Computational Selection and Experimental Validation of Allosteric Ribozymes That Sense a Specific Sequence of Human Telomerase Reverse Transcriptase mRNAs as Universal Anticancer Therapy Agents

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High expression levels of telomerase reverse transcriptase messenger RNAs in differentiated cells can be used as a common marker for cancer development. In this paper, we describe a novel computational method for selection of allosteric ribozymes that sense a specific sequence of human telomerase reverse transcriptase mRNAs. The *in silico* selection employed is based on computing secondary structures of RNA using the partition function in combination with a random search algorithm. We selected one of the ribozymes for experimental validation. The obtained results demonstrate that the tested ribozyme has a high-speed (~ 1.8 per minute) of self-cleavage and is very selective. It can distinguish well between perfectly matching effector and the closest expressed RNA sequence in the human cell with 10 mismatches, with a ~ 300 -fold difference under physiologically relevant conditions. The presented algorithm is universal since the allosteric ribozymes can be designed to sense any specific RNA or DNA sequence of interest. Such designer ribozymes may be used for monitoring the expression of mRNAs in the cell and for developing novel anticancer gene therapies.

Introduction

THERE IS A FUNDAMENTAL DIFFERENCE in the behavior between normal and tumor cells in a cell culture (Hanahan and Weinberg, 2000). Healthy cells divide for a limited number of times whereas cancer cells possess the ability to proliferate indefinitely under particular conditions and therefore, they can be regarded as immortal. To maintain their mortality, normal cells have a certain intrinsic molecular counting mechanism that has been lost in tumor development (Harley, 2008; Rankin et al., 2008; Shay and Keith, 2008). It has been evident for years that the progressive loss of the ends of chromosomes during genome replication can serve for cell division counting in the process of cellular aging (Aubert and Lansdorp, 2008).

The 3' ends of the human chromosomes, termed telomeres, contain many repeats of the TTAGGG sequence, which form a region between 3 and 20 kilobases in length (Poole et al., 2001). These repeats are shortened by 50–200 base pairs (bp) with each cell division until the telomeres become sufficiently short and reach their Hayflick limit (Hayflick and Moorhead, 1961). Normal human fetal cells in a culture usually divide between 40 and 60 times. After this, the cells enter growth arrest and die due to their senility. Usually cells become senescent and die before developing into cancerous ones.

Therefore, the cell division counting mechanism based upon the telomere length plays an important role in prevention of cancer development.

Human telomerase is a very important ribonucleoprotein complex that normally maintains the telomere length in the stem and reproductive cells by adding TTAGGG repeats onto the telomeres. It uses its own RNA as a template for reverse transcription (Smogorzewska and de Lange, 2004). Telomerase activity has been detected in about 90% of all human tumors (Ito et al., 1998); in contrast, the adjacent healthy cells do not express telomerase messenger (m) RNAs (Phatak and Burger, 2007). In the other 10% of human tumors, where telomerase is not expressed, the cells have found a special pathway to maintain normal telomere length termed alternative lengthening of telomeres (ALT) (Tinsley et al., 2007). The exact ALT mechanism is still unknown (Henson et al., 2002). It may include multiple recombinations between the telomeric regions (Wang et al., 2004). In most cases, the tumor cells maintain the length of their telomeres stable after many cell divisions by a steady expression of telomerase similar to that reported in stem and germ cells. This observation leads to the proposal for using the expression of telomerase in differentiated somatic cells as a universal marker for cancer development in at least 90% of the human tumors (Seki et al., 2001; Hindle et al., 2002; Waguri et al., 2003). An additional

role for telomerase includes upregulation of 70 genes known or suspected in cancer growth and spread through the body of malignant cells (Blackburn, 2005). The gene encoding the telomerase reverse transcriptase of the human telomerase complex is known as hTERT. The hTERT gene produces two mRNAs by alternative splicing. All these findings suggest that expression of hTERT mRNA can be used not only as a common marker for cancer, but also as a target for developing novel anticancer therapies.

