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# Agmatine-Containing Poly(amidoamine)s as a Novel Class of Antiviral Macromolecules: Structural Properties and *In Vitro* Evaluation of Infectivity Inhibition

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**Poly(amidoamine)s (PAAs) are multifunctional *tert*-amine polymers endowed with high structural versatility. Here we report on the screening of a minilibrary of PAAs against a panel of viruses. The PAA AGMA1 showed antiviral activity against herpes simplex virus, human cytomegalovirus, human papillomavirus 16, and respiratory syncytial virus but not against human rotavirus and vesicular stomatitis virus. The results suggest the contribution of both a polycationic nature and side guanidine groups in imparting antiviral activity.**

The development of antiviral molecules usually focuses on either preventing virus entry into the host cell or inhibiting virus replication following host infection. The first strategy may be based on antiviral polyanionic polymers capable of competitively blocking the interaction between viral proteins and cell surface heparan sulfate proteoglycans (HSPGs), which are exploited as attachment receptors by many viruses (1–4). Notwithstanding the large number of studies demonstrating their efficacy in preclinical models, polyanionic polymers somehow failed in clinical trials (5). Unlike polyanions, polycationic polymers have been less investigated as antiviral compounds. In principle, polycations may act as antivirals by electrostatically interacting either with the negatively charged cell membrane or with the envelope of lipid-enveloped viruses, thus preventing virus adsorption onto cell surfaces, or by directly inactivating the virus particle. In this context, it was shown that the cationic poly(acrylic ester) Eudragit E 100, endowed with a membrane-destabilizing activity, exerts antiviral activity against a panel of lipid-enveloped viruses (6, 7). Another study demonstrated that polyethylenimine, a cationic polymer able to condense DNA and mediate gene transfer into mammalian cells, inhibits infection by human cytomegalovirus (HCMV) and human papillomavirus (HPV), a lipid-enveloped virus and a non-enveloped virus, respectively (8).

Poly(amidoamine)s (PAAs) are multifunctional *tert*-amine polymers endowed with high structural versatility, obtained by Michael polyaddition of amines and *bis*-acrylamides (9). The repeating units of PAAs can be designed to be reminiscent of peptides. For instance, an amphoteric, prevalently cationic PAA named AGMA1 is a polymer mimic of the *arg-gly-glu* peptide (RGD) (10, 11).

In the search for new antiviral compounds, a minilibrary of PAAs was screened against a panel of seven viruses, namely, herpes simplex virus type 1 and 2 (HSV-1, HSV-2), HCMV, HPV-16, human respiratory syncytial virus (RSV), human rotavirus (HRV), and vesicular stomatitis virus (VSV), chosen as representative of different virus characteristics, such as the presence or absence of lipid envelope, a DNA or RNA genome, and HSPG dependency for virus attachment (12–16).

The minilibrary included three water-soluble PAAs, ISA1,

ISA23, and AGMA1, whose structures are reported in Fig. 1. The copolymeric PAA ISA1 (17), containing two randomly distributed repeating units present in equal amounts, and the homopolymeric PAAs ISA23 (17, 18) and AGMA1 (10) were prepared as previously reported. AGMA1 fractions with different average molecular weights were obtained by ultrafiltration against water using membranes with different nominal molecular weight cutoffs, as previously described (11). ISA1 is weakly cationic, but ISA23 and AGMA1 are amphoteric, with isoelectric points of  $\sim 5.2$  and  $\sim 10.3$ . As reported in Table 1, at pH 7.4, these PAAs have, respectively, +0.55,  $-0.55$ , and +0.55 average charges per unit. For ISA1, the reported value corresponds to the ionization degree of its *tert*-amine groups, with no other ionizable groups being present. For ISA23 and AGMA1, the reported figures correspond to the excess negative-over-positive charges and vice versa, that is, respectively,  $-1$  plus 0.45 and  $-1$  plus 1.55 per unit. Thus, at pH 7.4, the overall cationic charges of ISA1 and AGMA1 are superficially similar, but a deeper investigation reveals that their real charge distributions are different.

