Design and synthesis of artificial ribonucleases based on 1,4-diazabicyclo[2.2.2]octane and imidazole

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The review surveys the results of our studies devoted to the design of highly efficient catalysts of hydrolysis of the phosphodiester bonds in RNA. These catalysts contain the imidazole residue in the catalytic domain, one or several bis-quaternized rings of 1,4-diazabicyclo[2.2.2]octane as a polycationic RNA-binding domain, and a lipophilic radical. A versatile approach to artificial ribonucleases of this type was proposed, which allows one to vary not only the number of positive charges in the RNA-binding domain, the structure of the catalytic site, and their mutual arrangement but also the domain structure of the molecule as a whole. Analysis of the catalytic properties of the synthesized constructs makes it possible to optimize the domain structure and the geometry of the molecule ensuring its maximum ribonuclease activity.

Key words: artificial ribonucleases, simulation of enzyme catalytic sites, RNA hydrolysis, catalysis.

Deoxyribonucleic and ribonucleic acids are among the most important chemical compounds, which provide functioning of any living organism. These long-chain compounds are formed from nucleosides linked through phosphodiester bonds. Cleavage of internucleoside linkages is an ordinary and still very important biological process. Although phosphodiesters are generally stable under physiological conditions, they can be efficiently hydrolyzed in the presence of appropriate enzymes.

The design of small synthetic constructs, which are able to hydrolytically cleave RNA with high efficacy under physiological conditions, is of considerable interest for the solution of particular fundamental and applied problems in molecular biology. These compounds have come to be known as artificial or chemical ribonucleases. Investigation of their hydrolytic properties can help in revealing the role of the structural and dynamic factors responsible for high efficacy of catalysis performed by natural enzymes. Small molecules, which can nonspecifically interact with RNA thus inducing its hydrolysis, can be used in studies of the three-dimensional RNA structure, and their binding to antisense oligonucleotides opens up the way for the design of site-specific ribonucleases.

Artificial ribonucleases show promise as efficient antiviral pharmaceuticals or can be used in chemotherapy for treatment of malignant tumors.[1,2] However, these conjugates will be of practical interest in medicine only if the following conditions are met: the compounds should be non-toxic and chemically stable, possess high hydrolytic activity, and (in some cases) exhibit selectivity, and their synthesis should be simple and inexpensive.

Natural ribonucleases: active sites and mechanisms of action

Most of natural protein catalysts involve 20 major genetically coded amino acids. The functional groups of their side chains form catalytic sites. The residues of histidine, lysine, arginine, aspartic acid, glutamic acid, and their amides and also residues of hydroxy-containing amino acids are most often involved in catalytic sites of enzymes. The active sites of the majority of enzymes include ions of such metals as Mg, Ca, Zn, Co, Mn, Fe, and Ni coordinated by functional side groups of amino acids.[3—6] Enzymes whose active sites contain no metal ions occur much more rarely. Examples are ribonucleases A and T.

Progress in studies of the structures and mechanisms of functioning of natural enzymes hydrolyzing P—O bonds in different substrates was covered in recent reviews.[6—9] The general notion of enzymatic catalysis as a

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combination of coupled processes suggests that the active sites of natural ribonucleases contain several structurally functional blocks, which are responsible for activation of both the attacked and attacking species and also for stabilization of the transition state and the leaving group. It is generally assumed that hydrolysis of P—O bonds occurs due to one or other type of acid-base catalysis. Unlike hydrolysis of C—O bonds, the cleavage of P—O bonds through the mechanism of nucleophilic catalysis occurs very rarely.[10,11]

The water molecule most often serves as the attacking (nucleophilic) species. However, hydroxyalkyl groups, for example, the 2′-hydroxy group of ribose or the OH group of hydroxy-containing amino acids can also act as nucleophiles. Much more rarely, the function of the nucleophilic species is fulfilled by the SH group of cysteine.[12] According to the most probable mechanisms of phosphoester hydrolysis (EA; SN_2P),[13,14] the attack occurs on the phosphorus atom. The reaction proceeds *via* the formation of a negatively charged intermediate containing the pentacoordinated phosphorus atom.

Imidazole, guanidine, the amino group, the carboxylate anion, or metal complexes can serve as activators of nucleophilic species in catalytic sites of hydrolases. The protonated carboxy or amino group,[15] the imidazolium cations,[16] or the guanidinium cations[17] can activate the phosphorus atom by enhancing its electrophilicity or stabilize the intermediate state through compensation of the negative charge both on the phosphate group and the oxygen atom of the leaving group. Metal complexes can fulfill any one of these functions.

It should be noted that the presence of a catalytically active group (even a polyfunctional group) by itself is not necessarily sufficient for the manifestation of enzymatic activity. Thus, one catalytic event in hydrolysis of RNA by ribonuclease A proceeds with the simultaneous participation of two acid-base catalytic sites based on imidazole, which have different spatial arrangement with respect to the RNA target, one site serving as a proton donor and another site acting as a proton acceptor.

General principles of the design of artificial ribonucleases

The design of artificial ribonucleases is a rapidly developing field of the synthesis of enzyme mimetics, *viz.*, chemical constructs functionally analogous to natural enzymes.

Ribonucleases, like any enzyme, can be represented as a combination of the substrate-binding domain,* usually of protein nature, and the catalytic site. For ribonuclease to efficiently perform its function, *viz.*, the cleavage of phosphodiester bonds in RNA through either their direct hydrolysis or the transfer of the phosphate group, the catalytic site of artificial ribonuclease must possess at least one of the following properties (a combination of two or more such properties is desirable):

— Brønsted basicity (ability to accept the proton);
— Brønsted acidity (ability to donate the proton);
— ability to act as a nucleophilic catalyst;
— ability to form a hydrogen bond;
— ability to electrostatically stabilize the transition state by charge neutralization;
— Lewis acidity.

When designing a catalytic domain, it should be remembered that its activity can be enhanced by combining two or more active sites.

In recent studies, metal complexes or organic constructs containing one or more reactive groups have found the most extensive application as components of catalytic sites in artificial ribonucleases. Among these reactive groups generally involved as components in the active sites of natural ribonucleases are imidazole (RNases A and T1), guanidine (RNase S), carboxy groups (RNase T1), or amino groups (RNase A). Oligopeptides and polyamines are the simplest representatives of such artificial ribonucleases.

