Published in final edited form as:

Angew Chem Int Ed Engl. 2013 January 28; 52(5): 1462–1465. doi:10.1002/anie.201206888.

Small Molecule-Mediated Cleavage of RNA in Living Cells

Lirui Guan and Matthew D. Disney

The Department of Chemistry, The Kellogg School of Science and Engineering, The Scripps Research Institute, Scripps Florida, 130 Scripps Way, 3A1, Jupiter, FL 33458

Matthew D. Disney: Disney@scripps.edu

Abstract

Antisense oligonucleotides and small interfering RNAs (siRNAs) control gene expression by triggering the degradation of a mRNA via recruitment of RNase H or the RNA-induced silencing complex (RISC), respectively.^[1] These approaches are hampered, however, by the poor cellular permeability of oligonucleotides. A small molecule approach to cleave RNA targets could obviate uptake issues. Several compounds can induce RNA cleavage *in vitro*,^[2] however, to the best of our knowledge no small molecules have been previously described to cleave RNA in living cells. Herein, we describe the development of a potentially general approach to design small molecules that specifically cleave an RNA in a living cell, affecting biological function. Specifically, a designed, modularly assembled small molecule that binds the RNA that causes myotonic dystrophy type 1 (DM1)^[3] was appended with a moiety that generates hydroxyl radicals upon irradiation. Cleavage of the transcript improves DM1-associated defects in cell culture, and compounds are non-toxic at an efficacious dose as determined by a MTT viability assay. This approach may allow for the site-specific cleavage and inactivation of other cellular RNAs.^[4] Compounds that bind to and cleave RNA have the potential to serve as chemical genetics probes of function or lead therapeutics with spatial and temporal control.

Keywords

Nucleic acids; RNA; Chemical Biology; Bioorganic Chemistry; RNA recognition

Very few small molecule therapeutics bind RNA and modulate its function. The most notable exceptions target the bacterial ribosome or riboswitches. The ribosome comprises ca. 80–90% of the total RNA content of living cells and thus can be effectively targeted with small molecules even if they are not exquisitely selective.^[5] Small molecules that modulate riboswitch function can be designed by mimicking the riboswitch's natural metabolite, mirroring substrate mimicry approaches that have been a mainstay to target enzymes.^[6] Modulating the function of other cellular RNAs is therefore difficult due to their low expression levels (compared to ribosomal RNAs) and the lack of lead small molecules.^[7]

In an effort to identify lead small molecules to modulate RNA function, we have developed a bottom-up approach to target RNA. That is, the RNA secondary structural elements (motifs) that are preferred to bind small molecules are identified. These RNA motifs are then searched for in RNAs that cause or contribute to disease. In ideal cases, the disease-causing RNA has more than one targetable motif such that a multivalent ligand can be designed to increase ligand affinity and specificity. We have successfully applied this approach to

Correspondence to: Matthew D. Disney, Disney@scripps.edu.

This work is dedicated to Professor Dieter Seebach on the occasion of his 75th birthday.

Supporting information for this article is available on the WWW under <http://www.angewandte.org> or from the author.

modulate the toxicity of expanded repeating RNAs that cause or contribute to myotonic dystrophy type 1 (DM1) and Huntington's disease (HD).^{[4, 8] [9]}

DM1 is caused by an expanded r(CUG) repeat (50–1000+ repeats) in the 3' untranslated region (UTR) of the dystrophin myotonia protein kinase (*DMPK*) mRNA. The expanded repeat, or r(CUG)^{exp}, folds into an RNA hairpin that sequesters proteins, including muscleblind-like 1 (MBNL1), a pre-mRNA splicing regulator (Figure 1B).^[3, 10] Sequestration of MBNL1 by r(CUG)^{exp} leads to its inactivation, and hence alternative splicing defects in a subset of pre-mRNAs including the cardiac troponin T (cTNT), the insulin receptor, and the muscle-specific chloride ion channel (Figure 1A & B). These splicing defects explain the heart defects, insulin insensitivity, and myotonia that are experienced by DM1 patients. The disease mechanism for DM1 points to a therapeutic strategy in which a compound binds r(CUG)^{exp} and displaces MBNL1, allowing it to properly regulate pre-mRNA alternative splicing (Figure 1C). Several compounds have been used to validate this as a therapeutic strategy in both cellular and animal models of disease.^[9a, 9b, 11]

