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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.

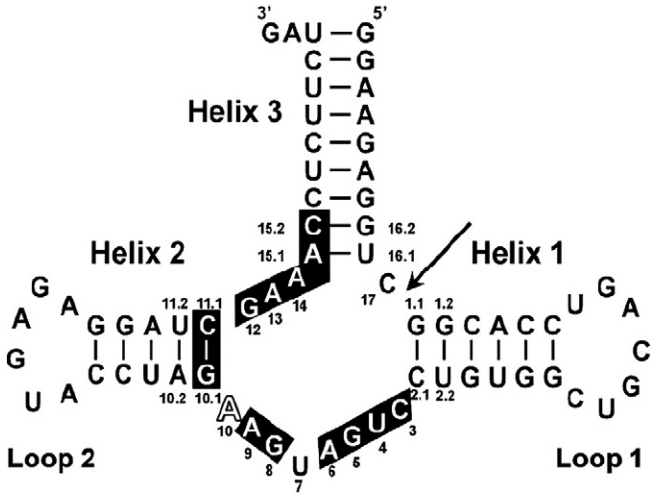

Fig. 1. Schematic representation of the 67 nucleotide-long CChMVd-HHR secondary structure. The nucleotide residues strictly or highly conserved in most natural HHR are in black background. Numbering is based on the standard criterion for the consensus hammerhead [36] with the exception of the extra A 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

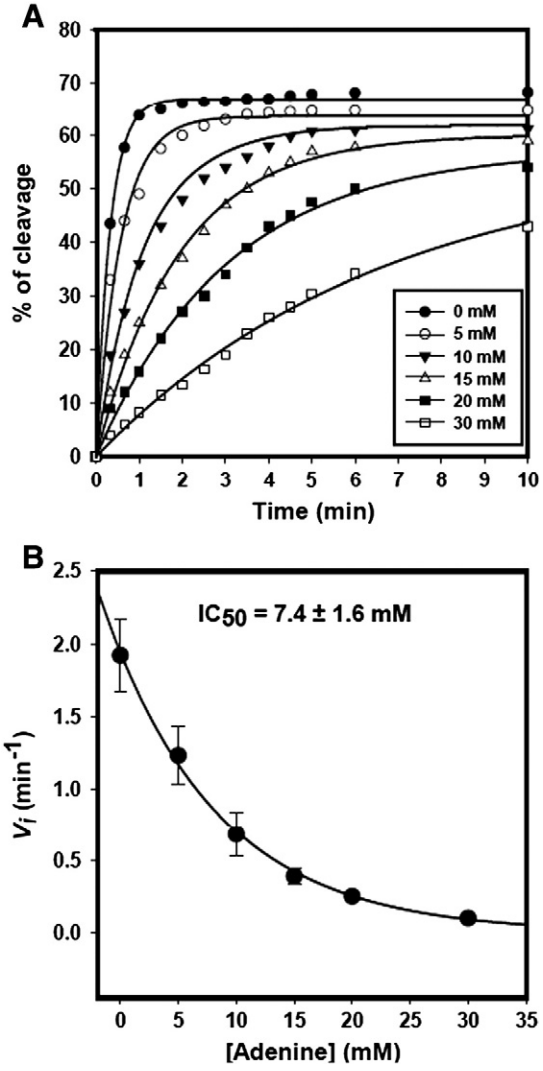

Fig. 2. Effect of adenine on CChMVd-HHR self-cleavage. (A) self-cleavage CChMVd-HHR kinetics in the presence of increasing concentrations of adenine. Kinetics were performed in 50 mM Tris, pH 7.5 and 0.6 mM Mg^{2+}, as described in Materials and methods. (B) Initial rates of self-cleavage as a function of adenine concentration.

presence at a concentration of 30 mM. Adenine possesses two functional groups: imidazole and amino. The effects of a series of molecules carrying the imidazole and/or the amino are shown in Fig. 3. Although the inhibition by these compounds is rather weak, the results indicate that the inhibitory effect is not due to the imidazole group but correlates with the presence of amino groups. This finding is consistent with results from our laboratory where metabolites such as cytosine, isocytosine, thiocytosine, 6-methyladenine, 6-dimethyladenine, 5-fluorouracil, lysine and phenylalanine do not have a significant inhibitory effect on the CChMVd-HHR (IC_{50} over 20 mM) while metabolites such as 3-methyladenine, 4-aminopyridine, 3,4-diaminopyridine and arginine have a rather small influence (data not shown). Our conclusion is also consistent with that of Stage et al. [24] who studied the inhibition of the hammerhead ribozyme by neomycin.