A broad spectrum of synthetic nucleases, which cleave nucleic acids by oxidative or photooxidative mechanisms, are presently known.[18] Many metal-containing artificial nucleases, particularly DNases, oxidize either certain nitrogen bases in the polynucleotide chains or the ribose rings. However, RNAs exhibit substantially higher resistance to oxidative cleavage as compared to DNAs due to higher stability of the glycoside bond in ribonucleotides as compared to deoxyribonucleotides.[19]

The introduction of the affinity site into synthetic ribonucleases, which ensures the formation of a stable complex of the RNA target with a catalyst, makes it possible to use the "drawing together" effect to increase the rate of RNA hydrolysis and/or to decrease the concentration of the catalyst by several times. Such RNA-binding domains can be targeted to interact both with nitrogen bases (for example, as intercalators through the stacking-interaction mechanism or through the formation of duplexes) and with the ribosephosphate backbone *via* an electrostatic mechanism (polycations).

Anchoring of the catalytic domain either to molecules, which serve as rather rigid spatial templates for RNA[20] (such as small cyclic peptides,[21] steroids,[22] or cyclodextrins[23]), or to oligonucleotides complementary to segments of the RNA target[24] enables one not only to use the "drawing together" effect but also to direct hydrolytically active groups at certain sites. The use of derivatives of antisense oligonucleotides, for example, their

* Hereinafter, the structural fragments of artificial ribonucleases, which are functionally analogous to particular domains of natural enzymes, will be referred to as domains by analogy with the nomenclature accepted in the protein chemistry.

methylphosphonate or phosphorothioate analogs, instead of antisense oligonucleotides as such hinders the self-cleavage of artificial nucleases under the conditions of hydrolysis.[25,26]

One of the main approaches to the construction of potential catalytic sites of synthetic ribonucleases is based on the design of transition metal complexes capable of cleaving RNA under mild conditions.[3,5] It is not surprising that ions of such metals as zinc, copper, iron, and cobalt, which are involved in catalytic sites of many natural enzymes, can successfully cleave RNA or related model substrates. However, lanthanide or thorium ions, which do not occur in natural enzymes, proved to be equally efficient catalysts of RNA hydrolysis. These metals are particularly efficient when involved in complexes with macrocyclic polyfunctional ligands. The design of these ligands has attracted considerable attention in recent years.

For many metal-containing artificial ribonucleases, the bi- or even polynuclear complexes of the above-mentioned metals demonstrated higher efficacy in hydrolysis of RNA or model substrates as compared to analogous lower-nuclearity complexes. In some cases, the introduction of the second metal (sometimes even hydrolytically inactive) into the catalytic domain gives rise to the pronounced synergistic effect.[27,28]

As expected, a combination of such metal-containing catalytically active groups and the substrate-binding domain (for example, small peptides, oligoamines, or cyclodextrins) within one molecule led to the enhancement of efficiency of the phosphodiester bond hydrolysis. The conjugates of catalytic domains based on zinc, copper, iron, manganese, or lanthanide complexes with antisense oligonucleotides actually demonstrated selective binding to the RNA substrate and caused its site-specific hydrolysis. Advances in the design of artificial nucleases based on metal complexes were surveyed in detail in reviews.[24,29—34]

Quite a different approach to the design of model RNA-hydrolyzing sites is based on a combination of organic functional groups involved in the active sites of natural nucleases. Compounds simulating the catalytic sites of natural RNases of this type most often involve imidazole, guanidine, amino groups, carboxy groups, or natural amino acids containing these fragments.

As mentioned above, efficient RNA hydrolysis is favored by the presence of more than one acid-base catalytic sites in the catalytic domain. Chemical RNases containing one catalytically active group hydrolyze RNA with a noticeable rate only under special conditions, for examples, in the presence of an imidazole buffer, which supplies the second component of the catalytic site.[35]

There are several examples of synergistic action of two organic active sites in the model catalytic domain. Thus, the synergism of action of the ammonium and carboxylate groups was exemplified by $tRNA^{Phe}$ hydrolysis by the glycine or iminodiacetic acid conjugates with anthraquinone.[36] The synergism of action of two imidazolyl groups or two guanidinium groups was demonstrated by hydrolysis of (3′—5′)-adenylyladenosine[37] or the model substrate, *viz.*, 2-hydroxypropyl *p*-nitrophenyl phosphate,[38] respectively. The synergism of action of the imidazole residue and the free amino group was observed in experiments on depolymerization of ribosomal RNA.[39]

The synergistic action of two or more amino groups is illustrated by the fact that RNA-hydrolyzing activity is exhibited by low-molecular-weight polyamines, such as ethylenediamine or triethylenetetramine,[40] as well as by a series of biogenic polyamines, such as spermine, spermidine, or putrescine,[41] whereas monoamines are absolutely inefficient. This observation gave impetus to the use of various polyamines as catalytic domains of artificial ribonucleases.

The simplest artificial ribonucleases are di- or oligopeptides containing histidine, arginine, or lysine as the catalytic domain. In enzyme mimetics of this type, the function of the RNA-binding domain can be performed by the same basic amino acids, such as lysine,[42,43] histidine, or arginine,[44] which possess an affinity for the ribosephosphate backbone of RNA, or hydrophobic amino acids structured to form β folds, which can be involved in stacking interactions with nitrogen bases.[45] The efficacy of RNA hydrolysis by even simple dipeptides, for example, by histidyl-lysine, was shown to be an order of magnitude higher than that of individual amino acids.[42]

As expected, artificial ribonucleases containing a catalytic domain along with intercalators, such as acridine,[44,46—48] phenazine, isoalloxazine, or ethidium[47,49] (which can form complexes with the polynucleotide chain of RNA through stacking interactions), may exhibit substantially higher hydrolytic activity. This enables one to decrease the concentration of the enzyme to millimole amounts with retention of efficiency of RNA hydrolysis.