In this study, we use methods of RNA synthetic biology to engineer allosteric ribozymes that can sense a common region for all hTERT mRNAs (Penchovsky and Breaker, 2005; Penchovsky, 2013a, 2013c). As a parental construct, we use the extended version of a ribozyme from the human parasite, *Schistosoma mansoni* (Osborne et al., 2005). The sequence of this particular hammerhead ribozyme possesses a high-speed of self-cleavage in the cell. Allosteric ribozymes are catalytic RNA molecules that can sense the presence of specific effectors that bind to their allosteric domain(s) and alter their function. The effectors can be small molecules, oligonucleotides, peptides, and others. Allosteric ribozymes can be obtained by various *in vitro* selection (Piganeau et al., 2001), rational design (Tang and Breaker, 1997), and computational methods (Penchovsky and Breaker, 2005). They have many applications of modern biotechnology, including molecular computing (Qian and Winfree, 2011; Penchovsky, 2012b), biosensing (Penchovsky and Breaker, 2005), as reporters in high-throughput screening (Okada et al., 2012) arrays for riboswitch-based antibacterial drug discovery (Blount et al., 2006; Penchovsky, 2013b, 2013d; Penchovsky and Stoilova, 2013), and in systems for exogenous control of gene expression (Penchovsky, 2012a).

To design hTERT mRNAs-sensing ribozymes, we have employed a highly accurate computational procedure for *in silico* selection of allosteric ribozymes based on modeling secondary structures with the partition function for RNA folding (Penchovsky, 2012b). The partition function does not compute just the minimal free energy structure but all possible secondary structures (McCaskill, 1990). This makes the approach very accurate and time-efficient. The presented procedure for computational selection is novel. It differs from the already published computational design of the allosteric ribozyme (Penchovsky and Breaker, 2005) in a very important point. The previously promulgated algorithms have produced ribozymes that sense randomly generated oligonucleotide sequences (Penchovsky and Breaker, 2005). Here, we describe a computational procedure that generates synthetic ribozymes with preselected specificity to a chosen oligonucleotide sequence. In this study, the obtained allosteric ribozymes are designed to sense the presence of hTERT mRNA under physiological relevant conditions. Therefore, they can be used as reporter systems for hTERT mRNA expression as well as novel and universal anticancer gene therapy tools (Penchovsky, 2012a).

Materials and Methods

Computational selection of oligonucleotide-sensing allosteric ribozymes with predefined effector

The ribozyme sequences presented throughout this paper were obtained by a computational algorithm for *in silico* selection of allosteric oligonucleotide-sensing ribozymes. The

algorithm predicts secondary structures based upon the partition function for RNA folding in the presence and absence of complementary oligonucleotide sequences. The partition function, in contrast to the minimal free energy function, computes all possible secondary structures as a function of temperature. For this goal, the Vienna RNA folding package (Gruber et al., 2008) and thermodynamic parameters for RNA (Mathews et al., 1999) were used.

The ribozymes were intended to have a NOT Boolean logic function. Therefore, they should be active only when an effector is not present. Two different states, either in the absence (ON state) or in the presence of effector (OFF state) were computed for each ribozyme. When the effector is present, the complementary oligonucleotides of the ribozymes are excluded from the ribozyme folding. The computational steps were as follows (Fig. 1d):

1. Substitute randomly one of the X nucleotides shown below over the alphabet of A, U, C, G by using a random search algorithm.
5'ggcagguacauacagcugaugagucccaauaggacgaacgX_nga
aaauguggguucuccaaXX_nuuuXXcguccugauuuccacugc,
where $n=1$ or 2 .
2. Fold the sequence obtained and calculate the free energy at 37°C using the partition function.
3. Determine whether nucleotides 40, 41, 42, 43, and 44 are base-paired with the 77, 76, 75, 74, and 73, respectively, with at least 85% probability. If not, reject the sequence and go to step 1.
4. Replace the oligonucleotide binding site (Kong et al., 2007) from the structure with the same number of artificial nucleotides (n) that are defined to have no binding properties.
5. Fold the sequence and compute the free energies based on the partition function. If a dominant OFF state with respectively with at least 85% probability stem IV is not formed, go to 1.
6. Compute the free energy of the dominant OFF state secondary structure based on the partition function and determine the gap between the OFF and ON state of free energies. If the energy gap is less than 6.0 kcal/mol or more than 8.5 kcal/mol, reject the sequence and go to 1.

