Polyamide amino acids: a new class of RNA ligands†

Vanessa Bonnard, Stéphane Azoulay, Audrey Di Giorgio and Nadia Patino*

Received (in Cambridge, UK) 2nd September 2008, Accepted 11th February 2009
First published as an Advance Article on the web 6th March 2009
DOI: 10.1039/b815324h

This communication introduces a new class of promising RNA ligands, named polyamide amino acids (PAA), which are able to bind a targeted bulged stem-loop RNA fragment (HIV-1 TAR RNA) with micromolar affinities and with specificity comparative to dsDNA and tRNA; both the affinity and the specificity of PAA for TAR depend on their length and on the nature of the amino acid residues.

Unlike regular double helix DNA, single-stranded RNA has the ability to fold into well-defined tertiary structures, which are often involved in biological functions *via* specific protein–RNA or RNA–RNA recognition. Synthetic molecules which are able to interact with high affinity and specificity with RNA structures, and thus to inhibit their function, constitute very attractive tools for molecular biology and medicine.[1]

In this context, we designed a new family of compounds named "polyamide amino acids" (PAA), constituted by an [oligo-*N*-(2-aminoethyl)glycine] backbone, onto which α-amino acid residues were bound (Fig. 1A). This oligomeric backbone, also found in peptide nucleic acids (PNA), could constitute a convenient scaffold for building new RNA ligands. As a mimic of the RNA sugar-phosphate moiety, it has enabled PNA to hybridize to complementary RNA with high affinity.[2] Most likely as complementary nucleic bases, amino acid residues are also prime RNA recognition motifs, as RNA–protein interactions play a central role in many biological processes. Therefore, PAA could become a new family of specific RNA ligands, recognizing their RNA target through both electrostatic and non-electrostatic interactions (H-bonding, π-stacking, Van der Waals), which could involve α-amino groups, amino acid side chains and backbone amide bonds of PAA, with the phosphates and nucleic bases of RNA.

The specificity of RNA–ligand interactions is typically assumed to be the result of non-electrostatic interactions, while electrostatic interactions are responsible for non-specific aspects.[3] Thus, finding the proper balance between these two types of interactions is a key element to identify specific ligands. On these bases and as a preliminary semi-empirical study, we designed hetero- (**1–4**) and homo- (**5–8**) tetra-PAA (Fig. 1C) incorporating four natural amino acids, *i.e.* arginine (R), lysine (K), phenylalanine (F) and alanine (A), and evaluated their potential to bind an RNA bulged stem-loop fragment with affinity and specificity. For this binding study,

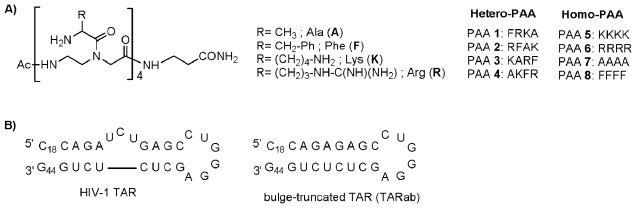
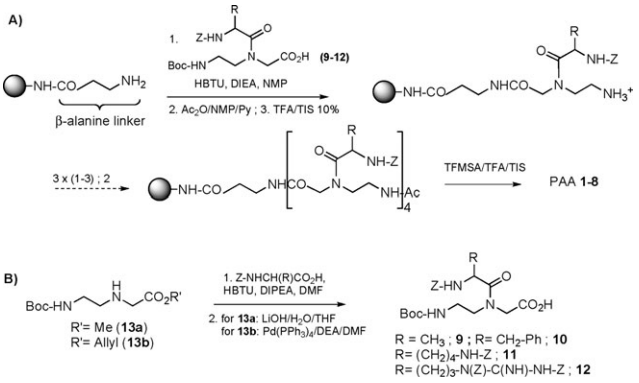


Fig. 1 (**A**) Structures of synthesized PAA. (**B**) Secondary structures of TAR RNA and of bulge-truncated TAR RNA fragments.

we selected the HIV-1 TAR hairpin fragment (Fig. 1B), often taken as a model RNA target to identify RNA ligands. The two basic residues (K and R) were introduced for promoting electrostatic interactions and/or hydrogen bonds. Moreover, among the wide variety of identified TAR synthetic ligands, peptides and peptidomimetics containing these basic amino acid residues were shown to bind tightly and specifically to TAR.[4] The aromatic and hydrophobic residues were selected for favoring π-stacking and van der Waals interactions.

