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Photochemical hammerhead ribozyme activation

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Abstract—We report the light-activation of allosteric *cis* and *trans* acting ribozymes via decaging of a small organic molecule ligand. To achieve this effectively, we introduce an optimized N-caging group based on a nitrobenzyl core structure. This approach can potentially be employed toward a light-induced control of gene function.

The hammerhead ribozyme is a small RNA motif consisting of three stems that intersect at a conserved catalytic core. It promotes the sequence-specific cleavage of RNA phosphodiester bonds with a rate of about $1\ min^{-1}$ at 25 °C and neutral pH. This represents a 10^6-fold rate enhancement compared to the non-catalyzed hydrolysis of RNA.[1,2] Using Watson–Crick base pairing, the hammerhead ribozyme has been engineered to cleave a variety of different RNA substrates.[3] Numerous studies have shown that *trans* acting ribozymes can be successfully employed inside living cells to knock down expression of specific genes and used as functional genomics tools.[4,5] Intracellular delivery of ribozymes can be achieved by either in vivo expression or microinjection. Ribozyme technology has found wide application in basic and applied research, and several ribozymes are currently being investigated in clinical trials.[6] Recently, the catalytic capabilities of RNAzymes have been further tuned via the integration of a small-molecule binding aptamer sequence to the ribozyme yielding an allosteric ribozyme.[7,3,8–14] For example, an allosteric hammerhead ribozyme composed of a catalytic domain connected to a theophylline binding domain via a communication module (see Fig. 1) has been created (other small organic ligands for allosteric ribozymes include, e.g., ATP, cAMP, TPP, FMN, and SAM).[7,3,8,15] Upon binding of the ligand, base pairing at the communication module linking the aptamer and the catalytic domain is stabilized. This activates the ribozyme leading to subsequent cleavage of the RNA substrate. This technology has been applied to the generation of RNA-based sensors for small organic molecules and to the evolution of new aptamers using an in vitro selection.[7,8] However, its utilization in the small-molecule mediated regulation of gene expression has not yet been exploited. To achieve control of gene function with a high temporal and spatial resolution

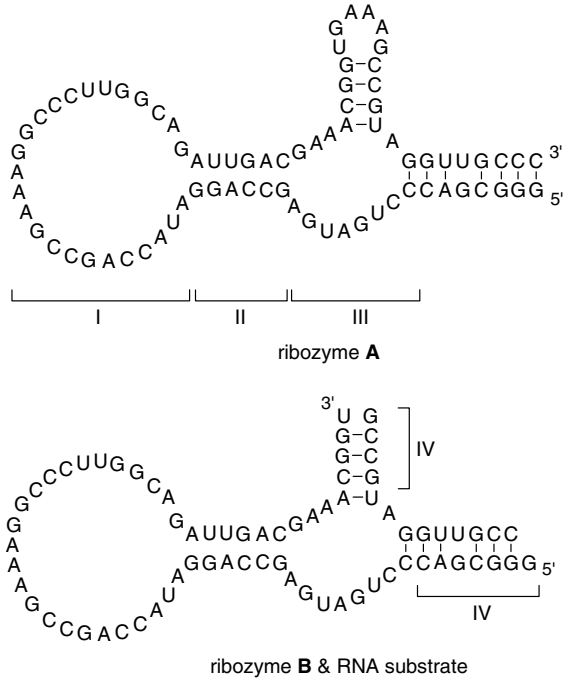

Figure 1. Theophylline responsive ribozymes that cleave RNA in a *cis* (intramolecular) (**A**) and a *trans* (intermolecular) (**B**) fashion. **B** is shown with its RNA substrate bound. They consist of an aptamer domain (I), a communication module (II), and a catalytic domain (III). The flanking regions (IV) define the RNA target.

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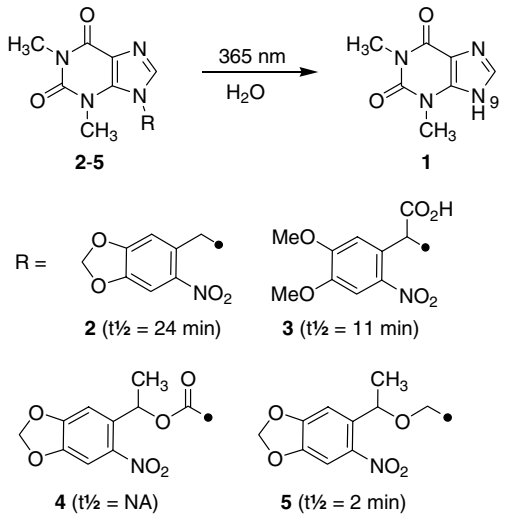
and with a minimum of secondary perturbations, light-mediated gene activation or deactivation is an ideal concept.[16] A photoactivatable ribozyme represents a potential photochemical gene control element. This system would be comprised of: (1) a photocaged small organic ligand[17–19] and (2) an allosteric ribozyme which is activated by this ligand. The principles of photocaging have previously been applied to ribozyme systems, but not to the extent where a potential control of gene function is achievable. Chaulk and MacMillan has previously reported photocaging of the RNA substrate of a ribozyme.[20] However, for obvious reasons this approach is impractical when attempting to achieve translational control of mRNA. Additionally, Breaker and co-workers have employed photocaged cAMP to assay activity of an allosteric ribozyme,[21] but this approach has no potential to be employed in gene control, as cAMP is an endogenous natural metabolite. As a result, the ribozyme would be constitutively activated by intracellular cAMP.