Preparations of nucleic acids

All DNA oligomers used for this paper, were purchased from Integrated DNA Technology. Oligomers up to 30 nt in length were purified by the manufacturer with high-performance liquid chromatography. Synthetic DNAs longer than 30nt were purified by denaturing (8 M urea) polyacrylamide gel electrophoresis (PAGE). In this case, DNA oligomers were visualized in the gel by UV shadowing. They were extracted from the gel by a crash and soak procedure and were recovered by ethanol precipitation.

To produce double-stranded (Yen et al., 2004) DNA templates for *in vitro* transcription, we used two overlapping single-stranded (ss) DNA strands at their 3'-ends, termed template and non-template (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/nat). The overlapping oligonucleotides were made double-stranded (ds) DNA by extension with SuperScriptII reverse transcriptase (Invitrogen) according to the manufacturer instructions. The reverse transcriptase was used instead Tag

FIG. 1. Computational selection of high-speed allosteric ribozymes that sense a specific sequence of the human telomerase reverse transcriptase (hTERT) gene. **(a)** A secondary structure of the extended version of the hammerhead ribozyme from the human parasite *S. mansoni*. The ribozyme cleaves itself at the position indicated by the arrowhead. The ribozyme has three stems (from I to III). There are tertiary interactions between the loop of stem II and the bulge loop of the stem I that make this RNA a high-speed ribozyme. All canonical (solid lines) and non-canonical (dashed lines) interactions are depicted. **(b)** The chosen complementary sequence to the hTERT mRNAs is depicted. **(c)** Secondary structure templates of a high-speed allosteric ribozyme. An oligonucleotide binding site (OBS) is embedded into stem III of the ribozyme and is designed to be complementary to a specific sequence of hTERT gene. This designing approach preserves all tertiary interactions between stems I and II. The X nucleotides are variable in different ribozymes. In the absence of specific effector, the ribozymes are supposed to fold into an active oligonucleotide (ON) state in which all three stems from I to III are properly formed. In the presence of a specific oligonucleotide sequence, the ribozymes are designed to fold into an inactive OFF state, in which stem IV is formed instead of stem III. **(d)** A schematic presentation of the computational selection procedure used for designing the allosteric ribozyme. All computational steps are described in details in the methods section. Note that the gray arrows indicate an unconditional move to the next computational steps while the dark ones show a conditional passage.

polymerase because it does not attach template independently adenine at the 3' ends. The non-template strand carried a T7 RNA polymerase promoter (5' TAATACGACTCAC TATA).

The synthetic dsDNAs were used as templates for *in vitro* transcription with a RiboMax kit (Promega) in the presence of antisense to the ribozyme core DNA oligomer (Supplementary Fig. S1), according to the manufacturer's instructions.

Instead of GTP, we used TAMRA (carboxyethylmethylrhodamine)-ApG, purchased from IBA. Note that the T7 promoter requires two or three guanine nucleotides at the start of transcription. As a result, all RNA molecules were labeled at their 5' terminus with TAMRA during the transcription (Supplementary Fig. S1). The transcribed RNAs were purified by 6% denaturing PAGE and recovered from the gel as described above for the DNA oligomers with only

TABLE 1. RESULTS OF A BLASTN SEARCH IN REFERENCE SEQUENCE RNA HUMAN DATABASE OF NATIONAL CENTER FOR BIOTECHNOLOGY INFORMATION

Genes from Homo sapiens RefSeq RNA database	BLASTN hit sequences:	Mismatches (number)	Thermodynamic stability (kcal/mol)
1. Homo sapiens telomerase reverse transcriptase (TERT), transcript variant 2, mRNA Sequence ID: ref NM_001193376.1	TTGGAAGAACCCACATTTTTC 22 TTGGAAGAACCCACATTTTTC 2965	0	35.09
2. Homo sapiens telomerase reverse transcriptase (TERT), transcript variant 1, mRNA Sequence ID: ref NM_198253.2	TTGGAAGAACCCACATTTTTC 22 TTGGAAGAACCCACATTTTTC 3154	0	35.09
3. Homo sapiens myosin, light chain 9, regulatory (MYL9), transcript variant 1, mRNA Sequence ID: ref NM_006097.4	3 GGAAGAACCCACA 16 289 GGAAGAACCCACA 302	8	25.00
4. Homo sapiens bridging integrator 1 (BIN1), transcript variant 10, mRNA Sequence ID: ref NM_139351.2	5. AAGAACCCACATTTTTC 22 1679 AAGAACCACACATTTTTC 1662	5	19.23