We previously reported a designed, modularly assembled compound that binds r(CUG)^{exp} and modulates its toxicity *in vivo*.^[9a] The compound consists of a peptoid scaffold that displays two copies of a *bis*-benzimidazole (H, Figure 2), separated by four spacing modules (2H-4, Figure 2). The H module binds a single 5' CUG/3' GUC motif displayed in r(CUG)^{exp} while 2H-4 binds two adjacent 5' CUG/3' GUC motifs.^[9c] We sought to improve the potency of 2H-4 *in vivo* by equipping the RNA-binding compound with a moiety that can induce degradation of r(CUG)^{exp}. The RNA-binding portion of the compound displaces MBNL1 from r(CUG)^{exp} and provides specificity while the cleavage-moiety leads to degradation of r(CUG)^{exp}. Both modes of action should lead to improvement of DM1-associated defects.^[11d]

We utilized a derivative of N-hydroxypyridine-2(1H)-thione that generates hydroxyl radicals upon irradiation to induce cleavage of r(CUG)^{exp}. Previous studies have employed N-hydroxypyridine- 2(1H)-thione to probe RNA structure *in vitro*.^[12] Thus, we synthesized 1-hydroxy-6-thioxo-1,6-dihydropyridine-2-carboxylic acid (N-HPT) that allows for conjugation of the hydroxyl radical-producing war head onto 2H-4 via an amide bond (2H-4-HPT) (Figure 2).^[13] A compound without the RNA-binding (H) module (2P-4-HPT) serves as a negative control.

The compounds were studied for cleaving r(CUG)₁₀ *in vitro* (Figure 3). These studies show that 2H-4-HPT cleaves r(CUG)₁₀ in the presence of UV light. Some cleavage is also observed when r(CUG)₁₀ is incubated with 2H-4, but it is less than what is observed for 2H-4-HPT. A slower mobility band was observed when r(CUG)₁₀ was incubated with both 2H-4 and 2H-4-HPT, suggesting a cross-link mediated by Hoechst occurs upon irradiation with light. No cleavage is observed for 2P-4-HPT as expected since 2P-4 does not bind r(CUG) repeats.^[9c]

Next, the *in vitro* potencies of the compounds for inhibiting the r(CUG)₁₀-MBNL1 complex were determined via a time-resolved fluorescence resonance energy transfer (TR-FRET) assay (Table 1).^[9b] Two sets of experiments were completed: (i) the RNA was incubated with the compound of interest and irradiated with light. MBNL1 was then added; and, (ii) the RNA was incubated with the compound of interest and then MBNL1 was added (no irradiation). The IC₅₀ for 2H-4 is similar whether the sample is irradiated or not. In contrast, a 6-fold increase in potency is observed for 2H-4-HPT when the sample is irradiated. The increase in potency of 2H-4-HPT is due to degradation of r(CUG)₁₀, as evidenced by *in vitro* cleavage (Figure 2). The decrease in potency of 2H-4 in the presence light irradiation is

presumably due to the degradation of ligand. (Figure S5). No effect on complex formation is observed with N-HPT, as expected.

Since 2H-4-HPT is a more potent *in vitro* inhibitor of a model r(CUG)^{exp}-MBNL1 complex than 2H-4 and cleaves r(CUG) repeats, it is possible that it has improved potency *in vivo*. Therefore, 2H-4-HPT was studied in a cell culture model for improving DM1-associated pre-mRNA splicing defects (Figure 4). HeLa cells were co-transfected with a DM1 mini-gene that expresses r(CUG)^{exp} and a mini-gene construct of cTNT alternative splicing. After transfection, the cells were dosed with compound in growth medium. After 6 h, cells were irradiated (+light). Total RNA was harvested 12–14 h later, and the amount of each alternatively spliced isoform of the cTNT mini-gene transcript was determined by RT-PCR. When cells were treated with 5 μM of 2H-4-HPT and irradiated with light, the levels of cTNT pre-mRNA splicing shifted towards what is observed in the absence of r(CUG)^{exp} (Figure 4). Furthermore, no toxicity is observed at these conditions as assessed by morphological changes and a MTT viability assay (Figure S7). This suggests that even though highly reactive species are being generated, they are localized to r(CUG)^{exp} and not damaging bystander, essential biomolecules. This specificity is afforded by 2H-4. Under identical conditions, 2H-4 does not improve pre-mRNA splicing defects at 5 μM, indicating that the improved bioactivity of 2H-4-HPT is due to cleavage of r(CUG)^{exp}. Additional control experiments show that 2H-4-HPT has no effect on the alternative splicing of *PLEKHH2* pre-mRNA, which is not controlled MBNL1 (Figure S6).^[10a]

To determine if 2H-4-HPT is cleaving r(CUG)^{exp} in living cells, total RNA was harvested and the amount of r(CUG)^{exp} relative to β-actin was measured by real-time RT-PCR (Figure 5).^[11d] These studies show that approximately 50% of r(CUG)^{exp} is cleaved upon irradiation while in the absence of irradiation none of the transcript is cleaved. These data support the hypothesis that the improvement in pre-mRNA splicing defects observed with 2H-4-HPT in the presence of light are due to specific cleavage of r(CUG)^{exp} and subsequent removal of the transcript by RNA degradation pathways. This cleavage frees MBNL1 from r(CUG)^{exp}, allowing it to regulate pre-mRNA splicing.