The Coulomb interactions between the polycation and the ribosephosphate backbone of the RNA substrate provide the basis for the design of conjugates of an imidazole-containing catalytic site with the biogenic polyamine spermine, which serves in this case as an RNA-binding domain.[49,50]

In some studies, the catalytic site of artificial ribonuclease was anchored to spatial RNA templates formed by the rigid frame of steroids[38,51,52] or cyclodextrins.[53]

Finally, it was demonstrated that site-specific artificial ribonucleases can be designed by conjugation of the above-described catalytic domains of organic nature with antisense oligonucleotides.[25,50,54—59] The introduction of polyamines into the middle of the DNA chain was proposed as a procedure for the pinpoint RNA scission.[60—66]

It should be noted that the classification of the catalytic domains into the metal-containing and organic types is somewhat arbitrary. The closer examination of the mechanism of action of artificial metal-containing ribonucleases revealed that (like in natural enzymes[67]) not only the metal ions but also various acidic or basic groups of complicated organic ligands are directly involved in the catalytic event. The synergistic action of two zinc atoms and two dimethylamino groups of the ligand was illustrated with binuclear zinc complexes with calix[4]arene ligands as an example. Thus, the Zn^{II} sites together activate the phosphoryl group, whereas the dimethylamino group acts as a base and deprotonates the hydroxy group of the model substrate, *viz.*, 2-hydroxypropyl *p*-nitrophenyl phosphate.[68] In catalysis of transesterification of phosphodiesters with terpyridine copper complexes, the catalytic event involved copper ions as the major catalyst along with either the hydroxo group bound to the metal atom or the peripheral tertiary amino group depending on pH of the medium.[69] Crystallographic evidence for the simultaneous involvement of metal ions and NH-acidic side chains of amino acids (the NH_4^+ groups of lysine, the guanidinium group of arginine, or the imidazole group of histidine) in the cleavage of phosphodiesters was obtained for copper-containing artificial RNases.[70]

The above-considered data account for two major principles of the design of artificial ribonucleases, *viz.*, a combination of two or more reactive groups of any nature in the catalytic domain and the introduction of an RNA-binding domain of any type into the enzyme molecule.

In spite of abundant data on the synthesis of compounds simulating the action of natural RNA-hydrolyzing enzymes, most of these studies proposed individual constructs containing different groups both in the RNA-binding and RNA-hydrolyzing domains. However, a lack of systematic studies performed under comparable conditions as well as the use of different RNAs or RNA-imitating substrates and non-comparable methods for the estimation of the extent of hydrolysis did not allow one to reveal the structure—activity correlations for synthetic ribonucleases.

We have demonstrated[71—77] * that efficient catalysts for the cleavage of the phosphodiester bonds in RNA can be designed by combining catalytically active imidazole-containing functional groups found in the active sites of natural ribonucleases, an RNA-binding group of polycationic nature based on 1,4-diazabicyclo[2.2.2]octane, which possesses a high affinity for the phosphate anions,[78] and a lipophilic hydrocarbon radical, which facilitates penetration through the cellular membrane. Our versatile approach to the synthesis of such artificial ribonucleases enables one to vary the structural parameters of both the RNA-binding and RNA-hydrolyzing sites as well as the geometry of the molecule as a whole over wide ranges and makes it possible to functionally improve the construct by introducing additional domains.

Design of artificial ribonucleases $XA^nB_iL^kC^m$

Generally, chemical ribonucleases $XA^nB_iL^kC^m$ consist of four domains, *viz.*, a normal aliphatic domain (**A**), an RNA-binding domain (**B**), a catalytic domain (**C**), and a polymethylene linker (**L**) joining the domains, and contain a terminal functional group **X** (Fig. 1).

Being the residue of histamine (C^1), methyl ester of histidine (C^2), or histidine (C^3), the catalytic domain C^m contains imidazole known as an important constituent of active sites of many natural RNA-hydrolyzing enzymes.

The multi-charged cationic domain B_i consisting of one ($i = 1$) or several fragments of bis-quaternized 1,4-diazabicyclo[2.2.2]octane linked through a standard bridge provides electrostatic binding of artificial ribonuclease to RNA. The cationic fragment may also participate in the catalytic event of the phosphodiester bond cleavage through stabilization of the negatively charged intermediate.

The polymethylene linker L^k (k is the number of methylene units) joins the catalytic and RNA-binding domains thus determining both the distance between these domains and their inter-orientation and, consequently, the orientation of the catalytic site toward the RNA substrate.

The normal aliphatic radical XA^n ($X = H$, n is the number of methylene units) was introduced into the molecule of artificial ribonuclease to facilitate the penetration of the latter through cellular membranes in the subsequent experiments on the *in vivo* RNA cleavage (lipophilicity of the construct is determined by the length of the aliphatic chain n). The functional group **X** (for example, $X = NH_2$, N_3, or PhtN) can be attached to the terminus of this aliphatic radical, which enables one either to impart an additional positive charge to the molecule or to use the construct as a monomer for building

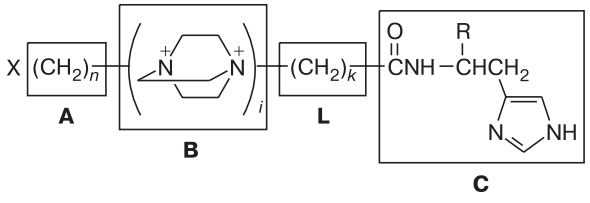


Fig. 1. Domain structure of artificial ribonucleases $XA^nB_iL^kC^m$.

* D. A. Konevetz, M. A. Zenkova, I. E. Beck, V. N. Silnikov, R. Giege, and V. V. Vlassov, *Artificial ribonucleases based on 1,4-diazabicyclo[2.2.2]octane and imidazole residues 5: the RNA cleaving activity depends on the RNA binding—to—RNA cleaving domains distance*, in preparation.

up the multi-charged RNA-binding domain. In addition, the functional group **X** can be used to introduce additional domains into chemical ribonucleases.

It could be assumed that the presence in the conjugate of only two domains, *viz.*, the catalytic domain (**C**) and the RNA-binding domain (**B**), joined through the linker **L** is the necessary and sufficient prerequisite to catalytic activity. Nevertheless, the domain **A** could also facilitate the catalytic event by imparting the surfactant properties to the chemical ribonuclease because it is known that some surfactants by themselves cleave RNA.[79]

