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Structure–activity relationships in new polycationic molecules based on two 1,4-diazabicyclo[2.2.2]octanes as artificial ribonucleases

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ABSTRACT

In the present study, we designed and synthesised new polycationic molecules based on two 1,4-diazabicyclo[2.2.2]octane (DABCO) moieties with hydrophobic groups connected by different linkers. The structure and the RNA-cleavage activity relationships of this novel series of artificial ribonucleases (aRNases) were investigated.

1. Introduction

The development of artificial ribonucleases (aRNases), which are synthetic organic molecules capable of cleaving RNA, has been the subject of numerous studies during the last two decades [1]. Such compounds can find important applications in molecular biology, biotechnology and medicine, for example in gene manipulations, the design of artificial *in vitro* restriction enzymes for RNA and the development of novel chemotherapeutic agents. Most of the aRNases described are complexes of metal-ion chelates, which have La^{3+} [2], Cu^{2+} [3–5] or Zn^{2+} [6–9] as the central ion. In addition, mimetic active centres of natural enzymes (containing imidazole, guanidinium, amino or carboxyl groups) [10–18] have been reported to catalyse sequence-selective phosphodiester hydrolysis, but their catalytic activity is rarely [19,20] compared to that of metal-ion-dependent cleavers.

We have previously described the design and synthesis of an aRNases based on polycationic derivatives of 1,4-diazabicyclo[2.2.2]octane (DABCO) [21]. These low-molecular-weight compounds were symmetrical polycationic molecules composed of two identical DABCO moieties, substituted at the bridge-nitrogen atom with a lipophilic alkyl chain (tail) of variable length (C_1, C_4, C_6 or C_{12}), which were connected by relatively rigid phenylenebismethylene linkers (substituent at the *para*, *meta*, or *ortho* position of the phenyl ring) (see Fig. 1). Combinatorial libraries of compounds with different hydrophobic residues were also synthesised [22]. Some aRNases have been found to have great cleaving activity that is comparable to that of the metalocomplexes [21–23].

We have shown that the alkyl-chain length and the distance between two DABCO groups affect the cleavage activity of synthesised aRNases [21,22]. With increasing length of the alkyl chain (increasing hydrophobic properties), the RNase activity is increased. The compounds with at least one 12-carbon alkyl chain (the longest studied fragment) and a p-phenylenebismethylene linker were found to have the greatest ribonuclease activity. One compound contained two DABCO residues substituted with dodecyl fragments, and another compound contained DABCO residues substituted with one ethyl and one dodecyl fragments in the *para* position of the benzene ring [22].

Two DABCO moieties of a polycation molecule can be expected to efficiently bind to the negatively charged phosphate groups of RNA (Fig. 2). The distance between two adjacent inter-nucleotide phosphate groups in RNA is 5.95 Å. The distances between the two nearest quaternized N^+ atoms of these compounds were calculated to be 4.34, 5.61 and 5.98 Å for *the ortho*, *meta* and *para* isomers, respectively. These values are similar and we have supposed that the polycationic compounds are capable of introducing the necessary distortion into the sugar–phosphate backbone, in order to stimulate the intramolecular phosphoester transfer reaction (i.e., accelerating the spontaneous cleavage of RNA by intramolecular transesterification) [21].

The present study is aimed at studying how the structure of the linker and the increased lipophilicity of the alkyl chain affect the cleavage reaction rate, with the aim to possibly obtain more active compounds. In an effort to find an optimal structure of DABCO-based aRNase, we designed and synthesised several series of polycationic derivatives of DABCO, differing in the structure of

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the linker or the hydrophobic fragment, and evaluated their ribonuclease activity in the cleavage of a synthetic 21-mer oligoribonucleotide under physiological conditions.