In summary, we have shown, for the first time, that small molecules can be designed to cleave a specific RNA in living cells and modulate function. This approach could be used in conjunction with photodynamic therapy to cleave specific cancer- or other disease-associated transcripts, to design chemical probes of RNA function or potentially selective therapeutics, or to probe RNA structure *in vitro* or *in vivo*,^[14] provided that the RNA-binding preferences of small molecules were sufficiently defined.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was funded by the national Institutes of Health (4RO1-GM079235-02S1) and The Scripps Research Institute. MDD is a Camille & Henry Dreyfus Teacher-Scholar

References

1. a) Crooke ST. Annu. Rev. Med. 2004; 55:61–95. [PubMed: 14746510] b) Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Nature. 2001; 411:494–498. [PubMed: 11373684]
2. Lonnberg H. Org. Biomol. Chem. 2011; 9:1687–1703. [PubMed: 21258754]
3. Day JW, Ranum LP. Neuromuscul. Disord. 2005; 15:5–16. [PubMed: 15639115]

4. a) Disney MD, Labuda LP, Paul DJ, Poplawski SG, Pushechnikov A, Tran T, Velagapudi SP, Wu M, Childs-Disney JL. *J. Am. Chem. Soc.* 2008; 130:11185–11194. [PubMed: 18652457] b) Childs-Disney JL, Wu M, Pushechnikov A, Aminova O, Disney MD. *ACS Chem. Biol.* 2007; 2:745–754. [PubMed: 17975888]
5. a) Kumar A, Park H, Fang P, Parkesh R, Guo M, Nettles KW, Disney MD. *Biochemistry.* 2011; 50:9928–9935. [PubMed: 21988728] b) Johnson LF, Williams JG, Abelson HT, Green H, Penman S. *Cell.* 1975; 4:69–75. [PubMed: 1078787]
6. a) Blount KF, Breaker RR. *Nat. Biotechnol.* 2006; 24:1558–1564. [PubMed: 17160062] b) Blount KF, Wang JX, Lim J, Sudarsan N, Breaker RR. *Nat. Chem. Biol.* 2007; 3:44–49. [PubMed: 17143270]
7. Guan L, Disney MD. *ACS Chem. Biol.* 2012; 7:73–86. [PubMed: 22185671]
8. Velagapudi SP, Seedhouse SJ, Disney MD. *Angew. Chem. Int. Ed. Engl.* 2010; 49:3816–3818. [PubMed: 20397174]
9. a) Childs-Disney JL, Hoskins J, Rzuczek SG, Thornton CA, Disney MD. *ACS Chem. Biol.* 2012; 7:856–862. [PubMed: 22332923] b) Parkesh R, Childs-Disney JL, Nakamori M, Kumar A, Wang E, Wang T, Hoskins J, Tran T, Housman DE, Thornton CA, Disney MD. *J. Am. Chem. Soc.* 2012; 134:4731–4742. [PubMed: 22300544] c) Pushechnikov A, Lee MM, Childs-Disney JL, Sobczak K, French JM, Thornton CA, Disney MD. *J. Am. Chem. Soc.* 2009; 131:9767–9779. [PubMed: 19552411]
10. a) Jiang H, Mankodi A, Swanson MS, Moxley RT, Thornton CA. *Hum. Mol. Genet.* 2004; 13:3079–3088. [PubMed: 15496431] b) Mankodi A, Logigian E, Callahan L, McClain C, White R, Henderson D, Krym M, Thornton CA. *Science.* 2000; 289:1769–1773. [PubMed: 10976074]
11. a) Warf MB, Nakamori M, Matthys CM, Thornton CA, Berglund JA. *Proc. Natl. Acad. Sci. U. S. A.* 2009; 106:18551–18556. [PubMed: 19822739] b) Wheeler TM, Sobczak K, Lueck JD, Osborne RJ, Lin X, Dirksen RT, Thornton CA. *Science.* 2009; 325:336–339. [PubMed: 19608921] c) Mulders SA, van den Broek WJ, Wheeler TM, Croes HJ, van Kuik-Romeijn P, de Kimpe SJ, Furling D, Platenburg GJ, Gourdon G, Thornton CA, Wieringa B, Wansink DG. *Proc. Natl. Acad. Sci. U. S. A.* 2009; 106:13915–13920. [PubMed: 19667189] d) Lee JE, Bennett CF, Cooper TA. *Proc. Natl. Acad. Sci. U. S. A.* 2012; 109:4221–4226. [PubMed: 22371589] e) Garcia-Lopez A, Llamusi B, Orzaez M, Perez-Paya E, Artero RD. *Proc. Natl. Acad. Sci. U. S. A.* 2011; 108:11866–11871. [PubMed: 21730182]
12. Chaulk SG, Pezacki JP, MacMillan AM. *Biochemistry.* 2000; 39:10448–10453. [PubMed: 10956035]
13. Agrawal A, de Oliveira CA, Cheng Y, Jacobsen JA, McCammon JA, Cohen SM. *J. Med. Chem.* 2009; 52:1063–1074. [PubMed: 19170530]
14. a) Mathews DH, Disney MD, Childs JL, Schroeder SJ, Zuker M, Turner DH. *Proc. Natl. Acad. Sci. U. S. A.* 2004; 101:7287–7292. [PubMed: 15123812] b) Watts JM, Dang KK, Gorelick RJ, Leonard CW, Bess JW Jr, Swanstrom R, Burch CL, Weeks KM. *Nature.* 2009; 460:711–716. [PubMed: 19661910]

