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Comparison of Small Molecule-Responsive RNA Aptazymes for Applications in Gene Control

Abstract

Modelling how genes act in both space and time is critical to understand animal development, which can potentially drive intervention in gene expression. Gene regulation is examined using many techniques; however, challenges such as cell delivery, invasiveness, toxicity, and efficacy limit our ability to fully probe gene networks. Recent advances have led to the development of tunable, titratable, and reversible tools that can be genetically-encoded into animal model systems to modulate genes with temporal and spatial control. This study compares such tools, testing several aptazyme-based switches that can be expressed inside cells and controlled through the addition of non-toxic small molecules. Three switches responsive to different small molecules were compared for switching activity in mammalian cells. The most efficient switches in terms of activity gauged by their modulation of gene expression were then further assayed. Finally, the specificity of the hypoxanthine switch was tested based on chemical structure and classification. The comparisons revealed the importance of both timing and small molecule concentrations on switch activity, while the specificity testing demonstrated switch activity inside the cell correlated to the aptamer binding properties that were measured biochemically. This work demonstrates the suitability of aptazyme-based switches for application in diverse genetic environments, and in controlling and studying gene networks in animals.

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Introduction

Measuring how genes act in both space and time is critical to understand animal developmental processes. Strategies such as gene knockouts[1], CRISPR mutations[2], tissue-specific promoters[3], and inducible and transcriptional activators[4] have been developed to allow for temporal regulation of gene expression. However, these tools are often limited by their delivery into cells, or do not result in long-term function[1,2,3,4]. Additionally, many of these strategies cannot be easily regulated, thus limiting their use to later developmental timepoints in animal studies. To address these limitations, we are testing nucleic acid tools known as RNA aptazymes that can be genetically-encoded into cells and organisms to control expression of a specific gene at any desired timepoint in any specific tissue.

Aptazyme switches precisely regulate the expression level of a desired gene through the activity of a natural self-cleaving ribozyme, which in our case is a hammerhead ribozyme isolated from the satellite tobacco ringspot virus (sTRSV)[5]. Ribozymes are catalytic RNA that, when placed in the 3' untranslated region (UTR) of a gene, are capable of cleaving and destabilizing mRNAs by removing the poly A tail, ultimately preventing protein translation[6,7,8]. To produce an RNA switch, the ribozyme is coupled to an aptamer[9]. Aptamers are synthetic oligonucleotides of DNA or RNA molecules that can tightly bind to a specific target molecule and change conformation[10]. Specifically, RNA aptamers upon conformational change can be incorporated into RNA aptazymes where target binding induces to destabilization of ribozyme activity[6,7,9,10]. These aptamer molecules can be effective in improving control of gene expression due to their high specificity, high affinity, and relatively small size[10]. In the switch system, when the aptamer's target molecule binds, it blocks ribozyme activity by interfering with the tertiary loop interactions required for ribozyme self-cleavage (**Figure 1**). More specifically, in an "ON" aptazyme switch, binding of the ligand to the aptamer prevents ribozyme cleavage, thus permitting translation – so the gene turns 'ON' in the presence of the molecule. Conversely, in the absence of the molecular input, the activity of the ribozyme results in mRNA degradation and the suppression of gene expression.

