Development of Artificial Ribonucleases Using Macrocyclic Lanthanide Complexes

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Abstract: The sequence-specific recognition and cleavage of ribonucleic acids (RNA) represents a fundamental challenge. Metal complexes are among the best synthetic catalysts for the cleavage of RNA. Covalent attachment of suitable macrocyclic lanthanide complexes to oligonucleotides allows the cleavage of complementary RNAs in a sequence-specific manner. The preparation, properties and use of such artificial ribonucleases are highlighted. In particular, the design and preparation of constructs cleaving RNA with multiple turn-over is described.

Keywords: Artificial ribonucleases · Lanthanide complexes · RNA cleavage · RNA recognition · Supramolecular chemistry

Introduction

Ribonucleic acid (RNA), one of nature’s most important biomolecules, exerts a large variety of different functions. Thus, messenger RNA serves as intermediate in gene expression, ribosomal RNA is an essential component of the translation machinery and transfer RNA constitutes the vehicle for specific delivery of amino acids during protein assembly. Furthermore, RNA has been shown to carry out many types of catalytic reactions [1]. Various types of ribonucleic acids, such as guide RNAs [2] or tm RNA [3], are involved in the processing and regulation of other RNAs. In addition, ribonucleic acid serves as the genetic material of many types of viruses.

Due to its significant role in all these biological processes, RNA is a subject of ongoing intensive investigation. Further elucidation of the principal rules of structural and functional behaviour of the different types of RNAs will lead to a better understanding of many elementary processes [4][5]. Positive impact may result e.g. in the design and development of new and better drugs targeting viral [6] and microbial [7] RNAs. In addition, with a rapidly increasing number of genes being deciphered, the understanding of structure and function of expressed mRNAs will be important for the field of functional genomics [8]. In this context, antisense oligonucleotides [9-11] have proven to be useful tools for validation of new therapeutic targets [12], since they offer a highly specific way of inhibiting gene expression by binding to the mRNA coding for a given protein.

The development of a method which allows the cleavage of an RNA molecule of interest at precisely one site offers potential benefits both for therapeutic and diagnostic purposes. In addition, it also means a valuable extension of the scientist’s toolbox for the study of RNA structure and function. With this background, it is not surprising that the sequence-specific recognition and cleavage of RNA has been a topic of great interest and several research groups have embarked to reach this goal [13][14].

Strategy

For the successful construction of an artificial ribonuclease two minimal requirements have to be fulfilled: i) the specific recognition and binding of the nucleic to the RNA target site and ii) the efficient chemical cleavage of the target’s phosphodiester backbone. A priori, several possibilities for the specific recognition of RNA exist, including antisense oligonucleotides and small molecule RNA binders. In light of the rapid development in the antisense technology, the use of a modified oligonucleotide as the recognition domain appeared most straightforward. The envisaged strategy is, thus, illustrated in Scheme 1.

The choice of the RNA cleaving moiety is less obvious. Many different chemical groups have been shown to catalyse
Synthesis of the terpyridine moiety and complex formation follows the procedure described by Constable and co-workers [19]. Terpyridine 1 can be synthesized from readily available starting materials in a small number of steps in high overall yield (see Scheme 3). Formation of the macrocyclic metal complexes (2) is achieved by treating 1 with a 2,6-diacyl-substituted pyridine in the presence of one equivalent of lanthanide (III) acetate and a small quantity of conc. hydrochloric acid [20]. Covalent attachment of the metal complexes to appropriately functionalized oligonucleotides via thiourea linkers is carried out using standard methods. Treatment of the amine-substituted terpyridine lanthanide complex with thiophosgene readily affords the intermediate isothiocyanate derivative which is subsequently reacted with an oligonucleotide carrying a 5'-linked amino group. This coupling procedure is preferably carried out while the oligonucleotide is still bound to the solid support on which it was synthesized. Due to the remarkable stability of the metal complex, oligonucleotide conjugates 3 can be cleaved from the solid support, deprotected and purified by standard chromatographic or electrophoretic methods.

RNA phosphodiester transesterification, including imidazoles, oligoamines, guanidines and metals, as well as combinations thereof [13][14]. The catalytic power of metals and their complexes has long been recognized. In particular, lanthanide salts and lanthanide complexes of type A are well known to promote cleavage of ribonucleic acid [15][16].

The mechanism by which metals catalyse the transesterification of RNA has been investigated in detail and can be illustrated as shown in Scheme 2 [17][18]. The principal driving force for a facile RNA strand cleavage upon coordination of the phosphate group by a Lewis acid, is the presence of the 2'-hydroxyl group which acts as the nucleophile in the displacement reaction. This transesterification reaction gives rise to two cleavage products, one fragment bearing a 2',3'-cyclophosphate, the other a free 5'-OH group. This knowledge in combination with other considerations, such as chemical stability and ease of synthesis, led us to pursue lanthanide metal complexes of type B as the compounds of choice.