Disease pathology for DM1 and the strategy that is used to target the causative agent, r(CUG)^{exp}. A, in wild type cells, MBNL1 regulates exon inclusion and exclusion by binding to various transcripts including cTNT pre-mRNA. In DM1-affected cells, MBNL1 is sequestered by r(CUG)^{exp} and is inactivated, resulting in a 40% increase in the cTNT exon 5 inclusion rate. C, the strategy used to cleave specifically r(CUG)^{exp}. We previously reported that a designed compound that displays two copies of a *bis*-benzimidazole (2H-4) improves DM1-associated defects *in vivo* with modest potency. Thus, 2H-4 was appended with 1-hydroxy-6-thioxo-1,6-dihydropyridine-2- carboxylic acid (HPT) to afford 2H-4-HPT. The

HPT moiety generates hydroxyl radicals upon irradiation. Therefore 2H-4-HPT can specifically cleave r(CUG)^{exp}.

Figure 2. The use of hydroxyl pyridine derivatives to photochemically generate hydroxyl radicals near targeted transcripts in living cells. Top, the mechanism by which hydroxyl radicals are generated by hydroxyl pyridines, as shown previously.^[11] Bottom, structures of the bis-benzimidazole (H) that binds the 5'CUG/3'GUC motif displayed multiple times in r(CUG)^{exp}; and, N-HPT that photochemically generates hydroxyl radicals. The synthetic schemes for producing 2H-4, 2H-4-HPT, and 2P-4-HPT are also shown.

Figure 3.

Studying the ability of 2H-4, 2H-4-HPT, and 2P-4-HPT to cleave r(CUG)₁₀ *in vitro*. Top, a plot of the amount of cleavage as a function of nucleotide position at 10 μM compound. As expected, the extent of cleavage by 2H-4-HPT is more than 2H-4 and 2P-4-HPT (does not bind r(CUG) repeats). Bottom, an autoradiogram of the gel that was quantified in the top panel. The concentrations of the compounds are 0.05, 0.1, 1, and 10 μM.

Figure 4.

2H-4-HPT improves a DM1-associated alternative pre-mRNA splicing defect in a cell culture model. In the presence of light, 2H-4-HPT shifts the cTNT alternative splicing pattern towards that observed in cells that do not express r(CUG)^{exp}. No improvement of the cTNT splicing defect was observed in the presence of light for 2H-4 or in the absence of light for 2H-4 or 2H-4-HPT, showing that HPT and light are required. The data represent the average of two transfected samples (*P < 0.05). Bottom, an autoradiogram of the gel that was quantified to produce the plot in the top panel.

Figure 5.

The results from real-time RT-PCR analysis of r(CUG)^{exp} in the presence of 2H-4-HPT with and without light. Top, schematic of the *DMPK* mini-gene that was used in these studies. Arrows indicate where primers are positioned.^[10d] Data show that 2H-4-HPT cleaves r(CUG)^{exp}. No effect on the amount of r(CUG)^{exp} is observed in the absence of irradiation. The data represent the average of two independent experiments (*P < 0.05).

Table 1

Potencies of 2H-4, 2H-4-HPT, and N-HPT for the inhibition of the r(CUG)₁₀-MBNL1 complex *in vitro* in the presence and absence of light.

Compound	IC ₅₀ (μM)	
	–light	+light
2H-4	54±6	83±12
2H-4-HPT	64±7	10±2
N-HPT	ND ^a	ND ^a

^aND means that no inhibition was detected at 120 μM, which was the highest concentration that could be tested due to solubility.