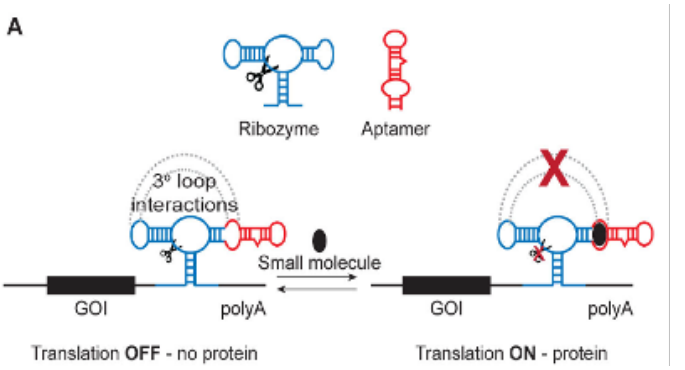


Figure 1. Aptazyme switch for gene expression control. The figure shows that RNA switches can control gene translation via mRNA degradation. In this study particularly, an "ON" aptazyme-based switch is applied. The full-length ribozyme has self-cleaving activity mediated by tertiary loop interactions at its catalytic core which leaves mRNA susceptible to degradation and preventing protein translation. Binding of the target to the aptamer inactivates the ribozyme catalytic activity, allowing for protein production. GOI = gene of interest

The goal of this work is to develop and test aptazyme-based switches within a genetic construct that can easily be ported into animal models. To date, RNA aptazymes have been tested in yeast and mammalian cells[9,11,12,13,14]. We hypothesize that RNA aptazymes will be useful tools in animals due to 1) the compact RNA-based composition avoids the use of immunogenic protein components[10]; 2) there is no molecular dependence of species-specific transcription factors ideally making them applicable to a wide range of animal species[10,12], and 3) the fast and direct action of the switches can allow for concise gene pathways to be tested[13,14].

Here, we examine the function of three small-molecule responsive aptazyme switches in a plasmid system in a common mammalian cell model. Two of the switches that demonstrated turn-on activity in the presence of their respective target molecules, hypoxanthine and folinic acid, were then tested with pre-treatment of increasing concentrations of small molecule under a range of incubation times post-transfection. Finally, the specificity of the hypoxanthine switch was tested by challenging the switch with similar purine molecules, adenine and guanine. This work demonstrates the portability of the switches in different genetic contexts and highlights the potential for applying these switches as tools in animal models.

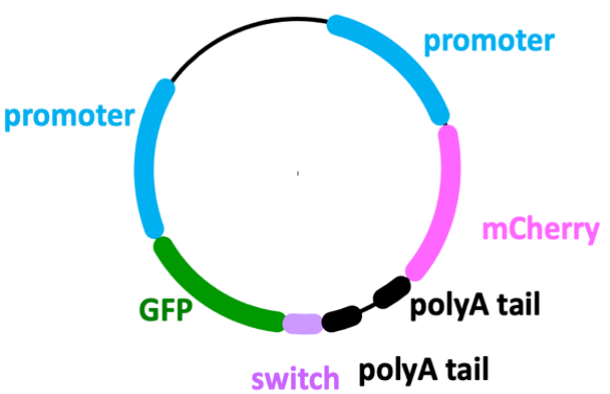
Methods

2.1 Materials

DMEM growth media was supplied from Thermo Fisher (Canada), derivative of human embryonic kidney (HEK293T) cells were generously provided by the Hébert lab at McGill University, all chemicals are from Sigma Aldrich, and kits used for assays are from New England Biolabs (NEB) unless otherwise noted. Oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). All sequences (**Figure 2a**) are available from the McKeague lab upon request.

Oligo Name	Sequences
PGP #10	CCTCTAGATTCTGCAGCCCTATAGCTAATCATAAATATACAAACAAAC AAAGCTGTCACC
PGP #11	TGTATCTTATCATGTCTGCTGATCAGCGGGTTTCCCGGTTTTTATTTTT CTTTTTGCTGT
PS #46	ATTCTGCAGCCCTATAGCTAATCA
PS #47	ATCTTATCATGTCTGCTGATCAGC
Neo-UGUAGCGG	AAACAAACAAAGCTGTCACCGGAGCTTGTCCTTTAATGGTCCTCCG GTCTGATGAGTCCTGTAGCGGGGACGAAACAGCAAAAAGAAAAAT AAAAA
Fol-UGGAG	AAACAAACAAAGCTGTCACCGGTGCTTGGTACGTTATATTCAGCCG GTCTGATGAGTCTTGGAGAGACGAAACAGCAAAAAGAAAAATAAA AA
Xan-ACGAG	AAACAAACAAAGCTGTCACCGGTGTATTACCCTAGTGGTCGACCGG TCTGATGAGTCTACGAGAGACGAAACAGCAAAAAGAAAAATAAAA A

(a)



(b)

Figure 2. (a) Table listing the oligonucleotide sequences of primers and plasmids used in this work. **(b)** Backbone plasmid (pMM100) is illustrated with reporter proteins and indicated location of switch insertion.

