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

Agmatine-containing poly(amidoamine)s as novel class of antiviral macromolecules: structural properties and in vitro evaluation of infectivity inhibition

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Running title: Antiviral properties of PAAs

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ABSTRACT

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 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, 2, 3, 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 could 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 directly inactivating the virus particle. In this context, it was shown that the cationic poly(acrylic ester) Eudragit E100, 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, prevailingly cationic PAA named AGMA1 is a polymer mimic of *arg-gly-glu* peptide (RGD) (10, 11).

In the search of 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 representatives of different virus characteristics such as presence or absence of lipid envelope, nature of genome (DNA or RNA) and HSPG dependency for virus attachment (12, 13, 14, 15, 16).

The minilibrary included three water-soluble PAAs, ISA1, ISA23 and AGMA1, whose structures

71 are reported in Figure 1. The copolymeric ISA1 (17), containing two randomly distributed repeating
72 units present in equal amounts, and the homopolymeric ISA 23 (17, 18) and AGMA1 (19) were
73 prepared as previously reported. AGMA1 fractions with different average molecular weights were
74 obtained by ultrafiltration against water using membranes with different nominal molecular weight
75 cut off, as previously described (20). ISA1, is weakly cationic, but ISA23 and AGMA1 are
76 amphoteric, with isoelectric points ~5.2 and ~10.3. As reported in Table 1, at pH 7.4, these PAAs
77 have, respectively, +0.55, -0.55 and +0.55 average charges per unit. For ISA1, the reported value
78 corresponds to the ionization degree of its *ter*-amine groups, no other ionizable groups being
79 present. For ISA23 and AGMA1, the reported figures correspond to the excess negative over
80 positive charges and vice-versa, that is, respectively, -1+0.45 and -1+1.55 per unit. Thus, at pH 7.4
81 the overall cationic charge of ISA1 and AGMA1 is superficially similar, but a deeper insight
82 reveals that their real charge distribution is different.
83 Antiviral assays were performed by infecting cell monolayers in presence of serial dilutions of
84 compounds for 2 hours at 37°C to generate dose-response curves and a selectivity profile of the
85 PAAs antiviral spectrum. The inocula were subsequently washed out and replaced with culture
86 medium containing the same concentration of compounds. The effect on HSV and VSV infections
87 was evaluated by a standard plaque reduction assay on pre-seeded Vero cells in 24-well plates (10 x
88 10^4 cells) infecting with 300 pfu/well of clinical isolates of HSV-1 and HSV-2 (21), and Vesicular
89 stomatitis virus (VSV) serotype Indiana; after incubation for 24 hours (HSV-2 and VSV) or 48
90 hours (HSV-1) at 37°C in 5% CO_2, cells were fixed and stained with 0.1% crystal violet in 20%
91 ethanol and viral plaques were counted. The mean plaque count for each drug concentration was
92 expressed as a percentage of the mean plaque count of the control.
93 In HCMV and RSV inhibition assays, infected cells were fixed and subjected to virus-specific
94 immunostaining as described previously (22, 23). In these assays, cells were pre-seeded at a density
95 of 6 x 10^3/well in 96-well plates. Hep-2 cells were infected with RSV strain A2 (60 pfu/well)

whereas HELF cells with HCMV strain AD169 (24 pfu/well). Three days (RSV) or five days (HCMV) post-infection viral immunostained plaques were microscopically counted.

HPV inhibition assays were performed on preplated 293TT cells (2 x 10^4/well in 96-well plates) using HPV-16 SEAP (secreted alkaline phosphatase) pseudoviruses (PsV) at the final concentration of 1 ng/ml L1; three days post infection the SEAP content in the clarified supernatant was determined as previously described (24). Plasmids used for PsV production were kindly provided by J. Schiller (NCI, Bethesda USA). Antiviral assays for rotavirus were carried out on preplated MA104 cells (1 x 10^4/well in 96-well plates) using human rotavirus strain Wa (200 pfu/well). After 16 hours, viral foci were determined by indirect immunostaining (24).