2. Results and discussion

2.1. Design of compounds

In the present work, compounds were designed based on the results of cleavage activity assaying of the compounds studied earlier. We speculated that any conformational alteration of the compounds, resulting from the presence of any bulky group in the benzene ring or changing distance between the two DABCO moieties, might influence its cleavage activity. To study the effects of the linker structure, we synthesised a novel compounds series composed of two DABCO moieties with covalently attached lipophilic chains that were connected by different linkers. To increase the lipophilic properties of the alkyl chain, we synthesised fluoro-containing compounds.

In order to know how the substituents on the benzene framework could affect the cleavage activity of the lead compounds, we prepared compounds **5** and **6** (analogues of **4b** and **4c**, respectively) bearing a nitro- or methylcarboxylate group on the benzene ring (see Table 1).

Owing to the efficient intramolecular aRNase–phosphate interaction that can be achieved by modulating the length and flexibility of the linkers, we synthesised compounds with relatively rigid linkers containing olefinic, acetylenic and aromatic functionalities, as well as flexible aliphatic linkers. Two compounds, **7** and **8**, contained rigid aromatic linkers, biphenyl and naphthalene fragments, respectively. The DABCO group of **7** and **8** contained either a dodecyl or a hexyl pendant tail (attempts to efficiently prepare the compound containing a naphthalene linker and a dodecyl pendant tail were unsuccessful). Three compounds, **9**, **10** and **11**, contained either acetylenic or olefinic linkers and were relatively rigid, such as the phenylenebismethylene linkers (see Table 1). Four compounds, compounds **12–15**, contained flexible aliphatic linkers (see Table 1), ranging from propylene (compound **12**) to octamethylene (compound **15**) groups.

As shown previously, changing the alkyl tail length from C_1 (compounds **1a–c**) to C_{12} (compounds **4a–c**) increased the RNase activity [20]. We are continuing our investigation into the variations in the nature of the lipophilic chain in an effort to optimise the hydrophobic fragments. Three fluorinated analogues **16–18** (see Table 1) were synthesised, with partly fluorinated substitutions of some methylene residues at the terminal 4, 6 or 8 carbon atoms. The presence of fluorine within a molecule can dramatically increase the hydrophobicity; as known, perfluorochemically modified aliphatic fragments are more hydrophobic than those that are not modified. As the RNAse activity is positively correlated with the hydrophobicity, we expected these compounds to display a higher activity than compound **4c**, because of the increasing hydrophobicity of the lipophilic chain.

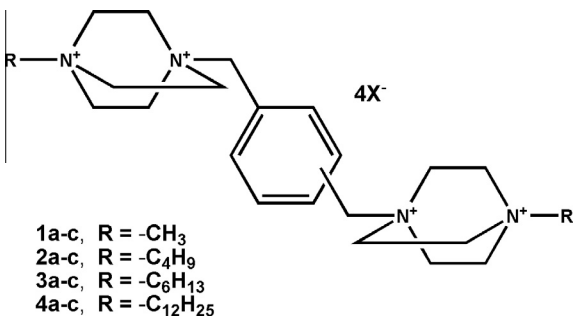


Fig. 1. Structures of the compounds studied earlier [21–23].

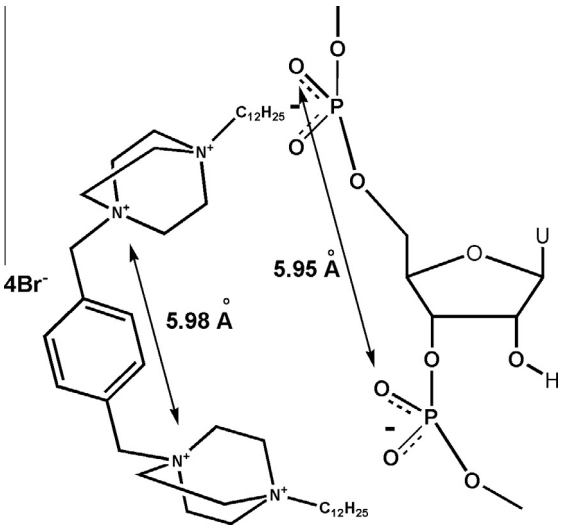


Fig. 2. DABCO moieties of a polycation molecule bind to the negatively charged phosphate groups of RNA.

