RNA Binding

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Small-Molecule-Mediated Cleavage of RNA in Living Cells**

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Dedicated to Professor Dieter Seebach on the occasion of his 75th birthday

Antisense oligonucleotides and small interfering RNAs control gene expression by triggering the degradation of mRNA by recruiting RNase H or the RNA-induced silencing complex, respectively.[1] These approaches are hampered by the poor cellular permeability of oligonucleotides. A small-molecule approach to cleave RNA targets could relieve these uptake issues. Several compounds can induce RNA cleavage in vitro,[2] however, to the best of our knowledge no small molecules have been described to cleave natural RNAs in living cells. Herein, we describe the development of a potentially general approach to design small molecules that cleave disease-associated RNAs 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 photolysis. Cleavage of the transcript improves DM1-associated defects in cell culture, and these compounds are non-toxic at an efficacious dose. This approach may allow for the site-specific cleavage and inactivation of other cellular RNAs by small molecules,[4] providing chemical genetic probes or therapeutic leads with spatial and temporal control.

Few small molecules can bind RNA and modulate cellular function. Notable cases target the bacterial ribosome or riboswitches. The ribosome comprises approximately 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 natural metabolites of the riboswitch, mirroring the substrate mimicry approaches that are a mainstay for targeting enzymes.[6] Modulating the function of other cellular RNAs is therefore difficult owing 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 developed a bottom-up approach to target RNA. That is, the RNA secondary structural elements (motifs) that preferentially bind small molecules were identified. These RNA motifs were then searched for in disease-causing RNAs. In ideal cases, the disease-causing RNA has multiple targetable motifs such that a multivalent binder can be designed to increase affinity and specificity. We have successfully applied this approach to modulate the toxicity of the expanded repeating RNAs that cause 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 dystrophia myotonica 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 inactiva-

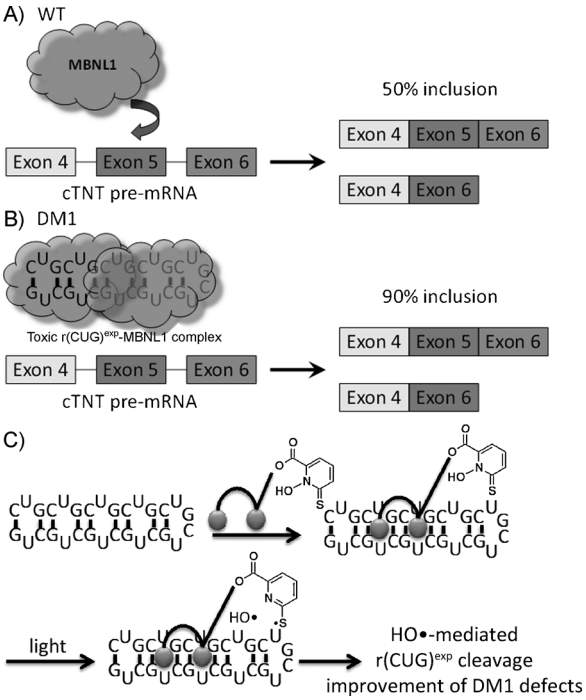

Figure 1. Disease pathology for DM1 and the strategy used to target the causative agent, r(CUG)^{exp}. A) In wild-type cells, MBNL1 regulates exon inclusion and exclusion by binding to pre-mRNAs (cTNT pre-mRNA, for example). B) In DM1-affected cells, MBNL1 is sequestered by r(CUG)^{exp} and inactivated, 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 2H-4 (for structure see Figure 2) improves DM1-associated defects in vivo with modest potency.[4b] 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 photolysis and therefore specifically cleaves r(CUG)^{exp}.

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tion, and hence alternative-splicing defects in a subset of pre-mRNAs including cardiac troponin T (cTNT), insulin receptor, and the muscle-specific chloride ion channel pre-mRNAs (Figure 1 A, B). These defects explain the heart defects, insulin insensitivity, and myotonia 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 1 C). Several compounds have validated this as a therapeutic strategy.[9a,b,11]

We previously reported a designed, modularly assembled compound that binds r(CUG)^{exp} and modulates its toxicity in vivo.[9a] This compound consists of a peptoid scaffold and two copies of a bis-benzimidazole (Ht, Figure 2), separated by four spacing modules (2H-4, Figure 2). The Ht 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 by equipping it 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 used a derivative of *N*-hydroxypyridine-2(1*H*)-thione that generates hydroxyl radicals upon photolysis to induce cleavage of r(CUG)^{exp}. Previous studies have used *N*-hydroxypyridine-2(1*H*)-thione to probe RNA structure in vitro.[12] Thus, we synthesized 1-hydroxy-6-thioxo-1,6-dihydropyridine-2-carboxylic acid (N-HPT) to conjugate a hydroxyl-radical-producing warhead onto 2H-4 using an amide bond (2H-4-HPT; Figure 2).[13] A compound without the RNA-binding (H) module (2P-4-HPT) served as a negative control.