one difference. Instead of UV shadowing for RNA visualization, we used a blue-light transilluminator, DarkReader-195M purchased from Clare Chemical Research, Inc., because all transcribed RNAs were 5'-TAMRA labeled.

Allosteric ribozyme assays

For various kinetic assays, allosteric ribozymes (250 nM) were incubated in a master mix solution containing 50 mM Tris-HCl with pH 8.5 at 37°C, 50 mM KCl, and 10 mM EDTA. After incubation for 5 minutes for ribozyme folding, an effector molecule is added if needed. Ribozyme reactions were initiated by adding MgCl₂ to a final concentration of 12 mM at 37°C. As a result, the free Mg²⁺ was 2 mM. During a 1-minute incubation, 5-μL aliquots from the master mix were withdrawn every 10 seconds and added to an equal volume of 2×loading buffer with 20 mM EDTA, pH 8.5, to stop the reaction. The obtained aliquots were run on 6% denaturing PAGE. The 5'-TAMRA-labeled RNAs were detected on the gel using the blue-light transilluminator, DarkReader-195M (excitation light length from 400 to 500 nm) and Nikon D5100 digital camera (Nikon Imaging Japan Inc.) equipped with a 520-nm optical filter. The images were analyzed with the ImageJ software (U.S. National Institutes of Health). We have estimated the initial kinetics of ribozyme self-cleavage with different effectors that is essential for the ribozyme function as an instant switch *in vitro* as well as *in vivo*. Longer kinetic studies of high-speed oligonucleotide-sensing ribozymes with NOT function are published elsewhere (Penchovsky and Breaker, 2005).

Results

Bioinformatics analysis of hTERT mRNAs

To identify a suitable effector domain of hTERT-mRNAs, which can be used to construct hTERT mRNA-sensing allosteric ribozymes, we compare the sequences of the two spliced variants of hTERT mRNA from the NCBI database. We have folded the two variants of hTERT mRNA applying the partition function for RNA folding (Supplementary Fig. S2). The effector domain should have the following characteristics:

1. The effector domain must be present in both variants of hTERT mRNAs.

2. It should be long minimum 20 and maximum 24 nucleotides to ensure its unique sequence.
3. It should be at least five mismatches between the perfectly matching effector and the closest expressed RNA sequences (Table 1). Most importantly, the thermodynamic stability between the perfectly matching dsRNA domain and the closest mismatching dsRNA should be at least 9 kcal/mol. This is more than the energy gap between the OFF and ON state of the ribozymes (at a maximum of 8.5 kcal/mol) and must guarantee the specific function of the ribozymes.
4. The whole region of hTERT mRNA selected to serve as an effector domain should not have more than 12 base-paired nucleotides in its secondary structure. Otherwise, the oligonucleotide binding site (OBS) of the ribozyme will not be able to bind effectively to the effector region.

Taking into account the selection criteria described above, we have chosen an effector domain that is 22 nt long (Fig. 1b). It is present in both hTERT mRNAs and satisfies all selection criteria. The sequence, which has the closest similarity to the selected effector belongs to myosin (Table 1). There are 8 mismatches between the two sequences. These mismatches are enough to guarantee significant deactivation of our ribozymes only in the presence of perfectly matching effector. The sequences are same from the 3rd to the 16th nucleotides. Most importantly, the thermodynamic stability of the 13-bp dsRNA is −25 kcal/mol, while the perfectly matching hybrid has −35 kcal/mol (Table 1). This makes a gap of 10 kcal/mol in thermodynamic stability between the hTERT RNA effector and the second closest expressed RNA. In fact, we have found a RNA sequence with bigger similarity (5 mismatches), which, however, forms a less stable hybrid (−19 kcal/mol) as shown in Table 1.