3.1. Effect of polyamines

On the basis of these observations different polyamines: diamine putrescine, diamine cadaverine, triamine spermidine and the tetramine spermine, were tested. The IC_{50} values of these molecules are shown in Fig. 4A. Although spermine and spermidine were shown to support the activity of hammerhead [25–27] and hairpin [28,29] ribozymes in the presence or even in the absence of the required divalent cations, it appears that, unexpectedly, spermine strongly inhibits the CChMVd-HHR self-cleavage activity. The inhibitory effect of these different polyamines increases with the number of amino groups and the length of these molecules. To obtain additional information about the influence of spermine on the activity of CChMVd-HHR, competition experiments between spermine and Mg^{2+} were performed. The Dixon plot obtained (Fig. 4B) yields a K_i of 17 ± 5 μM for this inhibitor and the profile of the Dixon plot shows a non-competitive inhibition, indicating that spermine does not bind to the ribozyme on the Mg^{2+} binding site(s).

3.2. Effect of cobalt hexamine and ruthenium hexamine

On the basis of previous results concerning the effects of cobalt hexamine $[Co(NH_3)_6]^{3+}$, a known efficient inhibitor of HHR self-cleavage [13], the influence of this compound, and that of its analog ruthenium hexamine $[Ru(NH_3)_6]^{3+}$ (generally used as an enzyme-reducing agent), on the activity of CChMVd-HHR was tested and the results are compared in Fig. 5A. These two molecules are rather good inhibitors of the ribozyme, with IC_{50} of 84 ± 6 μM and 25 ± 3 μM

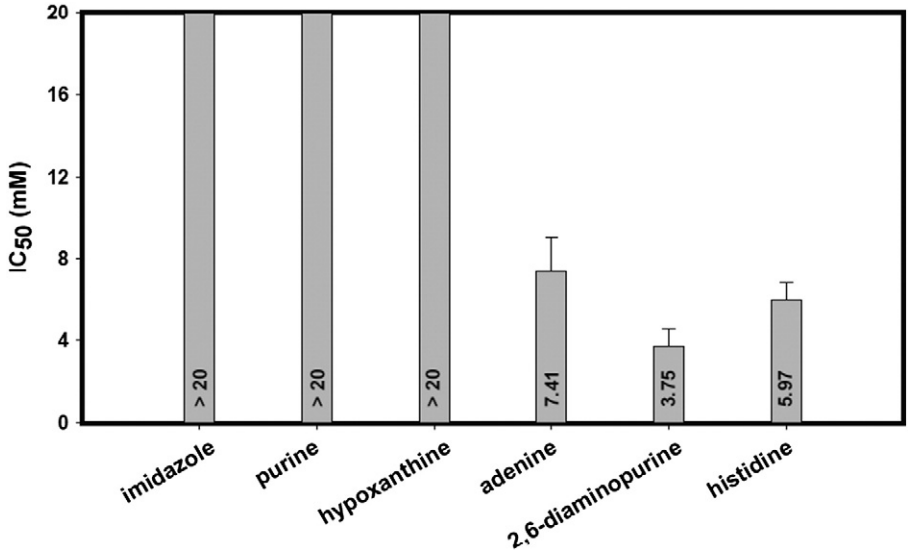

Fig. 3. Effect of imidazole and amino group-containing molecules on the self-cleavage activity of the CChMVd-HHR. IC_{50} values are shown for each molecule tested. They were determined by varying the inhibitor concentration (at least 5 concentrations). Kinetics were performed in 50 mM Tris, pH 7.5 and 0.6 mM Mg^{2+}.