Antiviral assays were performed by infecting cell monolayers in the presence of serial dilutions of compounds for 2 h at 37°C to generate dose-response curves and a selectivity profile of the PAAs' antiviral spectra. The inocula were subsequently washed out and replaced with culture medium containing the same concentration of compounds. The effect on HSV and VSV infections was evaluated by a standard plaque reduction assay on preseeded Vero cells in 24-well plates ( $10 \times 10^4$  cells) infected with 300 PFU/well of clinical isolates of HSV-1 and HSV-2 (19) and VSV serotype Indiana; after incubation for 24 h (HSV-2 and VSV) or 48 h (HSV-1) at 37°C in 5% CO<sub>2</sub>, cells were fixed and stained with

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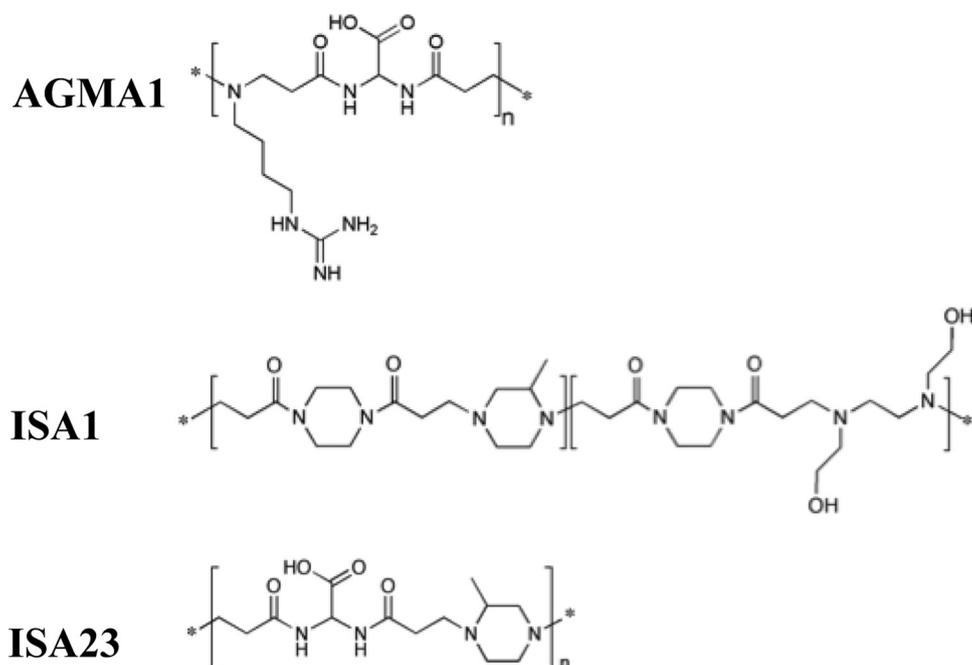


FIG 1 Chemical structures of the AGMA1, ISA1, and ISA23 repeating units.

0.1% crystal violet in 20% ethanol and viral plaques were counted. The mean plaque count for each drug concentration was expressed as a percentage of the mean plaque count of the control.

In HCMV and RSV inhibition assays, infected cells were fixed and subjected to virus-specific immunostaining as described previously (20, 21). In these assays, cells were preseeded at a density of  $6 \times 10^3$ /well in 96-well plates. Hep-2 cells were infected with RSV strain A2 (60 PFU/well), whereas HELF cells were infected with HCMV strain AD169 (24 PFU/well). Three days (RSV) or 5 days (HCMV) postinfection, immunostained viral plaques were microscopically counted.

HPV inhibition assays were performed on preplated 293TT cells ( $2 \times 10^4$ /well in 96-well plates) using HPV-16 secreted alkaline phosphatase (SEAP) pseudoviruses (PsV) at the final concentration of 1 ng/ml liter<sup>-1</sup>; 3 days postinfection, the SEAP content in the clarified supernatant was determined as previously de-

scribed (22). Plasmids used for PsV production were kindly provided by J. Schiller (NCI, Bethesda, MD, USA). Antiviral assays for rotavirus were carried out on preplated MA104 cells ( $1 \times 10^4$ /well in 96-well plates) using human rotavirus strain Wa (200 PFU/well). After 16 h, viral foci were determined by indirect immunostaining (22).