To be able to bind to the OBS of the ribozyme, the hTERT RNA effector domain must have less than half of its bases single-stranded. We have folded both spliced forms of hTERT RNA. We have found that in the first spliced forms, all 22 effector (Fig. S2a) nucleotides are not base-paired (Fig. S2b), while in the second spliced RNA 10 nucleotides are not base-paired (Fig. S2c). All these selection criteria are deliberately

chosen to ensure that the selected domain of the effector RNA has a unique sequence and is available for hybridization with the OBS of the designer ribozymes.

Computational selection of high-speed hTERT mRNA-sensing ribozymes. Having selected an effector domain that satisfies all criteria to serve as a ribozyme effector, we have calculated its reverse complementary sequence (Fig. 1b). We have inserted the reverse complement sequence into stem III (Fig. 1c) of the extended version of the hammerhead ribozyme from the human parasite *S. mansoni* (Fig. 1a). This variant of the hammerhead ribozyme possesses a high-speed of self-cleavage ($k_{\text{obs}} \sim 8$ per minute) under a physiological relevant concentration (1 mM) of Mg^{2+} . This is due to the canonical and non-canonical interactions between the nucleotides in the loop of stem II and those in the bulge loop in stem I. As a result of these interactions, stems I and II come close together, which

facilitates the ribozyme self-cleavage. We have preserved all these important connections by inserting the OBS in stem III and keeping intact stems I and II (Fig. 1c).

We have employed a novel approach for computational selection of allosteric ribozymes with NOT Boolean logic function (Fig. 1d) as described in the materials and methods (M&M) section. The computational procedure searches RNA sequences that fold into two different secondary structures in the presence or absence of a specific oligonucleotide effector that is complementary to the OBS based on the partition function for RNA folding. This algorithm computes all possible secondary structures. In the absence of effector, the allosteric ribozymes with NOT logic function are designed to fold into a dominant active state. In this conformation, all three stems (from I to III) are properly formed, and the ribozymes should undergo a self-cleavage. Otherwise, in the

FIG. 2. Computational design of the NOT-1 logic gate. **(a)** A secondary structure of a high-speed allosteric ribozyme with NOT Boolean logic function that senses a specific sequence of hTERT gene is depicted. An OBS (in gray) is embedded into stem III of the hammerhead ribozyme from the human parasite *S. mansoni*. In the absence of effector, the ribozyme is designed to fold into an active conformation in which all three stems from I to III are formed. Therefore, the ribozyme should cleave itself in the position indicated by the arrowhead. **(b)** A specific oligonucleotide effector is designed to hybridize with the OBS. As a result, stem IV is formed instead of stem III and the ribozyme's self-cleavage should be inhibited. **(c)** Shown is a dot matrix for RNA folding of the ribozyme sequence in the absence of effector. The upper right-hand triangle presents the base pairing probability based on the partition function while the down left-hand triangle depicts the secondary structure with the minimal free energy. If there is a base pairing between the corresponding bases, a black square is shown. The square's surface is as big as the base-pairing probability. Both secondary structures are almost identical, which indicates that there is one dominant secondary structure with all stems from I to III formed. **(d)** Dot matrix for RNA folding of the ribozyme sequence in the presence of effector where the stem IV is formed instead of stem III. In this state, the ribozyme is supposed to be inactive.

presence of a specific effector, the ribozymes are designed to fold into a dominant secondary structure where the stem IV is formed instead of stem III. As a result, the catalytic core of the ribozyme is not going to be formed, and the ribozyme will be inactive (Fig. 1c). To test different RNA sequences, we have applied a random search algorithm, which randomizes several nucleotides that connect stem III with the OBS of the ribozyme (Fig. 1c). After 30 minutes of computational time on a modern laptop computer, we have obtained three different sequences of allosteric ribozymes with NOT logic function (Figs. 2, 3, 4). The OBS for all ribozymes is the same. The ribozymes differ in several nucleotides that connect stem 3 with the OBS. This results in slightly different thermodynamic characteristics of the allosteric ribozymes. These parameters are the free energy of the active form of the ribozyme, the free energy of the inactive form, the difference between the free energies of the two states, and the thermodynamic stability of the nucleotides in the OBS which do not participate in base pairs when there is no bound effector to the ribozyme. The values of these parameters are given for each ribozyme sequence in Supplementary Table S1.

