A ligand-dependent hammerhead ribozyme switch for controlling mammalian gene expression

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The possibility to externally control gene expression is of fundamental importance in both basic and applied life sciences. Although there are some techniques available to regulate expression in mammalian cells, they rely on the presence of ligand-sensing transcription factors, making it necessary to generate cell lines or organisms that stably express these regulatory factors. In recent years, mechanisms relying on direct RNA-ligand interactions for controlling gene expression have been both discovered in nature and engineered artificially. Among the latter, RNA switches relying on catalytically active RNA have been described. In principle, ligand-dependent triggering of mRNA self-cleavage should be a universal mechanism in order to control gene expression in a variety of organisms. Nevertheless, no examples of such aptazymes acting as RNA-based switches have been reported so far in mammalian cells. Here we present the theophylline-induced activation of an mRNA-based hammerhead ribozyme, resulting in an off-switch of gene expression. Starting from an artificial aptazyme switch reported to function in bacteria, we identified and optimized important parameters such as artificial start codons and the communicating sequence connecting ribozyme and aptamer, resulting in an RNA switch that allows for controlling transgenic expression in mammalian cells without the need to express a corresponding ligand-sensing transcription factor.

Introduction

In the early 1990s, genetic systems for the conditional expression of transgenes became available, with tetracycline-inducible and -repressible promoters the most frequently used.¹ Although of tremendous use, these systems require to stably express a transcription factor that regulates the transgene of interest in response to an externally added chemical stimulus. On the other hand, recent years have seen a plethora of discoveries in the field of regulatory RNAs. Riboswitches are mRNA-embedded elements that sense the presence of a metabolite and respond with changes in gene expression without the necessity of protein co-factors. The initial recognition of the ligand is facilitated by an aptamer domain; this interaction is then communicated to an expression platform that changes gene expression *via* interfering with transcription elongation, translation initiation, or mRNA integrity. In one example, the glmS ribozyme, a phosphodiester cleavage of the mRNA is triggered upon binding of glucosamine-6-phosphate to the riboswitch.² Whereas such RNA switches seem to be wide-spread in bacterial regulation of gene expression, only a few examples of riboswitches are known to operate in eukaryotes.³ Nevertheless, several artificial systems have been constructed based on the described natural principles.^{4,5} Recently, catalytically active RNA elements such as the hammerhead ribozyme have been used as expression platforms in artificial riboswitches: ligand binding to an aptamer domain triggers changes

in the phosphodiester-cleaving ribozyme (the fusion of aptamer and ribozyme is called aptazyme), thereby changing gene expression *via* reducing mRNA stability in *Saccharomyces cerevisiae*^{6,7} or liberation of the ribosome binding site in *Escherichia coli*.^{8,9} In addition the ligand-controlled, ribozyme-mediated cleavage reaction should be useful to regulate other classes of RNAs besides protein-coding messages. We have recently shown that the usage of tRNAs can be regulated by utilizing ligand-dependent hammerhead ribozymes.¹⁰ Moreover, Yokobayashi and co-workers demonstrated the release of a miRNA in mammalian cell lines using a theophylline-dependent hammerhead developed in our group.^{8,11}

Ligand-regulated hammerhead ribozymes were first introduced by Breaker and co-workers.¹² By attaching varying aptamers to stem II of the ribozyme and optimization of the connecting sequence *via in vitro* selection, a theophylline-dependent ribozyme was generated. However, these ribozymes were based on the minimal motif and turned out to be impaired with respect to catalytic activity *in vivo*. With the discovery of the extended hammerhead ribozyme motifs that display stem I/stem II contacts, much faster cleavage reactions were observed.¹³

Meanwhile, an example of an extended but discontinued hammerhead ribozyme was discovered in mammalian mRNAs.¹⁴ On the other hand, Mulligan and co-workers have artificially introduced extended, fast-cleaving ribozymes into mammalian mRNAs, demonstrating that the ribozyme is able to efficiently cleave and hence knock down the expression of the respective message. Although the introduced ribozymes lacked an aptamer domain, an *in vivo* screen for inhibitors of the hammerhead cleavage reaction identified some compounds that allowed switching off the ribozyme and hence restoring gene expression in mammalian cell culture as well as in mice.

