate absorption, vaginas were pre-swabbed  
352 with a dry tipped swab immediately prior to instillation of 10-fold dilutions of viral stocks. Animals  
353 were then examined daily for clinical signs of infection that were graded according to a five-point  
354 scale: 0, no signs; 1, slight genital erythema and/or edema; 2, papules, ulcers and/or swelling; 3, fused  
355 ulcers, purulent genital lesions and/or hind limb paralysis; 4, death [47]. Titrations were performed  
356 using 5-8 animals/virus dilution. Lethal dose 50% (LD<sub>50</sub>) was calculated using the Reed-Müench  
357 method. One and 10 LD<sub>50</sub> roughly corresponded to 10<sup>6</sup> and 10<sup>8</sup> PFU, respectively. Animals that  
358 survived despite paralysis or other irreversible lesions were euthanized by cervical dislocation under  
359 anesthesia.

### 360 *2.11.2. Analysis of AGMA1 efficacy*

361 The antiviral activity of AGMA1 against HSV-1 and HSV-2 vaginal infections was assessed by  
362 dispensing AGMA1 (1 mg/ml) in a 2.4 % glycerol aqueous solution, as described previously. AGMA1  
363 (10 µl) was applied to pre-swabbed vaginas at varying time-points prior to infection (15 seconds to 30  
364 minutes). The “Vehicle” group, referring to the glycerol aqueous solution used to prepare the AGMA1  
365 solution, was treated the same way. Infections were performed with 1, 10 and 100 LD<sub>50</sub>. Animals were

366 monitored for clinical signs of infection for about 4 weeks post-infection. Immunosuppression was  
367 achieved with an intraperitoneal bolus of 350 mg/kg cyclophosphamide that depleted the circulating  
368 lymphocytes in a mouse by approximately 90% within 1 day, as described [38].

### 369 *2.11.3. Detection of HSV-2 DNA genome in nervous tissues*

370 Sacral nerves and genital ganglia were protease digested and the DNA extracted using the QIAamp  
371 DNA mini kit, as recommended by the manufacturer (Qiagen, Milan, Italy). Molecular analysis was  
372 carried out by performing a HSV-2 specific nested polymerase chain reaction (PCR) as previously  
373 described [48]. The outer and inner PCR primer pairs were: forward 6AF (5'-  
374 TCAGCCCATCCTCCTTCGGCAGTA-3') – reverse 6BR (5'-GATCTGGTACTCGAATGTCTCCG-  
375 3') and forward 6CF (5'-AGACGTGCGGGTCGTACACG-3') – reverse 6DR (5'-  
376 CGCGCGGTCCCAGATCGGCA-3'), respectively. The amplification profile (denaturation: 94°C for 2  
377 min; cycling: 94°C for 1 min, 56°C for 1 min, and 72 °C for 1 min – 5 cycles; cycling: 94°C for 45 sec,  
378 56°C for 30 sec, 72 °C for 1 min – 40 cycles; final extension 72°C for 15 min) was the same for both  
379 PCRs except that the second amplification profile was diminished from 40 to 30 cycles. Amplicons  
380 were examined by agarose gel (1%) electrophoresis.

### 381 *2.12. Statistical analysis*

382 All data were analyzed using GraphPad Prism 5.00 (GraphPad Software). Infectivity and measurement  
383 of plaque sizes in the presence and absence of AGMA1 were compared by one-way analysis of  
384 variance (ANOVA) followed by a Bonferroni test if *P* values showed significantly differences. Results  
385 were expressed as means ± standard deviations. Results of the direct binding test of the compound to  
386 the cell surface, were analyzed by Student's *t* test. The Fisher exact test was applied to evaluate the *in-*  
387 *vivo* test results. Differences in number of disease-free animals of AGMA1 vs vehicle and naïve groups  
388 were assessed for statistical significance using heterogeneity of contingency tables. A value of  $p < 0.05$   
389 was considered significant.

390 **3. Results.**

391 *3.1 AGMA1 solution characterization*

392 The AGMA1 solution showed a pH = 5.0, a viscosity = 1.07 cP and an osmolarity = 340 mOs, values  
393 suitable for a vaginal application. These parameters did not change after three months from the  
394 preparation.

395 *3.2. Antiviral activity of AGMA1 against HSV-1, HSV-2, and ACV-resistant strains in vitro*

396 AGMA1 was evaluated *in vitro* for antiviral activity against HSV-1, HSV-2, and two HSV-2 ACV-  
397 resistant strains by plaque reduction assays. Assays were performed by incubating cells in the presence  
398 of decreasing concentrations of compound (ranging from 100 µg/mL to 0.13 µg/mL) during and after  
399 viral adsorption. As shown in Table 1, AGMA1 was active against wild-type HSV-1 and HSV-2 with  
400 EC<sub>50</sub> values of 3.05 and 1.3 µg/ml, respectively, similar to previously reported values [28]. As  
401 expected, the resistant strains exhibited elevated EC<sub>50</sub>s for ACV [260 µM and 319 µM (58.5 µg/ml and  
402 71 µg/ml), respectively (data not shown in Table 1)]. By contrast, they were susceptible to AGMA1  
403 inhibitory activity. Microscopic inspection and cell viability assays showed that AGMA1 was not toxic  
404 to Vero cells up to the highest concentration tested (300 µg/ml), demonstrating that the antiviral  
405 activity was not a consequence of cell toxicity.

406

407 **Table 1. AGMA-1 antiviral activity against wild-type and ACV resistant HSV strains**

Virus	EC <sub>50</sub> <sup>a</sup> (µg/ml)	CC <sub>50</sub> <sup>a</sup> (µg/ml)	SI <sup>b</sup>
HSV-1	3.05 ± 1.22	> 300	> 98.36
HSV-2	1.30 ± 1.15	> 300	> 230.76
HSV-2 ACV-r1	0.69 ± 1.34	> 300	> 434.78
HSV-2 ACV-r2	1.00 ± 1,98	> 300	> 300.00

408

409 <sup>a</sup> The EC<sub>50</sub> (effective compound concentration that reduced viral plaque formation by 50%) and the  
410 CC<sub>50</sub> (50% cytotoxic concentration) are expressed as the mean (µg/ml) ± S.D. of three independent  
411 experiments. <sup>b</sup> SI= selectivity index, determined by the ratio of CC<sub>50</sub> to EC<sub>50</sub>

412

413 The antiviral effect of AGMA1 was confirmed further by means of the yield reduction assay (see  
414 Materials and Methods section), a stringent test that allows multiple cycles of viral replication to occur  
415 before measuring the production of infectious viruses. The dose-response curves reported in Figure 1  
416 show that AGMA1 effectively reduces the HSV-1 and HSV-2 yield, with EC<sub>50</sub> values equal to 0.74  
417 µg/ml and 1.14 µg/ml, respectively.

