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SUMMIT
SOFIA UNIVERSITY MARKING MOMENTUM FOR INNOVATION AND TECHNOLOGICAL TRANSFER

SOFIA UNIVERSITY -
MARKING MOMENTUM
FOR INNOVATION AND TECHNOLOGICAL TRANSFER

National Recovery and Resilience Plan
OF THE REPUBLIC OF BULGARIA

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Activity 3.4. Scientific research with potential for innovation and knowledge/intellectual property transfer

Creation of software systems for computer-aided design of rapid allosteric ribozymes that sense the presence of sequence-defined oligonucleotides and a database of clinically relevant human genetic variation

INTRODUCTION

According to a study by the World Health Organization, one of the most serious health problems in Bulgaria is cancer. It claims millions of lives annually. With 26% of deaths, it is the second most common cause of mortality in Europe. Statistics show that in Bulgaria there are over 200,000 people with malignant tumors. Their number increases every year by at least 26,000 new cases. Timely and accurate diagnosis, as well as correctly selected treatment with an innovative therapeutic agent, would reduce mortality and prolong patient survival, and why not their recovery. In search of new approaches to diagnosis and therapy, scientists discovered the diverse properties of ribozymes. They are RNA enzymes that have the properties of molecular biosensors, precisely sensing the presence/absence of certain substances in the cell. This property allows us to design specific, 100% efficient allosteric ribozymes, based on already patented and experimentally validated algorithms that have affinity for particular substances (oligonucleotides, tumor markers, etc.), ensuring the possibility of accurate, rapid and early diagnosis. Due to their specificity and fast kinetics, ribozymes can be used for control of gene expression, as well as for gene therapy.

PROJECT GUIDELINES

The main goal of grant 70-123-194/12.02.2024 is to create new, innovative, and original software systems that allow us to make 100% accurate designs of fast allosteric ribozymes that have an affinity for precisely defined molecules with a pre-selected and defined sequence. They are based on successfully validated algorithms with confirmed laboratory results (Penchovsky and Ackermann 2003, Penchovsky and Breaker 2005, Penchovsky and Kostova 2013, Penchovsky 2014, Kaloudas and Penchovsky 2022, Kaloudas, Pavlova et al. 2023), which we will modify. The results confirming their effectiveness confirmed the precise ligand specificity and their ability to function in molecular chains in which the product of self-cleavage of one RNA triggers the action of another. This engineering approach provides a fast and cheap way to create allosteric ribozymes to construct complex molecular systems. The first software program has a “YES” logic function, and the second has a “NO” logic function. Both recognize specific oligonucleotides. Allosteric ribozymes based on the extended version of the Hammerhead ribozyme can have high-speed cleavage kinetics under physiological conditions. The correct design implies the preliminary definition of a specific sequence of the oligonucleotide binding site, which is located in stem III of the extended version of the ribozyme. This allows the ribozyme to carry out synthetic control of gene expression in bacterial and eukaryotic cells, leading to the design of new agents that can be applied as therapeutic agents, including in the treatment of cancer patients. Fast allosteric ribozymes can be used in biotechnological and synthetic biology applications, even as biosensors for certain substances or as diagnostics for various diseases. In addition to the two software products, a system for searching for specific data will be created with a focus on the most common oncological diseases (lung cancer and breast cancer). It is designed for use in clinical centers and diagnostic laboratories.

METHODOLOGY

Two main methods for the design of allosteric ribozymes are known: *in vitro* selection and computational design. Due to the shortcomings of the first method (self-cleavage during transcription and selection or I nefficiency), we focus our efforts on computational design. The ability to create in silico fast ribozymes that exhibit specific activity in the presence of an effector molecule under physiological conditions can rapidly design fast ribozymes sensitive to oligonucleotides. In recent years, our team has published various algorithms for the computational design of allosteric ribozymes capable of sensing oligonucleotide targets and operating under physiological conditions, with experimentally proven efficiency (Penchovsky 2014), (Penchovsky and Kostova 2013). We have also developed programs for generating truncated Hammerhead allosteric ribozymes using RNA folding calculations, random search algorithms (Kaloudas and Penchovsky 2022), as well as those using the extended version of the Hammerhead ribozyme (Penchovsky and Ackermann 2003), which also serve us for the design of high-speed allosteric ribozymes, with a “YES” and “NO” logic function, with a variable oligonucleotide binding site (Penchovsky and Breaker 2005), (Kaloudas, Pavlova et al. 2023). The software systems, the subject of this project proposal, represent an upgraded and refined version of the already verified and proven algorithms for computer design of allosteric ribozymes using the extended version of the Hammerhead ribozyme.