These compounds were studied for cleaving r(CUG)$_{10}$ in vitro (Figure 3). As expected, 2H-4-HPT cleaved r(CUG)$_{10}$ upon photolysis. Some cleavage was also observed when r(CUG)$_{10}$ was incubated with 2H-4, but it was less than that of

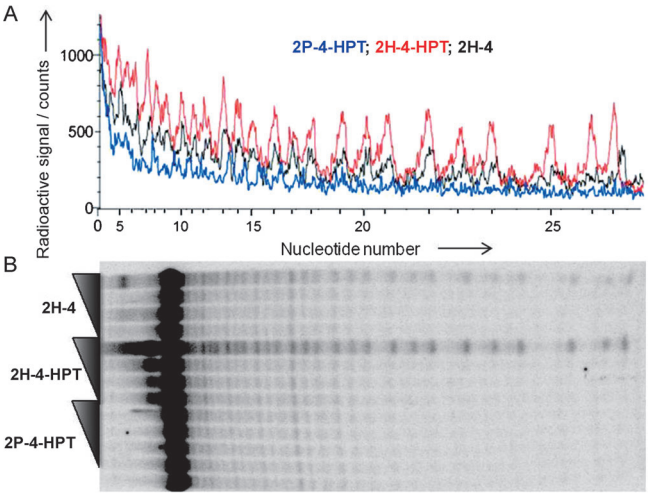

Figure 3. The ability of 2H-4, 2H-4-HPT, and 2P-4-HPT to cleave r(CUG)$_{10}$ in vitro. A) A plot of the amount of cleavage as a function of nucleotide position at 10 μM compound. As expected, the extent of cleavage by 2H-4-HPT is more than 2H-4 and 2P-4-HPT (which does not bind the r(CUG) repeats). B) An autoradiogram of the gel that was quantified in (A). The concentrations of the compounds are 0.05, 0.1, 1, and 10 μM.

2H-4-HPT. The small amount of cleavage induced by 2H-4 was mediated by Ht upon photolysis, indicating that reactive species were generated. This is supported by: 1) a slower-mobility band (corresponding to cross-linked species) that was observed when r(CUG)$_{10}$ was irradiated in the presence of 2H-4 and 2H-4-HPT but not 2P-4-HPT (Figure 3); 2) degradation of 2H-4 as a function of photolysis time was observed (Supporting Information, Figure S5); and, 3) Hoechst 33258, which is structurally related to Ht, induces strand breaks in DNA upon photolysis.[14] No cleavage was observed for 2P-4-HPT, as expected, because 2P-4 does not bind r(CUG) repeats and does not contain Ht.[9c]

Next, the in vitro potencies for inhibiting the r(CUG)$_{10}$-MBNL1 complex were determined using a time-resolved assay of fluorescence resonance energy transfer (Table 1).[9b] Two sets of experiments were completed: 1) RNA was incubated with the compound of interest and photolysed followed by addition of MBNL1; and, 2) RNA was incubated with the compound of interest followed by addition of MBNL1 and then photolysis. These experiments were also completed without photolysis (−light). The order of addition did not affect the IC$_{50}$