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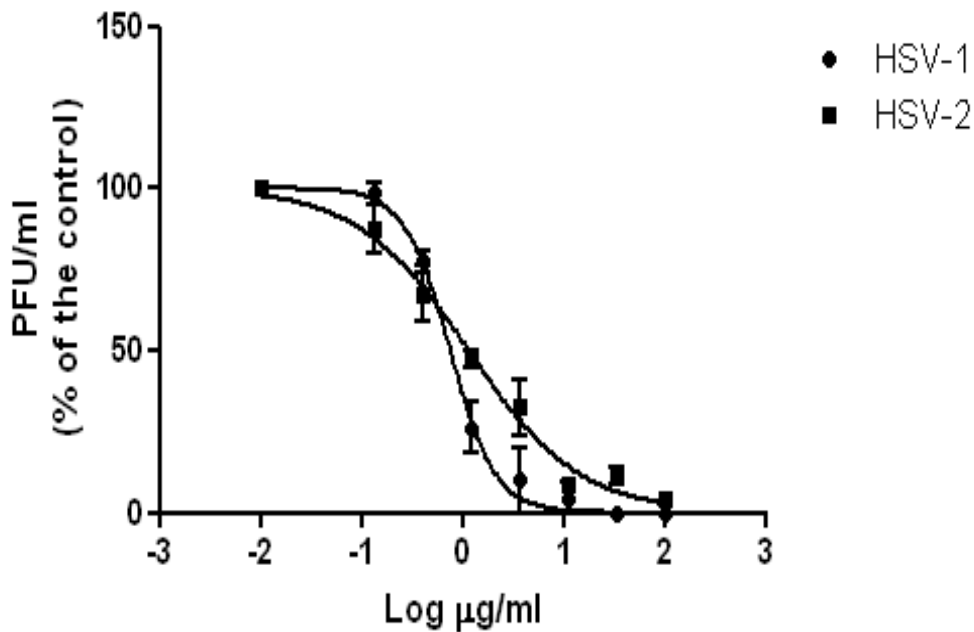
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431 **Figure 1. AGMA1 reduces virus yield in Vero cells.** Vero cells were infected at a MOI of 0.01 with  
432 clinical isolates of HSV-1 or HSV-2 and treated with increasing doses of AGMA1 during viral  
433 adsorption. Cells were exposed to the drug concentrations until an extensive viral cytopathic effect was  
434 observed in the untreated controls. The supernatants from cell suspensions were assayed for their

435 infectivity by standard plaque reduction assay. Values are the means  $\pm$  SD of three separate  
436 experiments performed in duplicate.

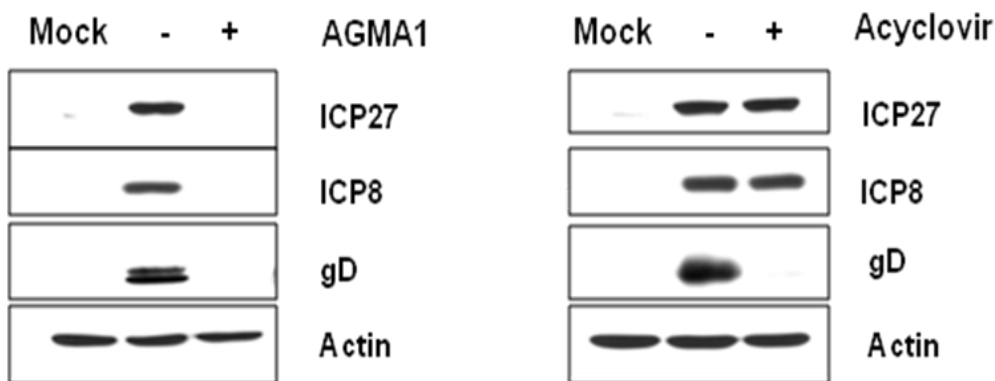
437

438

### 439 3.3. Investigation of AGMA1 mechanism of action

440 AGMA1's activity against ACV-resistant strains, as summarized in Table 1, may suggest that AGMA1  
441 acts through a different mechanism of action to that of ACV. To substantiate this hypothesis, the effect  
442 of AGMA1 and ACV on the expression of immediate-early, early and late viral proteins (ICP27, ICP8,  
443 and gD, respectively) was investigated by western blotting. As shown in Fig. 2, ACV completely  
444 suppressed the expression of the late protein gD. This finding was expected as ACV is a known  
445 inhibitor of viral replication, an event that occurs prior to late gene expression. In contrast, in addition  
446 to gD, AGMA1 also completely inhibited the expression of early viral proteins, indicating that  
447 AGMA1 may either inactivate the virus particle or inhibit an early step of the viral replication cycle  
448 that immediately precedes early gene expression (i.e. virus attachment or entry).

449



450

451

452 **Figure 2. AGMA1 inhibits early and late HSV gene expression.** Vero cells were infected with HSV-  
453 1 in the absence or presence of AGMA1 or Acyclovir during infection. Mock: uninfected cells.

454 Proteins were extracted and analyzed by western blotting using the following antibodies: anti-ICP27,  
 455 anti-ICP8, and anti-gD. Actin served as an internal control.

456

457 We first investigated whether the antiviral action of AGMA1 is exerted via the direct inactivation of  
 458 HSV-1 or HSV-2 virus particles. To this end, we performed the virus inactivation assay described in  
 459 section 2.8.4. As reported in Table 2, the virus titers of samples treated with AGMA1 did not  
 460 significantly differ from those determined for untreated samples ( $P < 0.05$ ), indicating that the compound  
 461 does not inactivate extracellular virus particles.

462

463 **Table 2. Effect of AGMA1 on virus infectivity.**

Incubation condition		AGMA1 <sup>a</sup>	Virus Titer (PFU/ml) <sup>b</sup>	
Temp (°C)	Duration (h)		HSV-1	HSV-2
37	0	-	$4.00 \times 10^5$	$1.19 \times 10^5$
37	0	+	$3.30 \times 10^5$	$1.68 \times 10^5$
37	2	-	$4.19 \times 10^4$	$4.50 \times 10^4$
37	2	+	$3.70 \times 10^4$	$3.54 \times 10^4$
4	2	-	$6.02 \times 10^5$	$1.57 \times 10^5$
4	2	+	$9.24 \times 10^5$	$9.82 \times 10^5$

464 <sup>a</sup> Concentration : 33 µg/ml

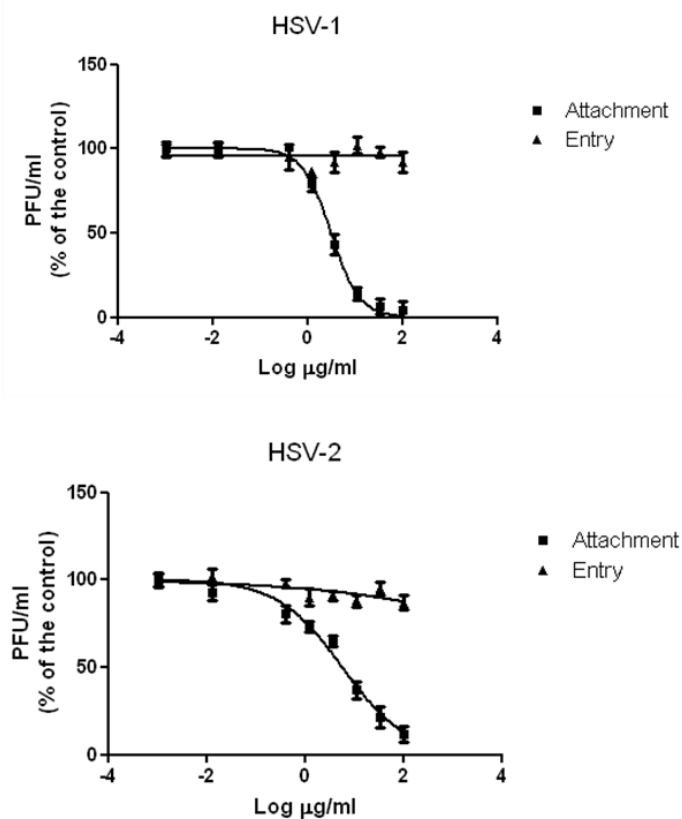
465 <sup>b</sup> Virus titers at high dilutions at which the compound was not active. The titers are mean values for experiments  
 466 performed in triplicate.