All ribozymes are designed to fold into dominant secondary structures in the presence or in the absence of specific effector. That is indicated by the dot matrices for RNA folding based on the partition function (Fig. 2, 3, 4). The first ribozyme, termed NOT-1 (Fig. 2), has the smallest thermodynamic gap (6.42 kcal/mol) between the ON (−29.49 kcal/mol) and the OFF (−23.07 kcal/mol) states. In the absence of

effector, the NOT-1 sequence is predicted to fold into a dominant active state where all three stems of the hammerhead ribozyme are properly formed (Fig. 2a). In this conformation, the ribozyme is supposed to cleave itself at the position indicated by the arrowhead (Fig. 1a). This is indicated by the dot matrix for RNA folding (Fig. 2c). Note that the upper-right triangle of the dot matrix depicts the RNA folding according to the partition function that computes all possible secondary structures. The lower left triangle shows the minimal free energy structure. When both triangles are mirror images to each other like in Fig. 2c, there is one dominant secondary structure of the NOT-1 ribozyme. In the presence of specific effector complementary to the OBS, the stem IV of the NOT-1 ribozyme (Fig. 2b) is going to be formed instead of stem III. In this case, we again have one dominant secondary structure, as depicted in the dot matrix in Fig. 2d, which is supposed to be inactive.

The NOT-2 ribozyme (Fig. 3) has a bigger dynamic range between the ON and OFF states (7.99 kcal/mol) than that of NOT-1. This is due to a bigger thermodynamic stability (−31.04 kcal/mol) of the NOT-2 ribozyme in ON state (Fig. 3a). The NOT-2 is designed to have dominant conformations in ON (Fig. 3a, c) as well as in OFF (Fig. 3b, d) states. The NOT-3 ribozyme (Fig. 4) has the biggest free energy gap of 8.14 kcal/mol between active (Fig. 4a) and inactive states (Fig. 4b). Again, this difference comes from the thermodynamic stability of the active states (−31.18 kcal/mol), because the stability of the inactive state in all ribozymes is very similar

FIG. 3. Computational design of the NOT-2 logic gate. (a) A secondary structure of a high-speed allosteric ribozyme that senses a specific sequence of hTERT gene is depicted. An OBS is depicted in gray. The ribozyme should cleave itself at the position indicated by the arrowhead. (b) A specific oligonucleotide effector is designed to hybridize with the OBS. As a result, stem IV is formed instead of stem III, and the ribozyme's self-cleavage should be inhibited. (c) A dot matrix for RNA folding of the ribozyme sequence in the absence of effector. (d) A dot matrix is shown for the folded ribozyme sequence in the presence of effector where the stem IV is formed instead of stem III. In this state, the ribozyme is supposed to be inactive.

FIG. 4. Computational design of the NOT-3 logic gate. **(a)** A secondary structure of an allosteric ribozyme that senses a specific sequence of hTERT gene is depicted. An OBS is depicted in gray. The ribozyme cleavage is indicated by the arrowhead. **(b)** A specific oligonucleotide effector is designed to hybridize with the OBS. As a result, stem IV is formed instead of stem III and the ribozyme's self-cleavage should be inhibited. **(c)** A dot matrix is shown for RNA folding of the ribozyme sequence in the absence of effector. **(d)** A dot matrix for RNA is shown for the ribozyme sequence in the presence of effector. In this case, the ribozyme is supposed to be inactivated.

FIG. 5. Kinetics of self-cleavage of a NOT-3 ribozyme. **(a)** Specific (DNA-1) and non-specific (DNA-2) effector sequences used in the kinetic experiments are presented. The gray nucleotides in DNA-1 are missing in DNA-2. **(b)** Gel images of self-cleavage of the NOT-3 in the presence of DNA-1 and DNA-2 oligomers and in the absence of any effector. **(c)** The results of the cleavage kinetics are depicted as a natural algorithm of the fraction remaining versus time.

(between -23.04 and -23.07 kcal/mol). The dot matrices of the NOT-3 ribozyme indicate dominant ON (Fig. 4b) and OFF (Fig. 4d) states.