The synthesis of new polycationic compounds was accomplished in a similar manner to that used for derivatives **1–4** [21]. For all new materials, ^1H and ^{13}C NMR spectra were measured along with quantitative elemental analyses, and they were found to be in accordance with the proposed structures; see Supporting Information for full experimental data.

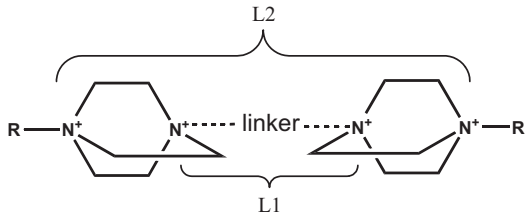
2.2. Concentration profiles of RNA cleavage by compounds 3–18

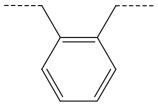
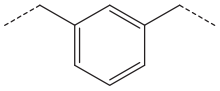
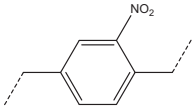
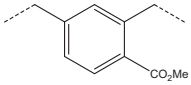
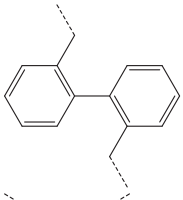
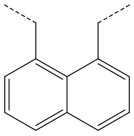
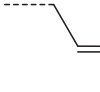
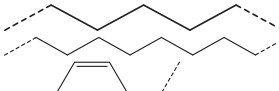
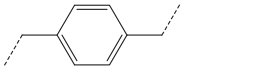
The concentration profiles for the compounds were in agreement with the results obtained earlier [23,24]. The compounds display the highest cleavage activity at concentrations far below the critical micelle concentration [24]. The compounds containing two DABCO residues, each substituted with a hexamethylene fragment (**3c** and **8**), have linear concentration dependence and a working concentration of 1 mM. The concentration profiles for most compounds containing dodecyl fragments are bell-shaped curves, with the maximal cleavage activity at 10 μM. However, compound **6**, containing methylcarboxylate residue in the benzene ring, possesses the maximal cleavage activity at 100 μM. This can possibly be explained by the presence of a methylcarboxylate group, which prevents RNA from binding to compound **6**. The concentration profiles of compounds **16–18** all have a bell-shaped form (Fig. 3), with a maximum at 10–50 μM.

2.3. Cleavage activity of compounds 3–18

The cleavage activity of the new compounds was measured as the extent of cleaved synthetic oligoribonucleotide r(UCGAAUUUCCACAGAAUUCGU) (ON 21) under standard conditions after 6, 10 and 24 h incubation with compounds in optimal concentrations. Oligoribonucleotide ON 21 forms hairpin with 7-member loop, containing UCCACAG sequence, which corresponds to nucleotides 60–66 of yeast $tRNA^{Phe}$ known to be particularly sensitive to cleavage by natural and artificial ribonucleases [25]. In all experiments the compounds cleaved the RNA target at C_{10}-A_{11} and C_{12}-A_{13} phosphodiester bonds located in the loop present in the secondary structure of this oligoribonucleotide (see Supp. Inf. Fig. S28). It is shown that the cleavage activity of compounds **4a–c** and **3a**, which were studied earlier and were taken as controls, are in agreement

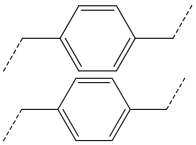
Table 1
The ribonuclease activity and the structural parameters of aRNases **3–18**.



