Bioorganic & Medicinal Chemistry Letters 28 (2018) 2794–2796

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Small molecule alteration of RNA sequence in cells and animals

Lirui Guan [a,c], Yiling Luo [a,c], William W. Ja [b,*], Matthew D. Disney [a,*]

[a] Department of Chemistry, The Scripps Research Institute, Jupiter, FL 33458, United States
[b] Department of Neuroscience, Center on Aging, The Scripps Research Institute, Jupiter, FL 33458, United States

ARTICLE INFO

Article history:
Received 20 August 2017
Revised 15 October 2017
Accepted 16 October 2017
Available online 18 October 2017

Keywords:
RNA
Small molecules
Chemical probes
Photochemistry
Myotonic dystrophy

ABSTRACT

RNA regulation and maintenance are critical for proper cell function. Small molecules that specifically alter RNA sequence would be exceptionally useful as probes of RNA structure and function or as potential therapeutics. Here, we demonstrate a photochemical approach for altering the trinucleotide expanded repeat causative of myotonic muscular dystrophy type 1 (DM1), r(CUG)^{exp}. The small molecule, 2H-4-Ru, binds to r(CUG)^{exp} and converts guanosine residues to 8-oxo-7,8-dihydroguanosine upon photochemical irradiation. We demonstrate targeted modification upon irradiation in cell culture and in Drosophila larvae provided a diet containing 2H-4-Ru. Our results highlight a general chemical biology approach for altering RNA sequence *in vivo* by using small molecules and photochemistry. Furthermore, these studies show that addition of 8-oxo-G lesions into RNA 3′ untranslated regions does not affect its steady state levels.

RNA plays important roles in cellular biology that are dictated by sequence and structure.[1] One way to interrogate RNA function is by using chemical probes.[2,3] Although antibacterials have been useful for probing the structure and function of the ribosome,[4] routine identification of ligands for diverse RNA targets has proven difficult. We previously developed a lead identification strategy in which the secondary structure of a disease-causing RNA is queried against an RNA motif-small molecule database.[2,3,5] We used this approach to identify a dimeric ligand (**2H-4**) that binds repeating secondary structural elements in r(CUG)^{exp} an expanded ("^{exp}") trinucleotide repeat implicated in myotonic muscular dystrophy type 1 (DM1).[6–8] **2H-4** targets r(CUG)^{exp} in cells and improves various aspects of DM1-associated defects.[9,10]

In subsequent work, we conjugated hydroxythiopyridine (HPT) to **2H-4** (**2H-4-HPT**) to deliver hydroxyl radicals to r(CUG)^{exp} and cleave it upon irradiation.[11] Although this approach was promising, only a single reactive hydroxyl radical is produced per HPT moiety. A potentially superior approach for photochemical control of RNA function would be to use Tris(bipyridine)ruthenium(II), which enables the catalytic production of singlet oxygen upon irradiation (Fig. 1A).[12] In a cell-free environment, singlet oxygen converts RNA guanosine (G) bases to 8-oxo-7,8-dihydroguanosine (8-oxoG) (Fig. 1B).[13,14] This approach would facilitate studies on the fate of oxidized RNA transcripts, which could include targeted degradation, as well as provide additional means to affect RNA function *in vivo*.

The trinucleotide repeat disorder DM1 is an incurable neuromuscular disease that is caused by an RNA gain-of-function mechanism in which non-coding r(CUG)^{exp} binds and sequesters proteins such as muscleblind-like 1 protein (MBNL1), knocking out its function and triggering disease.[6,8,16] To determine if we could alter the sequence of r(CUG)^{exp} by using a small molecule, we appended **2H-4** with Tris(bipyridine)ruthenium(II) [Ru(bipy)$_3$] to generate **2H-4-Ru** (Figs. 1A and S1). Briefly, an aminohexanoate linker was added to **2H-4** to provide a reactive amine handle onto which an activated ester of Ru(bipy)$_3$ could be attached. Exclusion of the RNA-binding module, **Ht**, produced the control compound, **2P-4-Ru** (Figs. 1A and S2), which does not bind to r(CUG)^{exp}. The **2H-4** compound has been shown to avidly recognize RNAs with two consecutive copies of 5′CUG/3′GUC 1 × 1 motifs that are present in r(CUG)^{exp} and shortened models thereof such as r(CUG)$_{10}$ as previously described.[9]