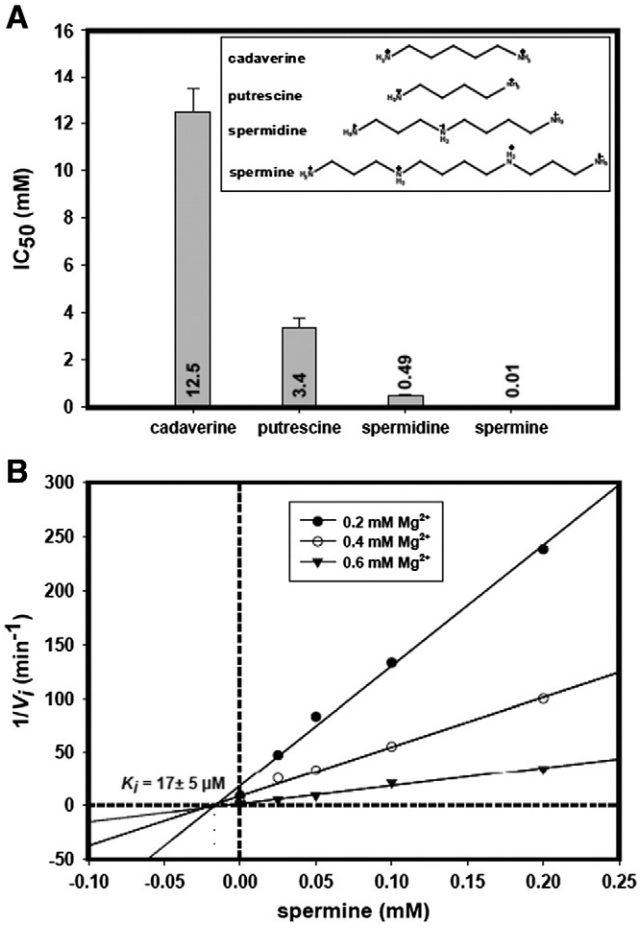

Fig. 4. Effect of polyamines on the self-cleavage activity of the CChMVd-HHR. (A) The IC_{50} values are shown for each polyamine used. Kinetics were performed in 50 mM Tris, pH 7.5 and 0.6 mM Mg^{2+}. The amino groups are positively charged since they are protonated under the pH conditions used here. (B) Dixon plot of the inhibition of the self-cleavage activity of CChMVd-HHR with spermine at different Mg^{2+} concentrations. Converging lines on the x-axis indicate that spermine does not bind competitively with Mg^{2+} on the ribozyme. K_i is 17 ± 5 µM.

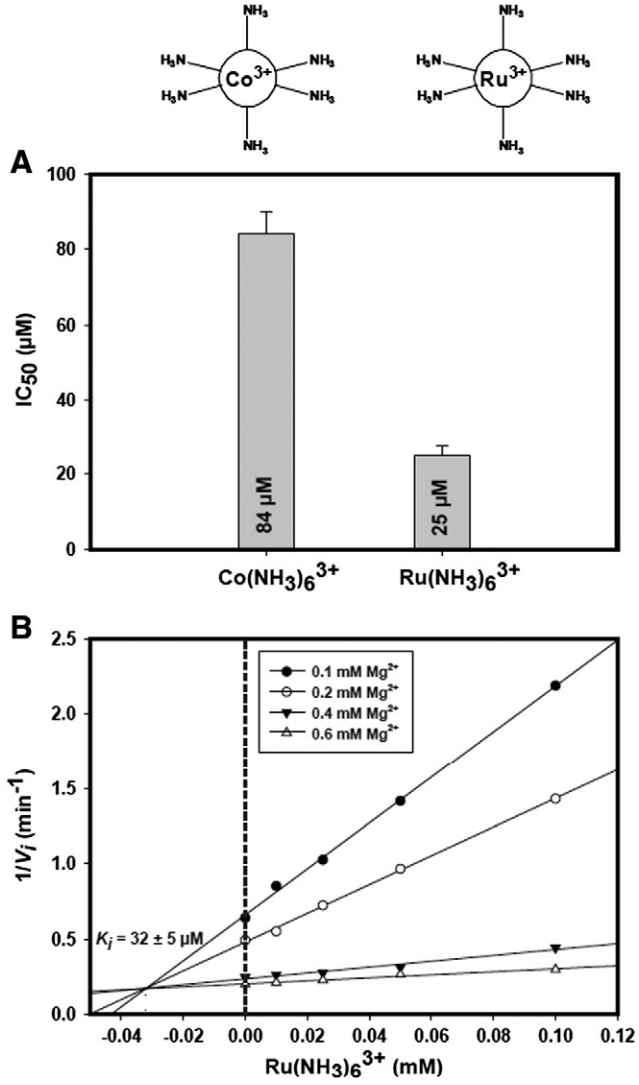

Fig. 5. Effect of ruthenium hexamine and cobalt hexamine on the self-cleavage activity of the CChMVd-HHR. (A) IC_{50} determined from kinetics performed in 50 mM Tris, pH 7.5 and 0.6 mM Mg^{2+}. The chemical structures are shown. (B) Dixon plot of the inhibition of the self-cleavage activity of CChMVd-HHR with ruthenium hexamine at different concentrations of Mg^{2+}. Converging lines above the x-axis indicate that ruthenium hexamine is competitive with Mg^{2+}. K_i is 32 ± 5 µM.

respectively. In spite of the fact that these two molecules bear three positively charged amino groups, ruthenium hexamine appears to be 3.5 times more efficient as inhibitor of the ribozyme than cobalt hexamine. This result shows not only that the inhibition of CChMVd-HHR self-cleavage is due to the positively-charged amino groups but also that it is metal-core dependent, suggesting that the ruthenium hexamine either can bind to an additional site(s) unreachable by the cobalt hexamine or binds to the same site(s) with a higher affinity. It is interesting to relate this observation with that of a study of the influence of the inner coordination sphere of cobalt and ruthenium hexamine on their interaction with thymus DNA in solution. This study showed that the hydrodynamic radius of ruthenium is much larger than that of cobalt [Rh (Co3) = 65 ± 5 nm and Rh (Ru3) = 440 ± 30 nm] although the two compounds exert an identical global influence on the structure of the macromolecule [30].