RESULTS

Allosteric RNAs operate as molecular switches that alter folding and function in response to ligand binding. Common types of natural allosteric RNAs are riboswitches, and designer RNAs with similar properties can be created by RNA engineering. We describe a computational approach for designing allosteric ribozymes triggered by binding oligonucleotides. Four universal types of RNA switches possessing AND, OR, YES, and NOT Boolean logic functions were created in a modular form, which allows ligand specificity to be changed without altering the catalytic core of the ribozyme. All computationally designed allosteric ribozymes were synthesized and experimentally tested in vitro. Engineered ribozymes exhibit>1,000-fold activation, demonstrate precise ligand specificity, and can function in molecular circuits wherein the self-cleavage product of one RNA triggers the action of a second. This engineering approach provides a rapid and inexpensive way to create allosteric RNAs for constructing complex molecular circuits, nucleic acid computing systems, and gene control elements.

Molecules with attributes of YES logic function must remain inactive unless receiving a single molecular impulse that triggers activity. The YES-1 construct is predicted to form the desired OFF- and ON-state structures in the absence and presence, respectively, of a 22-nt effector DNA (DNA 1; **Figure 1A**). In its inactive conformation, the nucleotides within the OBS are proposed to form a 'stem IV' structure. Stem IV involves extensive base-pairing interactions with portions of the hammerhead core and with most nucleotides that would otherwise form the stem II structure required for ribozyme activation. In the presence of DNA-1, a major portion of the nucleotides in stem IV would become sequestered by intermolecular base pairing, and nucleotides that can participate in stem II formation are liberated. The results of the computational assessment of the structure-forming potential of this construct are visually represented by dot matrix plots (**Figure 1A, right**).It is apparent from these plots that the probability of forming stem IV or several base-pairing alternatives is high, whereas there is no indication that stem II has any reasonable chance of forming. In contrast, repeating the computation in the presence of the DNA effector drastically reduces the probability that stem IV can form, and thus increases the probability that stem II will be formed. To estimate the dynamic range for allosteric activation, or the total range of rate constant enhancement brought about by effector binding, a time course for ribozyme self-cleavage was conducted in the presence and absence of the matched effector DNA (**Figure 1C**).

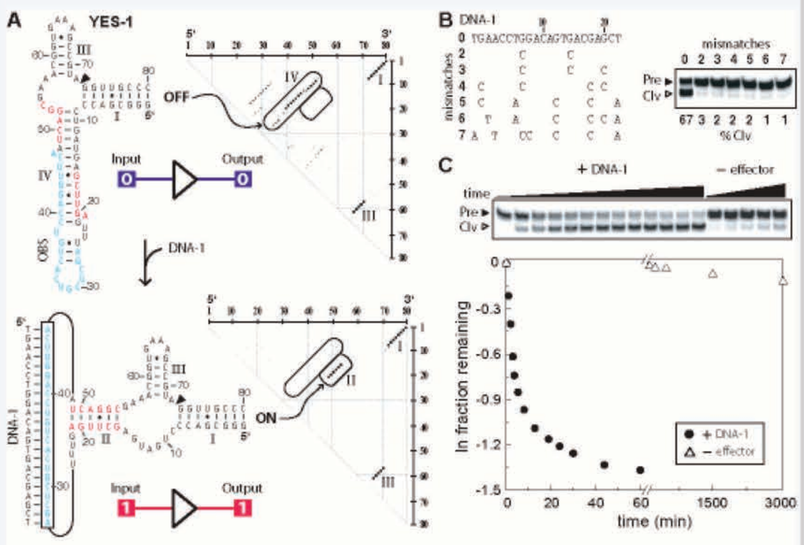

Figure 1. Design and characterization of an oligonucleotide-specific RNA switch possessing YES logic function. *(A) Secondary structure models for the most stable conformers as computed using the partition function algorithm in the absence (OFF) or presence (ON) of a 22-nucleotide DNA effector. The effector-binding site (light blue) is joined to nucleotides 10.1 and 11.1 of the hammerhead core via eight- and six-nucleotide linkers. In the ON state, most of these linker nucleotides are predicted to form an extended stem II structure (green). To the right of each model is a dot matrix plot wherein larger points reflect a greater probability of base pairing. Encircled points reflect the main differences in predicted structures between the OFF (stem IV) and ON (stem II) states. Nucleotides 1 through 79 are numbered from 5` to 3` across the top and right of the plots. Schematic representations of the logic states of the constructs are shown in this and subsequent figures. (B) Selective activation of ribozyme self-cleavage by an effector DNA complementary to the oligonucleotide binding site (OBS). Radiolabeled ribozymes (5` 32P, Pre) undergo self-cleavage only with the perfectly matched DNA effector (0 mismatches) and the resulting radiolabeled cleavage fragment (Clv) is separated from the precursor by denaturing 10% PAGE.*

