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Inhibition by polyamines of the hammerhead ribozyme from a Chrysanthemum chlorotic mottle viroid

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ABSTRACT

Background: Viroids are the smallest pathogens known to date. They infect plants and cause considerable economic losses. The members of the Avsunviroidae family are known for their capability to form hammerhead ribozymes (HHR) that catalyze self-cleavage during their rolling circle replication.
Methods: In vitro inhibition assays, based on the self-cleavage kinetics of the hammerhead ribozyme from a Chrysanthemum chlorotic mottle viroid (CChMVd-HHR) were performed in the presence of various putative inhibitors.
Results: Aminated compounds appear to be inhibitors of the self-cleavage activity of the CChMVd HHR. Surprisingly the spermine, a known activator of the autocatalytic activity of another hammerhead ribozyme in the presence or absence of divalent cations, is a potent inhibitor of the CChMVd-HHR with K_i of 17 ± 5 μM. Ruthenium hexamine and TMPyP4 are also efficient inhibitors with K_i of 32 ± 5 μM and IC_{50} of 177 ± 5 nM, respectively.
Conclusions: This study shows that polyamines are inhibitors of the CChMVd-HHR self-cleavage activity, with an efficiency that increases with the number of their amino groups.
General significance: This fundamental investigation is of interest in understanding the catalytic activity of HHR as it is now known that HHR are present in the three domains of life including in the human genome. In addition these results emphasize again the remarkable plasticity and adaptability of ribozymes, a property which might have played a role in the early developments of life and must be also of significance nowadays for the multiple functions played by non-coding RNAs.

1. Introduction

Viroids are the simplest RNA-based pathogens known to date. They consist of 246–401 single-stranded, circular, naked, and non-coding RNAs. They infect higher plants, induce serious diseases – such as Chrysanthemum chlorotic mottle, Avocado Sunblotch and Potato Spindle Tuber – and therefore cause considerable economical losses [1]. It has been shown recently that viroids can replicate in yeast and thus in other organisms than plants [2]. Approximately 30 species of viroids are currently known and classified into two families: Pospiviroidae and Avsunviroidae. The latter are characterized by the presence in their sequence of a hammerhead ribozyme (HHR) which is a small catalytic RNA motif involved in their replication through a symmetric rolling-circle mechanism (for recent reviews see Ding [3], Owens & Hammond [4] and Flores et al. [5]). Actually, the presence of hammerhead ribozymes is not restricted to the viroids and they are largely distributed in the genomes of all kinds of organisms including human [6–8].

Viroids were extensively studied in the past few years, and new insights not only into their propagation, *in vivo* replication, processing, trafficking, and pathogenesis, but also into their tertiary structure and interactions with cellular proteins or small RNA were reported. However, several questions regarding the mechanisms by which these viroids enter and leave the cell, the nucleus or the chloroplast and escape the host degradation system, remain unclear. Indeed the prevention of viroid infections in plants is so far based on barely biological means and no chemicals are available to control or prevent plant diseases caused by viroids. The current approaches used to combat viroids are the elimination of source inoculum, prevention of secondary spread, cross-protection, and the use of crops bearing resistance traits [9]. Nevertheless, one way of preventing Avsunviroidae infection could be to break their rolling circle replication by inhibiting their hammerhead self-cleavage, perturbing thus the equilibrium between linear, circular, and polymeric viroids in infected cells, and allowing the defense

Abbreviations: HHR, hammerhead ribozyme; CChMVd-HHR, hammerhead ribozyme from a Chrysanthemum chlorotic mottle viroid; TMPyP4, meso-5,10,15,20-tetrakis-(N-methyl-4-pyridinio)porphine; $[Co(NH_3)_6]^{3+}$, cobalt hexamine; $[Ru(NH_3)_6]^{3+}$, ruthenium hexamine; IC_{50}, apparent half maximal inhibitory concentration; K_i, apparent inhibition constant

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mechanism of the cell, in particular the nucleases, to recognize and degrade them. This idea has been put forward by Murray and Arnold who showed that tetracycline is a potent inhibitor of HHR [10].

Over the past two decades, many studies have reported on the inhibition of the HHR by variable metabolites such as aminoglycoside antibiotics [11], terbium (III) [12], and cobalt hexamine [13], *in vitro* as well as *in vivo* [14], but none of the ribozymes used in these studies was from a hammerhead viroid. In addition, most of these ribozymes were minimal *trans*-acting constructs although the physiological reaction is *cis*-acting. Furthermore, this reaction involves the native peripheral regions of the ribozyme [15,16] which, by interacting with each other, facilitate and stabilize folding into a single active structure. These regions are necessary for optimal activity in physiological conditions, although they are not directly involved in the catalysis [17]. Consequently, the present investigation made use of the HHR of the Chrysanthemum chlorotic mottle viroid (CChMVd). The structure and function of the peripheral loops of this ribozyme were previously studied using a combination of NMR spectroscopy, site-direct mutagenesis, kinetic studies and infectivity analyses [18]. The results obtained provided insights into the three-dimensional folding of the HHR and emphasize the importance of almost all the nucleotides in the terminal loops for self-cleavage of the ribozyme *in vitro* and for infectivity of the viroid *in vivo*. Recently, our laboratory investigated the *cis*-cleavage reaction of a CChMVd-HHR (Fig. 1) by the high pressure approach on the over-all cleavage reaction [19]. Two different conformations of active molecules were identified in the reaction mixture corresponding to fast and slow cleaving ribozymes.