2.2 Switch Insertion Design

The two reporter genes are GFP and mCherry. Each switch sequence was inserted into the 3' UTR region of GFP in plasmid pMM100 (available from McKeague lab upon request) (**Figure 2b**). Thus, GFP expression is dependent on switch activity, while mCherry expression should remain constant (to normalize for transfection efficiency).

2.3 Cloning RNA switches into the Plasmid

The backbone plasmid pMM100 was grown via overnight TOP10 culture, at 37°C for 16-18 hours. Following the overnight culture, the plasmid was prepped using Qiagen QIAprep Spin Miniprep Kit (250), quantified using a Thermo Scientific Nanodrop and stored at -20°C. PCR was performed using the forward primer PGP #10, reverse primer PGP #11, and the Phusion HF (high fidelity) enzyme from NEB as described by the manufacturer. PCR product was confirmed via 2% agarose gel electrophoresis, purified using the Monarch PCR Cleanup Kit according to manufacturer instructions, and quantified using Nanodrop. The backbone plasmid (pMM100) was digested using Xmal from NEB in CutSmart buffer. Following treatment with Quick-CIP, the cut plasmid was purified via 0.8% gel electrophoresis, then purified using the NEB Monarch Gel Extraction Kit according to the manufacturer instructions. Gibson assembly[15] was performed by incubating the digested pMM100 backbone plasmid and the PCR switch insert as previously described. The plasmid was plated on E. coli and ampicillin plates. Colonies were selected and tested via colony PCR using the forward primer PS #46, reverse primer PS #47, and enzyme Taq DNA polymerase[16,17]. Following the colony PCR, products underwent 2.5% agarose gel electrophoresis for 45 minutes and were purified using the Monarch PCR and DNA Cleanup Kit. Purified colony plasmids were sent for Sanger sequencing. Once sequences were confirmed, freezer stocks of the plasmid were made and stored at -80°C.

2.4 Target Molecules

Stock solutions of target small molecules for cell treatment were made prior to transfection. The various concentrations of small molecule tested in-vitro were serial-diluted down from their stocks: for a 10 mM neomycin stock, 0.5 g was dissolved into 10.0 mL of DNase/RNase nuclease free water; for the 10 mM folinic acid stock, 47.3 mg was dissolved into 10.0 mL of DMEM media; and for the 220 mM hypoxanthine stock, 2.99 g was dissolved into 99.8 mL of DMSO.

2.5 HEK-293 Cell Maintenance

HEK-293 cells were maintained by incubation at 37°C, 5% CO_2, and DMEM media with 5% Fetal Bovine Serum (FBS) and 1% Antibiotic-Antimycotic (AB/AM). These cells were passaged every 72-96 hours and discarded following 25-30 passages after initial thaw.

2.6 Mammalian Cell Transfection with Plasmid

A 24-well plate was seeded with 500 µL of cells at roughly 70% confluency, with an aim of 110,000 cells per well; at the time of transfection, cells were close to 100% confluency. For small molecule assays, cells were pre-treated with 500 µL of designated concentration of small molecule 1 hour prior to transfection. For transfection, OptiMEM, Lipofectamine 3000, and P3000 reagent from the Lipofectamine 3000 Kit from Sigma Aldrich were added to the plasmid and this suspension was transfected into the plated HEK-293 cells. In a 24-well plate, 50 µL of DNA-lipid complex was attainted, in addition to 500 ng of DNA per well.