To determine whether the ruthenium hexamine interferes with the binding of Mg^{2+}, the effect of this inhibitor was tested in the presence of varying concentrations of $MgCl_2$. The pattern of the Dixon plot indicates that $[Ru(NH_3)_6]^{3+}$ binds competitively with Mg^{2+} to the ribozyme (Fig. 5B). However this plot yields a K_i of 32 ± 5 µM, a value which is identical to that previously reported for cobalt hexamine (K_i = 30.9 ± 2.3 µM) [13], although the comparison concerns two different ribozymes. Interestingly, discrepancies in the effects of Co and Ru hexamines might relate to a recent study using RAMAN spectroscopy in which $[Co(NH_3)_6]^{3+}$ was reported to be competitive toward Mg^{2+} and Mn^{2+} for some but not all inner sphere metal-PO^{2-} sites [31].

3.3. Effect of meso-5,10,15,20-tertakis-(N-methyl-4-pyridinio)porphine (TMPyP4)

Finally and for comparison, the inhibitory potential of the porphine derivative meso-5,10,15,20-tetrakis-(N-methyl-4-pyridinio)porphine (TMPyP4) (Fig. 6B, inset) was tested. This compound has so far been known as the most powerful inhibitor of the ribozyme cleavage reaction [32]. The kinetics of the reaction in the presence of increasing concentrations of TMPyP4 and the determination of the IC_{50} are shown in Fig. 6A and B. The results show that indeed, TMPyP4 is also a potent inhibitor of the CChMVd-HHR self-cleavage activity with an IC_{50} of 177 ± 5 nM under the conditions used in these experiments. The k_{obs} of the reaction decreases from 4 min^{-1} in the absence of TMPyP4 to 0 in its presence at a concentration of 3 µM.