The end-points of the assays were the effective compound concentration that reduced the viral plaque/focus formation or SEAP activity by 50% (EC_{50}) in comparison to the untreated control. F test was used to compare LogEC_{50}s and two-way analysis of variance to analyze the significance between percentages of infection at the same doses of different compounds not able to generate EC_{50}s. Values of $p < 0.05$ were considered statistically significant. The EC_{50} values and all statistical analyses were calculated by using the program PRISM 4 (GraphPad Software, San Diego, California, U.S.A.). Cell viability assays were performed on cells pre-seeded in 96-well plates under identical culture conditions of antiviral assays (i.e. cell density and time of incubation with compounds) using CellTiter 96 Proliferation Assay Kit (Promega, Madison, WI, USA). The 50% cytotoxic concentrations (CC_{50}) were determined using Prism software and the selectivity index (SI) was calculated by dividing the CC_{50} by the EC_{50} (21). 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) (25, 26, 27, 28). 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); therefore their CC_{50} 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_{50} values of 3.04, 5.34, 0.76, 0.54 μg/mL respectively. Interestingly, AGMA1 was significantly more active than heparin against HSV-1 and HPV-16 infections, whereas was as active as heparin against HCMV infection ($p < 0.05$). By 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 $AGMA1_4$ ($\overline{M}_n$ 4500), $AGMA1_7$ ($\overline{M}_n$ 7800) and $AGMA1_{20}$ ($\overline{M}_n$ 20500) (Table 1). As depicted in Table 2, fractions with lower and higher molecular weights than polydisperse AGMA1 ($\overline{M}_n$ 10100) maintained inhibitory activity against HSV-1, HSV-2, HCMV and HPV-16 although to different extents. $AGMA1_4$ 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 EC_{50} of heparin and EC_{50}s of $AGMA1_4$ against HSV-2 and HCMV infections and between EC_{50} of heparin and EC_{50} of $AGMA1_{20}$ against HSV-1 infection.

Unlike polydisperse AGMA1, $AGMA1_4$, $AGMA1_7$ and $AGMA1_{20}$ were active also against RSV with EC_{50} values of 8.87, 7.44, and 1.37 μg/mL respectively. Both 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 AGMA1 fractions' size and 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 specie, but rather of families of homologous species differing for the number of repeating units. Therefore, it is considered inappropriate to adopt the molar concept describing their properties. Nevertheless, to compare activity across compounds,

Table 3 shows the EC_{50} values 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 AGMA1 fractions' size and anti-RSV potency, reported in text when data were expressed in terms of μg/ml, is preserved. Furthermore, $AGMA1_7$ and $AGMA1_{20}$ preserved a higher anti-HPV-16 activity than that of heparin ($p < 0.05$). By contrast, the antiviral activity of $AGMA1_4$ 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 to that of heparin against HSV-2 and HCMV ($p < 0.05$). This behavior might be ascribed to a more rigidity of the polymer with the lowest molecular weight. Being all the polymers polyelectrolytes, it is necessary to take into account that the charge density markedly affect 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 activity of ISA1 and ISA23 was assessed. Overall, whilst 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 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 (29, 30), 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 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 could be due to the

different real charge distribution on the macromolecules and to its side guanidine groups reinforcing membrane interactions, according to their well-known chaotropic properties (31). By 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 in respect to 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 preclinical development.

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

ACKNOWLEDGEMENTS

This work was supported by a grant from **"**Ricerca finanziata dall'Università degli Studi di Torino (ex 60%) 2012" to DL.