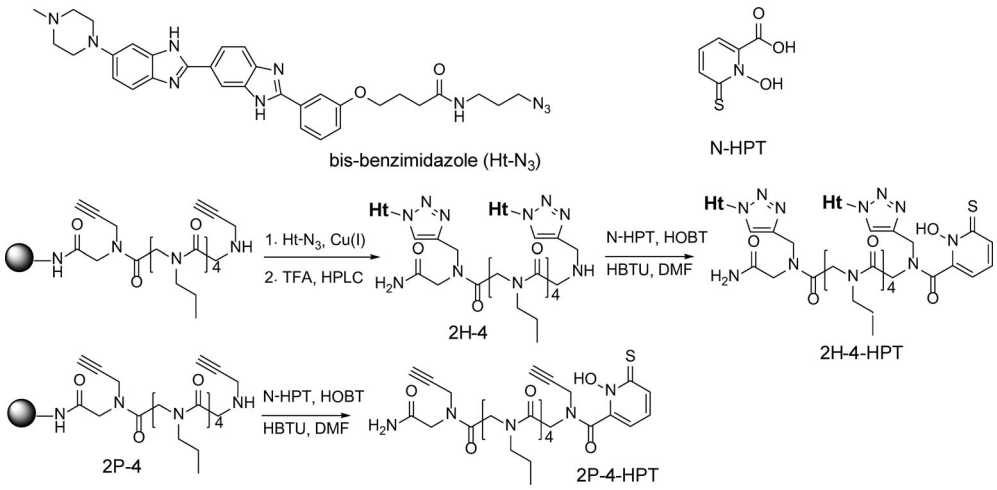

Figure 2. Top: Formulas of the bis-benzimidazole (Ht) that binds the 5′CUG/3′GUC motif displayed multiple times in r(CUG)^{exp} and N-HPT that photochemically generates hydroxyl radicals. Bottom: The synthesis for producing 2H-4, 2H-4-HPT, and 2P-4-HPT. The mechanism by which hydroxyl radicals are generated by hydroxypyridines is provided in Figure S1. TFA = trifluoroacetic acid; HOBT = 1-hydroxybenzotriazol; HBTU = 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; DMF = dimethylformamide.

Table 1: In vitro potencies of 2H-4, 2H-4-HPT, and N-HPT for the inhibition of the r(CUG)$_{10}$-MBNL1 complex in the presence and absence of light.

Compound	IC_{50} [μM][a]		IC_{50} [μM][b]	
	−light	+light	−light	+light
2H-4	54 ± 6	83 ± 12	56 ± 1	74 ± 2
2H-4-HPT	64 ± 7	10 ± 2	51 ± 2	23 ± 2
N-HPT	ND[c]	ND[c]	ND[c]	ND[c]

[a] Experiments were completed by incubating the ligand and RNA for 15 min followed by photolysis and then addition of MBNL1. [b] Experiments were completed by incubating the ligand and RNA for 15 min followed by addition of MBNL1 then photolysis. [c] ND = no inhibition detected at 120 μM, which was the highest concentration that could be tested owing to the solubility of N-HPT.

value for 2H-4 (approximately 55 μM in the absence of light and approximately 80 μM when samples are irradiated). The decrease in potency of 2H-4 when samples are irradiated was presumably due to the degradation of 2H-4 (Figure S5). In contrast, an increase in potency was observed for 2H-4-HPT upon irradiation; an approximately sixfold increase when the RNA and ligand were pre-equilibrated and photolysed prior to addition of MBNL1 and an approximately twofold increase when the sample was photolysed after addition of MBNL1. The increase in potency of 2H-4-HPT was due to degradation of r(CUG)$_{10}$, as evidenced by in vitro RNA cleavage (Figure 2). No effect on complex formation was observed with N-HPT, as expected.

Next, we used surface plasmon resonance spectroscopy to determine the affinities and the association and dissociation rates of 2H-4 and 2H-4-HPT for r(CUG)$_{10}$. 2H-4 bound to r(CUG)$_{10}$ 20-fold more tightly than 2H-4-HPT (K_{obs} of 70 nM and 1500 nM, respectively). Interestingly, the association rate (k_{on}) of 2H-4 was approximately tenfold faster than 2H-4-HPT ($3.7 \times 10^4\,M^{-1}s^{-1}$ and $4.1 \times 10^3\,M^{-1}s^{-1}$, respectively) while the dissociation rate (k_{off}) was approximately threefold slower ($2.5 \times 10^{-3}\,s^{-1}$ and $6.1 \times 10^{-3}\,s^{-1}$, respectively). The k_{on} and k_{off} of 2H-4-HPT and MBNL1 were similar ($3.28 \times 10^3\,M^{-1}s^{-1}$ and $2.03 \times 10^{-3}\,s^{-1}$, respectively). Taken together, the IC_{50} values and affinity measurements suggest that the increase in potency of 2H-4-HPT was due to cleavage of the RNA target. These studies also point to slower k_{off} and faster k_{on} as being key features that can be programmed into modularly assembled compounds targeting RNA to provide bioactivity.