To study the ability of **2H-4-Ru** to photochemically alter r(CUG)^{exp} sequences *in vitro*, the compound was incubated with 5′-end–labeled r(CUG)$_{10}$, irradiated, and treated with aniline, which cleaves RNA at positions containing 8-oxoG.[14] Polyacrylamide gel (PAGE) analysis showed aniline-mediated cleavage occurs at Gs when r(CUG)$_{10}$ was treated with **2H-4-Ru** and irradiated, but not with the control (**2P-4-Ru**) in the presence or absence of light (Fig. 2A), suggesting that Gs were specifically oxidized to 8-oxoG by **2H-4-Ru** upon photoactivation.[14] To further confirm that 8-oxoG was installed into r(CUG)$_{10}$, an ELISA-based approach with

* Corresponding authors.
E-mail address: disney@scripps.edu (M.D. Disney).
[c] These authors contributed equally to this work.

https://doi.org/10.1016/j.bmcl.2017.10.034
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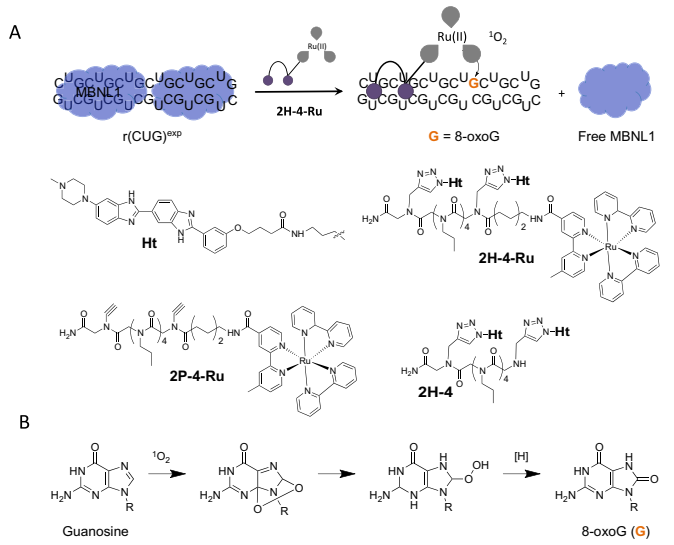

Fig. 1. A designer small molecule that targets r(CUG)^{exp}, the causative agent of DM1, was appended with Tris(bipyridine)ruthenium(II) [Ru(bipy)$_3$] to generate reactive oxygen species that can oxidize G residues to 8-oxoG. A, Scheme showing binding and oxidation of RNA by 2H-4-Ru and subsequent release of MBNL1. The Ru(bipy)$_3$ warhead produces singlet oxygen upon photoactivation. Other controls tested in this study include 2H-4, the binding module alone, and 2P-4-Ru, which lacks the RNA-binding component (Ht). The RNA binding modules are defined by the purple circles, and the linkers that span them and Ru(bipy)$_3$ indicated with gray lines. B, Singlet oxygen oxidizes guanine and produces 8-oxoG lesions.[15] The complete synthesis of these compounds is described in Schemes S-1 and S-2 (Supporting Information).