467

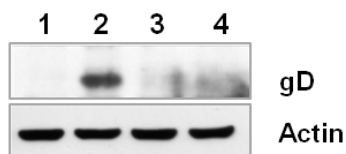
468 Next, we investigated whether AGMA1 could interfere with the early stages of viral infection. In a first  
 469 series of experiments, the viral attachment assays described in section 2.8.6. were performed. As shown  
 470 in Fig.3A, under these experimental conditions AGMA1 inhibited HSV1 and HSV-2 infection with  
 471 EC<sub>50</sub>s (3.09 µg/ml and 5.66 µg/ml, respectively) that are comparable to those measured in the classic  
 472 viral plaque assay suggesting that the antiviral activity of AGMA1 depends on its capacity to inhibit the  
 473 attachment of the viruses to the cell surface. To substantiate this interpretation, cells from the  
 474 attachment assay were lysed after washing and processed for immunoblotting, performed using a MAb

475 directed against the viral glycoprotein gD, to detect the amount of viral particles bound to the cell  
476 surface. Heparin was used in this assay as a positive control, being a known inhibitor of HSV  
477 attachment, which acts by competing with cell-surface HSPGs for virus binding [49,50]. As reported in  
478 Figure 3B, both AGMA1 and heparin inhibited HSV-1 infection. In a second series of experiments, we  
479 explored the ability of AGMA1 to prevent HSV entry using the entry assay described at section 2.8.7.  
480 As reported in Figure 3A, AGMA1 did not affect the capacity of prebound HSV-1 or HSV-2 virus to  
481 infect cells at any dose examined. Taken together, these data indicate that AGMA1 does not inactivate  
482 HSV-1 or HSV-2; instead it acts by inhibiting virus attachment, but not entry.

A



B



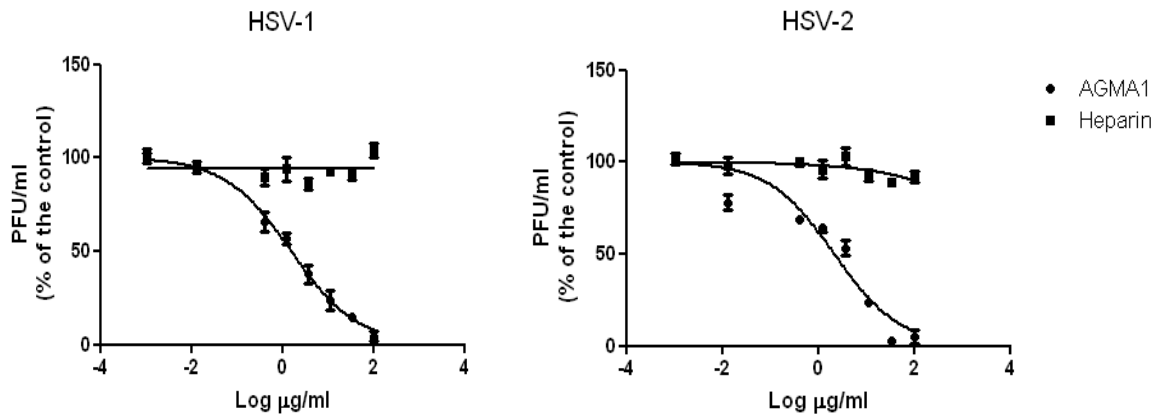
483

484 **Figure 3. AGMA1 prevents attachment but not entry of HSV to target cells.** (A) Anti-HSV-1  
485 activity and anti-HSV-2 activity in attachment and entry assays by Plaque Reduction Assay.  
486 Attachment: cells were pretreated with AGMA1 for 30 minutes at 4°C and then infected for 2 hours at  
487 4°C. Entry: prechilled cells were infected with viruses for 2 hours at 4°C, then washed and treated with  
488 AGMA1 for 3 hours at 37°C; unpenetrated virions were inactivated by acidic glycine treatment. Values  
489 are the means  $\pm$  SD of three separate experiments performed in duplicate. (B) Binding assay: cells were  
490 preincubated with AGMA1 or heparin (100  $\mu$ g/ml) for 30 min and then infected at an MOI of 5 with  
491 HSV-1 for 2 hours. Columns: (1) uninfected; (2) infected; (3) infected in presence of heparin; (4)  
492 infected in the presence of AGMA1. Attached virions were detected by Immunoblotting, using a Mab  
493 directed against the glycoprotein gD. Actin served as an internal control.

494

495 Antiviral compounds that block virus attachment to target cells mainly act by binding to (and  
496 sequestering) virions in the extracellular environment [16] or by binding (and masking) virus receptors  
497 on the surface of target cells [33]. To explore the possibility that AGMA1 acts directly on Vero cells,  
498 the pre-treatment assay described at section 2.8.5. was performed. As reported in Fig. 4, AGMA1  
499 inhibited infection by both HSVs in a dose response manner with  $EC_{50}$ s equal to 1.54  $\mu$ g/ml and 2.14  
500  $\mu$ g/ml for HSV-1 and HSV-2, respectively. As expected, heparin (that acts by binding directly to the  
501 virus) was inactive under these experimental condition. Taken together, these data suggest that  
502 AGMA1 reduces cells susceptibility to virus infection by tethering to the cell surface and possibly  
503 masking HSV receptors.





504

505 **Figure 4. Vero cells pre-treated with AGMA1 are less susceptible to HSV infection.** Cells were  
 506 pretreated with AGMA1 or heparin for 2 hours at 37°C before viral adsorption period. Values are the  
 507 means  $\pm$  SD of three separate experiments performed in duplicate.

508

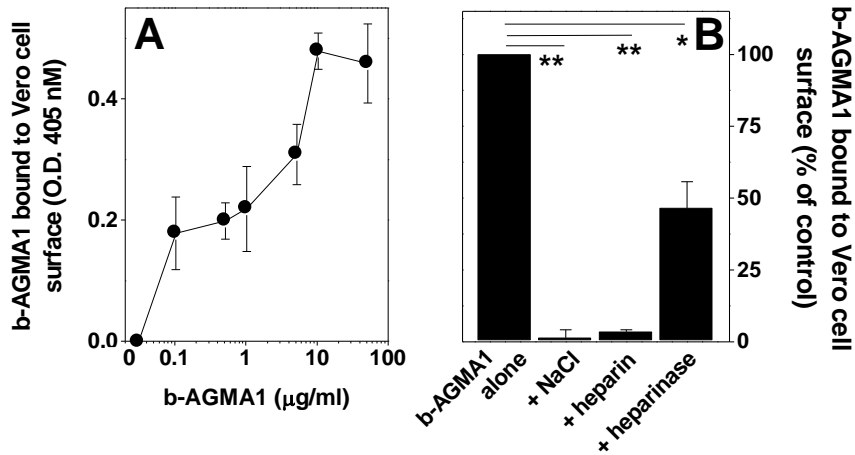
509 *3.4. AGMA1 interacts with the cell surface via HSPGs*

510 Based on the above results we investigated the effective capacity of AGMA1 to bind to the cell  
 511 surface of Vero cells *via* HSPGs (see methods, paragraph 2.7). As shown in Fig. 5A, AGMA1  
 512 effectively binds to the surface of Vero cells in a dose-dependent and saturable manner. Moreover,  
 513 binding could be disrupted by washing with 2 M NaCl (a treatment known to disrupt the binding of  
 514 cationic molecules to HSPGs [41]) and it could be prevented by a molar excess of heparin (a  
 515 structurally related antagonist of HSPGs) and by cell treatment with heparinase (an enzyme that  
 516 removes the heparan sulfate chains from cell surface-associated HSPGs) (Fig. 5B). Taken together,  
 517 these results provide strong evidence that AGMA1 interacts with the cell surface via HSPGs. However,  
 518 the partial inhibition of AGMA1 binding to heparinase II-treated cells also suggest that other receptors  
 519 beside HSPG may bind AGMA1.

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530 **Fig. 5. HSPGs contribute to AGMA1 binding to Vero cells.** Vero cells were incubated with  
531 increasing concentrations of b-AGMA1 alone (panel A) or subjected to the following treatments in the  
532 presence of b-AGMA1 at a fixed concentration (0.1 µg/ml) (panel B): i) incubated with b-AGMA1  
533 alone. ii) incubated with b-AGMA1 and then washed with PBS containing 2 M NaCl; iii) incubated  
534 with b-AGMA1 in the presence of a molar excess (10 µg/ml) of heparin. iv) pre-treated with heparinase  
535 before b-AGMA1 incubation. The amount b-AGMA1 bound to Vero cell surface was then measured  
536 and is reported in panel B. In panel A, each point is the mean ± SEM of 3 independent determinations  
537 in duplicate. In panel B, data are expressed as the percentage of b-AGMA1 bound to control cells and  
538 each point is the mean ± SEM of 2-4 independent determinations in duplicate. \* = p< 0.05, and \*\* = p<  
539 0.01 with respect to control treated with b-AGMA1 alone, Student's *t* test.