Supported synthesis of tetramers **1–8** was carried out on a β-alanine functionalized MBHA-LL resin, starting from fully *N*-protected PAA monomers **9–12**, and repeating a three-step procedure (Scheme 1A): (i) on-resin condensation of the selected PAA monomer, (ii) capping of the unreacted amino groups, and (iii) Boc cleavage by acidolysis. After *N*-acetylation of the last PAA residue and cleavage from the resin, crude PAA **1–8** were obtained in high yields (>95%) and high HPLC purity (from 80 to 90%). PAA **1–8** were purified by semi-preparative HPLC and fully characterized by HRMS, ^{1}H and ^{13}C NMR spectroscopy. Concerning the synthesis of the *N*-protected PAA monomer building blocks **9–12**, these synthons were prepared using a two-step procedure (Scheme 1B) consisting of the condensation of the *N*-Z protected amino acid residues onto the *N*-Boc methyl or



Scheme 1 (**A**) Solid-phase synthesis of tetra-PAA. (**B**) Synthesis of PAA monomers.

Laboratoire de Chimie des Molécules Bioactives et des Arômes, UMR 6001 CNRS UNSA, Institut de Chimie de Nice, Université de Nice Sophia Antipolis, 06108 Nice Cedex, France. E-mail: patino@unice.fr; Fax: (+33) 4 92 07 61 51; Tel: (+33) 4 92 07 61 46

† Electronic supplementary information (ESI) available: PAA synthesis and characterization, CD and fluorescence assays experimental sections. See DOI: 10.1039/b815324h

Table 1 Dissociation constants for PAA–RNA interactions in the absence or in the presence of nucleic acid competitors[a]

PAA sequence	K_d (TAR) (μM) without competitor	K'_d (TAR) (μM) with dsDNA[b]	K_d/K'_d	K''_d (TAR) (μM) with tRNA mix[c]	K_d/K''_d	K_d (TARab) (μM)	Specificity TAR *vs.* TARab[d]
FRKA (1)	0.75 ± 0.05	0.86 ± 0.09	1.1	1.6 ± 0.4	2.1	1.0 ± 0.3	1.3
RFAK (2)	1.8 ± 0.2	1.7 ± 0.2	1	3.3 ± 0.6	1.8	0.9 ± 0.7	0.5
KARF (3)	2.1 ± 0.3	3.0 ± 0.3	1.4	2.4 ± 0.2	1.1	2.2 ± 0.7	1.0
AKFR (4)	1.2 ± 0.2	2.2 ± 0.2	1.8	2.9 ± 0.9	2.4	3.4 ± 0.8	2.8
KKKK (5)	1.2 ± 0.4	2.6 ± 0.3	2.2	9.7 ± 0.5	8	5.2 ± 1.3	4.3
RRRR (6)	0.08 ± 0.02	0.10 ± 0.02	1.3	1.8 ± 0.9	22.5	0.22 ± 0.05	2.8
AAAA (7)	>1000	nd[e]	nd	nd	nd	>1000	nd

[a] All standard fluorescence measurements were performed in buffer A (20 mM HEPES (pH 7.4 at 25 °C), 20 mM NaCl, 140 mM KCl and 3 mM $MgCl_2$). [b] Measured in the presence of a 100-fold nucleotide excess of a 15-mer duplex DNA. [c] Measured in the presence of a 100-fold nucleotide excess of a mixture of natural tRNA ($tRNA^{mix}$). [d] Determined as K_d (TARab)/K_d (TAR) ratio. [e] Not determined.

allyl ester backbone **13a–b**, then carboxyl deprotection (*e.g.* saponification for methyl esters (→ **9–11**) or reaction with catalytic $Pd^0(PPh_3)_4$ for the allyl ester (→ **12**)).

We first studied by circular dichroism (CD) the possible PAA binding to the TAR fragment, as the interaction of TAR RNA with small ligands has been reported to induce TAR conformational changes, liable to be visualized by this method.[5] The CD spectra of PAA **1–8** alone did not reveal any of the characteristic shapes of secondary peptide structure, suggesting that PAA are either unstructured or adopt a non-canonical structure. In the presence of PAA **1–8**, a dose dependant modification of the molecular ellipticity intensity in the CD spectrum of TAR was observed (see ESI†). These modifications consisted of a decrease in the 265 nm band intensity (indicating a change in base stacking), and a concomitant increase in the negative Cotton effect at 208 nm (indicating a change in helicity). The magnitude of these changes depended on the overall positive charges of the PAA (see CD spectra of RRRR **6** *vs.* AAAA **7** in ESI†), but it was not strictly correlated with the number of amines on the PAA (hetero-PAA **1–4** *vs.* KKKK **5**).