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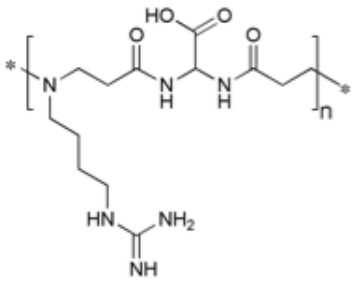
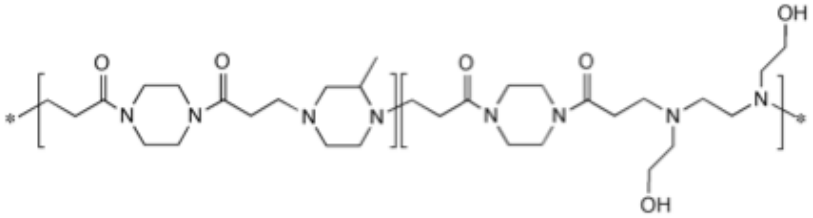
FIGURE LEGENDS

Figure 1. Chemical structure of AGMA1, ISA1 and ISA 23 repeating units

AGMA1



ISA1



ISA23

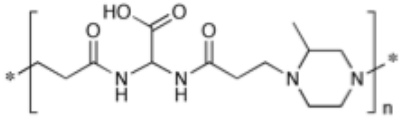


Figure 1. Chemical structure of AGMA1, ISA1 and ISA 23 repeating units

Table 1. Physico-chemical characteristics of PAAs

Polymer	$\overline{M}_n$	**Net average charge per unit at pH 7.4**	**Average negative charges per unit at pH 7.4**	**Average positive charges per unit at pH 7.4**
Polydisperse AGMA1	10100	+ 0.55	-1.00	+ 1.55
$AGMA1_4$	4500	+ 0.55	-1.00	+ 1.55
$AGMA1_7$	7800	+ 0.55	-1.00	+ 1.55
$AGMA1_{20}$	20500	+ 0.55	-1.00	+ 1.55
ISA1	13600	+ 0.55	0.0	+ 0.55
ISA23	16500	- 0.55	-1.00	+ 0.45

$\overline{M}_n$ = number average molecular weight. $\overline{M}_n = \frac{\sum_{i=1}^{n} N_i \times M_i}{\sum_{i=1}^{n} N_i}$, where N_i = number of macromolecules containing *i* repeating units, and M_i = weight of macromolecules containing *i* repeating units.

Table 2. Antiviral activities of PAAs and heparin

Compounds	virus	EC_{50} (μg/ml)[a] (95% C.I.)[b]	CC_{50} (μg/ml)[c]	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	n.a.[d]
	HRV	> 100	> 300	n.a.
	VSV	> 100	> 300	n.a.
$AGMA1_4$	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	n.a.
	VSV	> 100	> 300	n.a.
$AGMA1_7$	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	n.a.
	VSV	> 100	> 300	n.a.
$AGMA1_{20}$	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	n.a.
	VSV	> 100	> 300	n.a.
ISA1	HSV-1	> 100	> 300	n.a.
	HSV-2	> 100	> 300	n.a.
	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	n.a.
	VSV	> 100	> 300	n.a.
ISA23	HSV-1	> 100	> 300	n.a.
	HSV-2	> 100	> 300	n.a.
	HCMV	> 100	> 300	n.a.
	HPV-16	> 100	> 300	n.a.
	RSV	> 100	> 300	n.a.
	HRV	> 100	> 300	n.a.
	VSV	> 100	> 300	n.a.
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	> 6000
	HRV	> 100	> 300	n.a.
	VSV	> 100	> 300	n.a.

[a] EC_{50}: 50% effective concentration

[b] 95% CI: 95% confidence interval

[c] CC_{50}: 50% cytotoxic concentration

[d] n.a.: not assessable

Table 3. Antiviral activities of Poly(amidoamine)s expressed in terms of approximate molar values

Compounds	**virus**	**EC_{50} (μM)[a] (95% C.I.)[b]**	**CC_{50} (μM)[c]**
$AGMA1_4$	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
$AGMA1_7$	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
$AGMA1_{20}$	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

[a] EC_{50}: 50% effective concentration

[b] 95% CI: 95% confidence interval

[c] CC_{50}: 50% cytotoxic concentration