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The most active compound was an adenosine analogue, inhibiting the ribozyme *via* incorporation into cellular RNAs rather than binding to it. With respect to ligand-dependent ribozyme regulation, Breaker, Mulligan, and co-workers generated fast-cleaving, theophylline-dependent hammerheads by *in vitro* selection. Nevertheless, upon insertion of these elements into mammalian mRNAs no theophylline-dependent regulation of gene expression was observed.¹⁵

Here, we demonstrate the utilization of ligand-dependent hammerhead ribozymes and present possibilities for improving the aptazyme performance for controlling gene expression in

mammalian cell lines by *in vivo* screening. The immediate transfer of the theophylline-responsive aptazymes into the mammalian mRNA context resulted in only poorly switchable constructs, making it necessary to optimize these RNA switches for the use in higher eukaryotes.

Results

In Fig. 1A, the general principle of the envisioned hammerhead aptazyme switch is shown. Messenger RNA cleavage within the 5'-UTR results in loss of the 5'-cap, an important

Fig. 1 Ribozyme and aptazyme mediated *in vivo* regulation of gene expression. (A) Integration of aptazymes within the 5'-UTR of eukaryotic mRNA allows ligand-dependent regulation of gene expression. Binding of theophylline to the aptamer induces a conformational change that is communicated to the ribozyme resulting in autocatalytic self-cleavage. Loss of the 5'-cap destabilizes the functional transcript and turns off gene expression. (B) Nucleotide sequence of the used hammerhead ribozyme (HHR). The bold nucleotide highlights the position of an A to G point mutation resulting in ribozyme inactivation. (C) HHR location site within the two utilized reporter systems. (D) Gene expression levels of constructs relative to the parental vector lacking the ribozyme insert. Active HHRs inhibit reporter gene expression by mRNA cleavage. Ratios of expression of inactive versus active ribozymes are given.

element for mRNA stability and ribosome recruitment. Ligand binding to the aptamer domain should induce cleavage activity and hence destruction of the respective mRNA, resulting in reduced gene expression. In order to verify the activity of HHRs embedded in the 5'-UTR of human mRNA, we first tested a HHR sequence lacking an aptamer sequence reported earlier by Yen *et al.* (termed ribozyme N79).¹⁶ We introduced the HHR (see Fig. 1B) into the mRNA of two different reporter genes, namely eGFP and renilla luciferase (hRLuc). For eGFP we chose an integration site close to the start codon, whereas in the hRLuc construct the HHR was incorporated 230 nt upstream of the start codon (see Fig. 1C). To verify that reduction of gene expression was caused by cleavage activity of the ribozyme, a point mutation (A45 to G, bold in Fig. 1B) inhibiting ribozyme activity was introduced resulting in an inactive variant of the hammerhead ribozyme (inHHR). In order to identify the ability to regulate expression, plasmids harboring active as well as inactive HHRs were transfected into HEK293 cells. The expression of both reporters was normalized using control reporters (RFP and firefly luciferase, respectively) as described

in the methods section. The ribozyme-mediated change in reporter gene expression is represented by the ratio of active and inactive HHR versions. Interestingly, both reporter systems showed a comparable reduction of reporter gene expression with 22-fold and 16-fold for eGFP and hRLuc, respectively, see Fig. 1D.

In order to test whether the theophylline-dependent HHR (termed theo-HHAz) identified in the bacterial context⁸ is able to switch expression in mammalian cells, we included the theophylline aptamer and the respective connection sequence into stem III of the HHR, see Fig. 2A. Upon addition of theophylline to the medium, we detected a theophylline-dependent reduction of gene expression of 1.7-fold and 3-fold for eGFP and hRLuc, respectively, see Fig. 2B. Although these results represented a starting point for further optimization of the switches, all constructs harboring ribozyme sequences showed a general, drastic reduction of expression compared to the parental constructs (pEGFP-N1 and psi-CHECK2) lacking inserted ribozymes, see Fig. 1D and 2C. We therefore set out to optimize the expression of ribozyme-containing mRNAs.

Fig. 2 Ligand-dependent regulation of gene expression: Bacterial communication module. (A) Nucleotide sequence of the used theophylline-dependent aptazyme. A theophylline aptamer is fused to the hammerhead ribozyme via a connection sequence formerly identified in a bacterial screen (boxed sequence). (B) Comparison of switchability within different reporter systems eGFP (square) and hRLuc (circle). Values are normalized to 0 mM theophylline. (C) Absolute reporter activity of ribozyme-containing constructs is decreased for each reporter system, eGFP (square) and hRLuc (circle), respectively upon insertion of the ribozymes. Same dataset as in 2B is shown except that the values were normalized to expression of the parental vectors lacking the ribozyme insert in order to judge absolute expression levels of ribozyme-containing mRNAs.