2.7 Measuring Fluorescence

At each designated time point post-transfection, or following an incubation period, cells were treated with Glo-Lysis 1X buffer from Promega, left to lyse for 5-7 minutes at room temperature, and quantified for GFP and mCherry fluorescence using the BioTek Cytation 5 plate reader by 24 well and 96 well plate readings.

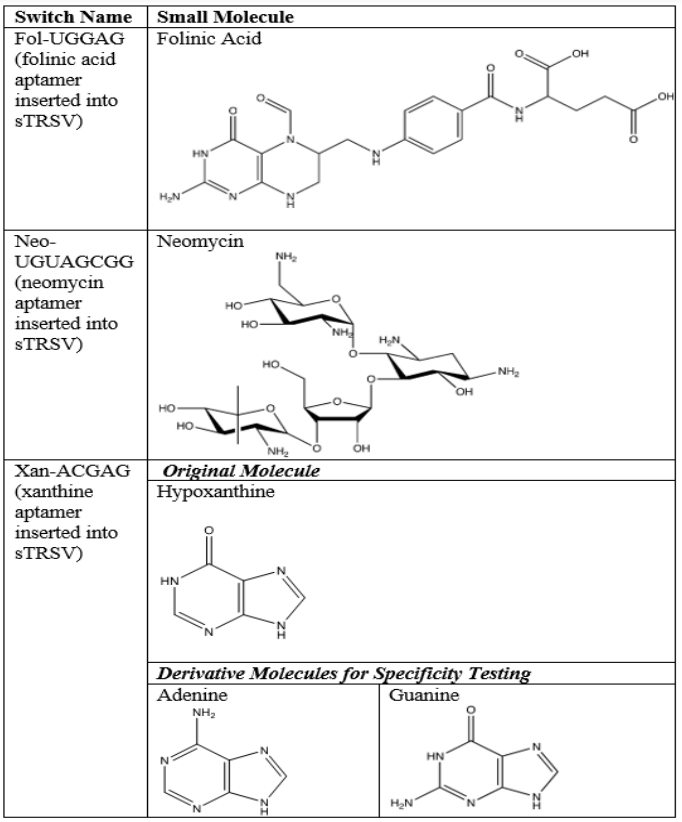
Results and Discussion

3.1 Preliminary comparison of xanthine, neomycin, and folinic acid switches.

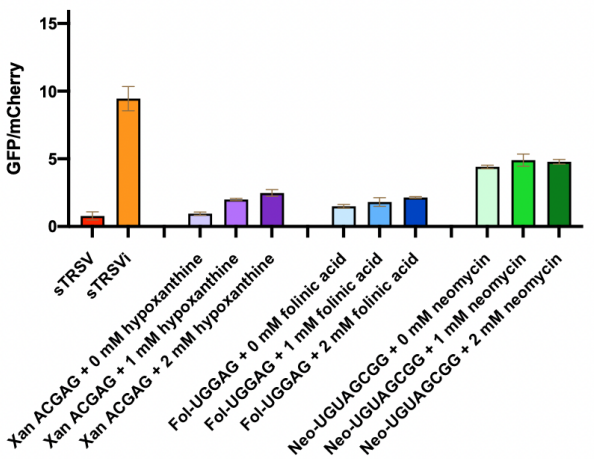
Numerous aptazyme switches[10,12] developed and tested in mammalian cells[9,11,12,13,14] have been reported to date. Small molecule-based aptazymes

are ideal since small molecules can be more easily delivered to cells and animals to modulate and turn on genes of interest. As such, we first tested switches developed to respond to small molecules that are expected to show no or little in vivo toxicity. Namely, we compared switches that interact with the FDA approved drug folinic acid[18]; the antibiotic neomycin[19]; and a purine found in cells, hypoxanthine[20]. Notably, each of these switches have previously demonstrated the ability to function in eukaryotes and show high "turn on" activity[13,14]. Each switch was cloned in a zebrafish-compatible plasmid to control GFP expression. Specifically, GFP is controlled by the Ubi-promoter which shows high function in zebrafish[21]. On the same plasmid, mCherry was included to serve as a transfection control.