Compound	R	Linker	L1[a] (Å)	L2[b] (Å)	Optimal concentration (M)	log*P*[c]	Cleavage extent (%)[d]
3c	C_6H_{13}		7.605	12.005	10^{-3}	0.02	48 (71)
4a	$C_{12}H_{25}$		5.429	10.184	10^{-5}	2.40	51 (75)
4b	$C_{12}H_{25}$		6.855	10.945	10^{-5}	2.41	63 (82)
4c	$C_{12}H_{25}$		7.610	12.017	10^{-5}	2.36	75 (94)
5	$C_{12}H_{25}$		7.521	11.787	10^{-5}	2.54	49 (70)
6	$C_{12}H_{25}$		6.709	10.776	10^{-4}	2.63	43 (65)
7	$C_{12}H_{25}$		5.615	7.518	10^{-5}	3.00	6 (7)
8	C_6H_{13}		5.509	10.013	10^{-3}	0.84	2 (5)
9	$C_{12}H_{25}$		6.311	11.350	10^{-5}	1.89	62 (90)
10	$C_{12}H_{25}$		6.203	11.514	10^{-5}	1.89	44 (76)
11	$C_{12}H_{25}$		6.305	10.465	10^{-5}	2.16	67 (93)
12	$C_{12}H_{25}$		5.046	9.457	10^{-5}	1.62	5 (10)
13	$C_{12}H_{25}$		6.420	11.548	10^{-5}	1.79	16 (25)
14	$C_{12}H_{25}$		7.615	12.504	10^{-5}	2.03	52 (93)
15	$C_{12}H_{25}$		11.524	16.501	10^{-5}	2.45	44 (68)
16	$(CH_2)_3C_4F_9$		7.615	12.056	5×10^{-5}	2.03	63 (88)

(*continued on next page*)

Table 1 (*continued*)

Compound	R	Linker	L1[a] (Å)	L2[b] (Å)	Optimal concentration (M)	log*P*[c]	Cleavage extent (%)[d]
17	$(CH_2)_3C_6F_{13}$		7.688	12.125	10^{-5}	2.46	61 (89)
18	$(CH_2)_3C_8F_{17}$		7.630	12.063	10^{-5}	3.14	72 (99)

[a,b] Calculated by using Chem 3D Ultra 10.0v program.

[c] Calculated using ALOGPS 2.1 online program: http://www.vcclab.org/web/alogps/. ALOGPS v 2.0 method for the assessment of n-octanol/water partition coefficient, log*P*, was developed on the basis of neural network ensemble analysis of 12908 organic compounds available from PHYSPROP database of Syracuse Research Corporation.

[d] Experimental conditions: 5′-labelled ON21 (5 μM) was incubated with one of the compounds at optimal concentrations for 10 (24) h at 37 °C. For details see "Experimental part".

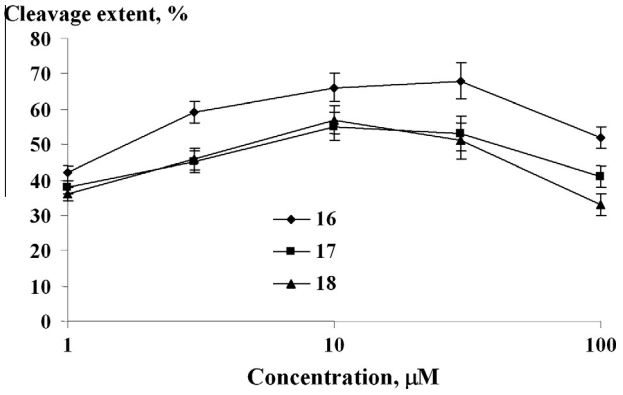


Fig. 3. Concentration profiles for compounds **16–18**.

with earlier studies (**4c** > **4b** > **4a** ⩾ **3c**). As the form of the kinetic curves of these compounds represents a saturation curve [20,21], the extent of RNA cleavage after 10 h was chosen to compare the compounds' cleavage activity. The ribonuclease activity (expressed as the extent of RNA cleavage after 10 h incubation, for each compound), the lipophilicity expressed in terms of the partition coefficient (log*P*), the distance (L1) between the two nearest nitrogen atoms of the compounds and the distance (L2) between the two farthest nitrogen atoms of the compounds are displayed in Table 1.