In the present study, the influence of a series of aminated molecules or metabolites on the activity of the CChMVd-HHR was investigated. Among them adenine was tested since this molecule appeared to bind to RNAs and modulate their activities. This is the case of the adenine-dependent hairpin ribozyme [20] and of the adenine riboswitch [21]. Interestingly, HHR and the adenine riboswitch present structural and mechanistic similarities. They fold into a very similar secondary structure formed by a central core, three stems and two loops and they are activated through the same loop–loop interactions ([15,16] for HHR and [22] for the adenine riboswitch).

The results obtained show that the apparent affinity of polyamines for the ribozyme increases with the number of their amino groups, suggesting that amino groups bind to the ribozyme phosphate backbone. Spermine, a known activator of HHR, appears to be a potent inhibitor of the CChMVd-HHR. In addition, a new inhibitor of this ribozyme, ruthenium hexamine was identified.

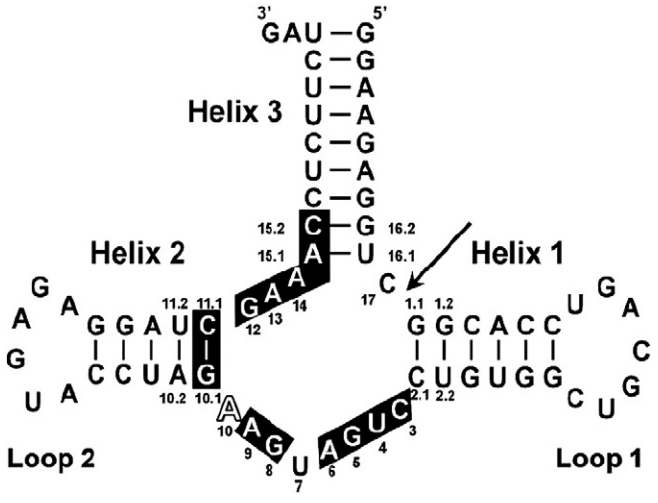

Fig. 1. Schematic representation of the 67 nucleotide-long CChMVd-HHR secondary structure. The nucleotide residues strictly or highly conserved in most natural HHR are in black background. Numbering is based on the standard criterion for the consensus hammerhead [36] with the exception of the extra A between residues A9 and G10.1, characteristic of CChMVd, which is referred to as number 10 (outlined font). Arrow indicates the self-cleavage site.

2. Materials and methods

2.1. Materials

DNA primers were synthetized by Proligo (Evry, France) and Eurofins MWG GmbH (Ebersberg, Germany), and Taq DNA polymerase and PCR buffer were purchased from Invitrogen (Carlsbad, CA, USA) and dNTPs from Promega (Madison, WI, USA). Transcription buffer, T7 RNA polymerase and rNTPs were obtained from Fermentas (St Leon-Rot, Germany). Most of the inhibitors tested were provided by Sigma-Aldrich (St. Quentin Fallavier, France). Ruthenium hexamine was kindly provided by Professor Olof Einarsdóttir from the University of California, Santa Cruz.

2.2. RNA preparation

The cDNA of the *cis*-acting CChMVd-HHR was PCR-amplified with Taq DNA polymerase as described in Ztouti et al. [23] using 5′-GTCG GCACCTGACGTCGGTGTCCTGATGAAGATCCATGAGAGGATCGAAACCTCT TCTAG-3′ as template, and 5′-TAATACGACTCACTATAGGAAGAGGTCGG CACCTGACGTCGG-3′ containing the T7 RNA polymerase promoter (underlined), as sense primer and 5′-CTAGAAGAGGTTTCGATCCTCTC-3′ as antisense primer. The CChMVd-HHR was synthesized by overnight *in vitro* transcription of the PCR products. To avoid cleavage during transcription, a deoxyribonucleotide (5′-CATGGATCTTCATCAGGACACC GAC-3′), complementary to a part of the HHR [15] was used in the transcription mixture at a concentration of 10 μM. The RNAs obtained were purified by denaturing (7 M urea) 15% polyacrylamide gel electrophoresis (PAGE) and eluted from the gel overnight in 300 mM sodium acetate, pH 5.2 at 4 °C. The RNAs were recovered from the solution by filtration through 0.22 μm diameter micro-filters and precipitation with ethanol. The purified ribozyme was finally resuspended in water and stored at −20 °C.

2.3. Self-cleavage kinetics

Inhibition experiments were performed in the presence of 0.6 mM $MgCl_2$ and 50 mM Tris–HCl pH 7.5, conditions under which the reaction is fast and reaches a plateau of 65% cleavage in 10 min. 50 μl of RNA for a final concentration of 0.5 μM, was denaturated in buffer (50 mM Tris–HCl) at 90 °C for 1 min, slowly cooled (3 °C·min^{-1}) to 23 °C, and then diluted in 400 μl of inhibitor solution containing the buffer. The cleavage reaction was initiated at 25 °C by addition of 50 μl of $MgCl_2$ in buffer for a final concentration of 0.6 mM. At appropriate times, aliquots of 35 μl (~400 ng of RNA) were withdrawn, the reaction was quenched with 35 μl of stop solution (50 mM EDTA, 7 M urea, 0.01% xylene cyanol) and the mixture was subjected to denaturing 15% PAGE. RNA bands were revealed using ethidium bromide and quantified with Image J (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997–2012). Curves of fractions of cleavage as a function of time were plotted using the SigmaPlot 11.0, and plots were fitted to a single-exponential equation. These exponential plots of the fraction of cleavage *versus* time were used to determine the k_{obs} and the initial rates that served to determine the apparent half maximal inhibition concentration (IC_{50}). All experiments were repeated at least twice.

3. Results

For the reasons indicated above, adenine was tested in a concentration range up to 30 mM. Fig. 2 shows that adenine has a rather weak inhibitory effect with an $IC_{50} = 7.4 \pm 1.6$ mM. The k_{obs} of the reaction decreases from 5 min^{-1} in the absence of adenine to 0.015 min^{-1} in its