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***In Vitro* Screening and *in Silico* Modeling of RNA-Based Gene Expression Control**

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Abstract

Molecular tools for controlling gene expression are essential for manipulating biological systems. One class of tools includes RNA switches that incorporate RNA-based sensors, known as aptamers. However, most switches reported to-date are responsive to toxic molecules or to endogenous metabolites. For effective conditional control, switches must incorporate RNA aptamers that exhibit selectivity against such endogenous metabolites. We report a systematic approach which combines a rapid *in vitro* assay and an *in silico* model to support an efficient, streamlined application of aptamers into RNA switches. Model predictions were validated *in vivo* and demonstrate that the RNA switches enable selective and programmable gene regulation. We demonstrate the method using aptamers that bind the FDA-approved small molecule (6*R*)-folinic acid, providing access to new molecular targets for gene expression control and much-needed clinically-relevant tools for advancing RNA-based therapeutics.

The ability to control gene expression using molecular targets is the cornerstone of synthetic biology allowing researchers to study and construct biological systems. Molecular tools that control gene expression may be composed of protein or RNA and typically require a sensor component that allows external or endogenous molecular targets to dynamically regulate gene expression.^{1, 2} Dynamic gene regulation has been applied in various biological and biochemical systems, including feedback control to optimize metabolic flux in a biochemical pathway,³ drug-responsive control over cell proliferation to increase safety of cell therapies,⁴ and agrochemical control over water use to improve drought tolerance in crops.⁵ The successful application of biological tools in these complex *in vivo* systems depends on their molecular *orthogonality*, defined as the ability of the sensor to selectively discriminate between the desired molecular target and similar intracellular molecules.

RNA switches exhibit a number of desirable characteristics as a tool for controlling gene expression, including design modularity,⁶ cross-species portability,⁷ and programmable activities for application in diverse biological systems.⁸ The sensor component, in the form

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Supporting Information Materials and Methods, seven additional figures (Figures S1–S7), six additional tables (Tables S1–S6), and an excel sheet (simulation raw data). This material is available free of charge via the Internet at <http://pubs.acs.org/journal/acbcct>

The authors declare competing financial interests in the form of a pending patent application.

of an RNA aptamer, can be generated *de novo* to bind to a desired molecular target.⁸ Although most small molecule-binding RNA aptamers are responsive to toxic targets (e.g., antibiotics, organic dyes) or biochemically essential targets that exist in all organisms (e.g., sugars, amino acids),^{9, 10} some aptamers have been generated to recognize non-toxic targets that are absent from commonly engineered organisms (e.g., plant-specific secondary metabolites, or new derivatives).^{11, 12} While these molecules may not be present in the intracellular environment, they often share structural features with naturally occurring metabolites.¹³ The selectivity of a RNA switch is compromised if its composite aptamer exhibits affinity to these naturally occurring metabolites. Thus, our capability to implement RNA switches for programmable gene control relies on the binding activity and selectivity of candidate aptamers to their target and to structurally similar molecules present in the cellular environment.

We report a systematic approach to probe and predict the responsiveness of selected RNA aptamers and the corresponding RNA switches to their target and structural analogs, collectively referred to as the molecules of interest (MOIs). The strategy uses a rapid *in vitro* surface plasmon resonance (SPR)-based assay to quantify the affinity and selectivity of the aptamers to a large panel of different MOIs.^{14, 15} A Monte Carlo simulation of a kinetic model using these *in vitro* parameters is applied to predict the *in vivo* activities of RNA switches harboring the characterized aptamers.¹⁶ We then characterized the corresponding RNA switches in yeast to verify the *in vivo* activities and cross-reactivities predicted from the model (Figure 1).

We demonstrate the utility of this method using a set of aptamers and corresponding RNA switches that bind an FDA-approved small molecule, (6*R*)-folinic acid ((6*R*)-FA). Folinic acid is a 5-formyl derivative of the naturally occurring tetrahydrofolic acid (THF), a key metabolite in folate metabolism (the folate-mediated one-carbon cycle). Thus, all the metabolites in this cycle may interact with the aptamers, causing cross-reactivity and reducing the ability to control corresponding RNA switches. To evaluate the potential interference of these naturally occurring metabolites, the MOIs in this study include the target (6*R*)-FA, the biologically-active diastereomer of the target (6*S*)-FA, all the precursors and intermediates in the one-carbon cycle (Figure S1), and other folate derivatives commonly used as antifolates in the treatment of cancer.