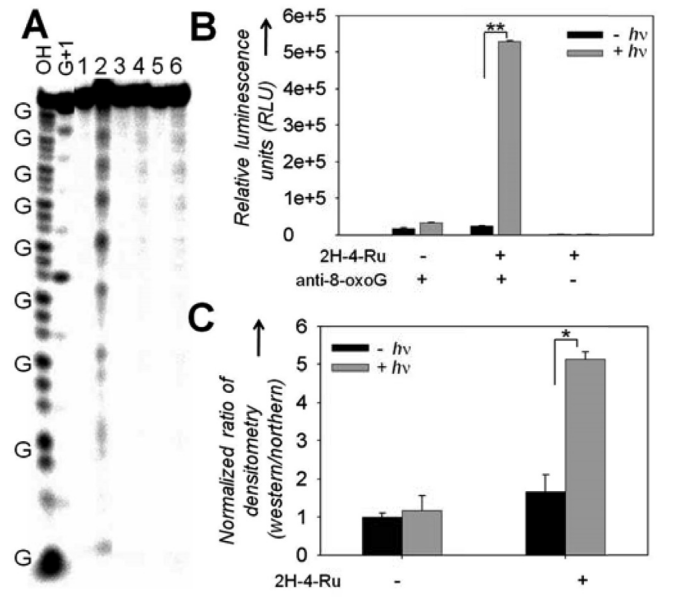

Fig. 2. *In vitro* studies of 8-oxoG formation in r(CUG)^{exp} by **2H-4-Ru** upon photoactivation. A, Gel electrophoresis of r(CUG)$_{10}$ shows 8-oxoG formation after incubation with **2H-4-Ru** and photoactivation in the presence of 50-fold excess total tRNA. Lanes: OH, alkaline hydrolysis; G+1, RNase T1 digest; 1, **2H-4-Ru** and light; 2, **2H-4-Ru** and light followed by aniline treatment; 3, **2P-4-Ru** and light; 4, **2P-4-Ru** and light followed by aniline treatment; 5, RNA only and light; 6, RNA only and light followed by aniline treatment. B, ELISA using an anti-8-oxoG antibody confirms the formation of 8-oxoG in r(CUG)$_{10}$ by **2H-4-Ru** upon photoactivation. C, Northwestern blot analysis shows an increase in the amount of 8-oxoG bases in r(CUG)^{exp} when cells were treated with **2H-4-Ru** and light (see also Fig. S3). *, $p < 0.05$; **, $p < 0.01$.

an anti-8-oxoG antibody was used. Biotinylated r(CUG)$_{10}$ was bound to a streptavidin-coated microtiter plate and treated with compound in the presence or absence of light. Upon probing with anti-8-oxoG antibody conjugated with horseradish peroxidase (HRP), 8-oxoG bases were only present in samples that were incubated with **2H-4-Ru** and irradiated (Fig. 2B). We also completed ESI-MS-MS analysis of r(CUG)$_{10}$ after treatment with **2H-4-Ru** or **2P-4-Ru** and nuclease digestion, using a previously studied RNA as a standard.[17] Indeed, G was only oxidized to 8-oxoG with **2H-4-Ru** (or riboflavin as positive control[18]) upon photoactivation (Figs. S-3 to S-6). Thus, all three independent studies show that small molecules can be used *in vitro* to alter the base composition of RNA transcripts.

After confirming that **2P-4-Ru** and **2H-4-Ru** are non-toxic and cell permeable (Fig. S-7), we tested the ability of **2H-4-Ru** to install 8-oxoG into r(CUG)^{exp} using a cellular model of DM1 (HeLa cells that express r(CUG)$_{960}$ repeats). After treatment with compound and irradiation, total RNA was isolated and analyzed via Northwestern blot. That is, a Northern blot was used to detect r(CUG)^{exp} and was then also probed with an 8-oxo-G antibody (Western blot) to detect 8-oxoG modifications in r(CUG)$_{960}$. Indeed, 8-oxoG lesions were found in r(CUG)^{exp} after **2H-4-Ru** treatment and irradiation (Figs. 2C and S-8). To further verify that r(CUG)^{exp} was selectively oxidized, immunoprecipitation (IP) experiments were carried out using anti-8-oxoG antibody-coated magnetic beads, which capture 8-oxoG-containing transcripts harvested from cells (Fig. S9). Quantification of captured RNA by RT-qPCR showed enrichment of r(CUG)$_{960}$ only after drug and light treatment (Fig. S6). Apparently, photochemistry can be used to alter RNA sequence in a cellular system. A RT-qPCR analysis of the r(CUG)$_{960}$-containing mRNA shows that its steady state levels are not affected in cells (Fig. S10). Interestingly, a previous study showed that 8-oxoG lesions induce No-Go decay of the corresponding transcript.[17] In those studies, the lesions were present in a coding region. It is possible that levels of the r(CUG)$_{960}$-containing mRNA were unaffected because it is present in a UTR. Collectively, RNA repeat expansions in coding regions, such as the expanded r(CAG) repeats that cause Huntington's disease, could be targeted and destroyed by introduction of 8-oxoG lesions and subsequent induction of No-Go decay.