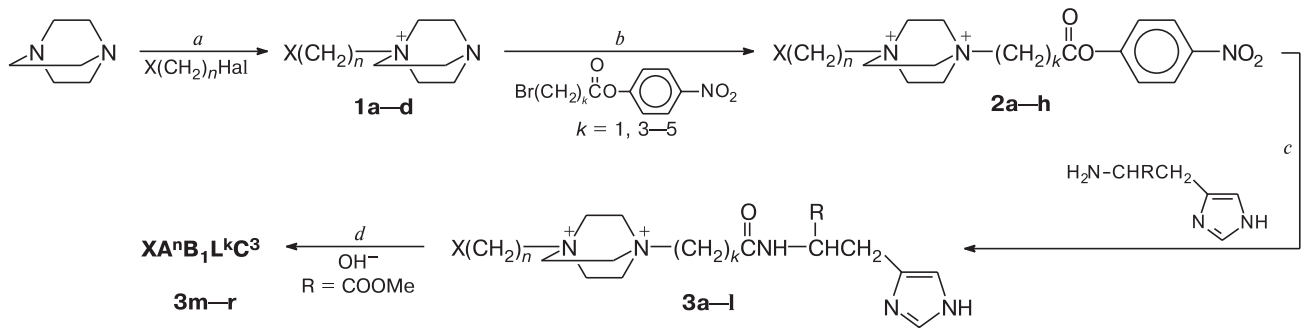
When developing approaches to the synthesis of chemical ribonucleases, we made provision for the variations in both their domain structure and the nature of the domains, as well as in the molecular geometry over wide ranges. In this case, the synthesis of series of chemical ribonucleases with certain domain structures and a comparative analysis of their catalytic properties becomes possible. The homologs in each series differ only by one parameter (for example, by the number of positive charges in the RNA-binding domain **B**, the structure of the catalytic site **C**, the length of the polymethylene linker **L**, or lipophilicity of the aliphatic radical **A**). Besides, we designed a series of model "truncated" compounds by excluding one or several structural elements from artificial ribonuclease $\mathbf{XA}^n\mathbf{B}_i\mathbf{L}^k\mathbf{C}^m$ and studied their catalytic properties, which allowed us to examine the role of each domain in detail. In this way, we intended to reveal the necessary and sufficient set of domains and to determine the optimum structure of chemical ribonuclease of this type, which most efficiently cleaves RNA.

Synthesis of artificial ribonucleases $\mathbf{XA}^n\mathbf{B}_1\mathbf{L}^k\mathbf{C}^m$

General synthesis strategy. When designing the synthesis of artificial ribonucleases with the domain structure $\mathbf{XA}^n\mathbf{B}_1\mathbf{L}^k\mathbf{C}^m$, we proceeded from the requirements of the versatility and simplicity of experimental techniques and commercial availability of the precursors bearing necessary functional groups. It seemed reasonable to start the building-up of the RNA-binding site from inexpensive 1,4-diazabicyclo[2.2.2]octane. To form the catalytic site, we chose commercial histamine and methyl ester of histidine as the most accessible imidazole-containing compounds.

Since both histamine and methyl ester of histidine are aliphatic amines, we believed that it was most reasonable to use the activated ester method for linking domains. This method was developed in detail in the peptide chemistry. Hence, we synthesized all the ribonucleases (Schemes 1 and 2) with the use of the general approach consisting in the design of activated ester $\mathbf{XA}^n\mathbf{B}_1\mathbf{L}^k$**—Np** (**Np** = 4-$NO_2C_6H_4$), which contained the RNA-binding domain anchored to the acylating functional group through the linker $\mathbf{L}^k$ of the required length (steps *a* and *b*) followed by its aminolysis with imidazole-containing amine (steps *c* and *f*).

Scheme 1

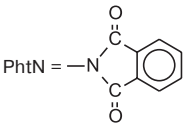


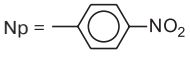
1		X	*n*
PhtNA^{2}B$_1$	(**a**)	PhtN	2
N$_3$A^{3}B$_1$	(**b**)	N_3	3
HA^{2}B$_1$	(**c**)	H	2
HA^{14}B$_1$	(**d**)	H	14

2		X	*n*	*k*
HA^{14}B$_1$L^1—Np	(**a**)	H	14	1
HA^{14}B$_1$L^3—Np	(**b**)	H	14	3
HA^{14}B$_1$L^4—Np	(**c**)	H	14	4
HA^{14}B$_1$L^5—Np	(**d**)	H	14	5
HA^{2}B$_1$L^1—Np	(**e**)	H	2	1
HA^{2}B$_1$L^3—Np	(**f**)	H	2	3
PhtNA^{2}B$_1$L^1—Np	(**g**)	PhtN	2	1
N$_3$A^{3}B$_1$L^1—Np	(**h**)	N_3	3	1

3		X	*n*	*k*	R
HA^{14}B$_1$L^{1}C^1	(**a**)	H	14	1	H
HA^{14}B$_1$L^{3}C^1	(**b**)	H	14	3	H
HA^{14}B$_1$L^{4}C^1	(**c**)	H	14	4	H
HA^{14}B$_1$L^{5}C^1	(**d**)	H	14	5	H
N$_3$A^{3}B$_1$L^{1}C^1	(**e**)	N_3	3	1	H
PhtNA^{2}B$_1$L^{1}C^1	(**f**)	PhtN	2	1	H
HA^{14}B$_1$L^{1}C^2	(**g**)	H	14	1	COOMe
HA^{14}B$_1$L^{3}C^2	(**h**)	H	14	3	COOMe
HA^{14}B$_1$L^{4}C^2	(**i**)	H	14	4	COOMe

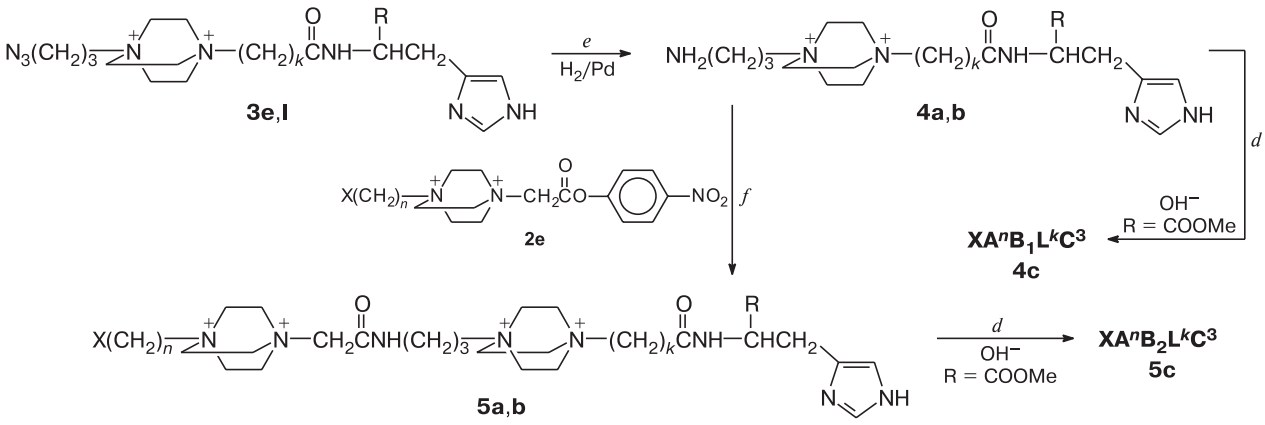
3		X	*n*	*k*	R
HA^{14}B$_1$L^{5}C^2	(**j**)	H	14	5	COOMe
HA^{2}B$_1$L^{3}C^2	(**k**)	H	2	3	COOMe
N$_3$A^{3}B$_1$L^{1}C^2	(**l**)	N_3	3	1	COOMe
HA^{14}B$_1$L^{1}C^3	(**m**)	H	14	1	COOH
HA^{14}B$_1$L^{3}C^3	(**n**)	H	14	3	COOH
HA^{14}B$_1$L^{4}C^3	(**o**)	H	14	4	COOH
HA^{14}B$_1$L^{5}C^3	(**p**)	H	14	5	COOH
HA^{2}B$_1$L^{3}C^3	(**q**)	H	2	3	COOH
N$_3$A^{3}B$_1$L^{1}C^3	(**r**)	N_3	3	1	COOH