Experimental validation of the NOT-3 ribozyme and its proposed application as a gene control element in novel anticancer therapies

We have used the NOT-3 ribozyme for biochemical experiments, which have to validate its kinetics of cleavage and specificity of deactivation under physiologically relevant conditions. We decided to test experimentally the NOT-3 because this ribozyme possesses the biggest free energy gap between ON and OFF states. This thermodynamic gap (8.14 kcal/mol) should ensure a big dynamic range in the apparent contacts of cleavage in the presence and absence of effector. At the same time, this thermodynamic gap is smaller than the difference (10 kcal/mol) in the free energies between the perfectly matching effector and a mismatching effector with the highest stability expressed in the human cell (Table, 1 line 3). This should ensure high specificity of ribozyme deactivation.

We have synthesized and purified NOT-3 ribozyme as described in M&M and depicted in Supplementary Fig. S1. Two overlapping DNA oligomers were made double stranded (Yen et al., 2004) by reverse transcriptase (Supplementary Fig. S1a). The obtained dsDNA (Supplementary Fig. S1b) was used as a template for *in vitro* transcription of RNA in the presence of 5'-TAMRA-ApG and antisense to the ribozyme's core DNA oligomer (Supplementary Fig. S1c). As a result, the all ribozymes were 5'-TAMRA-labeled (Supplementary Fig. S1d). Despite the presence of antisense DNA oligomer, 20% of the transcribed ribozyme was self-cleaved (Supplementary Fig. S1e). Note that the antisense oligonucleotide dissociated easily from the ribozyme sequence due to its relatively short length (17 nt) compared with the oligonucleotide effector (22 nt). It was inappropriate to use a longer antisense oligonucleotide because it will diminish the yield of the *in vitro* transcription reaction. The non-cleaved ribozyme was extracted from the gel as described in M&M. The ribozyme was used for various biochemical arrays (Fig. 5). As a non-specific effector, we used a sequence of the myosin gene, which is the closest expressed mRNA sequence to our specific hTERT effector (Fig. 5a). The kinetic experiments were performed under physiologically relevant conditions as detailed in M&M. We conducted separate experiments to demonstrate the cleavage kinetics of the ribozyme without effector, with a specific effector, and with the non-specific effector as shown in the fluorescence images (Fig. 5b). Each kinetic experiment was performed three times, and the average results of the cleavage kinetics are depicted as a natural algorithm of the fraction remaining versus time in Fig. 5c. The standard deviation of these experiments was within $\pm 5\%$.

Based on the slope of the curves, we have calculated the apparent rate constant (k_{obs}) for each experiment. In the absence of effector or in the presence of the non-specific effector, the NOT-3 ribozyme cleaves itself at $k_{\text{obs}} = 1.8$ per minute. The non-specific effector does not deactivate the ribozyme self-cleavage at all. We have demonstrated that the presence of a non-specific effector does not deactivate the ribozyme, which undergoes self-cleavage at the same rate as in the test without effector. In contrast, the ribozyme function is significantly deactivated (300 fold) in the presence of the specific hTERT

effector ($k_{\text{obs}} = 0.006$ per minute). The difference of 10 kcal/mol between the specific and the non-specific effector reveals < what > to be sufficient for the specific function of the effector.

We removed several terminal bases from the effector sequences because we wanted to prove the specificity of ribozyme inactivation between that ideally matching effector of hTERT mRNA the closest RNA expressed in the human cells. Otherwise, we have already proven that oligonucleotide-sensing ribozymes can distinguish very well between a perfectly matching effector and similar oligonucleotide over a length of 22 nt (Penchovsky and Breaker, 2005). The established high speed of self-cleavage and specificity of deactivation of the NOT-3 ribozyme under physiologically relevant conditions make it suitable for applications as a gene control element in the cell. One of the most promising applications for developing novel anticancer therapies is depicted in Supplementary Figs. S3, S4. The NOT-3 ribozyme can be embedded into the 5' and 3' UTR region of exogenous mRNA that encodes p53. The exogenous mRNA can be expressed in the cell by a viral vector. If the differentiated cell is normal, hTERT mRNA will not be expressed. As a result, the NOT-3 will cleave itself, the exogenous mRNA will be rapidly decayed, and the p53 will not be expressed (Supplementary Fig. S3). In contrast, if the cell is cancerous, hTERT mRNA will be present. It will deactivate the NOT3 ribozyme. The exogenous mRNA will be stabilized, and the p53 will be overexpressed (Supplementary Fig. S4). As a result, the cancer cell will undergo apoptosis and will die. This approach may be used as a universal anticancer therapy, since hTERT mRNA is expressed in most cancer types.