First, an SPR-based assay^{14, 15} was used to rapidly and thoroughly characterize two high-affinity aptamers (t8-4, t8-11) that were previously selected to bind (6*R*)-FA.¹⁷ The SPR-based assay supports the characterization of the kinetic and equilibrium binding properties of these aptamers under various magnesium (Mg^{2+}) concentrations. As the selection for these aptamers was performed at 5 mM Mg^{2+} , previous analysis performed at this same Mg^{2+} concentration indicated that the t8-4 aptamer ($K_D = 19.2$ nM) exhibited a higher affinity than the t8-11 aptamer ($K_D = 40.5$ nM).¹⁷ However, our characterization of the binding properties of these aptamers at different Mg^{2+} concentrations indicated that the t8-4 aptamer is highly dependent on Mg^{2+} concentrations and exhibits a substantially reduced affinity at physiologically relevant (0.5 mM) Mg^{2+} concentrations ($K_{D,\text{kinetic}} = 185$ nM; Table S1; $K_{D,\text{kinetic}}$ values calculated as the ratio of the binding rates $K_{D,\text{kinetic}} = k_{\text{off}}/k_{\text{on}}$).^{14, 18, 19} In contrast, the t8-11 aptamer was found to be less dependent on Mg^{2+}

concentration, resulting in no statistically significant change in the measured K_D at 0.5 mM Mg^{2+} from 5 mM Mg^{2+} (Table S1). Our results, as well as previous selection analyses,²⁰ suggest that aptamer selections performed at low Mg^{2+} concentrations (e.g., 0.5–1 mM) may be beneficial for downstream aptamer applications intended for use at physiological conditions.

These (6*R*)-FA-specific aptamers are potential candidates for sensor components of selective RNA switches, which can be activated by the exogenous addition of non-naturally occurring (6*R*)-FA to cells as a molecular target. However, to act as an effective gene expression control tool, these aptamers must not exhibit cross-reactivity to the biologically-active diastereomer of the target, (6*S*)-FA and structurally similar folate derivatives that naturally exist in cells.^{21, 22} These metabolites may be present up to mM concentrations in some cell types (Table S2) and the selection of these aptamers did not incorporate any counterselections to folate derivatives.¹⁷ Thus, we further applied the SPR-based assay to rapidly examine the structure-activity relationship of the aptamer-target binding: specifically to quantify the selectivity of the t8-4 and t8-11 aptamers against the isomer (6*S*)-FA and other MOIs (Figure S2) at physiological Mg^{2+} concentrations. Both aptamers recognize the (6*S*)-diastereomer with an approximate 3-orders of magnitude reduction in binding. Metabolites in the one-carbon cycle typically have 6*S* stereochemistry,²³ thus explaining why the aptamers also display at least 3-orders of magnitude selectivity against most of the MOIs studied (pink group, Table 1). A notable exception is dihydrofolinic acid (DHF) which is achiral. The target, (6*R*)-FA, is composed of a pterin ring, *p*-benzoate (PABA), glutamate, and formyl moieties; all except the glutamate have been identified as important for aptamer recognition.¹⁷ Thus, the additional reduction in aptamer binding (greater than 3-orders of magnitude reduction of affinity) observed for some analogs results from the absence or modification of the formyl group (e.g., (6*R*)-THF; (6*R*)-5-methyltetrahydrofolate ((6*R*)-5mTHF); (6*R*)-methylenetetra-hydrofolate ((6*R*)-CH₂THF); methotrexate (MTX)) or absence of the PABA moiety (e.g., (6*R*)-tetrahydrobiopterin ((6*R*)-THB)). Finally, the precursors, PABA and 7,8-dihydroneopterin (DHN), which are composed of only one of the required moieties for aptamer recognition, display little-to-no binding (blue group, Table 1). These data indicate that both aptamers bind to (6*R*)-FA with high affinity and strong selectivity against structurally similar MOIs, and are thus good sensor components for RNA switches to act as selective gene expression control tools. Furthermore, the remarkable selectivity of these aptamers that were selected without deliberate counterselections highlights the powerful capabilities of *in vitro* selection to generate aptamers with selectivity to its target.