These results prompted us to investigate the binding of tetra-PAA **1–8** to TAR RNA by monitoring the fluorescence change of a fluorescently-labelled (Alexa 488) TAR fragment. This method, broadly used to study interactions between RNA and small ligands,[6] allowed us to determine dissociation constants (K_d) (Table 1) and thermodynamic parameters (Table 3) of PAA–TAR complexes. All curves fitted well a 1 : 1 stoichiometry model, except for FFFF **8**, which seems to follow a 2 : 1 (ligand : RNA) behavior, although the final stoichiometry could not be unambiguously deduced. Apparent dissociation constants ranged from 80 nM to 2 mM, octa-cationic RRRR **6** and tetra-cationic AAAA **7** displaying the highest and the lowest affinities, respectively, highlighting the importance of electrostatic interactions for binding to TAR RNA. However, affinities were not strictly correlated with the number of cationic charges. Indeed, hexa-cationic hetero-PAA **1–4** and octa-cationic KKKK **5** bound to TAR with similar affinities. Moreover, octa-cationic RRRR **6** displayed a K_d value one order of magnitude lower than that of octa-cationic KKKK **5**. Altogether, these results indicate that the formation of the PAA–TAR complexes probably results both from electrostatic and non-electrostatic interactions.

To estimate the TAR RNA *vs.* dsDNA specificity of PAA **1–6**, binding affinities (K'_d) were measured in the presence of a 100-fold nucleotide excess of a 15-mer DNA duplex (Table 1). For all tested compounds, only a slight affinity decrease was observed (maximum 2.2-fold for PAA **5**), demonstrating a high TAR RNA *vs.* dsDNA specificity of PAA, even in the case of the highly charged PAA **5**. The PAA **1–6** specificity for TAR RNA was also assessed by measuring binding affinities (K''_d) in the presence of a 100-fold nucleotide excess as a mixture of natural tRNA (Table 1). As expected, the octa-cationic PAA **5** and **6** were less specific than the hexa-cationic PAA **1–4**. Indeed, in the presence of tRNA, the affinities of PAA **5–6** for TAR decreased 8- and 22-fold, respectively, while those of PAA **1–4** were only slightly affected (Table 1). The diminution in the competitive effect of tRNA as the number of amines decreases likely reflects a decrease in the non-specific electrostatic binding energy. However, this energy loss may be compensated for by a binding energy increase resulting from non-ionic interactions, as illustrated in the cases of PAA **6** and PAA **4**, which display similar K''_d values.

We also investigated the interaction of tetra-PAA **1–6** with a bulge-truncated TAR fragment (TARab; Fig. 1B) (Table 1). Octa-cationic PAA **5** and **6** displayed specificity for TAR comparative to TARab, their affinity for TAR being approximately 4- and 3-fold higher than for TARab, respectively. Concerning hetero-PAA **1–4**, their specificity was shown to be sequence-dependent. Indeed, PAA **4** was more specific for TAR than for TARab (3-fold) while conversely, PAA **2** was found to be 2-fold more specific for TARab than for TAR.

Moreover, we compared the affinity and specificity of tetra-PAA **1**, **2** and **6** for TAR RNA with those of truncated derivatives (tri-PAA **1′**, **2′** and **6′**) (Table 2).

As expected, RNA affinity increased when going from the tri- to the tetra-PAA, either by adding an A or R residue. However, this affinity gain was not strictly correlated with the

Table 2 Comparison of tri- and tetra-PAA

PAA	Affinity gain (tri→tetra) TAR	Affinity gain (tri→tetra) TARab	Binding specificity[a] TAR *vs.* TARab
FRK (1′)	× 9	× 13	1.9
FRKA (1)			1.3
FAK (2′)	× 38	× 70	0.9
RFAK (2)			0.5
RRR (6′)	× 175	× 33	0.5
RRRR (6)			2.8

[a] Measured by K_d (TARab)/K_d (TAR).