Removal of ribozyme-based alternative start codons

In eukaryotes, the translation initiation site is selected *via* a scanning mechanism with the small (40S) ribosomal subunit binding at the 5'-cap of the mRNA and scanning linearly along the 5'-UTR until recognition of the first start codon.¹⁷ Alternatively, upstream start codons are often recognized by the small ribosomal subunit depending on the position and flanking regions.^{18,19} This can lead to a drastic reduction in translation of the main ORF.¹⁷ The HHR embedded in the 5'-UTR of the reporter gene mRNAs contains two potential start codons, see Fig. 2A. The first AUG is located in the extended stem I and is not in frame with the main ORF, whereas the second AUG is found in the catalytic core of the ribozyme and is in frame with the main ORF. Although removal of the first AUG seems to be straightforward, mutations in the catalytic core are restricted due to a high degree of conservation. Nevertheless, U26 of the AUG sequence in the catalytic core (corresponding to U7 according to the hammer-head nomenclature, see ref. 20) has been reported to be variable.²¹ Removal of both start codons was performed by point mutations (U6 to G and U26 to C, respectively) as reported as ribozyme N107 by Yen *et al.*¹⁶ Although the N107 variant has been described already by Mulligan and co-workers, no information about the influence of AUG removal on the absolute levels of gene expression were available. Interestingly, the reporter activity of the inactive variant of HHR increased to levels comparable to the parental luciferase reporter activity. Even more important, the difference between the active and inactive ribozyme variants increased to changes of 75-fold and 91-fold for HEK and HeLa cells, respectively, see Fig. 3A. As expected, removal of the start codons within the aptamer-containing sequence theoHHAz as well restored reporter activity while retaining the ligand-dependent reduction in expression, see Fig. 3B.

Improving theophylline-dependence

Following the optimization of the expression levels *via* removal of artificial start codons, we aimed at the identification of sequences that showed more pronounced inactivation ratios upon addition of theophylline. Two different strategies were followed for improving the switchability of the system. On the one hand we randomized four nucleotides in the region connecting the aptamer and HHR domain to perform an *in vivo* screen for improved RNA switches. On the other hand we modified the connection module in a rational way by computing the thermodynamic stability of different variants of the theo-HHAz using the *mfold* algorithm.²² Using the first approach, we screened 192 clones (representing a statistical 53% coverage of the sequence space of the four-nucleotide randomized pool¹⁹) in HEK293 cells. For this purpose, a library of isolated plasmids was transfected in the presence and absence of theophylline and clones displaying the highest reduction of reporter activity in response to the ligand were sequenced. Surprisingly, 16% of all clones showed a change in reporter expression of more than 25% upon addition of theophylline and covered a broad distribution of operational ranges. The sequence of the most responsive clones of both *in vivo* selection and rational design strategy, namely P1-F5

Fig. 3 Influence of upstream start codons within the aptazyme sequence. (A) Absolute reporter activity is increased to parental vector (white) after removal of upstream start codons (grey) compared to HHR sequence containing start codons (black). The psi-CHECK2 control gives 100% in both cell lines due to the normalization procedure. (B) Absolute reporter activity (hRluc) is observed for the theoHHAz variants containing (black) and lacking ribozyme-based start codons (grey).

and 5.3, are shown in Fig. 4A. Both P1-F5 and 5.3 showed similar switching ratios. In HEK cells, a 4.5-fold reduction was found with both sequences and a 6-fold change in HeLa cells, see Fig. 4B and C.