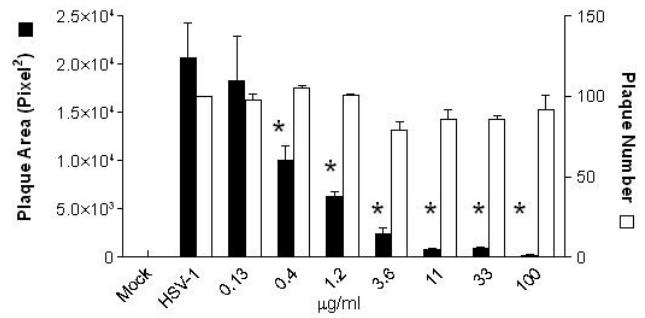
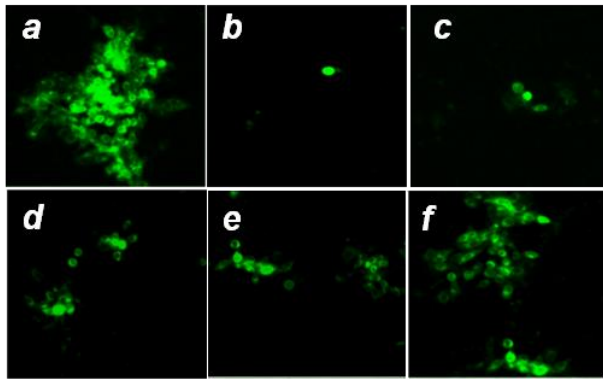
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### 541 3.5. Effect of AGMA1 on the cell-to-cell spread of HSV

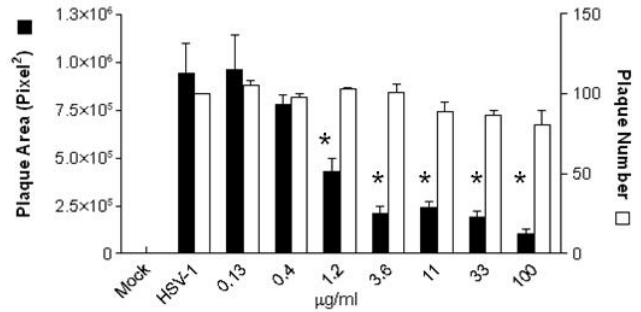
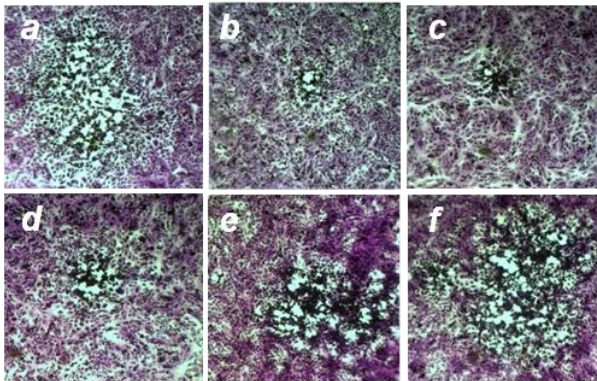
542 To determine whether AGMA1 interferes with cell-to-cell virus spreading, post-entry assays, described  
543 at section 2.8.9., were performed. As shown in Figure 6A, the area of HSV-1(GFP) plaques, assessed  
544 by fluorescence microscopy, decreased in a dose-dependent manner in AGMA1-treated cells, and at a  
545 concentration of 100 µg/ml singly infected cells were mainly seen. In contrast to the significant

546 reduction in plaque size, quantified using ImageJ software, no significant reduction in the number of  
547 HSV plaques was observed. Similar results were obtained for wild-type HSV-1 (Fig. 6B) and HSV-2  
548 (Fig. 6C). A process of fusion of plasma membrane of an infected cell with that of a neighboring  
549 uninfected cell, is thought to occur during cell-to-cell spread. Recently, syndecans, single  
550 transmembranous heparan sulfate proteoglycans, have been demonstrated to contribute to HSV-1  
551 induced cell-to-cell fusion and lateral spread [51]. Inhibition of cell-to-cell spread of HSV by AGMA1,  
552 it's probably due to its ability to interact with HSPG and consequently mask the core protein of  
553 syndecan-1, involved in membrane fusion. Viral yield reductions assays also demonstrated that  
554 addition of AGMA1 after infection heavily affected viral production (data not shown) with EC50s of  
555 6.54  $\mu\text{g/ml}$  (HSV-1) and 3.98  $\mu\text{g/ml}$  (HSV-2).

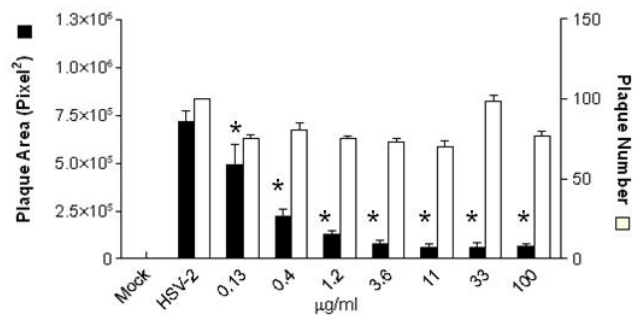
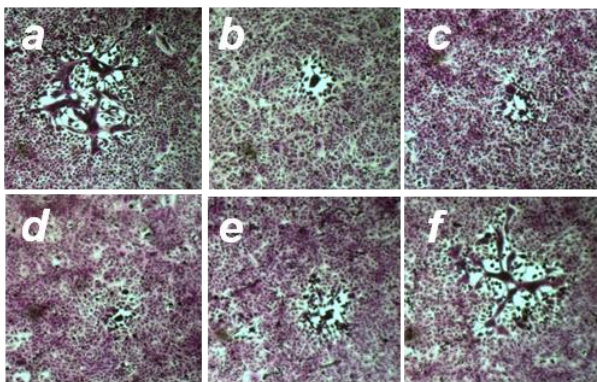
A



B



C



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**Figure 6. AGMA1 inhibits HSV at a post-entry level.** Monolayers of Vero cells were infected with HSV-1(GFP) (A), clinical isolates of HSV-1 (B), or HSV-2 (C) in the absence of AGMA1. The inoculum was removed at 2 hours post-infection, and cells were left untreated (a) or incubated in the presence of the following concentrations of AGMA1: (b) 100 µg/ml, (c) 33 µg/ml, (d) 11 µg/ml, (e) 3.6

561  $\mu\text{g/ml}$ , or (f)  $0.13 \mu\text{g/ml}$ . Plaque formation was assessed 24 or 48 hours after infection. The bar charts  
562 show the plaque area and the plaque count of HSV-1(GFP), HSV-1 and HSV-2, as a function of  
563 AGMA1 concentration. The data presented are means plus standard deviations for triplicates. \*,  $P <$   
564  $0.05$ .