In the presence of the effector, the apparent rate constant observed for ribozyme activity (apparent kobs) is ~1.1 x 10-1 min-1, whereas the apparent kobs for the ribozyme in the absence of effector DNA is ~1.6 x 10-5 min-1. These results indicate that the allosteric dynamic range is nearly 7,000 fold, and the maximum rate constant is within 10-fold of the typical maximum activity for the unmodified hammerhead ribozyme core (~1 min-1) measured under similar conditions. Furthermore, the stability of the OFF-state structure is not so extreme or so rapidly adopted that activation by the effector DNA is precluded when a reaction buffer (**Figure 1B**) and DNA are introduced simultaneously to the YES-1 ribozyme.

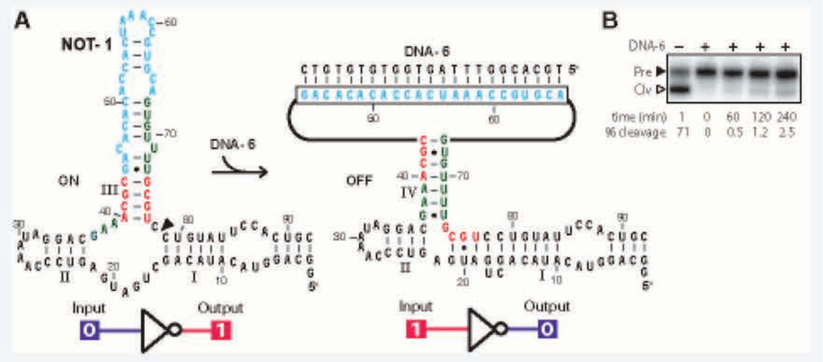

Figure 2. Design and characterization of NOT-1 based on an extended hammerhead ribozyme

An extended natural hammerhead ribozyme from *Schistosoma mansoni*, was used as the parent construct for the design of a ribozyme that is deactivated by allosteric interactions with oligonucleotides. This ribozyme exhibits faster RNA cleavage kinetics and requires lower concentrations of Mg2+ to trigger activity. Allosteric constructs derived from parental ribozymes with these properties are more likely to function in vivo where divalent ion concentrations are low and where fast ribozymes might be needed. Extended hammerhead ribozymes exhibit improved function because they form a tertiary structure between the loop sequences of stem II and a bulge within stem I. Therefore, the OBS was relocated to stem III for the design of a construct that functions as a NOT gate so this critical ribozyme tertiary-structure contacts would not be disrupted. The resulting design, termed NOT-1 (**Figure 2A**) is predicted to form a single major ON state structure (Ep = -30.55 kcal mol-1) in the absence of effector DNA-6 (23 nt), and is predicted to form a single major structure in its effector-bound OFF state that has an extended stem I and a disrupted stem III (Ep = -25.46 kcal mol-1). If the NOT-1 construct functions as predicted and self-cleaves in the absence of effector DNA, then the preparation of the RNA is expected to pose a problem because the ribozyme could undergo self-cleavage during transcription in vitro. To avoid this outcome, DNA templates corresponding to the NOT-1 RNA were transcribed in the presence of 10 uM DNA-6 and 10 uM of the antisense oligonucleotide CTCATCAGC. The latter DNA is complementary to nucleotides 15 through 23 of the NOT-1 hammerhead core. Although the NOT-1 RNA exhibited only ~25% self-cleavage when produced by transcription under these conditions (data not shown), the RNA exhibits robust self-cleavage activity (kobs >1 min-1) when incubated in the absence of DNA-6 (**Figure 2B**). Similarly, addition of excess effector oligonucleotide to a NOT-1 ribozyme assay causes strong inhibition.

CONCLUSION

The two programs will be formatted as a patent application. These developments could support the scientific competence and development of young scientists working on the project and expand their knowledge and skills in RNA biology, ribozyme design, anti-cancer therapy, and diagnostics. The results achieved within the framework of this project will be presented on the official website of Prof. Dr. Robert Penchovsky (https://penchovsky.atwebpages.com/). This will lead to increased awareness of the Competition for Funding Scientific Research with Potential for Innovation or Knowledge/Intellectual Property Transfer - SUMMIT, among established scientists and young colleagues in Bulgaria.

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Contract BG-RRP-2.004-0008 for the financing of project "Sofia University - Marking Momentum for Innovation and Technological Transfer" under pillar 2 "Establishing a network of research higher education institutions in Bulgaria", component "Innovative Bulgaria" from National Recovery and Resilience Plan part of the program to accelerate economic recovery and transformation through science and innovation