In vitro characterization of ribozyme kinetics

In order to demonstrate that the ligand-dependent changes of gene expression observed *in vivo* are indeed mediated *via* theophylline-dependent changes of ribozyme activity, we performed *in vitro* cleavage kinetics utilizing the identified aptazyme sequences. For this purpose, cis-cleaving ribozymes were prepared by *in vitro* transcription as described.²³ The cleavage reactions were initiated by addition of Mg^{2+} to a final concentration of 0.2 mM (in order to mimic low, physiological Mg -concentrations) in the presence or absence of 5 mM theophylline. In the case of the parental HHR lacking the theophylline aptamer domain there was only little rate enhancement observed upon addition of theophylline (from 0.23 min^{-1} to 0.29 min^{-1}), whereas the cleavage rates of the identified variants P1-F5 and 5.3 were increased 5.2-fold and

Fig. 4 Switchability of theophylline-dependent aptazymes in HEK and HeLa cells. (A) Sequences of mutants optimized by *in vivo* screening and rational design. Two mutants P1-F5 and 5.3 are shown. (B) Theophylline dependence of P1-F5 (diamond) and 5.3 (circle) compared to theozHHaz (square) in HEK cells. Values are normalized to 0 mM theophylline. (C) Same as B but assayed in HeLa cells.

3.0-fold in the presence of theophylline (see Fig. 5 and Table 1). Furthermore, high cleavage efficiencies were observed for all ribozymes in the presence of theophylline.

Conclusions

Since ribozyme-mediated cleavage irreversibly changes the integrity of a given mRNA, the principle of using ribozyme-mediated switches to conditionally regulate gene expression should be transferable from one organism to another. There is one example of a successful utilization of a hammerhead-based regulation in the apicomplexan parasites *Toxoplasma gondii* and *Plasmodium falciparum*.²⁴ Here, we achieved the transfer of a ligand-regulated ribozyme that acts as a switch from bacteria to mammalian cells. In our case, although the original bacterial sequence showed moderate switching behavior in the tested cell lines, the simple transfer of bacterial sequences to eukaryotes seems limited. In contrast to prokaryotic mRNA, the stability of eukaryotic mRNA is regulated by 5'-cap and 3'-poly(A) tail elements. Ribozyme cleavage results in loss of the 5'-cap and leads to reduction of mRNA stability

and incapability of recruiting the translational machinery. Different mRNA fates such as spatial and temporal decoupling of transcription and translation, post-transcriptional processing reactions, mRNA-bound proteins, and different conditions such as divalent metal ion concentrations in mammalian cells compared to bacteria impose altered requirements for the catalytic range of the switchable ribozymes. These fundamental differences seem to make it necessary to fine-tune the connection module of aptamer and ribozyme. In order to optimize the theophylline-dependent aptazyme, we pursued two different strategies: We have demonstrated that due to the fundamental differences in translation initiation, the removal of artificial start codons in the inserted ribozyme sequences is beneficial for ensuring high translation efficiencies. Another possibility would be the implementation of ribozymes in the 3'- rather than the 5'-UTR. On the other hand, studies carried out in the Mulligan and Smolke groups hint at less pronounced effects on gene expression if the mRNAs are cleaved in the 3'-region.^{7,16} In order to improve the switching ratios, we applied an optimization technique that relies on *in vivo* screening of mutant pools containing randomized sequences at the ribozyme/aptamer

Fig. 5 *In vitro*-cleavage kinetics of optimized theophylline-dependent aptazymes. Theophylline-dependence of self-cleavage reaction of *in vitro*-transcribed ribozymes was assayed at 0.2 mM MgCl_2 concentration. Black: absence of theophylline; grey: 5 mM theophylline. (A) Parental HHR lacking the aptamer. (B) Aptazyme P1-F5. (C) Aptazyme 5.3.

connection site. While this approach resulted in significantly enhanced switching rates, the procedure is rather cumbersome and characterized by low throughput in comparison to screening of bacterial mutants. Recently, Ellington and co-workers introduced a combined *in vitro/in vivo* selection protocol that might be well suited to overcome these limitations in

Table 1 *In vitro* cleavage activity of ribozymes at 0.2 mM MgCl_2

	Theophylline ^a	$k_{\text{obs}}/\text{min}^{-1}$	Cleavage efficiency	Induction rate
HHR	–	0.23 ± 0.01	82%	1.20
	+	0.30 ± 0.02	82%	
Theo 5.3	–	0.08 ± 0.01	86%	3.0
	+	0.23 ± 0.01	86%	
Theo P1-F5	–	0.05 ± 0.01	56%	5.2
	+	0.27 ± 0.01	72%	
Theo-HHAz ^b	–	1.3	> 80%	2.8
	+	3.6	> 80%	

^a 0 and 5 mM respectively. ^b Taken from ref. 8.

identifying powerful ribozyme-based switches.²⁵ Noteworthy, the transfer of *in vitro* selected aptazymes to *in vivo* applications seems even more complicated and mostly these aptazymes show decreased or no switching behavior *in vivo*.^{5,15} Breker, Mulligan, and co-workers have argued that factors other than high switching ratios of fast-cleaving ribozymes might be important. In addition, Ellington and co-workers have demonstrated that in some cases ribozymes with slower cleavage kinetics are able to efficiently cleave in mammalian cells.²⁵ In this light, *in vivo* screening and selection protocols seem to be suited to identify RNA-based, ligand-dependent switches of gene expression.