The control called sTRSV represents the ribozyme lacking the aptamer that allows for constant self-cleavage of the GFP RNA transcript and thus should result in very little expression. In all experiments, sTRSV showed excellent negative control (off) activity (**Figure 3**). Conversely, sTRSVi is a positive control in that the ribozyme cannot function. As such, GFP should be constitutively expressed. Indeed, sTRSVi in all experiments showed high gene expression (**Figure 3**). For each molecule, we tested the switches by incubating high concentrations of the target molecule for 72 hours (**Figure 3b**). Following incubation, switch activity was compared by measuring GFP fluorescence normalized to mCherry. Importantly, when comparing these three aptazyme switch designs head-to-head, the hypoxanthine switch showed the most switching activity, turning on 2-fold in the presence of 2 mM of target molecule compared to in its absence (**Figure 3b**). The neomycin switch showed no switching activity, and folinic acid showed a small increase in gene expression with 2 mM target molecule. Considering K_d for each of the switches[13,14], the hypoxanthine aptamer exhibits micromolar binding affinity to its ligand while that of folinic acid is nanomolar. Given these results, we focused on further characterizing the hypoxanthine and folinic acid switches.



(a)



(b)

Figure 3. (a) Table outlining the switches and their associated target small molecule(s) tested in this work. **(b)** Preliminary in-vitro testing of hypoxanthine, folinic acid, and neomycin switches to detect the highest measure of fluorescence. The plot (**Figure 3b**) shows the change in normalized GFP/mCherry fluorescence upon addition of small molecule target to the three distinct switches at 72 hours post-transfection. For the control switches, sTRSV (red) and sTRSVi (orange), three and six replicates were prepared, respectively. Control switch sTRSVi was treated with different concentration of respective small molecule in duplicate. Our control switch sTRSV was not treated with any molecule as it acts as our negative control. For each switch tested, different concentrations of the molecules were used in duplicates. Results are the mean and standard deviation of the number of replicates described: hypoxanthine data is in purple, folinic acid in blue, and neomycin in green.

3.2 Testing the hypoxanthine-responsive switch under various parameters

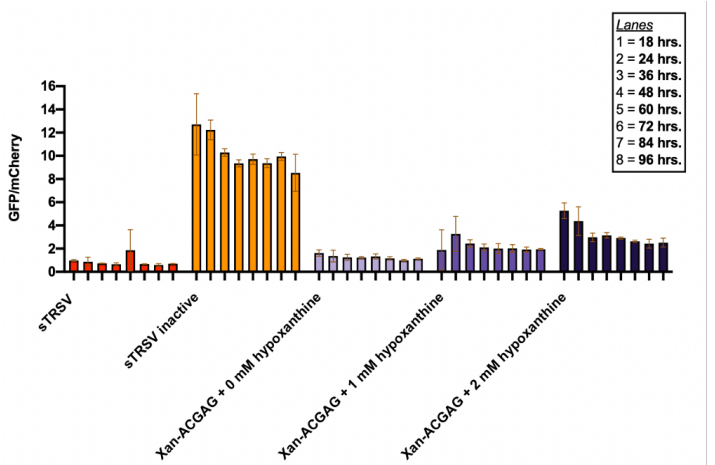
To further investigate the hypoxanthine-responsive switch, we conducted experiments testing activity of the switch using high concentrations over various time points post-transfection, as well as a large concentration study. Since cells had to be lysed, one 24 well plate was transfected per timepoint. To minimize uncertainties associated with different results for gene expression, fluorescence was normalized to sTRSV and sTRSVi control switch activity.