PhtN = 

Np = 

Note. Compounds **3a**—**e**,**g**—**r** were tested for ribonuclease activity.

Scheme 2



Compound	X	*n*	*k*	R
$HA^2B_1L^1$—Np (**2e**)	H	2	1	—
$N_3A^3B_1L^1C^1$ (**3e**)	N_3	3	1	H
$N_3A^3B_1L^1C^2$ (**3l**)	N_3	3	1	COOMe
$NH_2A^3B_1L^1C^1$ (**4a**)	NH_2	3	1	H
$NH_2A^3B_1L^1C^2$ (**4b**)	NH_2	3	1	COOMe

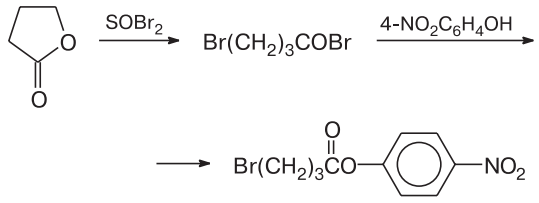
Compound	X	*n*	*k*	R
$NH_2A^3B_1L^1C^3$ (**4c**)	NH_2	3	1	COOH
$HA^2B_2L^1C^1$ (**5a**)	H	2	1	H
$HA^2B_2L^1C^2$ (**5b**)	H	2	1	COOMe
$HA^2B_2L_1C^3$ (**5c**)	H	2	1	COOH

Note. Compounds **4a**,**b** and **5a**,**b** were tested for ribonuclease activity.

Design of activated esters containing the RNA-binding domain. The monoquaternary salts of 1,4-diazabicyclo[2.2.2]octane **XA^nB_1** (**1**), most of which (with **X** = H) are well-known, can be readily prepared by alkylation of the starting 1,4-diazabicyclo[2.2.2]octane with normal or ω-functionalized alkyl halide (Scheme 1, step *a*). The reactions were generally carried out in acetone or methanol, the temperature and reaction time being governed by the reactivity of the alkyl halide employed. After washing with an excess of the solvent, the precipitates of the monoquaternary salts were used in the subsequent synthesis without additional purification.

Taking into account the goal to be sought, the second quaternizing radical must contain either an activated ester group, which is suitable for the direct reaction with imidazole-containing amine, or its precursor. Although monoquaternary salts **1** are readily alkylated with ω-halogen-containing carboxylic acids, we failed to transform the resulting acids into activated esters. At the same time, most of ω-halogen-containing 4-nitrophenyl carboxylates have been described in the literature. Generally, these esters are prepared by condensation of acids with 4-nitrophenol in the presence of dicyclohexylcarbodiimide. This procedure was used for the synthesis of activated ester of bromoacetic acid. However, we decided that it is more practicable to synthesize esters of higher acids starting from more accessible and inexpensive cyclic compounds containing the required number of carbon atoms. Thus, 4-nitrophenyl γ-bromobutyrate was easily prepared by the opening of the γ-butyrolactone ring under the action of thionyl bromide followed by acylation of 4-nitrophenol with the resulting γ-bromobutyryl bromide (Scheme 3).

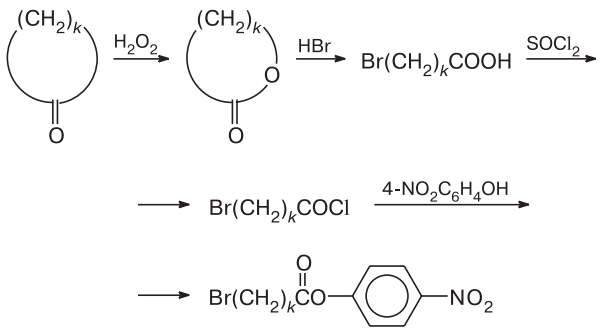
Scheme 3



The synthesis can be carried out without isolation of half-product. 4-Nitrophenyl δ-bromovalerate and 4-nitrophenyl ε-bromocaproate were prepared from the corresponding cyclic ketones as follows. Cyclopentanone or cyclohexanone were subjected to Baeyer—Villiger oxidation under the action of hydrogen peroxide. The resulting mixture of hydroxy acids and their derivatives was transformed into ω-bromoacids by treating with HBr and then into ω-bromoacyl chlorides under the action of thionyl chloride followed by acylation of 4-nitrophenol (Scheme 4). After chromatographic purification, the activated esters were obtained in 50—60% yields.

The monoquaternary salts of 1,4-diazabicyclo[2.2.2]octane were alkylated with 4-nitrophenyl ω-bromocarboxylates (see Scheme 1, step *b*) in acetone or acetonitrile. The choice of the solvent was governed by the solubility of the starting monoquaternary salts in this solvent (bis-quaternary salts are insoluble in both

Scheme 4



acetone and acetonitrile). It appeared that the monoquaternary salts containing a higher aliphatic radical are more convenient to alkylate as suspensions in acetone in which these salts are partially soluble. In other cases, acetonitrile proved to be more suitable because both reacting compounds were readily dissolved in this solvent. Since the reactions often proceeded slowly, the absence of water in the solvents was of fundamental importance; otherwise partial hydrolysis of activated ester took place. We also attempted to reduce the reaction time through an increase in temperature by fusing the reagents or performing the reaction under pressure in a sealed tube. Although the target products could be obtained in this way, their yields were substantially lower due to resinification of the reaction mixture, and procedures for isolation and purification became unavoidable.

It should be noted that activated esters $\mathbf{XA^nB_1L^k}$—**Np** (**2**) containing an RNA-binding domain are rather stable and keep quite well at room temperature without decomposition for several months when stored under dry atmosphere.