The binding properties of the *in vitro* selected (6*R*)-FA-specific aptamers (t8-4, t8-11) are more suitable for programmable gene control compared to the recently discovered natural THF riboswitch^{18, 24, 25} for several reasons. First, the THF riboswitch exhibits substantially lower affinity to (6*R,S*)-FA ($K_D = 22 \pm 2 \mu M$)¹⁸ compared to the synthetic aptamers described in our work. Furthermore, the THF riboswitch has been shown to bind to the majority of the metabolites in the one carbon cycle with similar affinities (i.e., in the μM range).²⁴ Finally, the THF riboswitch also binds purines, which are primary metabolites; e.g., experiments demonstrated binding to 7-deazaguanine with a K_D of 38 μM at 0.5 mM Mg^{2+} .¹⁸ In comparison, the t8-11 and t8-4 aptamers bind 7-deazaguanine with K_D s of 1.30

± 0.06 and 10 ± 5 mM, respectively. In summary, compared to the *in vitro* selected (6*R*)-FA aptamers, the natural THF riboswitch does not exhibit sufficient selectivity against cellular metabolites and is thus less useful for synthetic *in vivo* gene control.

The (6*R*)-FA-specific aptamers (t8-4, t8-11) were previously incorporated into RNA switches¹⁷ by coupling them to a hammerhead ribozyme switch based on a tertiary interaction mechanism.^{26, 27} In this mechanism, binding of the target to the aptamer disrupts tertiary loop interactions within the ribozyme, preventing ribozyme self-cleavage and thus allows gene expression. We implemented the switches for gene expression control by cloning them into the 3' untranslated region (3' UTR) of a gene of interest.⁶ Binding of the target to the aptamer inhibits cleavage of the RNA switch and thus stabilizes the transcript, resulting in higher levels of the protein of interest. In the absence of the target, cleavage of the RNA switch leads to rapid degradation of the transcript and reduced protein levels. The ratio of protein levels (in the presence and absence of target) is defined as the activation ratio (AR), which is used to quantify RNA switch activities *in vivo*.

Before testing the switches *in vivo*, we built an *in silico* model to predict the activities of RNA switches based on the *in vitro*-measured K_D values of composite aptamers to each MOI. The model captures the predicted expression level of the protein of interest (P) regulated by an RNA switch that can adopt two states – RNA aptamer without target binding (R) and target-bound RNA (RL) (Figure 2a). A Monte Carlo sampling technique (with $N = 1000$) was used to generate ARs by randomizing mRNA-associated kinetic parameters in the model while keeping other parameters constant (see Supporting Information). We performed this simulation for both RNA switches, targeting five MOIs spanning a wide range of K_D values (nM-level: (6*R*)-FA; μ M-level: (6*R*)-THF, (6*S*)-FA; and mM-level to no binding: folic acid, DHN). The simulation results indicate that for an aptamer binding to a target with a K_D in the nM-level, the predicted ARs of the corresponding switch range between 3.0 to 8.0; aptamers binding to targets in the μ M- or mM-level, the predicted ARs of the corresponding switch range between 1.1 to 1.3 or 1 to 1.1, respectively (Figure 2b).

To validate the predictions of the model, the *in vivo* activity and selectivity of each (6*R*)-FA-responsive RNA switch was characterized in *S. cerevisiae* via a flow cytometry assay, by inserting the switches into the 3' UTR of an EGFP reporter gene (Figure S3).⁶ We first verified that feeding each MOI to yeast has no significant toxicity effects on cell viability (Figure S4). The ARs were measured for the two RNA switches (FA_ON_tert11, tert32), two non-ligand responsive RNA ribozymes (as controls for RNA structural effects on gene expression), and an EGFP expression control against the five selected MOIs (see Supporting Information). Both RNA switches exhibit significantly higher ARs to the target molecule (6*R*)-FA compared to the other MOIs ($p < 10^{-4}$; one-way ANOVA), while the two switch controls exhibit no significant response to either the target or other MOIs (Figure S5). FA_ON_tert11 exhibits a better *in vivo* AR compared to FA_ON_tert32, suggesting an inverse relationship between the activities of RNA switches and the binding affinity of the composite aptamer ($AR_{tert11} = 6.3$, $K_{D,t8-11} = 61$ nM; $AR_{tert7} = 3.4$, $K_{D,t8-4} = 530$ nM). Furthermore, the *in vivo* measured ARs of each switch to the MOIs align with the AR ranges predicted from the *in silico* simulation, indicating that the kinetic model is able to predict the *in vivo* activities of RNA switches through Monte Carlo sampling. Previous aptamer

measurements performed at 5 mM Mg^{2+} suggested that the t8-4 aptamer would result in the best *in vivo* gene expression control, resulting in poor predictability of *in vivo* activity. Thus, our results indicate that aptamer binding information measured *in vitro* at physiologically relevant Mg^{2+} was critical for accurate prediction of *in vivo* switch activity (Figure S6). Our results further suggest that low Mg^{2+} concentrations should be employed in selections for aptamers intended for *in vivo* applications to ensure high affinity and selectivity of the resulting aptamers at physiologically relevant Mg^{2+} concentrations.