The endpoints of the assays were the effective compound concentration that reduced the viral plaque/focus formation or SEAP activity by 50% (EC<sub>50</sub>) in comparison to that in the untreated control. The F test was used to compare logEC<sub>50</sub>s, and two-way analysis of variance was used to analyze the significance between percentages of infection at the same doses of different compounds not able to generate EC<sub>50</sub>s. *P* values of <0.05 were considered statistically significant. The EC<sub>50</sub>s were calculated and all statistical analyses were performed by using the program PRISM 4 (GraphPad Software, San Diego, CA, USA). The viability of cells preseeded in 96-well plates was determined under identical culture conditions in antiviral assays (i.e., cell density and time of incubation with compounds) using a CellTiter 96 proliferation assay kit (Promega, Madison, WI, USA). The 50% cytotoxic concentrations (CC<sub>50</sub>) were determined using Prism software, and the selectivity index (SI) was calculated by dividing the CC<sub>50</sub> by the EC<sub>50</sub> (19). All data were generated from duplicate wells in at least three independent experiments. Heparin was included in the study as a positive control, being a known inhibitor of HSPG-dependent viruses (e.g., HSV-1, HSV-2, HCMV, RSV, and HPV-16) (23–26). As expected, heparin blocked infection by HSPG-dependent viruses but not that by VSV and HRV, which are not dependent on HSPG (Table 2).

Data reported in Table 2 prompt the following observations. The PAA antiviral effect was not a consequence of cytotoxicity, since none of the screened compounds significantly reduced cell viability at any concentration tested (i.e., up to 300 μg/ml); there-

TABLE 1 Physicochemical characteristics of PAAs

Polymer	$\bar{M}_n^a$	Net avg charge per unit at pH 7.4	Avg negative charge per unit at pH 7.4	Avg positive charge per unit at pH 7.4
Polydisperse	10,100	+0.55	-1.00	+1.55
AGMA1				
AGMA1 <sub>4</sub>	4,500	+0.55	-1.00	+1.55
AGMA1 <sub>7</sub>	7,800	+0.55	-1.00	+1.55
AGMA1 <sub>20</sub>	20,500	+0.55	-1.00	+1.55
ISA1	13,600	+0.55	0.0	+0.55
ISA23	16,500	-0.55	-1.00	+0.45

<sup>a</sup>  $\bar{M}_n$ , number average molecular weight.

$$\bar{M}_n = \frac{\sum_{i=1}^n N_i \times M_i}{\sum_{i=1}^n N_i}$$

where  $N_i$  is the number of macromolecules containing *i* repeating units and  $M_i$  is the weight of macromolecules containing *i* repeating units.

TABLE 2 Antiviral activities of PAAs and heparin<sup>a</sup>

Compound	Virus	EC <sub>50</sub> (μg/ml) (95% CI)	CC <sub>50</sub> (μg/ml)	SI
AGMA1	HSV-1	3.04 (1.75–5.28)	>300	>98.7
	HSV-2	5.34 (1.85–15.4)	>300	>56.2
	HCMV	0.76 (0.40–1.47)	>300	>395
	HPV-16	0.54 (0.53–0.55)	>300	>556
	RSV	>100	>300	NA
	HRV	>100	>300	NA
	VSV	>100	>300	NA
AGMA1 <sub>4</sub>	HSV-1	1.93 (1.43–2.61)	>300	>155
	HSV-2	1.35 (0.57–3.17)	>300	>222
	HCMV	0.39 (0.11–1.30)	>300	>769
	HPV-16	0.92 (0.53–1.58)	>300	>326
	RSV	8.87 (6.51–12.1)	>300	>33.8
	HRV	>100	>300	NA
	VSV	>100	>300	NA
AGMA1 <sub>7</sub>	HSV-1	17.0 (11.4–25.4)	>300	>17.6
	HSV-2	4.80 (3.13–7.35)	>300	>62.5
	HCMV	4.45 (3.28–5.90)	>300	>67.4
	HPV-16	0.79 (0.44–1.44)	>300	>380
	RSV	7.44 (3.11–17.8)	>300	>40.3
	HRV	>100	>300	NA
	VSV	>100	>300	NA
AGMA1 <sub>20</sub>	HSV-1	5.10 (3.21–8.10)	>300	>58.8
	HSV-2	2.82 (1.72–4.64)	>300	>106
	HCMV	4.14 (2.50–6.86)	>300	>72.5
	HPV-16	0.72 (0.50–1.06)	>300	>417
	RSV	1.37 (1.11–1.68)	>300	>219
	HRV	>100	>300	NA
	VSV	>100	>300	NA
ISA1	HSV-1	>100	>300	NA
	HSV-2	>100	>300	NA
	HCMV	1.26 (0.79–2.00)	>300	>238
	HPV-16	3.55 (1.97–6.40)	>300	>84.5
	RSV	9.54 (5.51–16.5)	>300	>31.4
	HRV	>100	>300	NA
	VSV	>100	>300	NA
ISA23	HSV-1	>100	>300	NA
	HSV-2	>100	>300	NA
	HCMV	>100	>300	NA
	HPV-16	>100	>300	NA
	RSV	>100	>300	NA
	HRV	>100	>300	NA
	VSV	>100	>300	NA
Heparin	HSV-1	5.22 (4.22–6.45)	>300	>57.5
	HSV-2	0.67 (0.39–1.18)	>300	>448
	HCMV	0.38 (0.24–0.64)	>300	>789
	HPV-16	2.88 (1.81–4.57)	>300	>104
	RSV	0.05 (0.04–0.06)	>300	>6,000
	HRV	>100	>300	NA
	VSV	>100	>300	NA