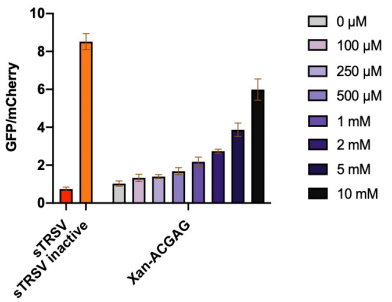
We tested the hypoxanthine switch at concentrations with 0 mM, 1 mM, and 2 mM at eight different timepoints post-transfection ranging from 18-96 hours (**Figure 4a**). Following the various incubation times (post-transfection), switch activity was compared by measuring GFP fluorescence normalized to mCherry. Comparing the switch activity over the various timepoints, incubation periods between 18 to 24 hours showed the highest "turn on" activity, but later timepoints (36-72 hours) resulted in reduced variability. Given these results, we confirmed that shorter than 72-hour transfection incubation periods for our given switch are indeed enough time to allow for gene expression, though periods within this range closer to 72 hours may be more useful for future comparisons.

We next performed a concentration gradient experiment using concentrations of hypoxanthine ranging from 100 μM to 10 mM (**Figure 4b**). Following the incubation time (post-transfection), switch activity of the switches treated with various concentration of molecule was compared by measuring GFP fluorescence normalized to mCherry. Comparing the switch activity, increasing "turn on" activity was observed across increasing concentrations of small molecule, where the largest change in gene expression was noted between 500 μM and 10 mM concentration.

3.3 Testing the folinic-acid responsive switch under various parameters

While the folinic acid switch showed less activity than the hypoxanthine switch in the initial experiments (3.1), we nevertheless sought to measure the importance of incubation time (post-transfection time points) and concentration of folinic acid switch activity. As per control switch confirmation, sTRSVi showed a higher normalized GFP/mCherry relative expression while sTRSV remains low as expected (Figure 5). We tested the





(b)

Figure 4. (a) Hypoxanthine-responsive switch demonstrated increased GFP/mCherry relative expression in the presence of hypoxanthine in vitro at early time points post-transfection. For the control switches, sTRSV (red) and sTRSVi (orange), three and six replicates were prepared respectively. Control switch sTRSVi was treated with different concentration of hypoxanthine in duplicate. Our control switch sTRSV was not treated with any molecule as it acts as our negative control. Each other condition represents the mean and standard deviation of four replicates. Results show that the switch activity occurs rapidly in the first 18-24 hours, and then later likely starts to be reduced with time due to cell division. **(b)** The hypoxanthine-responsive switch shows an increased GFP/mCherry relative expression in vitro with increasing concentrations of hypoxanthine. For the control switches, sTRSV (red) and sTRSVi (orange), four and eight replicates were prepared respectively. Control switch sTRSVi was treated with different concentration of hypoxanthine in duplicate. Our control switch sTRSV was not treated with any molecule as it acts as our negative control. Each other condition represents the mean and standard deviation of four replicates.

folinic acid switch with 0 mM, 2 mM, 5 mM, and 10 mM at six different time points post-transfection ranging from 24-84 hours. Following the various incubation times (post-transfection), switch activity was compared by measuring GFP fluorescence normalized to mCherry. "Turn on" activity was shown across increasing concentrations. However, there was no increased switching observed at time points later than 24 hours.

3.4 Specificity testing of the hypoxanthine-responsive switch

Aptamers are typically highly selective for their cognate targets but, nevertheless, can bind similar derivatives in some cases. As an example, the xanthine aptamer, which was tested in vitro with small molecule hypoxanthine (and used in our platform as a "hypoxanthine switch") is a derivative of the original target ligand xanthine. Indeed, Kiga et al.[3] demonstrated that the aptamer, which they refer to as XBA (xanthine-binding aptamer), could also target purines with several modifications to the ring structure. Here, we wanted to assess whether the selectivity demonstrated directly on the aptamer was maintained when incorporated into our aptazyme switch. In particular, adenine was bound to the aptamer with a large K_d (indicative of low affinity) while guanine had a very small K_d (indicative of high affinity) for XBA. As such, we tested these two molecules for the ability to activate the hypoxanthine-responsive aptazyme switch.