Table 3 Thermodynamic parameters for PAA–TAR associations

PAA sequence	ΔG° (kJ mol^{-1})	ΔH° [a] (kJ mol^{-1})	$T\Delta S^\circ$ [a] (kJ mol^{-1})	$T\Delta S^\circ/\Delta H^\circ$	ΔG°nel[b] (kJ mol^{-1})	$T\Delta S^\circ$nel (kJ mol^{-1})	ΔG°el = $T\Delta S^\circ$el[c] (kJ mol^{-1})
FRKA (1)	−35.1 ± 0.2	−70.3 ± 1.9	−34.7 ± 0.4	0.50	−26.1 ± 0.9 (74%)	−44.2 ± 2.8	−9.0 ± 1.1
RFAK (2)	−32.6 ± 0.3	−70.7 ± 5.6	−38.1 ± 1.1	0.54	−26.2 ± 0.9 (80%)	−44.5 ± 6.5	−6.4 ± 1.2
KARF (3)	−32.2 ± 0.5	−69.9 ± 2.7	−37.7 ± 2.7	0.54	−25.8 ± 0.5 (80%)	−44.1 ± 3.6	−6.4 ± 1.0
AKFR (4)	−33.9 ± 0.6	−76.6 ± 1.4	−42.7 ± 1.4	0.56	−28.8 ± 1.0 (85%)	−47.8 ± 2.4	−5.1 ± 1.6
KKKK (5)	−33.9 ± 1.1	−59.0 ± 1.1	−25.1 ± 5.8	0.43	−19.7 ± 1.3 (58%)	−39.3 ± 2.4	−14.2 ± 2.4
RRRR (6)	−40.6 ± 1.1	−77.0 ± 0.9	−36.4 ± 5.0	0.46	−24.1 ± 2.8 (58%)	−52.9 ± 3.7	−16.5 ± 3.9
AAAA (7)	−14.6 ± 0.2	−85.4 ± 2.4	−70.8 ± 20.4	0.79	−8.4 ± 0.2 (57%)	−77.0 ± 2.6	−6.2 ± 0.4

[a] Determined by temperature effect experiments using the $\Delta G^\circ_T = \Delta H^\circ_{Tr} + \Delta C_P\,(T - Tr) - T\Delta S^\circ_{Tr} - T\Delta C_P \ln(T/Tr)$ (see ESI†). [b] Determined by salt effect experiments using the equation $\log(K_d) = \log(K_{nel}) - Z\Psi \log[KCl]$ (see ESI†). In brackets: percentage of non-electrostatic interactions (ΔG°nel/ΔG°). [c] ΔG°el = $\Delta G^\circ - \Delta G^\circ$nel = $T\Delta S^\circ$el.

number of cationic charges, since for example, adding a PAA(R) monomer to PAA **2′** led to a 38-fold affinity increase for TAR, while adding the same residue to PAA **6′** resulted in a 175-fold increase. Concerning its impact on TAR *vs.* TARab (nearly 6-fold), whereas adding the same residue to **2′** led to a 2-fold decrease in specificity. Lastly, addition of a PAA(A) monomer to **1′** led to a slight decrease in the specificity for TAR *vs.* TARab. Altogether, these results unambiguously show that affinity and specificity for a RNA target may be modulated by the length and the nature of the PAA sequence.

In order to have further insight into the binding mode of the PAA–TAR interactions, ΔH° and ΔS° thermodynamic parameters associated with the formation of the complexes were determined from ΔG° *versus* temperature curves (T° from 278 to 308 K) (Table 3). Non-electrostatic (ΔG°nel, ΔH°nel ($\approx \Delta H^\circ$), ΔS°nel) and electrostatic (ΔG°el, ΔH°el (≈ 0), ΔS°el) components were obtained by examining the dependency of the dissociation constants on the ionic strength of the solution.[7] In any case, the Gibbs energy ($\Delta G^\circ = \Delta G^\circ$el + ΔG°nel) reflects a balance of one unfavorable and two favorable contributions. The unfavorable contribution ($T\Delta S^\circ$nel) mainly stems from the entropic cost of bimolecular complex formation. This factor is overwhelmed by two favorable contributions, which stem from the polyelectrolyte effect (ΔG°el = $-T\Delta S^\circ$el) and non-covalent ligand–RNA interactions (ΔH°nel), the latter providing the predominant driving force for complex formation. As expected, the non-electrostatic contribution of the total binding (ΔG°nel/ΔG°) was lower for octa-cationic PAA **5** and **6** (<60%) than for hexa-cationic PAA **1–4**, for which the non-electrostatic part clearly dominates the binding (from 74 to 85%). Concerning the lowest charged AAAA **7**, the significant favorable enthalpic factor ($\Delta H^\circ = -85$ kJ mol^{-1}) was strongly balanced by a highly unfavorable entropic factor ($T\Delta S^\circ = -70$ kJ mol^{-1}; $T\Delta S^\circ/\Delta H^\circ = 0.79$), leading to the highest ΔG° value of the series (-14 kJ mol^{-1}). So, even if the electrostatic part (ΔG°el) of the total Gibbs energy for PAA **7** was 2-fold lower than for the highest charged PAA **5** and **6** (-6.2, -14.2 and -16.5 kJ mol^{-1}, respectively), the ΔG°nel/ΔG° ratio was the same for the three compounds.