Joining of RNA-binding and catalytic sites. Aminolysis of the resulting activated esters **2** with imidazole-containing amines, *viz.*, histamine or methyl ester of histidine, gave rise to the target artificial ribonucleases with the structure $\mathbf{XA^nB_1L^kC^m}$ (**3a—l**) (see Scheme 1). Imidazole-containing amines, which are commercially available as hydrochlorides, were preliminarily transformed into free bases. Aminolysis can be successfully carried out also by the reactions of the activated esters with salts of amines in the presence of an excess of a base. However, in this case, the target products contained mixtures of counterions rather than were obtained as individual compounds. The reactions were carried out in DMF or *tert*-butyl alcohol. The choice of the solvent was governed by the ease of isolation of the products. All reactions of this type can be successfully carried out in DMF, but distillation of its excess from the reaction mixture is a time-consuming procedure. Low-lipophilic activated esters were poorly soluble in readily distillable *tert*-butyl alcohol.

It should be noted that the final step of isolation of the products of amide condensation, which was employed in the study, involved precipitation with acetone. However, the starting compounds, which are also bis-quaternary salts, are insoluble in acetone as well. In spite of the possibility to recrystallize the reaction products (for example, from large amounts of isopropyl alcohol), though with substantial losses, it was impossible to separate the starting reagents from the products in this way. To resolve this problem, the reaction conditions were chosen so that the reactions proceeded virtually to completion. In this case, the yields of the reaction products were determined only by the completeness of product precipitation.

Chemical ribonucleases $\mathbf{XA^nB_1L^kC^3}$ (**3m—r**) containing the free carboxy group in the catalytic domain were prepared by saponification of the corresponding methyl esters $\mathbf{XA^nB_1L^kC^2}$ (**3g—l**) with an aqueous-ethanolic solution of triethylamine (step *d*). Saponification proceeded rather rapidly to give products in quantitative yields and no additional purification was required.

Synthesis of multi-charged artificial ribonucleases $\mathbf{XA^nB_iL^kC^m}$ (see Scheme 2). We tried to develop a versatile procedure, which could enable one to prepare polycationic compounds based on the bis-quaternary salts of 1,4-diazabicyclo[2.2.2]octane bearing any number of charges and containing a catalytic site.

This problem could be solved by performing successive alkylation reactions to form a linear molecule consisting of the quaternized rings of 1,4-diazabicyclo[2.2.2]octane linked through polymethylene fragments. In spite of the obviousness of this approach, one will meet with essential experimental difficulties. Thus if the polymeric molecule contains more than two quaternary ammonium groups, the chemical properties of the starting reagents and alkylation products become virtually identical, making them hardly separable. At the same time, the goal to be sought is in many ways similar to the peptide synthesis, as in the above-considered case. In both cases, one has to prepare a polymeric molecule consisting of chemically similar monomers. This monomer could be amino acid containing bis-quaternized 1,4-diazabicyclo[2.2.2]octane with a protected amino group and a carboxy group ready to condensation owing to its transformation into activated ester. However, we faced with serious experimental difficulties when preparing such monomers.

We succeeded in synthesizing the only compound of this type, *viz.*, 4-(4-nitrophenoxycarbonyl)methyl-1-(2-phthalimidoethyl)-1,4-diazoniabicyclo[2.2.2]octane dibromide (**2g**) (see Scheme 1). The reaction of free 1,4-diazabicyclo[2.2.2]octane with *N*-(2-bromoethyl)phthalimide afforded compound **1a** (step *a*). Alkylation of this half-product at the second nitrogen atom with 4-nitrophenyl bromoacetate gave rise to the target

product **2g** (step *b*). Aminolysis of activated ester **2g** with imidazole-containing amines, in particular, with histamine, to produce compound **3f** was also trouble-free (step *c*). However, an attempt to remove the protective phthaloyl group using hydrazine hydrate in ethanol led to deep destruction of the molecule.

We also failed to replace the protective phthaloyl group by the more easily removable *tert*-butoxycarbonyl group. An attempt to acylate 1-(2-aminoethyl)-1-azonia-4-azabicyclo[2.2.2]octane (**$NH_2A^2B_1$**) with di-*tert*-butyl pyrocarbonate also led to destruction of the bicycle.

An alternative procedure involves the insertion of another nitrogen-containing group instead of a protected amino group into the activated monomer. Such groups as the nitrile, amide, nitro, or azido groups could serve as chemical precursors of the amino group. We believed that the azido group is most suitable for this purpose. On the one hand, the azido group, unlike the aliphatic nitro group, can be readily inserted into the molecule and, on the other hand, this group, unlike the nitrile and amide groups, can be easily reduced to the target amino group under mild conditions.

Alkylation of 1,4-diazabicyclo[2.2.2]octane with crude 1-azido-3-chloropropane, which was prepared by the reaction of 1-bromo-3-chloropropane with sodium azide, afforded the monoquaternary salt **$N_3A^3B_1$** (**1b**) as very hygroscopic crystals in high yield (see Scheme 1, step *a*). It should be noted that when the reaction solution contained even traces of moisture, the reaction product was isolated as an oil, which we failed to crystallize.

Compound **1b** was easily alkylated at the tertiary nitrogen atom with 4-nitrophenyl bromoacetate (see Scheme 1, step *b*). Being activated ester containing the azido group, the reaction product, *viz.*, the bis-quaternary salt **$N_3A^3B_1L^1$—Np** (**2h**), represents a monomer suitable for the synthesis of multi-charged RNA-cleaving constructs. The ester group of the key half-product **2h** was easily subjected to aminolysis with imidazole-containing aliphatic amines (step *c*) giving rise to the compounds **$N_3A^3B_1L^1C^m$** (**3e**,**l**).

Catalytic hydrogenation of the compounds **$N_3A^3B_1L^1C^m$** (**3e**,**l**) (see Scheme 2, step *e*) enabled us to prepare the dipeptide-like compounds **$NH_2A^3B_1L^1C^m$** (**4a**,**b**). The use of the Lindlar catalyst, which is generally employed to reduce azides, was unsuccessful. The required amines were reproducibly prepared in high yields under mild conditions with the use of the much more active catalyst, *viz.*, palladium on carbon.

Acylation of amines **4a**,**b** with one more molecule of activated ester **2h** followed by reduction of the azido group can be used for building up the oligopeptide chain containing the imidazole residue at the C-terminus. Any activated ester **$XA^nB_1L^k$—Np** (**2**) can be used as the N-terminal residue.