In summary, the combination of the SPR-based assay and the *in silico* simulation provides a method for streamlining and expediting the application of RNA aptamers as gene expression control tools. The predictions of the model were validated with *in vivo* characterization assays to five MOIs, including the target and four potentially interfering endogenous compounds (metabolites of the one-carbon cycle and synthetic analogues), indicating that the described RNA switches result in selective and programmable gene-regulatory activity. Our results suggest that, for exogenously-fed molecules, achieving high switching response *in vivo* may require RNA switches incorporating sensors that display at least a 3-orders of magnitude selectivity to *in vivo* interfering metabolites. Finally, the minimal toxicity profile of folinic acid in clinical studies²⁸ suggests that the RNA switches described in this study can be broadly applicable for controlling gene expression across different cell types and organisms, as well as in the context of clinical applications including cellular and gene therapies.

METHODS

For detailed descriptions of the *in vitro* assays and biological methods, see the Supporting Information

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Experimental design for developing RNA switches for selective control of gene expression. Two aptamers (t8-4 and t8-11) were characterized using a rapid and quantitative SPR-based assay at physiological Mg^{2+} concentrations. Nucleotides that were previously identified for effective binding are depicted in the aptamer structures; stable stems for both aptamers are also necessary for target binding, but the exact base-pairs can be altered.¹⁷ An *in silico* kinetic model with Monte Carlo simulation was employed to predict the corresponding *in vivo* switch activity, which was verified with *in vivo* target feeding experiments and flow cytometry analysis.

Figure 2.

A kinetic model with Monte Carlo sampling for predicting *in vivo* activities of RNA switches from *in vitro* binding parameters and experimental validation. a) A simple kinetic model describing an EGFP reporter regulated by an RNA switch incorporating the characterized aptamers. Five mRNA-associated kinetic rates (production rate: k_f ; degradation rates: k_{dmRNA_R} , k_{dmRNA_RL} ; exchange rates between R and RL states: k_{1f} , k_{1r}) and two protein-associated kinetic rates (production rate: k_p ; degradation rate: k_{dp}) are used in the model. b) Activities of two RNA switches (left: FA_ON_tert11; right: FA_ON_tert32), incorporating two different aptamers regulating EGFP expression under five different MOIs spanning three levels: nM, μ M, and mM-level to no binding (NB). *In silico* simulation ARs (activation ratios; defined as the ratio of protein levels in the presence and absence of MOI) are generated using Monte Carlo sampling ($N = 1000$) through randomizing mRNA-associated kinetic parameters (box plots). *In vivo* experimental ARs are determined based on the ratio of normalized median fluorescence in the presence and absence of each MOI (dots). (Note: Q1, Q2, and Q3 represent the first, second, and third quartile, respectively)

Table 1

In vitro characterization of FA aptamers with all three groups of MOIs. Reported $K_{D, \text{equilibrium}}^{\dagger}$ values are the mean and standard deviation of at least three independent experiments. The schematic of the folate metabolism is on the left.

Molecules of interest (MOIs)		Aptamers	
		t8-11	t8-4
Target	(6 <i>R</i>)-FA	61 ± 9 nM	530 ± 250 nM
Precursors	PABA	NB	NB
	DHN	NB	2.7 ± 2 mM
One-carbon cycle	folic acid	40 ± 30 mM	3 ± 2 mM
	DHF	21 ± 8 μM	25 ± 10 μM
	(<i>S</i>)-CH ₂ THF*	370 ± 270 μM	670 ± 260 μM
	(6 <i>S</i>)-THF	NB	NB
	(6 <i>S</i>)-5mTHF*	220 ± 60 μM	340 ± 80 μM
	(6 <i>S</i>)-FA	126 ± 2 μM	140 ± 10 μM
Analogues	(6 <i>R</i>)-THB	NB	NB
	(6 <i>R</i>)-THF	40 ± 10 μM	70 ± 10 μM
	MTX	NB	NB

Note: NB = no binding detected with the SPR-based assay up to 1 mM of MOI.

* Compounds only available as mixture of two diastereomeric isomers, (6*S*) and (6*R*).

$K_{D, \text{equilibrium}}^{\dagger}$ values measured using the steady-state affinity model.¹⁴