In conclusion, considering that only four different amino acid residues were selected for this preliminary study, our results are very promising, as they demonstrate the potential of PAA to behave as specific RNA ligands, *via* electrostatic and non-electrostatic interactions. Both the affinity and the specificity of PAA for an RNA target may be modulated by their length and by the nature of the amino acid residues. Further work will focus on the preparation of PAA libraries containing a wide variety of natural or non-natural amino acids in order to identify ligands capable of binding with high affinity and specificity to biologically relevant RNA targets.

This research was supported by ANRS, Sidaction, CG 06, FRM and CAMPL. V. Bonnard is a recipient of a MENRT PhD fellowship. We thank E. Margeat for helpful discussions and for his assistance with the BIOEQS program.

Notes and references

1 J. R. Thomas and P. J. Hergenrother, *Chem. Rev.*, 2008, **108**, 1171.
2 A. Porcheddu and G. Giacomelli, *Curr. Med. Chem.*, 2005, **12**, 2561; E. Uhlmann, A. Peyman, G. Breipohl and D. W. Will, *Angew. Chem., Int. Ed.*, 1998, **37**, 2796.
3 Y. Takeda, P. D. Ross and C. P. Mudd, *Proc. Natl. Acad. Sci. U. S. A.*, 1992, **89**, 8180; A. I. Dragan, J. Klass, C. Read, M. E. Churchill, C. Crane-Robinson and P. L. Privalov, *J. Mol. Biol.*, 2003, **331**, 795.
4 C. W. Lee, H. Cao, K. Ichiyama and T. M. Rana, *Bioorg. Med. Chem. Lett.*, 2005, **15**, 4243; S. Hwang, N. Tamilarasu, K. Kibler, H. Cao, A. Ali, Y. H. Ping, K. T. Jeang and T. M. Rana, *J. Biol. Chem.*, 2003, **40**, 39092; M. A. Gelman, S. Richter, H. Cao, N. Umezawa, S. H. Gellman and T. M. Rana, *Org. Lett.*, 2003, **5**, 3563; V. Kesavan, N. Tamilarasu, H. Cao and T. M. Rana, *Bioconjugate Chem.*, 2002, **13**, 1171; N. Tamilarasu, I. Huq and T. M. Rana, *Bioorg. Med. Chem. Lett.*, 2001, **11**, 505; N. Tamilarasu, I. Huq and T. M. Rana, *Bioorg. Med. Chem. Lett.*, 2000, **10**, 971; S. Hwang, N. Tamilarasu, K. Ryan, I. Huq, S. Richter, W. C. Still and T. M. Rana, *Proc. Natl. Acad. Sci. U. S. A.*, 1999, **96**, 12997; T. Klimkait, E. R. Felder, G. Albrecht and F. Hamy, *Biotechnol. Bioeng.*, 1999, **61**, 155–168; F. Hamy, E. R. Felder, G. Heizmann, J. Lazdins, F. Aboul-ela, G. Varani, J. Karn and T. Klimkait, *Proc. Natl. Acad. Sci. U. S. A.*, 1997, **94**, 3548; V. Ludwig, A. Krebs, M. Stoo, U. Dietrich, J. Ferner, H. Schwalbe, U. Scheffer, G. Dürner and M. W. Göbel, *ChemBioChem*, 2007, **8**, 1850.
5 B. J. Calnan, S. Biancalana, D. Hudson and A. D. Frankel, *Genes Dev.*, 1991, 201; R. Tan and A. D. Frankel, *Biochemistry*, 1992, **31**, 10288.
6 J. Haddad, L. P. Kotra, B. Llano-Sotelo, C. Kim, E. F. Azucena, M. Liu, S. B. Vakulenko, C. S. Chow and S. Mobashery, *J. Am. Chem. Soc.*, 2002, **124**, 3229; J. R. Thomas, X. Liu and P. J. Hergenrother, *J. Am. Chem. Soc.*, 2005, **127**, 12434; N. W. Luedtke, Q. Liu and Y. Tor, *Biochemistry*, 2003, **42**, 11391.
7 M. T. Record, W. Zhang Jr and C. F. Anderson, *Adv. Protein Chem.*, 1998, **51**, 281.