**Synthesis of Macrocyclic Lanthanide Complexes and Conjugation to Oligonucleotides**

Synthesis of the terpyridine moiety and complex formation follows the procedure described by Constable and co-workers [19]. Terpyridine 1 can be synthesized from readily available starting materials in a small number of steps in high overall yield (see Scheme 3). Formation of the macrocyclic metal complexes (2) is achieved by treating 1 with a 2,6-diacyl-substituted pyridine in the presence of one equivalent of lanthanide (III) acetate and a small quantity of conc. hydrochloric acid [20]. Covalent attachment of the metal complexes to appropriately functionalized oligonucleotides via thiourea linkers is carried out using standard methods. Treatment of the amine-substituted terpyridine lanthanide complex with thiophosgene readily affords the intermediate isothiocyanate derivative which is subsequently reacted with an oligonucleotide carrying a 5'-linked amino group. This coupling procedure is preferably carried out while the oligonucleotide is still bound to the solid support on which it was synthesized. Due to the remarkable stability of the metal complex, oligonucleotide conjugates 3 can be cleaved from the solid support, deprotected and purified by standard chromatographic or electrophoretic methods.
The ability of the obtained conjugates to cleave ribonucleic acids was tested by incubating them with a complementary synthetic oligoribonucleotide. We found that the conjugates efficiently induce RNA cleavage [21]. Typically, the artificial ribonucleases cleave the target with a half-life of 6–10 h at 37 °C (pH 7.4), using a 10-fold excess of the ribonuclease. Cleavage occurs in the single stranded region of the RNA strand, as illustrated with the cleavage of oligoribonucleotide 4 by conjugate 3 (see Fig. 1). Detailed studies subsequently showed that the efficiency of the cleavage reaction is dependent on several factors [22]. In decreasing order of importance, these factors are: i) the type of metal; ii) the chemical composition and substitution of the ligand and iii) the sequence of the RNA target [23]. Europium conjugates are most efficient, lanthanum derivatives show no or only moderate cleavage. With many different ligand systems synthesized and tested, it was also found that substituent $R$ in conjugates of type 3 played a significant role. Cleavage efficiencies are generally better if $R$ is hydrogen than if it is methyl. Finally, cleavage by the lanthanide complexes is influenced by the base composition of the target RNA, occurring predominantly between 5'-purine-purine and 5'-pyrimidine-purine sites. An especially preferred site is the 5'-GA dinucleotide [23].

2'-MOE-modified cleaver conjugates (i.e. each of the sugar residues in the oligonucleotide is substituted with a 2'-O-(2-methoxyethyl) group, see Fig. 1) cleave RNA with equal efficiency and at essentially the same site as unmodified (deoxy) conjugates. However, due to the increased RNA binding affinity observed with the 2'-MOE modification (ΔTm of +1.1 °C per modified nucleotide) [24] it is possible to use conjugates of much shorter length to achieve comparable cleavage efficiencies. Thus, the 10-nucleotide long, 2'-MOE modified artificial ribonuclease 5 cleaves target RNA 6 with almost identical efficiency as the unmodified 20mer 3. 2'-MOE modified oligonucleotides have been shown to possess significant advantages over unmodified oligonucleotides regarding target affinity and nuclease resistance.
[9]. Therefore, the use of 2'-MOE modified conjugates seems particularly promising for applications in cellular systems.

**Artificial Chemical Ribonucleases Acting with Multiple Turnover**

The results described above are limited to examples in which the cleavage takes place in the single stranded region of the RNA. Furthermore, an excess of cleaver conjugate is required to achieve efficient cleavage. The potency of such artificial ribonucleases might be raised if the cleavage process could be extended into the duplex region. This would not only increase the number of possible cleavage sites but, more importantly, offer the possibility of catalytic turnover. A fundamental requirement of catalysis is a rapid dissociation of the two RNA fragments from the nuclease after the cleavage process in order to avoid product inhibition. Hence, whereas formation of a stable duplex between the nuclease and the target is required in a first step, the final complex between the nuclease and the RNA fragments after the reaction should be sufficiently destabilised to allow rapid release of the nuclease. This condition is not fulfilled if the target is cleaved in the single strand (Scheme 4A) but it could arise if cleavage occurred within the duplex (Scheme 4B).

On the other hand, it has been reported that double stranded RNA is considerably more resistant to metal ion promoted transesterification than its single stranded counterpart [25] rendering cleavage of RNA within a duplex difficult or impossible. The poor reactivity of ribonucleotides to transesterification/cleavage in the duplex is attributed to the rigid conformation of the phosphodiester backbone, preventing the required in-line arrangement of the nucleophilic 2'-OH and the 5'-OH leaving group around the pentacoordinate phosphate intermediate [26]. This limitation might be overcome by the introduction of bulges and internal loops in the RNA target strand [27]. To test this hypothesis, several artificial nucleases were designed and synthesized. Lanthanide complexes were linked to the oligonucleotides at appropriate positions either at the ribose or a nucleobase such that the bulge can be reached through the minor (Fig. 2A) or the major (Fig. 2B) groove, correspondingly [28]. Efficient cleavage was observed in both cases at or near the bulge. Even a 5'-end linked metal complex (Fig. 2C) effected strong cleavage almost exclusively at the bulge.
– with only a trace of cleavage taking place in the single stranded region.