565

### 566 *3.6 . AGMA1 antiviral activity is not affected by acidic pHs*

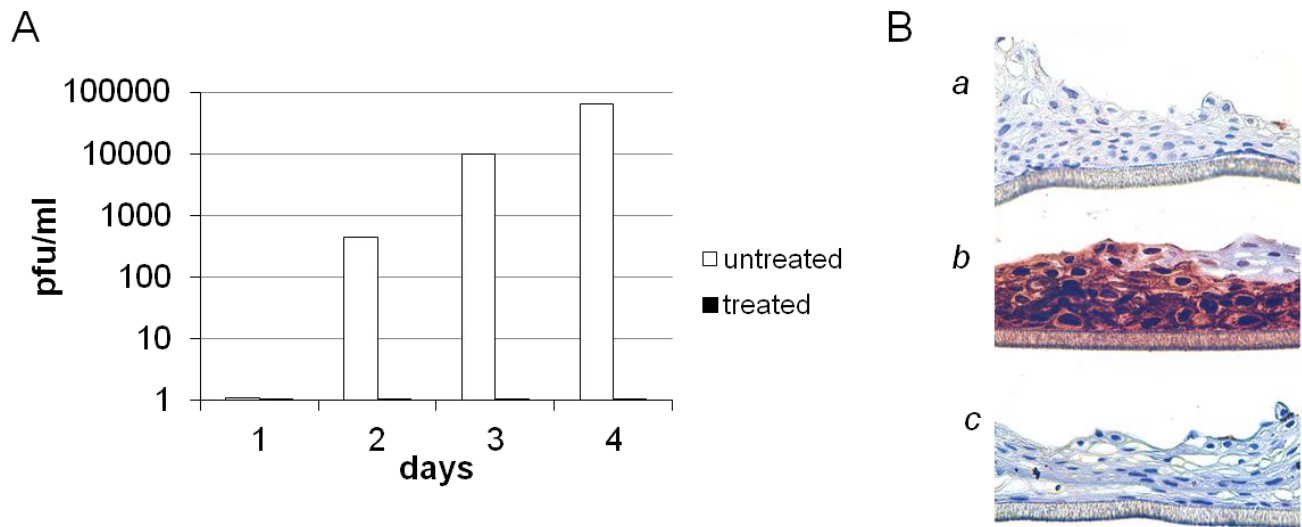
567 Analysis of the mechanism of action of AGMA1 demonstrated its ability to prevent HSV infection. To  
568 evaluate its potential as candidate microbicide for preventing genital HSV-2 infections, the antiviral  
569 activity in presence of specific physiological properties of the vagina, such as acidic pHs, was  
570 considered. To this end, AGMA1 was incubated in buffers of different pHs for 2 hours at  $37^\circ\text{C}$ , and the  
571 antiviral activity was evaluated by viral plaque reduction assays at physiological pH. Results  
572 demonstrated that the acidic treatment did not affect the activity of AGMA1, since the inhibitory effect  
573 against HSV-2 at pH 3 ( $\text{EC}_{50}$ :  $3.86 \mu\text{g/ml}$ ) and at pH 5 ( $\text{EC}_{50}$ :  $2.28 \mu\text{g/ml}$ ) was similar to that observed  
574 for compound incubated at neutral pH ( $\text{EC}_{50}$ :  $2.32 \mu\text{g/ml}$ ).

575

### 576 *3.7. Antiviral activity of AGMA1 in EpiVaginal tissue*

577 To investigate the effects of AGMA1 in a model that more closely resembles the in vivo environment,  
578 the EpiVaginal system was employed. Briefly, this system consists of human-derived ectocervical  
579 epithelial cells grown on a collagen-coated membrane to form a multilayered and highly differentiated  
580 tissue that closely resembles the vaginal mucosa. EpiVaginal cultures were treated apically with  $100$   
581  $\mu\text{g/ml}$  AGMA1 for two hours, and then infected with  $1000$  pfu HSV-2. AGMA1 totally inhibited the  
582 virus emerging from the apical surface at different days post infection (Fig.7A). Complete inhibition of  
583 viral infection was confirmed by immunohistochemistry, using an HSV-2-specific antibody, at 3 days  
584 post-infection. As shown in Figure 7B, sections derived from the infected tissue exhibited strong

585 staining for the expression of HSV-2 antigens (Fig. 7Bb). In contrast, no HSV-2 positive cells were  
586 observed in the uninfected tissue (Fig. 7Ba). AGMA1-treated samples did not show a HSV-2 signal  
587 (Fig. 7Bc). In addition, pre-treatment of tissues with AGMA1 reduced viral infection at 2 days post  
588 infection (84% inhibition; data not shown).



589  
590 **Figure 7. AGMA1 inhibits HSV-2 infection in EpiVaginal tissue.** (A) Antiviral activity of AGMA1  
591 in EpiVaginal tissue infected with 1000 pfu of HSV-2. (B) Immunohistochemistry of control tissue (a),  
592 HSV-2-infected tissue (1000 PFU) (b), and HSV-2-infected tissue treated with 100  $\mu$ g/ml of AGMA1  
593 at 3 days post-infection (c) using a specific antibody to HSV-2 (brown signal). The pictures shown are  
594 representative of analyzed sections (5 to 12 sections analyzed per condition).

595  
596 Since reconstituted tissues are ideally suited for toxicology studies [52], we also tested biocompatibility  
597 and the inflammatory potential of AGMA1. Briefly, AGMA1 (100  $\mu$ g/ml) was applied to the apical  
598 surface at the air-tissue interface for 1, 4, or 18 hours at 37°C, and tissues were subsequently analyzed  
599 for (i) the reduction of tetrazolium salt (MTT) to colored formazan compounds in order to study the  
600 metabolic activity of the living cells; (ii) lactate dehydrogenase (LDH) release, to measure the  
601 accumulation of dead cells; and (iii) the release of interleukin-1 alpha (IL-1 alpha) to evaluate the

602 inflammatory activation of cells (see Materials and Methods for further details). As reported in Table 3,  
 603 AGMA1 did not affect viability, and Effective-Time 50 (ET-50), i.e. the time necessary to reduce cell  
 604 viability by 50% was greater than 18 hours and indistinguishable to that observed in naïve cells.  
 605 Furthermore, no difference in the release of LDH cytoplasmic enzyme was observed between AGMA1-  
 606 treated and untreated tissues, suggesting that no cytoplasmic damage had occurred. Finally, there was  
 607 no significant difference in the level of the proinflammatory cytokine IL-1 alpha (Table 3) compared to  
 608 untreated samples.

609

610 **Table 3. Evaluation of the irritation potential of 100 µg/ml of AGMA1 in the EpiVaginal tissue**  
 611 **model.**

612

Conditions	% Viability	LDH release (A)	IL-1 alpha release (pg/ml)
Untreated (1 h)	100	0.75 ± 0.06	8.4 ± 2.1
AGMA1 (1 h)	115.07 ± 12.90	0.74 ± 0.02	8.2 ± 1.9
Untreated (4 h)	100	0.76 ± 0.03	10.8 ± 1.0
AGMA1 (4 h)	109.59 ± 0.04	0.73 ± 0.03	9.9 ± 1.3
Untreated (18 h)	100	1.80 ± 0.04	31.5 ± 6.2
AGMA1 (18 h)	67.04 ± 10.75	1.46 ± 0.02	32.9 ± 1.4

613 3.8. Assessment of AGMA1 antiviral activity *in vivo*

614 Finally, we sought to confirm our *in vitro* findings and assess AGMA1 efficacy *in vivo* by analyzing  
615 HSV infection by venereal spread, the chief route of HSV transmission in industrialized and  
616 developing countries [11,53]. Here, we used an established murine animal model of HSV genital  
617 infection [38] and 100 µg/ml AGMA1, a concentration that was well-tolerated in mice and able to  
618 abolish viral infectivity in the EpiVaginal tissue. Tests were aimed to: 1. Determine the best timing  
619 of administration before infection; 2. Evaluate efficacy against HSV-1 and HSV-2 strains; 3. Assess  
620 the breadth of antiviral activity toward escalating infectious doses; 4. Investigate whether animals  
621 that exhibited no visible signs of infection had subclinical infection. All experiments were  
622 performed using 6-12 animals/group, a number suitable for statistical analysis, and lasted about four  
623 weeks, i.e. a time sufficient to monitor the complete course of the disease. Depending on infectious  
624 dose and ability of immune system to restrain viral spread, infection is usually self-limited,  
625 clinically manifests at day 5-6, and disappears within two-three weeks [38]. Clinical signs can be  
626 negligible (subclinical or asymptomatic infection), severe and rapidly progressing to paralysis and  
627 death, or evolve in a persistent disease lasting several weeks and usually culminating in the death of  
628 the animal. Clinical outcome was scored according to a standard five-point scale [38,47] as  
629 described in Material and Methods.