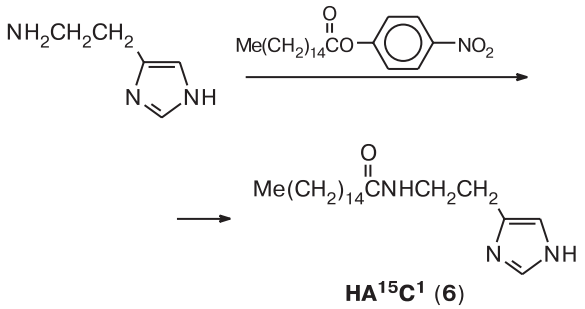
However, we restricted ourselves to the synthesis of "tripeptides" **$HA^2B_2L^1C^m$** (**5a**,**b**) containing two rings of 1,4-diazabicyclo[2.2.2]octane and one imidazole residue using the specially prepared activated ester **$HA^2B_1L^1$—Np** (**2e**) as the N-terminal residue (step *f*).

Design and synthesis of "truncated" artificial ribonucleases. We designed a series of model compounds by excluding one or several structural elements from the molecule of artificial ribonuclease **$HA^{14}B_1L^kC^m$**. A comparison of the catalytic properties of these compounds and hydrolytic activity of parent chemical ribonucleases allowed us to examine the role of all domains in detail.

We synthesized model "truncated" chemical ribonucleases **$HA^2B_1L^2$—NH_2**, **$HA^{14}B_1L^2H$**, **$HA^{15}C^1$**, and **$HA^2B_1L^3C^3$**, which lack either one or several domains that are present in the parent ribonuclease **$HA^{14}B_1L^kC^m$** containing the lipophilic, RNA-binding, and catalytic domains. In the conjugate **$HA^2B_1L^3C^3$** (**3q**), the tetradecyl radical of the parent ribonuclease **$HA^{14}B_1L^3C^3$** (**3n**) was replaced by the ethyl fragment, whithout lipophilic properties. Being histamine acylated at the aliphatic amino group with fatty acid, the compound **$HA^{15}C^1$** (**6**) differs from the parent construct by the absence of the RNA-binding domain **B**. The construct **$HA^{14}B_1L^2H$** (**7**), *viz.*, the bis-quaternary salt of 1,4-diazabicyclooctane bearing one lipophilic alkyl radical, is lacking in the catalytic domain. The compound **$HA^2B_1L^2$—NH_2** (**8**), which bears neither a catalytic domain nor a lipophilic radical, simulates the RNA-binding domain. In this compound, the amino group involved in one of the quaternized radicals emulates the basic properties of imidazole.

The nonpolar lipophilic histamine derivative **$HA^{15}C^1$** (**6**) was prepared by aminolysis of 4-nitrophenyl palmitate (Scheme 5).

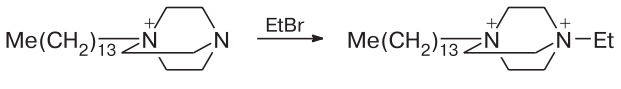
Scheme 5



$HA^{15}C^1$ (**6**)

The bis-quaternary salt of 1,4-diazabicyclo[2.2.2]octane **$HA^{14}B_1L^2H$** (**7**), with one of the aliphatic radicals being lipophilic, was prepared by alkylation of the monoquaternary salt **$HA^{14}B_1$** (**1d**) with ethyl bromide (Scheme 6).

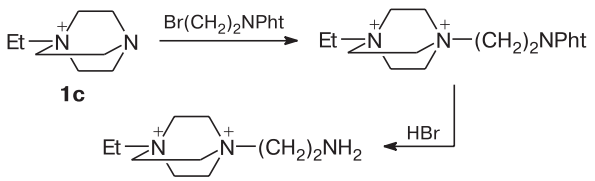
Scheme 6



1d **$HA^{14}B_1L^2H$ (7)**

The compound **$HA^2B_1L^2$—NH_2 (8)** was prepared by alkylation of the monoquaternary salt **HA^2B_1 (1c)** with *N*-(β-bromoethyl)phthalimide followed by removal of the protective group with an aqueous solution of HBr (Scheme 7).

Scheme 7



1c

$HA^2B_1L^2$—NH_2 (8)

Varying the domain structure, the nature of domains, and the molecular geometry provided within the framework of the proposed synthesis strategy. We developed the procedure for building up molecules of artificial ribonucleases based on 1,4-diazabicyclo[2.2.2]octane and imidazole from standard monomers by the peptide synthesis techniques (see Schemes 1 and 2). This procedure allows one to arbitrarily vary the set of domains and the distances between them.

The lipophilicity of the molecule is determined by the length of the aliphatic radical inserted into the structure in the first step of the synthesis (*a*) (alkylation of free 1,4-diazabicyclo[2.2.2]octane). This parameter can be varied over a wide range due to the availability of alkyl halides of virtually any length.

The positive charge of the molecule is determined by the number of the 1,4-diazabicyclo[2.2.2]octane rings in the oligopeptide chain *i*, which is specified in the synthesis. However, this procedure enabled us to prepare constructs only with even numbers of positive charges 2*i*. It should be noted that the presence of an additional primary amino group at the end of the aliphatic radical, which was generated from the azido group of the oligomer **$N_3A^nB_iL^kC^m$** by reduction, allows one to increase the formal positive charge of the molecule by unity resulting in the odd number of the positive charges. There are no principal obstacles to the building-up of an odd-charged construct, with the charge being determined exclusively by the quaternized amino groups, through activated ester **$R'_3N^+A^nB_1L^k$—Np**. The latter can be used as a precursor of the oligomer N-terminal unit. The distance between the 1,4-diazabicyclo[2.2.2]octane ring and the quaternary amino group dictated by the length of the aliphatic chain of 1-bromo-ω-chloroalkane can also be arbitrarily varied.

We used only histamine, histidine, and methyl ester of histidine as the catalytic domains of the constructs. However, the synthesis procedure imposes the only limitation on the structure of this domain, *viz.*, the presence of an additional aliphatic amino group along with a hydrolytically active grouping.