Discussion

The presented computational method builds upon the already published computational design of allosteric ribozymes by applying the partition function for RNA folding in conjunction with a random search algorithm. However, these two algorithms are quite distinct despite using common selection criteria based on the partition function for RNA folding that are experimentally proven to be very accurate. They have different dataflow and objectives. While the computational design method generates ribozymes with random OBSs, the presented approach for computer-based selection produces RNA switches with predefined OBSs. In fact, the most useful approach is to design ribozymes, which sense a specific predefined oligonucleotide sequence.

The tested NOT-3 ribozyme possesses a high-speed of cleavage ($k_{\text{obs}} = 1.8$ per minute) and large dynamic range (300-fold)—the difference in the cleavage kinetics between ON and OFF states. This means that within 35 seconds the ribozyme cleaves itself. This high speed of cleavage is very important, since in the cell all molecules are constantly made and degraded. Moreover, the NOT-3 ribozyme is a rapid switch, which means that it changes its function immediately after addition of the effector molecule. In addition, the ribozyme is deactivated only in the presence of a specific oligonucleotide sequence and is active in the presence of the closest RNA sequence expressed in human cells. Since telomerase expression is highly prevalent in human cancers (Okada et al., 2012), it would be easy to inhibit the ribozyme *in vivo*. The RNA transcription in the eukaryotic cell is taking place in the nucleus. Our ribozymes are designed to work immediately after

their synthesis in the nucleus and after their transport in the cytosol. Note that the effector hTERT mRNA sequence is present in both the nucleus and in cytosol since this sequence is chosen to be single-stranded in pre mRNAs and in the spliced mRNA variants.

The extended version of the hammerhead ribozyme from *S. mansoni* has already been proven to work in cell culture *in vitro*, as well as in mouse (Yen et al., 2004). All these findings made the presented designer ribozyme a promising tool for metabolic engineering of exogenous control of gene expression. In addition, RNA-sensing ribozymes based on hammerhead ribozyme from *S. mansoni* have been shown to work *in vivo* (Klauser and Hartig, 2013). Such approach may be used for reprogramming the cell fate by exogenous control of p53 expression and its potential applications in novel anticancer gene therapies.

Conclusions

The presented algorithm for computational selection of mRNA-sensing ribozymes is proven to be very accurate, time and labor efficient. If we compare the time and the labor needed for *in vitro* selection of oligonucleotide-sensing allosteric ribozymes with this and other computer-based methods, the advantages are clear on the side of the computational approaches. The computational selection, presented within this paper, produces the ribozyme sequences within several min on an average personal computer whereas at least several weeks are needed to obtain allosteric ribozyme sequences through *in vitro* selection. In fact, all *in vitro* selection methods are based on repeating cycles of many biochemical steps that make the process very time and labor consuming. The presented method is universal, since we can alter the ribozyme specificity by replacing the OBS, and therefore, it may be applied to any specific RNA sequence expressed significantly in the cell. We have already published a computational design and experimental validation of a high-speed NOT-ribozyme that senses a randomly generated oligonucleotide effector with similar biochemical properties (Penchovsky and Breaker, 2005). The presented strategy for a novel anticancer approach based on RNA-sensing ribozymes is advantageous because it combines two main functions. On one hand, it senses the presence of cancer indicative RNAs in real time in the cell, and on the other hand, it acts immediately to kill the cell if proven malignant. The advance of RNA nanotechnology and its application to future therapeutics is an important avenue for novel research (Guo et al., 2012). This approach will give us new possibilities for metabolic engineering of complex circuits of exogenous control of gene expression and reprogramming the cell fate.

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