Introduction of nitro- or methylcarboxylate groups into compounds **4c** or **4b** led to compounds **5** and **6**, which displayed a lower catalytic activity (48% and 43%, respectively), than the activity of **4c** (75%) or **4b** (63%). The reason for the reduced activity of these analogues appears to result from steric hindrance effects and restricted rotation of the DABCO fragments, leading to no optimal geometry molecules as a whole.

Alteration of the phenylenebismethylene linker to a biphenyl or naphthalene linker had detrimental effects on the ribonuclease activity. Although log*P* of compounds **7** and **8** are higher than compounds **4c** and **3c**, they give significantly lower cleavage activity, resulting in 6% and 2%, respectively, versus the control compounds **4c** (75%) and **3c** (48%). Negligible interactions between compounds **7** or **8** and the RNA target explain this. However, the L1 distance of these compounds is less than the L1 distance of compounds **4c** and **3c** (5.615 Å: **7**, 5.910 Å: **8**, 7.610 Å: **4c** and **3c**), and they are comparable with the L1 distance of compounds **4a**, which have moderate activity. Thus, the essential geometry of compounds **7** or **8** is not provided, in order to catalyse RNA cleavage.

In general, the ribonuclease activity of compounds with aromatic linkers decreased in the order: **4c** > **4b** > **4a** ⩾ **3c** ≈ **5** ≈ **6** ≫ **7** ≈ **8**.

Compounds with unsaturated linkers (**9–11**) contain the same number of carbon atoms. Although, the log*P* value and the L1 distance of these compounds are close, their ribonuclease activities differed and decreased in the order: **9** (67%) ∼ **11** (62%) > **10** (44%). The cleavage extent of these compounds is close to that of compounds **4c** and **4b** (for **9** and **11**) and **4a** (for **10**). It can be a representative example of the importance of the essential geometry for high cleavage activity of a compound.

The activity of the compounds with a flexible linker (**12–15**) strongly depended on the length of the linker. Analyses showed that an alkyl chain length of $n = 5$ exhibited the most activity (52% cleavage for compound **14**), whereas $n < 5$ or $n > 5$ decreased the ribonuclease activities; compounds **12**, **13** and **15** possessed less cleavage activity (8%, 40% and 22%, respectively). This could be explained, because no optimal distance between two DABCO residues, in the cases of the last compounds, formed stable complexes with inter-nucleotide phosphates according to the scheme in Fig. 2. However, in the case of **14** (L1 = 7.615 Å compared with **4c**), the distance is optimal for binding. In comparison to the compounds with unsaturated linkers (**9–11**), compound **13** (bearing a C_4 linker) exhibited much lower activity. A possible explanation for this fact is that the fixed conformation of **9** and, to less extent **10** and **11**, provides a higher affinity to inter-nucleotide phosphates compared to the free conformation of **13** and even **14**.

The results showed that, although the lipophilicity of the linker group did not play a role, the structure of the linker group significantly influenced the ribonuclease activity. The ability of a molecule to take the essential geometry is important in providing the high affinity of inter-nucleotide phosphates. The optimal geometry of the compound for cleavage RNA can be found by further computer modelling and by calculating the distances and angles between two DABCO residues more accurately.

In the investigation into variations of the nature of the lipophilic chain, we identified that fluorinated compound **18** was the most active. Compounds **18** (72% RNA cleavage) possess almost the same cleavage activity as **4c** (75%) after 10 h incubation; and the activity of compounds **16** and **17** are lower (63% and 61%, respectively). The largest difference between cleavage activities can be observed at the middle of the linear part of the kinetic curves of these compounds. After 6 h incubation, the extent of ON21 cleavage by **18** (48%) is 1.5 times higher than by **16** (29%), which is even higher than by **4c** (38%). Compounds from the perfluorinated series contained phenylenebismethylene linkers, as in compound **4c**. The distance between the nitrogen atoms of these compounds is the same distance as in compound **4c**, whereas the hydrophobic properties (the log*P* value) are increased with increasing perfluorinated chain length.