630 The most effective timing of AGMA1 administration was determined using four groups of animals  
631 (six animals/group) that were infected via the vagina with 1 LD<sub>50</sub> of HSV-1 and were either left  
632 untreated (naïve control) or treated with AGMA1 30 minutes, 15 minutes, or 15 seconds before  
633 infection. As shown by Fig. 8A, which depicts the percent of animals that remained disease-free  
634 throughout the observation period, all naïve controls developed infection, manifesting overt  
635 symptoms from day 6, and two animals died on day 10-11. Of the 4 surviving animals, 3 had  
636 recovered by day 13, and one was still sick when the experiment was terminated. Of the animals  
637 treated with AGMA1 30 minutes before infection, 3 were transiently infected and fully recovered by



638 day 12, 1 died on day 9, and 2 showed no symptoms throughout the course of the follow-up period.

639 In contrast, of the groups of animals pretreated with AGMA1 at 15 minutes and 15 seconds before

640 infection, 3 and 4 animals remained disease-free, respectively, and 3 and 2 developed a transient and

641 mild disease (clinical score  $\leq 2$ ). Although groups were too small to draw firm conclusions,

642 pretreatment at 15 seconds and 15 minutes clearly delayed and reduced clinical manifestations (Fig.

643 8A). Compared to the naïve group, percent of disease-free animals of these two groups reached

644 statistical significance at day 7 post infection ( $p < 0.002$ , data not shown). This result indicates that

645 AGMA1 exerts similar antiviral activity when applied within this period of time. In all subsequent

646 experiments, we thus applied AGMA1 15 minutes before infection.

647 We next assessed whether AGMA1 protects against both HSV-1 and HSV-2 strains. For these

648 experiments we used 36 animals that were split in three groups: naïve, AGMA1, and Vehicle, i.e.

649 animals treated with AGMA1 carrier. After administration of AGMA1 and Vehicle, animal groups

650 were further subdivided into two groups and infected with 1 LD<sub>50</sub> HSV-1 or HSV-2. Five animals of

651 the naïve/HSV-1 group become overtly infected and 1 showed no symptoms. Of the infected

652 animals, 2 died on day 11 and 3 fully recovered. All naïve/HSV-2 animals acquired infection, 3 died

653 on day 11 and 2 still showed disease symptoms at the end of observation period (Fig. 8B). No

654 significant differences were observed between Vehicle and Naïve groups. AGMA1 reduced the

655 outcome of disease of the two infections. In both AGMA1/HSV-1 and AGMA1/HSV-2 groups, 2

656 animals showed no symptoms, 3 animals were transiently infected, and 1 animal died. Compared to

657 the naïve group, the difference in numbers of disease-free animals was statistically significant for

658 AGMA1/HSV-2 group ( $p < 0.05$ ), this was not the case for HSV-1 as only 5/6 naïve animals became

659 sick and, in general, showed a milder course of infection (Fig. 8B).

660 Because of similar efficacy against the two strains, higher virulence of HSV-2 strain, higher

661 incidence of genital HSV-2 infections in humans, and to limit *in vivo* tests, analysis of AGMA1

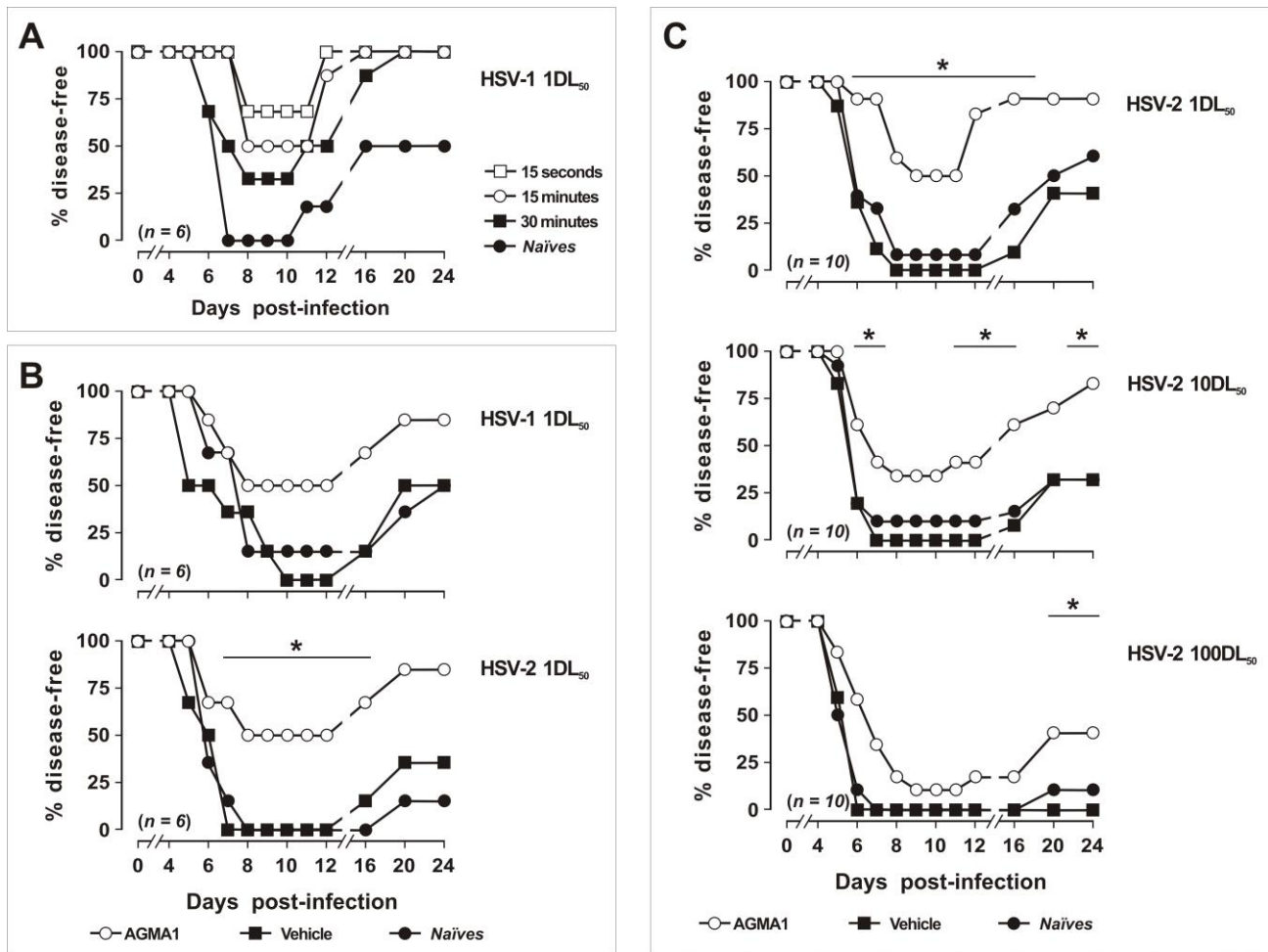
662 potency against escalating doses was performed with HSV-2. For this experiment we used 10

663 animals/group and 1, 10, and, 100 HSV-2 LD<sub>50</sub>. As expected, clinical grading and mortality rate  
664 increased with infectious dose; 1 LD<sub>50</sub> infected 9/10 and killed 3/10 animals of Naïve group, and  
665 infected and killed 9/9 and 5/9 animals of Vehicle group (one animal was found dead at day 2 post  
666 infection for unknown reasons); 10 LD<sub>50</sub> infected all animals of both groups and killed 4/10 and  
667 6/10 animals of Naïve and Vehicle groups, respectively; 100 LD<sub>50</sub> infected and killed all animals of  
668 both groups except 1 naïve that fully recovered at day 18 post infection. The AGMA1 group  
669 challenged with 1 LD<sub>50</sub> yielded: 5 animals totally protected, 4 mildly and transiently infected, and 1  
670 still sick at the end of observation period. Statistical analysis showed that this group performed  
671 significantly better compared to Naïve and Vehicle at  $p<0.05$  (Fig. 8C). Pretreatment with AGMA1  
672 and challenging with 10 LD<sub>50</sub> resulted in 3 animals fully protected, 4 transiently infected, 1  
673 chronically infected, and 1 death. These results were, at same time, significantly different compared  
674 to control groups at  $p<0.0001$ . Finally, AGMA1 pretreatment did not spare animals from infection  
675 with 100 LD<sub>50</sub> but, among the 6 surviving animals, 4 were transiently infected and 2 still sick at the  
676 end of the observation period. Whereas the difference in percent disease-free animals reached  
677 statistical significance only at onset of disease and end of experiment, this was statistically  
678 significant by comparing mortality rate by day 10 post infection ( $p<0.01$ ) (Fig. 8C and data not  
679 shown). This experiment demonstrated that AGMA1 protects against disease at low to moderate  
680 infectious doses, and lessens clinical consequences of a very high input dose (100 LD<sub>50</sub>), an  
681 infectious load unlikely to find in human transmission.