The distance between the RNA-binding and catalytic sites in the molecule of artificial ribonuclease is determined by the length of the aliphatic chain in 4-nitrophenyl ω-halogenocarboxylate, which is used in the synthesis of the monomer **$XA^nB_1L^k$—Np**, and can also be varied arbitrarily. Many acids of the same type are commercially available and other acids can be easily prepared. Their 4-nitrophenyl esters can be readily obtained either by direct condensation of acid and 4-nitrophenol in the presence of dicyclohexylcarbodiimide or *via* acid halide as a half-product. We faced the only limitation of the method. Thus, we failed to synthesize activated esters **$XA^nB_1L^2$—Np** (**2**, $k = 2$) with sufficiently high purity. Alkylation of monoquaternary salt **1** with 4-nitrophenyl β-bromopropionate led to elimination of hydrogen halide from the ester molecule instead of the formation of the bis-quaternary salt. We succeeded in partially shifting the equilibrium toward activated ester **2** ($k = 2$) by adding a large excess of 4-nitrophenyl acrylate. The formation of the target product was detected by spectroscopy. However, we failed to isolate this product from the reaction mixture in the pure form.

The length of the chain between the 1,4-diazabicyclo[2.2.2]octane rings is determined, on the one hand, by the parameter *n* of the dipeptide **$NH_2A^nB_1L^kC^m$** and, on the other hand, by the parameter *k* of the activated ester **$XA^nB_1L^k$—Np** that acylates the dipeptide. Apparently, the structure of this ester can also be varied over a wide range although we did not make such attempts.

Hence, the use of the above-described procedure for the synthesis of artificial ribonucleases **$XA^nB_iL^kC^m$** enables one to vary virtually all structural parameters of the construct.

RNA hydrolysis by artificial ribonucleases $XA^nB_1L^kC^m$: main features

The catalytic activity of artificial ribonucleases was examined with the use of the following transport RNAs as substrates: yeast $tRNA^{Phe}$, the *in vitro* transcript of yeast $tRNA^{Asp}$, the *in vitro* transcript of human mitochondrial $tRNA^{Lys}$ with the point mutation A9→C, as well as the *in vitro* mRNA transcript of the influenza virus M2 protein, and the synthetic decaribonucleotide

UUCAUGUAAA. The use of various RNAs enabled us to reveal the general features of RNA hydrolysis and exclude special effects caused by specific interactions between the conjugates and the unique fragments of the spatial structure of the particular RNA target. In the general case, the [^{32}P]-labeled substrate was incubated under physiological conditions in the presence of chemical nuclease ($1 \cdot 10^{-3}$—$1 \cdot 10^{-5}$ mol L^{-1}) for 1—24 h.* At certain intervals, the composition of the hydrolysis products was analyzed by polyacrylamide gel electrophoresis. The extent of RNA hydrolysis was determined as the ratio of depolymerized RNA to the total amount of radioactivity deposited on the gel. The assignment of the hydrolysis sites was made by comparing with the products of partial hydrolysis of the tRNA substrate by RNase T1 and imidazole or by RNase A. For each concentration of chemical nuclease, the kinetics was determined from eight time points. The conclusions about the relative efficacy of RNA hydrolysis by different conjugates were made based on the results obtained in the same experiment.

In the present review, we restrict ourselves to the qualitative discussion of the characteristic features of the RNA cleavage by the conjugates synthesized.

The biological assays demonstrated that virtually all conjugates $\mathbf{XA^nB_1L^kC^m}$ taken in millimole concentrations exhibited rather high hydrolytic activity (up to 80—90% of the RNA substrate was depolymerized for 8—10 h). The rate of hydrolysis of the RNA substrate was only ~5000 times lower than that observed in the case of natural RNase A.[76] All conjugates exhibited similar specificity, *viz.*, they cleaved the phosphodiester bonds predominantly in the CA and UA sequences located in the single-stranded regions of RNA.

The study of the hydrolytic properties of the "truncated" artificial ribonucleases showed that all domains are necessary for the efficient functioning of the construct. Thus, the exclusion of any domain led to a substantial decrease (in some cases, to the complete loss) in catalytic activity. Thus, the construct $\mathbf{HA^2B_1L^2—NH_2}$ (**8**) containing only the RNA-binding domain, as expected, did not exhibit any ribonuclease activity. The compounds $\mathbf{HA^{15}C^1}$ (**6**) (the RNA-binding cationic domain is excluded) and $\mathbf{HA^{14}B_1L^2H}$ (**7**) (the catalytic domain is excluded) were essentially inferior to "full-sized" conjugates in activity. The $\mathbf{HA^2B_1L^3C^3}$ construct (**3q**) (the lipophilic domain is excluded) was even less active. The analysis of the experimental data led to a rather unexpected conclusion that the presence of the lipophilic domain in the conjugate $\mathbf{ABL^kC^m}$, particularly, in combination with a cationic group imparting surfactant properties to the conjugate, is important for efficient RNA hydrolysis.

* In the absence of cleaving agents, the RNA substrate was rather stable.

The study of the dependence of the catalytic activity on the positive charge of the RNA-binding domain (the series $\mathbf{HA^2B_1L^1C^m—NH_2A^3B_1L^1C^m—HA^2B_2L^1C^m}$, $m = 1$ or 2) demonstrated that an increase in the number of the cationic sites from +2 to +4 and, as a consequence, the enhancement of the affinity of artificial RNases for the substrate leads to an increase in catalytic activity by approximately 30 times.

The nature of the catalytic domain has a substantial effect on hydrolytic activity of the construct. In most cases, the conjugates containing the imidazole group along with the other functionally important group, *viz.*, the methoxycarbonyl ($\mathbf{HA^{14}B_1L^kC^2}$) or, particularly, carboxyl ($\mathbf{HA^{14}B_1L^kC^3}$) group, appeared to be much more active than the corresponding histamine-containing analogs ($\mathbf{HA^{14}B_1L^kC^1}$). It should be noted that the latter proved to be more sensitive to the presence of an imidazole buffer in the reaction mixture, which, apparently, serves as the second acid-base component of the catalytic site in the absence of this site in the molecule of artificial ribonuclease.

The dependence of the catalytic activity on the distance between the RNA-binding and catalytic domains appeared to be more complicated and related to the structure of the catalytic domain. For the conjugates with histamine ($\mathbf{HA^{14}B_1L^kC^1}$) or methyl ester of histidine ($\mathbf{HA^{14}B_1L^kC^2}$), an increase in the length of the linker k from 1 to 5 led to an increase in hydrolysis efficacy, whereas the artificial ribonuclease $\mathbf{HA^{14}B_1L^3C^3}$ exhibited the maximum activity in the series of the histidine-containing conjugates ($\mathbf{HA^{14}B_1L^kC^3}$).

* * *

To summarize, the analysis of the above-considered corrleations between the structure of the conjugates $\mathbf{XA^nB_iL^kC^m}$ and their catalytic activity allows one to design more active artificial ribonucleases.

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