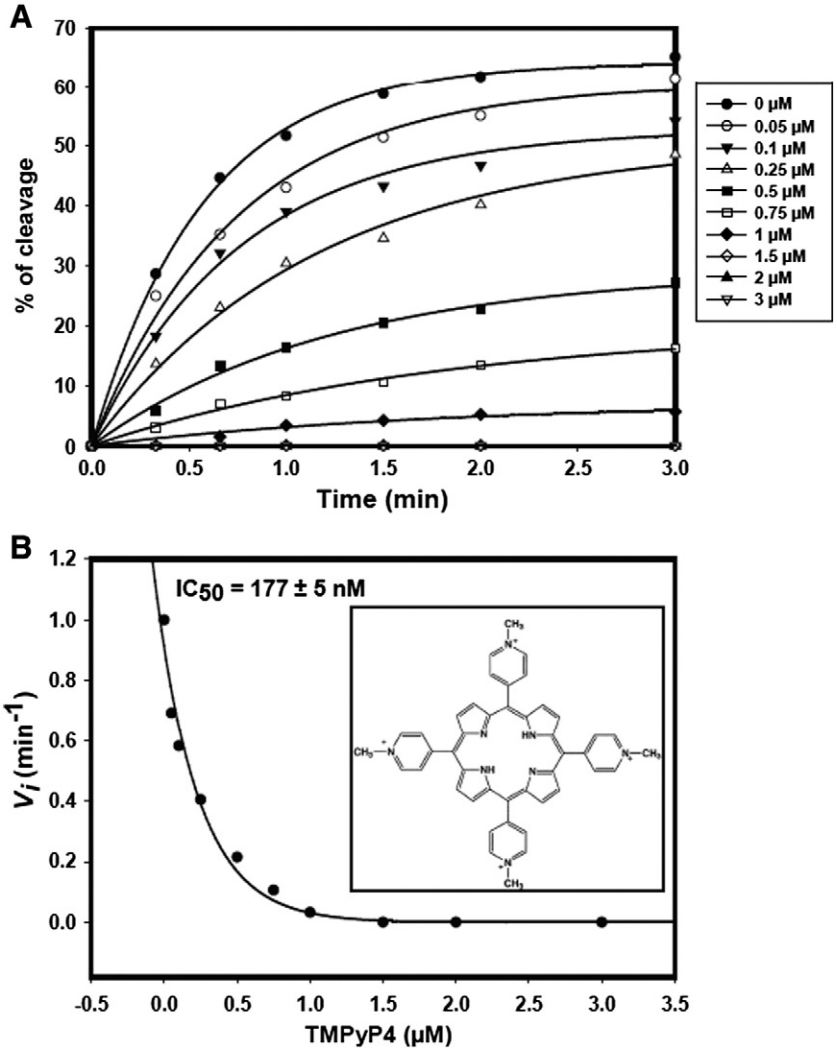

Fig. 6. Effect of TMPyP4 on the self-cleavage activity of the CChMVd-HHR. (A) self-cleavage CChMVd-HHR kinetics in the presence of increasing concentrations of TMPyP4. Kinetics were performed in 50 mM Tris, pH 7.5 and 0.6 mM Mg^{2+}. The symbols of 1.5, 2 and 3 µM of TMPyP4 are superimposed on the x-axis indicating 100% inhibition. (B) Initial rates of the self-cleavage kinetics as a function of TMPyP4 concentration. The IC_{50} determined is 177 ± 5 nM. Inset: Chemical structure of TMPyP4.

4. Discussion

The aim of this investigation was to search for compounds able to inhibit HHR self-cleavage. Among the various molecules tested, those bearing multiple amino groups that are protonated at physiological pH appear to be among the most efficient to date. This study also led to the identification of ruthenium hexamine as a new potent inhibitor of HHR. It also revealed an interesting contradiction in the mode of action of spermine on hammerhead ribozymes. Spermine known to support the cleavage activity of HHR both in the presence and in the absence of Mg^{2+} [25,26] is shown here to be a strong inhibitor of the CChMVd-HHR. Such a discrepancy concerning the effect of polyamines on RNA conformation has been already reported. For example, spermine can switch a *Neurospora* VS ribozyme from a slow *cis*-cleavage to a fast *trans*-cleavage reaction [33]. It can also activate some variants of the hairpin ribozyme but inhibit others [29]. In the present case there are several possible explanations for this discrepancy. First, the previously reported results concerning HHR were not obtained like in the present studies using a full HHR possessing the tertiary interactions between the two peripheral loops 1 and 2 (Fig. 1) whose importance was previously demonstrated [15,16]. In addition, the previously reported results concern trans-cleavage reactions involving the catalytic core of the viroid and an independent small RNA as substrate, while the reaction tested in the present study is the *cis*-self-cleavage which is much closer to the physiological one. It might also relate to the fact that CChMVd differs from most other HHR by the presence of an additional A nucleotide (A10 in Fig. 1) as well as larger stem 2 and loop 2. This last possibility is presently investigated using ELVD HHR, in the frame of a more general comparison between the catalytic properties of viroids and their isolated catalytic cores.

Spermine could have also altered the kinetics of the cleavage through increasing the rate of the ligation reaction. This is very unlikely since that would have shifted the value of the reaction equilibrium, an effect which is not observed. In addition, it was shown previously that the 65% maximal cleavage at the end of the reaction did not change upon addition of an excess of the large product of cleavage to the incubation mixture, indicating that the plateau does not correspond to an equilibrium but to the presence of an inactive conformation of the ribozyme. It might be that this inactive conformation is stabilized by the polyamine.

Although it can be expected that spermine interacts with the phosphate groups of the RNA backbone, the results reported here show that this molecule is a non-competitive inhibitor towards Mg^{2+}, indicating that they do not interact with the same phosphate groups. This interpretation is in agreement with that of the localization of spermine binding sites in 23S rRNA [34] where the authors proposed, that the structural and functional changes caused by polyamines may be different from those induced by divalent metal ions. All these observations

illustrate the high degree of plasticity and adaptability of the ribozymes, a property that might have played a role in the early developments of life.

Finally, the polyamines studied here are ubiquitous in plant cells at concentrations varying from micromolar to more than millimolar depending greatly on environmental conditions, in particular stress [35]. It is thus plausible to envisage a way of fighting viroids by modulating the polyamine biosynthesis or by adding exogenous polyamine. A decrease in the activity of ribozymes could affect the equilibrium between linear, circular, and polymeric genomes in infected cells and thus allow nucleases to eliminate the viroid before it completes its replication cycle.

Author contributions

The manuscript was written contributions from all authors. All authors have approved the final version of the manuscript.

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Notes

The authors declare no competing financial interests.

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