We selected theophylline (**1**) as a small organic ligand, due to its absence in bacterial, yeast, and mammalian cells, its high solubility in an aqueous environment, its low toxicity, and its ability to bind selectively to certain RNA sequences.[22,23] Since **1** is not present in animal cells, it can be used as an exogenous regulator of gene control in model organisms. Hence, we synthesized the photocaged compounds **2**–**5**, blocking the 9-NH position with a variety of caging groups,[17–19] as previous studies have demonstrated that this position is crucial for efficient binding to a known RNA aptamer.[22,23] In **2**–**5**, we caged this position with photoremovable nitrobenzyl groups (Scheme 1).

Irradiation of **2**–**5** (0.1 mM) with non-photodamaging UV light (365 nm, 23 W, hand-held UV lamp) irreversibly removes the photoprotecting group and delivers free theophylline (**1**). Our studies commenced with **2**, which is easily synthesized and decages under UV irradiation.

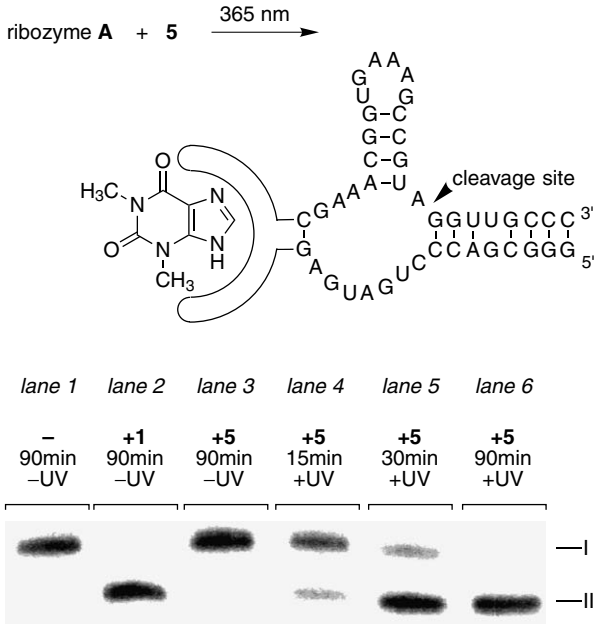


Scheme 1. Caged theophyllines **2**–**5**. Half-life times under irradiation conditions (0.1 mM, 365 nm, 23 W, hand-held UV lamp) are given in parentheses. NA, not applicable.

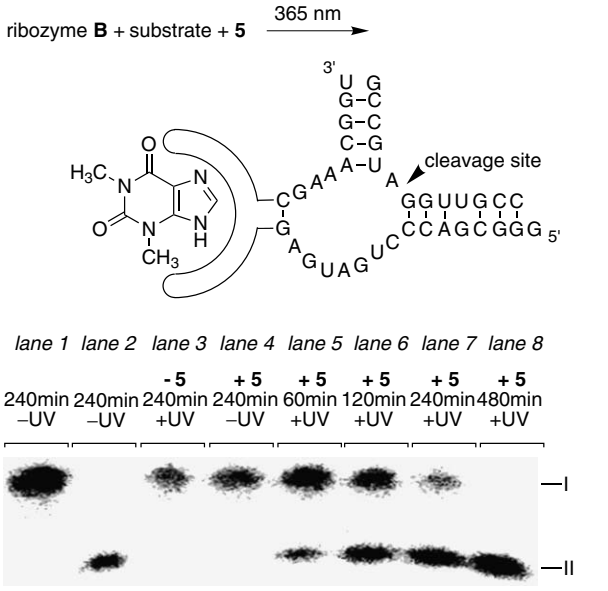
However, it produces a potentially toxic benzaldehyde as a byproduct in the decaging reaction and displays low solubility in an aqueous environment. This problem was solved by synthesizing the carboxy-modified molecule **3**. It displayed high aqueous solubility but still relatively slow decaging properties. In **4** we employed a carbamate protecting group which has been proven to provide rapid decaging. However, **4** was highly unstable in an aqueous environment and hydrolyzed on a minute time scale. Finally, with **5** we introduced the (2-nitrovaleryl)oxymethyl (NVOM) group as a new amino photoprotecting group. Related caging groups have previously been used for the photoprotection of hydroxy functionalities.[24] The NVOM group is prepared via methyl addition to nitropiperonal, followed by formation of the chloromethyl ether. The chloromethyl ether is then utilized in the caging of theophylline (Cs_2CO_3, DMF, rt). The NVOM caging group has several advantages over other amino caging groups: it decages rapidly via irradiation with non-photodamaging UV light, it produces a less toxic acetophenone byproduct in the photodecaging, the caged molecule is highly stable in an aqueous environment at various pH, and the caging step proceeds in high yield. Moreover, the caged theophylline **5** is non-toxic and easily penetrates cell membranes. This has been validated in both *Escherichia coli* and *Danio rerio*, as 0.1 mM incubations with **5** yielded no alterations in growth rate or phenotype. These incubations resulted in intracellular concentrations of approximately 0.06 mM in both, bacterial cells and zebrafish embryos, as determined by LC–MS analysis of the cell lysates.

