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A cellular high-throughput screening approach for therapeutic *trans*-cleaving ribozymes and RNAi against arbitrary mRNA disease targets

Edwin H. Yau [c, b, 1], Mark C. Butler [b], Jack M. Sullivan [a, b, c, d, e, f, g, *]

[a] *Research Service, VA Western New York Healthcare System, Buffalo, NY 14215, USA*
[b] *Department of Ophthalmology (Ira G. Ross Eye Institute), University at Buffalo- SUNY, Buffalo, NY 14209, USA*
[c] *Department of Pharmacology/Toxicology, University at Buffalo- SUNY, Buffalo, NY 14209, USA*
[d] *Department of Physiology/Biophysics, University at Buffalo- SUNY, Buffalo, NY 14209, USA*
[e] *Neuroscience Program, University at Buffalo- SUNY, Buffalo, NY 14209, USA*
[f] *SUNY Eye Institute, University at Albany- SUNY, USA*
[g] *RNA Institute, University at Albany- SUNY, USA*

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ABSTRACT

Major bottlenecks in development of therapeutic post transcriptional gene silencing (**PTGS**) agents (e.g. ribozymes, RNA interference, antisense) include the challenge of mapping rare accessible regions of the mRNA target that are open for annealing and cleavage, testing and optimization of agents in human cells to identify lead agents, testing for cellular toxicity, and preclinical evaluation in appropriate animal models of disease. Methods for rapid and reliable cellular testing of PTGS agents are needed to identify potent lead candidates for optimization. Our goal was to develop a means of rapid assessment of many RNA agents to identify a lead candidate for a given mRNA associated with a disease state. We developed a rapid human cell-based screening platform to test efficacy of hammerhead ribozyme (**hhRz**) or RNA interference (**RNAi**) constructs, using a model retinal degeneration target, human rod opsin (*RHO)* mRNA. The focus is on RNA Drug Discovery for diverse retinal degeneration targets.

To validate the approach, candidate hhRzs were tested against NUH↓ cleavage sites (N = G,C,A,U; H = C,A,U) within the target mRNA of *secreted* alkaline phosphatase (***SEAP***), a model gene expression reporter, based upon *in silico* predictions of mRNA accessibility. HhRzs were embedded in a larger stable adenoviral VAI RNA scaffold for high cellular expression, cytoplasmic trafficking, and stability. Most hhRz expression plasmids exerted statistically significant knockdown of extracellular *SEAP* enzyme activity when readily assayed by a fluorescence enzyme assay intended for high throughput screening (**HTS**). Kinetics of PTGS knockdown of cellular targets is measureable in live cells with the SEAP reporter. The validated *SEAP* HTS platform was transposed to identify lead PTGS agents against a model hereditary retinal degeneration target, *RHO* mRNA. Two approaches were used to physically fuse the model retinal

Abbreviations: anova, analysis of variance; bp, base pair; CV, coefficient of variation; EGFP, enhanced green fluorescent protein; FWHM, full width half maximum; HEK293S, human embryonic kidney cells-suspension adapted; HEK293S-*RHO*-IRES-*SEAP*, HEK293S cells stably expressing *RHO*-IRES-*SEAP*; HEK293S-*SEAP*, HEK293S cells stably expressing *SEAP*; hhRz, hammerhead ribozyme; HTS, high-throughput screening; IRES, internal ribosome entry site; MFE, minimum folding energy; mppRNA, multi-parameter prediction of RNA accessibility; 4-MUP, 4-methylumbelliferyl-phosphate; nt, nucleotide; NUH↓, ribozyme cleavage motif where N is any nucleotide, U is uridine, and H is any nucleotide excluding guanosine; PLAP, placental alkaline phosphatase; PTGS, post transcriptional gene silencing; *RHO*, rod opsin; RISC, RNA-induced silencing complex; RNAi, RNA interference; *SEAP*, secreted alkaline phosphatase; SEM, standard error of mean; shRNA, short-hairpin RNA; siRNA, short interfering RNA.

* Corresponding author. Veterans Administration Western New York Healthcare System, Medical Research, Building 20, Rm 245, 3495 Bailey Ave, Buffalo, NY 14215, USA.
E-mail addresses: js354@buffalo.edu, jackmsullivanmdphd@yahoo.com (J.M. Sullivan).
[1] Current Affiliation: University of California San Diego, Dept. of Hematology and Oncology, USA.

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gene target mRNA to the *SEAP* reporter mRNA. The most expedient way to evaluate a large set of potential VAI-hhRz expression plasmids against diverse NUH↓ cleavage sites uses cultured human HEK293S cells stably expressing a dicistronic *Target*-IRES-*SEAP* target fusion mRNA. Broad utility of this rational RNA drug discovery approach is feasible for any ophthalmological disease-relevant mRNA targets and any disease mRNA targets in general. The approach will permit rank ordering of PTGS agents based on potency to identify a lead therapeutic compound for further optimization.

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1. Introduction

Development of therapeutic nucleic-acid based PTGS agents is a challenging task as evidenced by the fact that only two agents have been FDA-approved for human clinical use during the last three decades of academic and corporate research (Anderson et al., 1996; Jabs and Griffiths, 2002; Merki et al., 2008). The dense secondary and tertiary structures of the target mRNA, RNA-protein association, and expected molecular dynamics severely restrict the regions that are accessible to essential second-order annealing reactions with smaller PTGS ligands in *trans* (Sullivan et al., 2008). Additional biocomplexity results from cellular compartmentalization of the target mRNA, on both gross and fine scales, which promotes spatial and temporal distributions of target mRNAs within the cell. To be effective the PTGS agent must journey through the same cellular locales and have residence, stability, and kinetic performance that overlaps with the target mRNA lifetime in each spatial compartment. However, these challenges due to biocomplexity can be addressed by new approaches that attack bottlenecks in RNA drug development (Sullivan et al., 2008, 2012). Here we developed a human cell-based screening platforms to rapidly and reliably identify lead hhRz or RNAi candidate agents ("hits") from substantial sets of potential agents.