Materials and methods

Plasmid construction

All eGFP and hRluc reporter gene plasmids were generated by site directed mutagenesis using primer pairs encoding the respective aptazyme or HHR sequence.²⁶ Nomenclature was as follows: HHR describes the hammerhead ribozyme N79 as described previously;¹⁶ theoHHAz describes the theophylline-dependent HHR obtained previously by screening in bacteria;⁸ P1-F5 describes a variant of theoHHAz obtained by additional screening and 5.3 is a rationally designed variant of theoHHAz with a longer connection module between aptamer and ribozyme domain. The term ‘inactive’ describes aptazymes harboring a point mutation A14G (corresponding to the hammerhead nomenclature, see ref. 20) within the catalytic core of the HHR domain which was reported to inactivate HHR self-cleavage.^{8,16} In the case of the eGFP reporter gene plasmids, aptazyme variants respective HHR or inactive variants were introduced at nucleotide position 90 within the transcription unit of eGFP corresponding to position 670 within the pEGFP-N1 vector. In the case of the hRluc reporter gene plasmids, ribozymes were introduced at nucleotide position 230 upstream of the start codon of hRluc within the psiCHECK-2 vector (Promega).

Oligonucleotides used for PCR reactions

HHR: 5'-d(CGAAACGCGCTTCGGTGCCTGCTGGATT-CCACTGCTATCCACCGCCACCATGGTGAGCAAG) and 5'-d(PTCCTATTGGGACTCATCAGCTGGATGTACCTGC ATCTCAGACCGGTGGATCCCGGGC)
 theoHHAz: 5'-d(CGAAATACATACCAGCCGAAAGGCCCTTGGCAGGTGCTCTGGATTCCACTGCTATCCA-

CCGCCACCATGGTGAGCAAGG) and 5'-d(pTCCTATT-TGGGACTCATCAGCTGGATGTACCTGCATCTCAGACCGGTGGATCCCGGGC)

5.3: 5'-d(AAAAATCATACCAGCCGAAAGGCCCTTG-GCAGGA TTTCCTGGATTCCACTGCTATCCACCGC-CACCATGGTGAGCAAGG) and 5'-d(pCGTCCTATTG-GGACTCATCAGCTGGATGTACCTGCATCTCAGACC-GGTGGATCCCGGGC)

P1-F5: 5'-d(CGAAANNCATACCAGCCGAAAGGCCCTTGGCA GGNNCTCTGGATTCCACTGCTATCCACC-GCCACCATGGTGAGCAAGG) and 5'-d(pTCCTATTG-GGACTCATCAGCTGGATGTACCTGCATCTCAGACCG-GTGGATCCCGGGC) whereas N represents an equimolar mixture of all four nucleotides. Sequences of clones were obtained by sequencing following screening.

C2_HHR: 5'-d(pCGAAACGCGCTTCGGTGCCTCCT-GGATTCCACTGCTATCCACAAGTTGGTCGTGAGGC-ACTG) and 5'-d(TCCTATTGGGACTCATCAGCTGGA-TGTACCTGCATCTCAGCTGCAGCTTAAGTTTCGAGAC)

C2_HHR_woAUG: 5'-d(pCAGCTGACGAGTCCCAAA-TAG) and 5'-d(GATGTACCTGCACCTCAG)

C2_Theo: 5'-d(pAAGGCCCTTGGCAGGTGTCCTGG-ATTCCACTGC) and 5'-d(TCGGCTGGTATGTATTTCG-TCCTATTGGGACTC)

C2_5.3: 5'-d(pAGGCCCTTGGCAGGATTTCCTGGAT-TCCACTGC) and 5'-d(TTCGGCTGGTATGATTTTTCG-TCTATTGGGACTC)