<sup>a</sup> EC<sub>50</sub>, 50% effective concentration; 95% CI, 95% confidence interval; CC<sub>50</sub>, 50% cytotoxic concentration; SI, selectivity index; NA, not assessable.

fore, their CC<sub>50</sub> values may be considered to be higher than 300 μg/ml in all the cell lines tested.

Polydisperse AGMA1 strongly inhibited infections by HSV-1, HSV-2, HCMV, and HPV-16, generating dose-response curves

with EC<sub>50</sub>s of 3.04, 5.34, 0.76, and 0.54 μg/ml, respectively. Interestingly, AGMA1 was significantly more active than heparin against HSV-1 and HPV-16 infections, whereas it was as active as heparin against HCMV infection ( $P < 0.05$ ). In contrast, polydisperse AGMA1 was inactive against RSV, HRV, and VSV.

To evaluate the influence of molecular weight on antiviral potency, three additional linear AGMA1 fractions were prepared, namely, AGMA14 ( $\bar{M}_n$ , 4,500), AGMA17 ( $\bar{M}_n$ , 7,800), and AGMA120 ( $\bar{M}_n$ , 20,500) (Table 1). As depicted in Table 2, fractions with lower and higher molecular weights than that of polydisperse AGMA1 ( $\bar{M}_n$ , 10,100) maintained inhibitory activity against HSV-1, HSV-2, HCMV, and HPV-16 although to different extents. AGMA1<sub>4</sub> showed a stronger anti-HSV-1 activity than that of heparin, and all fractions were more active than heparin against HPV-16 infection ( $P < 0.05$ ). No statistically significant differences were observed between the EC<sub>50</sub> of heparin and the EC<sub>50</sub>s of AGMA14 against HSV-2 and HCMV infections and between the EC<sub>50</sub> of heparin and the EC<sub>50</sub> of AGMA120 against HSV-1 infection.

Unlike polydisperse AGMA1, AGMA14, AGMA17, and AGMA120 were also active against RSV, with EC<sub>50</sub> values of 8.87, 7.44, and 1.37 μg/ml, respectively. Both of the polydisperse AGMA1 and AGMA1 fractions failed to display any significant inhibitory effect against HRV and VSV. The antiviral activity of AGMA1 seems not to be dependent on its molecular weight for HSV-1, HSV-2, HCMV, and HPV-16; instead, there is a clear relationship between the AGMA1 fractions' sizes and their anti-RSV potency. Explaining why polydisperse AGMA1 did not exert a detectable anti-RSV activity while all of the size fractions did demands further investigation.

Polymers do not consist of a single molecular species but rather of families of homologous species differing in their numbers of repeating units. Therefore, it is considered inappropriate to adopt the molar concept describing their properties. Nevertheless, to compare activities across compounds, Table 3 shows the EC<sub>50</sub>s of AGMA1 fractions and heparin expressed in terms of molarity instead of μg/ml, considering the average molecular weight reported in Table 1. It was not possible to convert the average molecular mass of polydisperse AGMA1 in terms of molar equivalents, since its molecular mass is not univocally defined. Interestingly, the relationship between the AGMA1 fractions' sizes and their anti-RSV potency, reported in the text where data are expressed in terms of μg/ml, is preserved. Furthermore, AGMA1<sub>7</sub> and AGMA1<sub>20</sub> preserved a higher anti-HPV-16 activity than that of heparin ( $P < 0.05$ ). In contrast, the antiviral activity of AGMA1<sub>4</sub> in terms of molarity is lower than that in terms of μg/ml; its activity is similar to that of heparin against HSV-1 and HPV-16 infections and is lower than that of heparin against HSV-2 and HCMV ( $P < 0.05$ ). This behavior might be ascribed to a greater rigidity of the polymer with the lowest molecular weight. Because all of the polymers are polyelectrolytes, it is necessary to take into account that the charge density markedly affects the dynamic rheological properties, the flexibility, and the chain entanglements. Increased polymer charge density results in intermolecular electrostatic repulsion and increased polymer solubility.