Thus, the lipophilicity of the hydrophobic groups and the distance between the two DABCO moieties, as reflected by the length and structure of the linker, are important requirements for the ribonuclease activity. A better understanding of the structure and the RNA-cleavage activity relationship might assist in the design of new aRNases. The reaction mechanism for RNA cleavage, using

these compounds, is not well understood and requires further study.

3. Conclusions

In the present work, it was shown that the linker moiety plays a key role in RNA cleavage by DABCO-contained aRNases. Although the alteration of the benzene linker to a biphenyl or naphthalene linker inactivated the nuclease activity, the compounds with multiple-bond linkers (**9–11**) and compounds with flexible linkers (**14**) possessed a high cleavage activity. The presence of a bulky group in the benzene ring decreased the cleavage activity. Fluorinated aRNase **18**, containing the optimal phenylenebismethylene linker (as in **4c**) and more hydrophobic fragments, possessed even higher activity than **4c**.

4. Experimental section

4.1. Synthesis

See Supporting Information for full experimental data.

4.2. Miscellaneous chemicals, enzymes and RNA

Chemicals for electrophoresis were purchased from Sigma (USA), and [γ-^{32}P]-ATP was from Biosan (Russia). Solutions for RNA handling were prepared using Milli-Q water that had been filtered through membranes with a 0.2 μm pore size (Millipore, USA) and autoclaved. T7 RNA polymerase and T4 polynucleotide kinase were obtained from Fermentas (Lithuania). Ribonuclease T1 and bovine alkaline phosphatase were purchased from Sigma (USA). Oligonucleotide ON21 r(UCGAAUUUCCACAGAAUUCGU) used as RNA substrate was synthesised by Dr. M. Repkova (this institute) using solid-phase H-phosphonate method and purified by successive ion-exchange and reverse-phase HPLC.

4.3. End-labelling of RNA

5′-[^{32}P]-ON21 was labelled using γ-[^{32}P]-ATP and T4 polynucleotide kinase according to published protocol [26]. After labelling, the RNAs were purified by electrophoresis in 8% denaturing polyacrylamide gel. The labelled RNAs were eluted from the gel by three portions (300 μL each) of 0.5 M ammonium acetate, which contained 0.5 mM EDTA and 0.1% SDS, and were then precipitated with ethanol. RNA pellets were centrifuged, rinsed twice with 80% ethanol, dissolved in water and stored at −20 °C.

4.4. Cleavage experiments

The 5′-ends of labelled ON21 were diluted with unlabelled ON21 to a final concentration of 5 μM. Kinetic and concentration experiments were carried out under standard reaction conditions. A standard reaction mixture (10 μL) contained 50,000–100,000 Cherenkov cpm of [5′-^{32}P]-RNA substrates (stabilised by 5 μM ON21), and 5–1000 μM of the compounds in 50 mM Im buffer at pH 7.0, 0.2 M KCl and 0.5 mM EDTA. In other experiments one or more parameters were varied to assay the influence of reaction conditions. Reaction mixtures were incubated at 37 °C for different times (from 1 to 24 h); reactions were quenched by RNA precipitation with 100 μL of 2% lithium perchlorate in acetone. The RNA pellets were collected by centrifugation and dissolved in the loading buffer (7 M urea and leading dyes: 0.01% xylene cyanol and 0.01% bromphenol blue). The cleavage products were analysed in 8 or 15% PAAG, containing 8 M urea and TBE × 1 as the running buffer. The cleavage sites were assigned by comparison of the electrophoretic mobilities of the cleavage products with the products of RNA cleavage, using RNase T1 and the statistical hydrolysis in 2 M imidazole buffer (pH 7.0). Quantitative data were obtained using Molecular Imager FX (Bio-RAD, USA). Typical gels were depicted in Supporting Information.

Appendix A. Supplementary material

Supplementary material associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bioorg.2014.10.002.

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