C2_P1-F5: 5'-d(pAGGCCCTTGGCAGGGTTCTTGGA-TTCCACTGC) and 5'-d(TTCGGCTGGTATGGCTTTCG-TCTATTGGGACTC)

p represents a 5'-phosphate group

Cell culture and transfection

HEK293 cells and Hela229 cells were maintained in a 5% CO₂-humidified incubator at 37 °C in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (PAA), 100 U ml⁻¹ penicillin, and 100 µg ml⁻¹ streptomycin (Invitrogen). In case of eGFP reporter gene plasmids, cells were trypsinized one day before transfection, diluted with fresh medium and transferred to 96-well plates (15 000 cells per 100 µl). Cotransfection of pEGFP-N1 vector variant and pTagFP-635 was carried out using Turbofect (Fermentas) according to the manufacturer's instruction with modifications. Specifically, 25 ng of pEGFP-N1 vector variant and 50 ng of pTagFP-635 were used with 0.5 µl of Turbofect reagent per well. After incubation in the respective medium supplemented with the indicated concentrations of theophylline for 48 h, fluorescence measurement was performed for eGFP as described below. In the case of using hRLuc reporter gene plasmids, cells were trypsinized one day before transfection, diluted with fresh medium and transferred to 96-well plates (15 000 cells per 100 µl). Then, cells were transfected with 50 ng plasmid using Lipofectamine 2000 reagent (Invitrogen). Upon incubation in a 5% CO₂-humidified incubator at 37 °C in the respective medium supplemented with the indicated concentrations of theophylline for 24 h, luminescence measurements were performed for hRLuc as described below.

Reporter activity measurements

For quantifying the change in eGFP gene expression, eGFP fluorescence and TagFP fluorescence were detected using an Infinite microplate reader (Tecan) according to the manufacturer's instructions. Before measuring the fluorescence intensity, medium was removed and cells were washed twice with each 50 µl prewarmed (37 °C) phosphate-buffered saline (PBS). Measurement conditions were as follows: excitation 488 nm for eGFP and 588 nm for TagFP, emission 535 for eGFP and 635 for TagFP. Data were processed as follows: First, background signal (well with untransfected cells) was subtracted from both eGFP and TagFP signal. Then, fluorescence values of eGFP were divided by those of TagFP to obtain eGFP/TagFP (TagFP-635 fluorescence was used for normalization of transfection efficiency) and the average values and standard deviations of eGFP/TagFP from triplicate samples were calculated. For quantifying the change in hRLuc gene expression, luciferase activity was detected using the Promega Dual Luciferase Assay Kit according to the manufacturer's instructions. Then, values of hRLuc were divided by those of hluc+ to obtain hRLuc/hluc+ and the average values and standard deviations of hRLuc/hluc+ were calculated from each independent experiments performed in duplicates.

In vivo library screening

In vivo screening was conducted in 96-well plates using eGFP as the reporter gene. First, an aptazyme library was generated by site directed mutagenesis as described above by using primer pairs with randomized connection modules. Then, XL-10 Gold cells (Stratagene) were transformed and single colonies were transferred and grown in 96-well plates. Upon plasmid preparation using NucleoSpin Multi-96 Plus Plasmid Kit (Macherey Nagel) HEK293 cells were co-transfected with the clone library in 96-well scale as described before and cultured in the presence or absence of 5 mM theophylline. Finally, reporter gene activity was measured for each clone as described before and theophylline-dependent ratios were calculated.

In vitro transcription and cleavage kinetics

All five aptazyme variants were generated by PCR using DNA templates containing a T7 promoter and the respective aptazyme or HHR sequence. Upon purification of the PCR products by agarose gel electrophoresis we performed *in vitro* transcription reactions using T7 RNA polymerase. Reactions were conducted in the presence of 25 µM blocking anti-sense oligonucleotides (5'-ATTTGGGACTCATCAGCTGG-3') in order to prevent self-cleavage and of traces α-³²P-labeled ATP. Full-length RNA products were then purified by 8% denaturing PAGE and then substituted to *in vitro* cleavage reactions. Aptazymes were annealed by incubating at 95 °C for 2 min in 50 mM Tris-HCl (pH 7.5) followed by cooling down to 37 °C within 30 min. Self-cleavage reactions in the presence or absence of 5 mM theophylline were carried out by addition of 0.2 mM MgCl₂ at 37 °C. Aliquots were removed and stopped by addition into formamide loading buffer. Cleavage fragments were then separated by a 10% denaturing

polyacrylamide gel and cleavage percentages were quantified by PhosphorImaging.

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