Next, to investigate whether the activity of AGMA1 was specifically due to the structure of its repeating unit, the antiviral activities of ISA1 and ISA23 were assessed. Overall, while AGMA1 was active against HSV-1, HSV-2, HCMV, RSV, and HPV-16 infection, ISA1 was active only against HCMV and RSV, with a lower activity than that of heparin, and was as active as heparin against

TABLE 3 Antiviral activities of poly(amidoamine)s expressed in terms of approximate molar values<sup>a</sup>

Compound	Virus	EC <sub>50</sub> (μM) (95% CI)	CC <sub>50</sub> (μM)
AGMA1 <sub>4</sub>	HSV-1	0.43 (0.30–0.61)	>66.67
	HSV-2	0.30 (0.11–0.80)	>66.67
	HCMV	0.33 (0.09–1.27)	>66.67
	HPV-16	0.20 (0.12–0.33)	>66.67
	RSV	1.97 (1.44–2.69)	>66.67
	HRV	>22.22	>66.67
	VSV	>22.22	>66.67
AGMA1 <sub>7</sub>	HSV-1	2.18 (0.65–7.33)	>38.46
	HSV-2	0.61 (0.38–1.00)	>38.46
	HCMV	0.56 (0.41–0.76)	>38.46
	HPV-16	0.10 (0.06–0.18)	>38.46
	RSV	0.95 (0.40–2.28)	>38.46
	HRV	>12.82	>38.46
	VSV	>12.82	>38.46
AGMA1 <sub>20</sub>	HSV-1	0.25 (0.16–0.40)	>14.63
	HSV-2	0.14 (0.08–0.23)	>14.63
	HCMV	0.20 (0.12–0.33)	>14.63
	HPV-16	0.04 (0.02–0.51)	>14.63
	RSV	0.07 (0.05–0.08)	>14.63
	HRV	>4.87	>14.63
	VSV	>4.87	>14.63
Heparin	HSV-1	0.38 (0.30–0.49)	>21.90
	HSV-2	0.04 (0.03–0.07)	>21.90
	HCMV	0.03 (0.02–0.05)	>21.90
	HPV-16	0.21 (0.13–0.36)	>21.90
	RSV	0.01 (0.00–0.01)	>21.90
	HRV	>7.30	>21.90

<sup>a</sup> EC<sub>50</sub>, 50% effective concentration; 95% CI, 95% confidence interval; CC<sub>50</sub>, 50% cytotoxic concentration.

HPV-16 ( $P < 0.05$ ). ISA23 was inactive in all cases. At pH 7.4, both AGMA1 and ISA1 are positively charged, whereas ISA23 is negatively charged. It is known that polycationic polymers establish ionic interactions with the cell surface HSPG (27, 28), a feature that may impart antiviral activity to these compounds. This feature, along with the finding that the active PAAs have the same antiviral activity spectrum as heparin, supports the hypothesis that PAAs may exert their antiviral action, at least in part, by interacting with HSPG, thus preventing virus attachment. However, notwithstanding the fact that AGMA1 and ISA1 carry the same density of positive charges, i.e., +0.55, AGMA1 showed a greater activity for HSV-1, HSV-2, and HPV-16. This may be due to the different real charge distributions on the macromolecules and to their side guanidine groups reinforcing membrane interactions, according to their well-known chaotropic properties (29). In contrast, the guanidine side group does not seem to be necessary for the anti-HCMV activity. Furthermore, a different chain entanglement might explain the different activity of AGMA1 with respect to that of RSV. Overall, these results provide a starting point to tailor a macromolecule with enhanced antiviral activity against a selected virus. Future work will be focused on narrowing the molecular mass distribution of PAA samples to assist in pre-clinical development.

Studies are ongoing to elucidate the mechanisms of action of the active PAAs and their antiviral potential and biocompatibility profile in preclinical models.

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