Invertebrates such as *Drosophila melanogaster*, the fruit fly, have emerged as powerful models for studying the genetic mechanisms and pathophysiology of neurodegenerative diseases.[19] Their small body size, low cost of maintenance, and short lifespan also make them attractive platforms for *in vivo* testing of potential therapeutics. Previously, a *Drosophila* model of DM1 was developed by expression of r(CUG)$_{250}$ in the 3′ untranslated region (UTR) of a control gene, DsRed.[20] We took advantage of this model to test whether **2H-4-Ru** would show efficacy *in vivo*. *Drosophila* 1st instar larvae developed on food dosed once with **2H-4-Ru**. After several days, 3rd instar live larvae were irradiated once and total RNA was harvested. Immunoprecipitation of 8-oxoG-containing transcripts showed that DsRed is enriched upon **2H-4-Ru** treatment and irradiation, suggesting that r(CUG)$_{250}$ was selectively modified (Fig. 3). Again, the r(CUG)$_{250}$ transcript upon photoactivation is stable without significant alteration in RNA levels (Fig. S11).

Importantly, these approaches demonstrate another approach to validate the cellular and animal targets of small molecules directed at RNA. For example, Chemical cross linking and isolation by pull down (Chem-CLIP) and small–molecule nucleic acid profiling by cleavage applied to RNA (Ribo-SNAP) have provided cross-linking and cleavage approaches, respectively, to read out the RNA targets of small molecules.[11,21–23] The ability to iummunopreciptate RNAs with 8-oxo-G lesions as introduced via small molecules

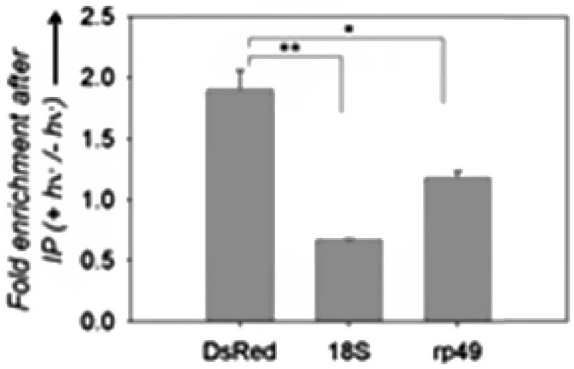

Fig. 3. 2H-4-Ru modified the sequence of r(CUG)$_{250}$ in a *Drosophila* model of DM1. RT-qPCR analysis of DsRed-r(CUG)$_{250}$ and the control genes, *18S* and *rp49*, after anti-8-oxoG immunoprecipitation of RNA from fly larvae treated with **2H-4-Ru**. Greater enrichment of DsRed with irradiation suggests that DsRed-r(CUG)$_{250}$ is selectively modified. Pull-down of 18S and rp49 may suggest a lack of exquisite selectivity of the antibody.

offers an additional approach to target validation and also validates r(CUG)^{exp} as a target for small molecules in animals for the first time.

In conclusion, we have demonstrated that small molecules that bind to RNA targets can alter RNA sequence when a photosensitizer is added to the compound. In addition, these studies have facilitated an understanding of the fate of oxidized RNAs in cells and animals, showing that abundance of modified RNAs do not significantly change, at least with the over-expressed targets used in these cases. This approach may have application to other targets for studying and manipulating RNA function both *in vitro* and *in vivo*. Since many neurological disorders produce reactive oxygen species that modify RNA bases, our approach may inform future studies to determine the specific effect of these modifications on disease-associated RNAs and to establish whether they are associative or causative of disease.

Acknowledgments

This work was funded by the US National Institutes of Health (grants DP1NS096898, MDD, and R01AG045036, WWJ) and the Muscular Dystrophy Association (grant 380467).

A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bmcl.2017.10.034.

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