Using a system as shown in Fig. 2C, it was possible to find 2'-MOE conjugates acting with multiple turnover. Several conjugates of various lengths and base composition were tested against bulged RNAs and were found to cleave more than one equivalent of target RNA [23]. One example is shown in Scheme 5 [29]. An excess of oligoribonucleotide 7 is treated at 37 °C with different ratios of cleaver conjugate 8. Cleavage of 7 takes place at the bulge and, to a lesser extent, also at the 3'-site adjacent to the bulge. Considerably more than one equivalent of RNA is cleaved after 64h. Under optimal conditions, 39 equivalents of RNA (relative to the cleaver conjugate) are cleaved. This finding demonstrates that it is possible to design artificial chemical ribonucleases acting in a catalytic way by the bulge approach.

Outlook

The field of artificial ribonucleases has seen substantial progress over the last years. In combination with the rapid development in oligonucleotide technology, a variety of practical applications is becoming feasible. One of the most important aspects is the use of such artificial ribonucleases in cellular systems. A recent report, describing the use of lanthanide oligonucleotide conjugates in cells, underlines the potential for the use of artificial ribonucleases in biological systems [30].

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[1] T. Tuschl, J.B. Thomson, F. Eckstein, Curr. Opin. Struct. Biol. 1995, 5, 296–302.
[2] B.K. Adler, S.L. Hajduk, Curr. Opin. Genet. Dev. 1994, 4, 316–322.
[3] A. Muto, C. Ushida, H. Himeno, Trends Biochem. Sci. 1998, 23, 25–29.
[4] J.A. Douzina, J.H. Cate, Curr. Opin. Struct. Biol. 1997, 7, 310–316.
[5] G.L. Conn, D.E. Draper, Curr. Opin. Struct. Biol. 1998, 8, 278–285.
[6] W.D. Wilson, K. Li, Current Medicinal Chemistry 2000, 7, 73–98.
[7] K. Michael, Y. Tor, Chem. Eur. J. 1998, 4, 2091–2098.
[8] D.J. Ecker, R.H. Griffith, Drug Discov. Today 1999, 4, 420–429.
[9] A. De Mesmaeker, R. Häner, P. Martin, H.E. Moser, Acc. Chem. Res. 1995, 28, 365–374.
[10] E. Uhmann, A. Peymann, Chem. Rev. 1990, 90, 543–584.
[11] S.T. Crooke, Biotechnol. Genet. Eng. Rev. 1998, 15, 121–157.
[12] C.F. Bennett, L.M. Cowsert, Biochimica et Biophysica Acta-Gene Structure and Expression 1999, 1489, 19–30.
[13] R. Häner, J. Hall, Antisense Nucleic Acid Drug Dev. 1997, 7, 423–430.
[14] B. Trawick, A.T. Danher, J. K. Bashkin, Chem. Rev. 2000, 98, 939–960.
[15] E. Bamann, Angew. Chem. 1939, 52, 186–188.
[16] J.R. Morrow, L. Buttrey, V. Shelton, K. Berback, J. Am. Chem. Soc. 1992, 114, 1903–1905.
[17] F. Westheimer, Acc. Chem. Res. 1968, 1, 70–78.
[18] M. Ovianen, S. Kiusela, Linnberg, H. Chem. Rev. 1998, 98, 961–990.
[19] E. Constable, J. Holmes, Polyhedron 1988, 7, 2531–2536.
[20] R. Häner, J. Hall, G. Rihs, Helv. Chim. Acta 1997, 80, 487–494.
[21] J. Hall, D. Huesken, U. Pieles, H.E. Moser, R. Häner, Chem. Biol. 1994, 1, 185–190.
[22] J. Hall, D. Huesken, R. Häner, Nucleosides Nucleotides 1997, 16, 1357–1368.
[23] J. Hall, D. Huesken, R. Häner, unpublished work.
[24] P. Martin, Helv. Chim. Acta 1995, 78, 486–504.
[25] K. Kolasa, J.R. Morrow, A. Sharma, Inorg. Chem. 1993, 32, 3983–3984.
[26] D. Usher, A. McHale, Proc. Natl. Acad. Sci. U.S.A 1976, 73, 1149–1153.
[27] D. Huesken, G. Goodall, M.J. Blommers, W. Jahnke, J. Hall, R. Häner, H. E. Moser, Biochemistry 1996, 35, 16591–16600.
[28] J. Hall, D. Huesken, R. Häner, Nucleic Acids Res. 1996, 24, 3522–3526.
[29] R. Häner, J. Hall, A. Pfützer, D. Hüsken, Pure and Applied Chemistry 1998, 70, 111–116.
[30] B.F. Baker, S.S. Lot, J. Kringle, S. Cheng-Flournoy, P. Viliet, H.M. Samor, A.M. Siwkowski, L.L. Chappell, J.R. Morrow, Nucleic Acids Res. 1999, 27, 1547–1551.