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686 **Figure 8. AGMA1 reduces the burden of infection of HSV-2 genital infection in mice.** Plots  
 687 show the percent of animals that remained disease-free throughout the observation period. **A.**  
 688 Definition of timing of administration before infection. 6 animals/group were pretreated with  
 689 AGMA1 at times indicated in the legend and then infected with 1 LD<sub>50</sub> HSV-1. Animals pretreated  
 690 with AGMA1 15 seconds and 15 minutes before infection were fully protected or manifested milder  
 691 clinical signs compared to naïve animals. **B.** Analysis of antiviral efficacy against HSV-1 and HSV-  
 692 2 infections. 6 animals/group were either untreated (Naïve) or pretreated with AGMA1 or carrier  
 693 (Vehicle) 15 minutes before infection with 1 LD<sub>50</sub> HSV-1 (top graph) or HSV-2 (lower). Asterisk  
 694 indicates significant differences relative to Naïve and Vehicle groups at  $p \leq 0.05$ . **C.** Antiviral  
 695 efficacy against escalating infectious doses of HSV-2. 10 animals/group were either untreated  
 696 (Naïve) or pretreated with AGMA1 or carrier (Vehicle) 15 minutes before infection with 1 (top

697 graph), 10 (middle), and 100 (lower) LD<sub>50</sub>. Asterisk indicates significant differences relative to  
698 Naïve and Vehicle groups at  $p \leq 0.05$ .

699

700 The last set of in vivo experiments was aimed to assess whether the animals that had no clinical  
701 signs underwent subclinical (nearly or completely asymptomatic) infection as it frequently occurs in  
702 nature [53,54]. To this end, Naïve, Vector, and AGMA1 groups (11 animals/each) were challenged  
703 with 10 LD<sub>50</sub> HSV-2, monitored for four weeks, left untreated for two months, and finally  
704 immunosuppressed with a bolus of cyclofosfamide to induce reactivation of latent infection. At four  
705 weeks post infection, 4 AGMA1 and 1 naïve mice resisted or underwent subclinical infection;  
706 remaining animals were either dead or still sick (Table 4). One AGMA1 mice died at day 3 post  
707 infection for unknown reasons as it showed no clinical symptoms. As observed here, as well as in a  
708 previous study [38], cyclofosfamide treatment depleted circulating lymphocytes by approximately  
709 90% within 1 day and left the animals strongly leukopenic for over two weeks (data not shown). Six  
710 out of seven naïve animals showed clinical lesions by day 3 post-cyclofosfamide treatment and half  
711 of them died between day 6-8. The naïve animal that showed no clinical lesions following infection  
712 also had no symptoms after immunosuppression, suggesting that this animal resisted infection.  
713 Clinical relapse also occurred in 5/5 Vehicle animals, 3 of which died between day 8-11. In the  
714 AGMA1 group, 3/7 mice that remained disease-free following infection also showed no signs upon  
715 immunosuppression; 4/7 mice had clinical relapse that was milder, delayed, and shorter compared to  
716 control animals. Of note, three of them were transiently infected and one showed no signs of disease  
717 following primary infection (Table 4). At the end of the experiment, animals were sacrificed, and  
718 their sciatic nerves and cervical ganglia assayed for HSV-2 genome. All animals that underwent  
719 clinical reactivation were PCR positive as opposed to animals that were disease-free after  
720 immunosuppression and tested negative (Table 4).

721

722 **Table 4. Analysis of viral reactivation in Naïve, Vehicle, and AGMA1-treated mice infected with 10**  
 723 **LD<sub>50</sub> HSV-2 and, three months later, immunosuppressed with a bolus of Cyclofosfamide.**

Animal group	Disease status at week 4 post-infection				Disease status at week 4 post-immunosuppression				
	No. treated	Dead	Sick <sup>a</sup>	Healthy <sup>b</sup>	No. treated	Dead	Sick	Healthy	HSV-2 genome in nervous tissues <sup>d</sup>
<i>Naïve</i>	11	4	6	1	7	3	3	1	3/2
<i>Vehicle</i>	11	6	5	0	5	3	2	0	0/0
<i>AGMA1</i>	11	3 <sup>e</sup>	4	4	7	1	3	3	5/2

724 <sup>a</sup> Animals that were still sick at the end of follow-up or developed transient infection.

725 <sup>b</sup> Animals that remained disease-free throughout the follow-up.

726 <sup>c</sup> No. examined/no. positive animals for HSV-2 genome. Nested PC analysis was performed in the  
 727 sciatic nerve and cervical ganglia collected at week 4 post-cyclophosphamide treatment.

728 <sup>e</sup> One death was likely unrelated to HSV infection as the animal died at day 3 post-infection and  
 729 showed no clinical symptoms.

731

732

#### 733 4. Discussion

734 This study reports on the anti-herpetic activity of AGMA1, a prevailingly cationic PAA that exerts  
 735 antiviral activity with a mode of action that differs from that of acyclovir. Indeed, immunoblotting  
 736 analysis revealed that AGMA1 blocks infection before the expression of immediate early viral genes,  
 737 whereas acyclovir prevents late viral genes expression. The antiviral activity of AGMA1 against  
 738 acyclovir-resistant strains supports this conclusion further. These features prompted us to perform  
 739 further studies in order to explore the therapeutic potential of AGMA1 as an anti-herpetic compound.

740 Synthetic polycations have recently become the subject of much interest as candidates for the  
 741 prevention of viral infections. They can inactivate the virus particle directly, as demonstrated for  
 742 polyethylenimine (PEI) against a panel of viruses, including HSV [44,55-58], and for the poly(acrylic

743 ester) Eudragit E100, histidine peptides, polylysine, and arginine, all of which are endowed with  
744 membrane-destabilizing activity against HSV [59-62]. Although AGMA1 shares a polycationic nature  
745 with the above mentioned compounds, here we demonstrate that it does not inactivate the virus  
746 particles. The lack of a direct effect of AGMA1 on the virus and its capacity to inhibit the expression of  
747 immediate-early viral proteins suggest that AGMA1 could act directly on target cells by interfering  
748 with a very early event in HSV infection, possibly corresponding to virus attachment and/or entry.  
749 Indeed, our results demonstrate that AGMA1 prevents HSV-1 and HSV-2 attachment. Attachment  
750 assays showed that AGMA1 treatment prevents viral particles from binding to the cell surface; this was  
751 further demonstrated by immunoblotting the lysates from treated cells. The initial interaction between  
752 HSV and the cell membrane is mediated by interactions between the positively charged domains on  
753 viral glycoproteins gC and gB and the negatively charged HSPGs on the target cell membrane [63].  
754 Others findings have revealed that AGMA1 acts by binding to virus receptors on the surface of target  
755 cells [36]. Of note, we have previously reported that AGMA1 exerts antiviral activity against other  
756 HSPG-dependent viruses [28]. Moreover, we have previously shown that, due to its polycationic  
757 nature, AGMA1 is endowed with heparin-binding capacity and, accordingly, tethers to HSPGs present  
758 on the surface of different epithelial cell types, thereby masking these receptors and preventing HPV  
759 attachment [36]. Indeed, the data reported in this study demonstrate that AGMA1 binds to Vero cells in  
760 a HSPG-dependent manner. However, they do not rule out other interactions occurring between  
761 AGMA1 and the cell surface. To this regard, it is important to point out that the side guanidine groups  
762 of AGMA1 might reinforce membrane interactions, thanks to their well-known chaotropic properties  
763 [28,36,64]. Interestingly are also the observation that the binding of HSV-1 and 2 glycoproteins gD to  
764 nectin-1 depends on several basic amino acids, including L25, R36, R134 and R222 [65] and that  
765 HSV-2 infection can be mediated by  $\alpha_v\beta_3$  integrin [66] that is well known to bind its physiological or  
766 pathological ligand via basic domains [67-69]. Taken together, these data suggest that the high positive