Because 2H-4-HPT was a more potent in vitro inhibitor of a model r(CUG)^{exp}-MBNL1 complex than 2H-4 and it cleaved r(CUG) repeats, it is possible that it has improved potency in vivo. Therefore, 2H-4 and 2H-4-HPT were studied for improving a DM1-associated pre-mRNA splicing defect (Figure 4). 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 was observed in the absence of r(CUG)^{exp} (Figure 4). Furthermore, no toxicity was observed at these conditions (Figure S7). This suggests that even though highly reactive species were generated, they were localized to r(CUG)^{exp}, not damaging bystanding, essential biomolecules. Under identical conditions, 2H-4 did not improve pre-mRNA splicing defects, indicating that the improved bioactivity of 2H-4-HPT was due to cleavage of r(CUG)^{exp}. Additional control experiments showed that 2H-4-HPT had no effect on the alternative splicing of *PLEKHH2* pre-mRNA, which is not controlled by MBNL1 (Figure S6).[10a]

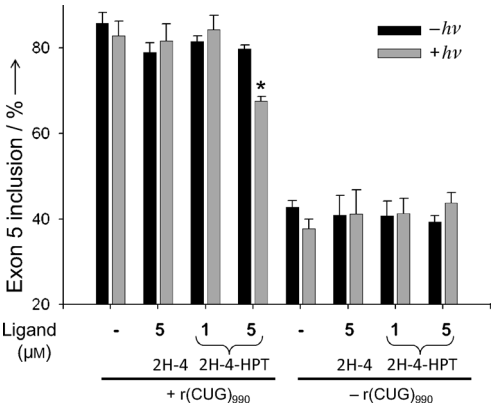

Figure 4. 2H-4-HPT improved a DM1-associated alternative pre-mRNA splicing defect in a cell-culture model. Upon photolysis, 2H-4-HPT shifted the cTNT alternative splicing pattern towards that observed in cells that do not express r(CUG)^{exp}. No improvement of the cTNT splicing defect was observed in 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. Increasing the concentration of 2H-4-HPT up to 15 μM did not further improve the pre-mRNA splicing defects (* = $p < 0.05$).

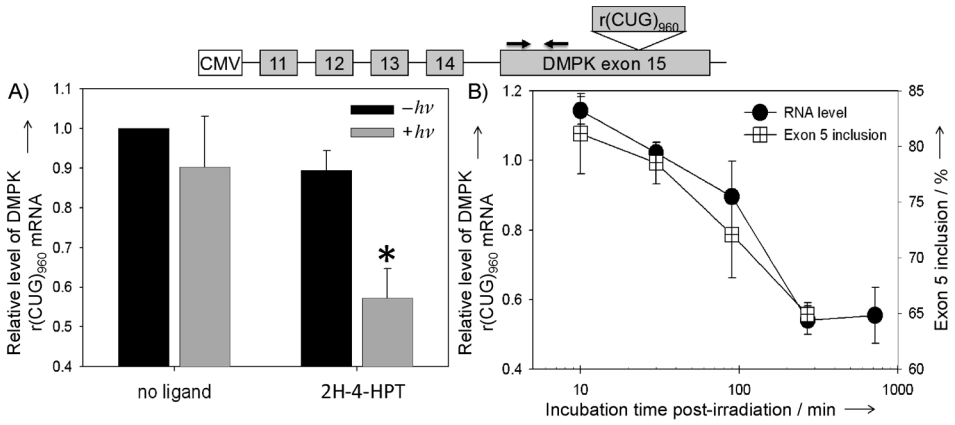

Figure 5. Results from real-time RT-PCR analysis of r(CUG)^{exp} in the presence of 2H-4-HPT with and without light. Top: Scheme of the *DMPK* mini-gene used in these studies. Arrows indicate where primers are positioned.[11d] A) 2H-4-HPT cleaved r(CUG)^{exp} after photolysis and incubation for 12 h. No effect on the amount of r(CUG)^{exp} was observed in the absence of light (no photolysis). Data represent the average of two independent experiments (* = $p < 0.05$). B) Cleavage of r(CUG)^{exp} and the amount of cTNT alternative splicing in the presence of 2H-4-HPT as a function of time after photolysis.

To determine if 2H-4-HPT cleaved r(CUG)^{exp} in living cells, total RNA was harvested from treated cells, and the amount of r(CUG)^{exp} relative to β-actin was measured by real-time RT-PCR (Figure 5).[11d] Approximately 50% of r(CUG)^{exp} was cleaved at 12–16 hours post photolysis. Moreover, a decrease in the amount of r(CUG)^{exp} transcript was observed over time and plateaued at 300 minutes (Figure 5). (This time is indicative of the rate of recruitment of the RNA degradation machinery.) The amount of r(CUG)^{exp} cleaved and the improvement in pre-mRNA splicing as functions of time were well correlated (Figure 5), further suggesting that cleavage and changes in alternative splicing are correlated. Importantly, cleavage studies validate r(CUG)^{exp} as a target for 2H-4.

In summary, we have shown that small molecules can be designed to cleave RNA in living cells and modulate their 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 to develop methods to probe RNA structure,[15] provided that the RNA-binding preferences of the small molecules were sufficiently defined.

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