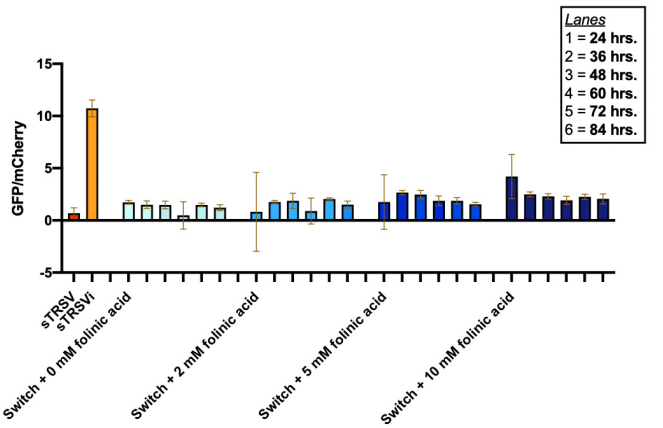


Figure 5. Folinic acid-responsive switch results in increased GFP/mCherry relative expression in presence of folinic acid in vitro with increasing time post-transfection. The plot shows the change in fluorescence with increasing concentrations of folinic acid at six distinct time points post-transfection. For the control switches, sTRSV (red) and sTRSVi (orange), three and eight replicates were prepared respectively. Control switch sTRSVi was treated with different concentration of folinic acid in duplicate. Our control switch sTRSV was not treated with any molecule as it acts as our negative control. All other conditions represent the mean and standard deviation of four replicates.

We tested the hypoxanthine switch with 0 mM, 1 mM, and 2 mM of hypoxanthine, guanine, or adenine at a constant incubation time of 72 hours post-transfection. Similar to the aptamer binding experiments, the switch was activated in the presence of guanine but not adenine. Interestingly, even though guanine has a similar affinity to the aptamer as xanthine, the switching activity was less than with hypoxanthine. Nevertheless, these results confirm that the selectivity of the aptamer is maintained despite it being incorporated into a switch and expressed inside a cell.

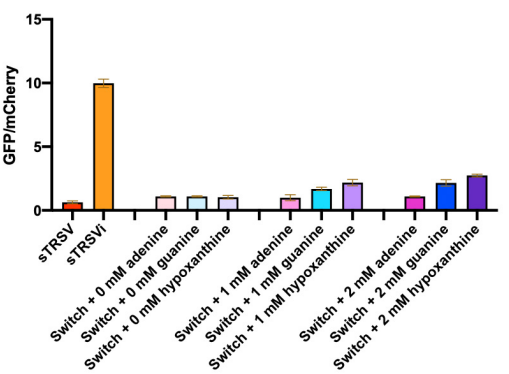


Figure 6. Hypoxanthine-responsive switch specificity in the presence of guanine and adenine. The plot shows the change in fluorescence with addition of derivative small molecule to Xan-ACGAG at 72 hours post-transfection. For the control switches, sTRSV (red) and sTRSVi (orange), three and six replicates were prepared, respectively. Control switch sTRSVi was treated with different concentration of respective small molecule in duplicate. Our control switch sTRSV was not treated with any molecule as it acts as our negative control. All other conditions are the mean and standard deviation of four replicates.

Conclusion

Several small-molecule responsive aptazyme-based switches were compared[22]. The hypoxanthine switch displayed robust "turn on" activity in the presence of low concentrations of molecule relatively quickly following transfection. Interestingly, the hypoxanthine-responsive switch specificity was demonstrated and is comparable to biochemical aptamer affinity studies. Future work will examine additional early switch time points as well as other possibly confounding molecules found inside cells. The long-term goal of this work is to generate a suite of switches that can be easily incorporated into animal models and integrated into the genome to study genes relevant to development.

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Author Contributions

J.S. conducted the experiments. J.S. and M.M. analyzed data and wrote the manuscript.

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