767 charge of AGMA1 may mediate its binding to receptors different from HSPGs, conferring to the  
768 polymer a “multitarget” mechanism of action, as already demonstrated for cationic dendrimer-like  
769 compounds [70].

770 An important feature of the AGMA1 antiviral activity that most probably derives from its capacity to  
771 bind to and mask HSPGs, thus preventing virus interaction, is its ability to diminish a cell’s  
772 susceptibility to HSV when administered before virus infection. By contrast, we show that heparin, a  
773 known attachment inhibitor that interacts directly with the virus particle rather than with the cells, did  
774 not show any inhibitory activity in the pre-treatment assay.

775 This feature prompted us to focus our studies on AGMA1 as a potential microbicide for the prevention  
776 of the sexual transmission of HSV infections.

777 The development of effective, safe, and topically applied microbicides is an apt strategy to prevent  
778 STIs that cannot be contained with pre-exposure immunization strategies or systemic antiviral  
779 treatments.

780 The lack of a protective vaccine against HSV, the observation that genital herpes increases  
781 susceptibility to HIV and other STIs [8,71], and the inherent ability of herpesviruses to establish latent  
782 infections underline the importance of topical microbicides to block HSV mucosal transmission by  
783 inhibiting virus attachment [72].

784 In recent years, numerous preclinical studies have been performed mainly focused on negatively  
785 charged polyanions able to bind to the viral envelope and block attachment, but none of these  
786 compounds have passed phase III clinical trials [16,17]. Many dendrimers have been screened for  
787 potential antiviral activity and selected for development as candidate microbicides [18-20].

788 Beside the already mentioned cationic dendrimers whose main mechanism of action is by binding and  
789 masking HSPGs to virus attachment (see introduction), other compounds have been developed among  
790 which the polyanionic sulfonated and carboxylated polylysine dendrimers, shown to exhibit inhibitory

791 activity against HSV-1 and -2 infection *in vitro* and *in vivo* and protecting animals against an  
792 intravaginal HSV-2 challenge [19].

793 Accordingly, SPL7013, a dendrimer with highly anionic charged branches, has been developed by  
794 Starpharma Pty Ltd (Melbourne, Australia) as microbicide against vaginal bacteriosis (marketed as  
795 VivaGel) is currently under Phase 3 testing for its capacity to prevent HIV and HSV infections [20,21].

796 Unlike these previous studies, we recommend a cationic PAA – AGMA1 – for further development as  
797 an active ingredient of topical microbicides due to several important properties. First, AGMA1 shows  
798 antiviral activity in an organotypic model of cervicovaginal epithelial tissue, i.e. the main target of  
799 HSV-2 infection. In this system, a total inhibition of HSV, emerging from the apical surface, was  
800 observed at different days post infection.

801 A second important property of AGMA1 is that, despite being positively charged and in contrast with  
802 other polycationics (e.g. PEI) it is not toxic, it is not hemolytic in the pH range 5.5-7.4 [22], and it does  
803 not lead to an inflammatory response in the tissue model. Third, when it was administered two hours  
804 pre-infection, AGMA1 prevented infection in Epivaginal tissues, as observed *in vitro*. Fourth, AGMA1  
805 did not affect the growth of *Lactobacillus gasseri* and *Lactobacillus acidophilus*, two components of  
806 the normal vaginal flora (data not shown). Fifth, AGMA1 antiviral activity was not affected by acidic  
807 treatments (pH 3 and pH 5), that simulate physiological vaginal environment.

808 Finally, it must be pointed out that severe HIV infection-driven immunodeficiency causes a well  
809 documented increase in HSV as well as HPV infection [73,74]. Conversely, HSV-2 infection clearly  
810 enhances the transmission of HIV-1 infection [8]. Relevant to this point, AGMA1 has been already  
811 demonstrated to prevent HPV infection suggesting the possibility to obtain a formulation with a  
812 multitarget mechanism of action that can control and/or prevent multiple sexually transmitted  
813 infections simultaneously.



814 The in vitro results prompted us to test AGMA1 as a topical microbicide against genital HSV infection  
815 in vivo. For this task we used two virulent isolates shown to be difficult to contain by immunological  
816 means [38], a well-validated animal model, and a clinical scoring largely used for HSV genital  
817 infection [47]. AGMA1 showed some antiviral efficacy even when applied 30 minutes before infection,  
818 a time lapse that compares favorably with other chemical compounds for which antiviral activity has  
819 been shown to fade very rapidly [75]. All in vivo tests were carried out by applying AGMA1 15  
820 minutes before infection, which was performed with high input loads of HSV-1 and HSV-2. AGMA1  
821 significantly reduced infection rate and clinical grading even against 10 LD<sub>50</sub> HSV-2, an infectious  
822 dose that induced severe disease and high mortality rate in controls. Finally, at 100 LD<sub>50</sub>, which killed  
823 19/20 controls, AGMA1 reduced casualty to 4/10 animals and the 6 surviving animals infection healed  
824 in three weeks. Since herpetic infections establish life-long persistency in the host, a crucial matter is  
825 viral reactivation upon appropriated stimuli. This was addressed in animals that partially or apparently  
826 resisted initial infection. Here, animals were treated with a potent chemotherapeutic drug that  
827 reactivated HSV-2 infection in 92% controls versus 57% AGMA1-treated animals. Further, clinical  
828 relapse in the latter group was milder and transient suggesting that AGMA1 reduces the number of  
829 latently infected cells and the potential for virus reactivation. In all, in vivo tests indicate that AGMA1  
830 provides significant protection against HSV infection and disease and compares favorably well with  
831 dendrimers and polyanions considered good candidate topical microbicides [18-21,34,58,75-76].

832

## 833 **5. Future study.**

834 The main aim of this work was the evaluation of the activity and toxicity of AGMA1. Considering the  
835 efficacy and safety results obtained, the next step of the research will concern the development of an  
836 improved AGMA1 preparation intended for vaginal administration as a microbicide. Formulation  
837 considerations and product design will considered the regulatory aspects and will mainly comprise the

838 choice of excipients, the buffer capacity, the viscosity, the stability and the shelf-life as well as the  
839 volume to be administered. The rheological properties and the vaginal distribution will be also  
840 evaluated to obtain a desirable microbicide product.

841

## 842 **6. Conclusion**

843 AGMA1 prevents HSV infection *in vitro*, *ex vivo* and *in vivo* and shows a good biocompatibility  
844 profile. Of consequence, AGMA1 is a highly promising candidate for development as a topical  
845 microbicide for the prevention of sexually transmitted HSV and HPV infections. Further studies and  
846 the validation of the product in a pharmaceutical formulation will be required to advance it for clinical  
847 testing.

848

## 849 **Acknowledgements**

850 This work was supported by a grant from Ricerca Locale Finanziata dall'Università degli Studi di  
851 Torino (ex 60%) 2012 to D.L.

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