The allosteric ribozymes we selected are comprised of the catalytic domain of a hammerhead ribozyme coupled with a theophylline aptamer via a communication module shown in Figure 1.[21] The *cis* acting ribozyme **A** cleaves itself in an intramolecular fashion and is used as a model system. The *trans* acting ribozyme **B** can be utilized to cleave virtually any RNA by tailoring its flanking sequences.

In the absence of the small organic molecule, the allosteric ribozyme **A** is inactive. However, in the presence of its effector **1**, binding of the aptamer and structural reorganization occur. This event is transmitted to the catalytic domain via the communication module, activating the ribozyme to induce intramolecular cleavage. The RNA degradation was detected by SDS–polyacrylamide gel electrophoresis (PAGE) of a radiolabeled oligonucleotide. The gel shown in Scheme 2 displays the photoactivation event: in the absence of the small organic ligand, the ribozyme is inactive and remains uncleaved even after 90 min of incubation (lane 1). Presence of theophylline (**1**) induces complete self-cleavage after 90 min (lane 2). In contrast, the photocaged theophylline **5** does not activate the ribozyme as lane 3 shows, verifying the importance of the 9-NH group for catalytic activity. However, lanes 4–6 display the progressing cleavage of the photoactivated ribozyme by irradiation with a hand-held UV lamp (365 nm, 23 W) for just 5 min. Complete RNA cleavage was observed after 90 min.



Scheme 2. Photochemical activation of the allosteric ribozyme **A** by irradiation for 5 min, followed by complete intramolecular RNA cleavage within 90 min. PAGE of light-activated ^{32}P labeled ribozyme visualized using a phosphorimager. Lane 3 shows uncleaved ribozyme (I) in the presence of **5** but absence of UV light. Lane 6 shows the same reaction after brief UV irradiation, indicating complete RNA cleavage yielding a truncated ribozyme (II). The other lanes are described in more detail the text.



Scheme 3. Light-activation of the *trans* acting allosteric hammerhead ribozyme **B** followed by cleavage of a single-stranded 5′-^{32}P labeled RNA oligomer. Lane 4 shows uncleaved RNA substrate (I) in the presence of **5** but absence of UV light. Lane 8 shows the same reaction after 5 min of UV irradiation followed by incubation for 480 min, indicating complete cleavage to a shorter RNA strand (II). The other lanes are described in more detail in the text.

In order to employ an allosteric ribozyme for gene silencing studies, the RNA cleavage event needs to be conducted in a *trans* rather than a *cis* fashion. This has been demonstrated for non-allosteric ribozymes directed against the mRNA of a variety of targeted genes;[25–30] however, we describe a photochemically activatable system leading to RNA cleavage in a *trans* fashion. As it is possible to introduce modified sequences in the flanking regions of the ribozyme, this method can be specifically tailored to knock down virtually any gene of interest. The sequence of the ribozyme **B** and the target RNA is depicted in Figure 1. Rapid decaging of **5**, via irradiation with a hand-held UV lamp (365 nm, 23 W) for 5 min, leads to activation of the allosteric ribozyme **B**, formation of a ternary complex consisting of the ribozyme, **1**, and the RNA substrate, followed by complete RNA substrate degradation within 480 min. This photoactivated RNA cleavage is documented by SDS–PAGE (Scheme 3). Only the 5′-^{32}P endlabeled RNA substrate (13 nucleotides) is visible in lane 1. In the presence of a non-allosterically regulated hammerhead ribozyme (5′-GGGCGACCCUGAUGA GGCCUUCGGGCCGAACCGGU-3′) complete cleavage of the substrate is achieved in 240 min (lane 2). In the presence of ribozyme **B** but absence of theophylline (**1**) (lane 3), or in the presence of caged theophylline **5** but in the absence of UV light (lane 4), no ribozyme activity is detected and the RNA substrate remains intact even after 240 min of incubation. As expected, presence of **5** and brief UV irradiation (5 min) induce complete RNA degradation over the course of 480 min (lanes 5–8).

In summary, we have demonstrated the photochemical activation of *cis* and *trans* acting allosteric ribozymes by irradiation of a photocaged exogenous ligand. This enables photoactivation of ribozyme function in a precise temporal and spatial control. Future developments will demonstrate the in vivo activity of this system and the application of the NVOM amino caging group in the photoactivation of other biological macromolecules.

Acknowledgments

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