Both hhRz and RNAi catalyze the sequence specific cleavage of target mRNAs. HhRzs are small RNA sequences capable of enzymatic cleavage of polyribonucleotides (Bertrand et al., 1994; Vaish et al., 1998; Amarzguioui and Pyrdz, 1998; Hauswirth and Lewin, 2000; Lewin and Hauswirth, 2001; Sullivan et al., 2011). Originally discovered as intramolecular self-cleaving (*cis*) sequences in self-replicating plant viroids (Flores et al., 2012), the hhRz consists of three helices surrounding an evolutionarily conserved catalytic core. *Trans*-cleaving hhRzs are readily constructed by embedding the hhRz core enzyme into a target annealing sequence which gives the unimolecular RNA the capacity for both molecular recognition and enzymatic cleavage of an independent target mRNA. The target molecular recognition arms of the *trans* hhRz are designed to provide antisense complementarity (Watson-Crick) to a defined accessible region of an independent target mRNA (Uhlenbeck, 1987). After 2nd-order collision-based interaction and kissing complex formation, full annealing with the target may occur over the antisense spans to form a complete hhRz: target hybrid structure. This hybrid undergoes conformational changes to align specific bases within the RNA enzyme core that mediate proton transfer chemistry and accelerate target mRNA cleavage at a specific phosphodiester bond. The *trans* design strategy allows for potential realization of hhRzs that possess potent sequence-specific endoribonuclease activity against any given target RNA (Haseloff and Gerlach, 1988). HhRz target motifs are **NUH↓** triplets, where N is any nucleotide (**nt**), U is a uridine, and H can be any nucleotide except guanosine (Perriman et al., 1992; Ruffner et al., 1990; Zoumadakis and Tabler, 1995; Shimayama et al., 1995; Birikh et al., 1997). Given this versatility, any moderately sized mRNA target has numerous potential NUH↓ cleavage sites. For example, in *SEAP* mRNA (1777 nt) there are a total of 180 NUH↓ cleavage sites and in the dominant polyadenylated form of human *RHO* mRNA there are 236 potential NUH↓ cleavage sites. Similarly, RNAi agents can be designed for cellular expression as short hairpin RNAs (**shRNA**). These are processed intracellularly by Drosher and Dicer into short-interfering RNAs (**siRNA**) that associate as guide sequences within the RNA-induced silencing complex (**RISC**), built upon the endonuclease Ago2, which anneal with the target mRNA and drive cleavage by protein-mediated catalysis (Brummelkamp et al., 2002; Rossi, 2008). While this might seem to make PTGS therapeutics a straightforward endeavor, in live cells most potential NUH↓ cleavage sites and RNAi target sites within any mRNA are inaccessible due to strong secondary and tertiary structures and protein binding (Amarzguioui et al., 2000; Brown et al., 2005; Lima et al., 1992; Patzel and Sczakiel, 1998; Patzel et al., 1999; Scherr and Rossi, 1998; Scherr et al., 2000). Identifying the optimum site for targeting is a daunting task, yet critical for successful RNA drug discovery.

We employ a mutation-independent approach to hhRz development for RNA Drug Discovery for autosomal dominant retinal degenerations (Montgomery and Dietz, 1997; Millington-Ward et al., 1997; Sullivan et al., 2002; Farrar et al., 2002; Gorbatyuk et al., 2005, 2007; Cashman et al., 2005; Sullivan et al., 2011). In this approach one works to identify the most potent hhRz or shRNA that can maximally suppress a given disease target mRNA and protein. In the context of a genetic disease such as an autosomal dominant retinitis pigmentosa, a mutation independent hhRz will suppress not only the mutant mRNA but also the WT mRNA. Such a single therapeutic agent would be expected to be useful for treatment of most if not all of the known mutations in a given gene as the optimum targeting location within the mRNA is likely to harbor relatively few, if any, random mutations. Prevention of haploinsufficiency due to suppression of the intrinsic WT mRNA is achieved in a combined gene therapy paradigm in which the knockdown hhRz agent is expressed in concert with an allelic variant of the WT target which transcribes a "hardened" WT mRNA which cannot be cleaved by the potent therapeutic agent yet encodes the WT protein (Sullivan et al., 2011; Millington-Ward et al., 1997; Gorbatyuk et al., 2007).

The rationale for this study is that efficient and timely realization of potent lead candidates in the RNA drug discovery process requires initial approaches to identify regions of accessibility in a target mRNA, and the means to rapidly screen the efficacy (potency) of sets of agents designed against accessible regions and (control) inaccessible regions in live cells in order to identify the lead candidate on the basis of rank-ordered activity. In this study we addressed both issues and exploited the *SEAP* reporter protein to establish a platform for rapid and reliable assessment of the efficacy of *trans*-cleaving hhRz and shRNA constructs. The stable *SEAP* reporter protein is secreted into culture medium in proportion to its steady-state intracellular mRNA levels, which makes it an ideal "model" target mRNA to assay the immediate and long term kinetic impact of PTGS agents on gene expression in live cell cultures (Berger et al., 1988). We first developed a HTS fluorescence plate assay for secreted *SEAP* enzyme and used computational RNA