RNA Targeting

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A Toxic RNA Catalyzes the In Cellulo Synthesis of Its Own Inhibitor**

*Suzanne G. Rzuczek, HaJeung Park, and Matthew D. Disney**

Abstract: *Potent modulators of RNA function can be assembled in cellulo by using the cell as a reaction vessel and a disease-causing RNA as a catalyst. When designing small molecule effectors of function, a balance between permeability and potency must be struck. Low molecular weight compounds are more permeable whereas higher molecular weight compounds are more potent. The advantages of both types of compounds could be synergized if low molecular weight molecules could be transformed into potent, multivalent ligands by a reaction that is catalyzed by binding to a target in cells expressing a genetic defect. It was shown that this approach is indeed viable in cellulo. Small molecule modules with precisely positioned alkyne and azide moieties bind adjacent internal loops in r(CCUG)^{exp}, the causative agent of myotonic dystrophy type 2 (DM2), and are transformed into oligomeric, potent inhibitors of DM2 RNA dysfunction by a Huisgen 1,3-dipolar cycloaddition reaction, a variant of click chemistry.*

RNA dysfunction causes disease through various mechanisms, including microRNA silencing of pro-apoptotic proteins,[1] translation of aberrant protein,[2] and gain of function.[3] It has been difficult, however, to design small molecule chemical probes of RNA function or lead therapeutics. If broadly applicable methods were developed to drug non-ribosomal RNAs with small molecules, they could have important applications in chemical biology and medicinal chemistry.[4] One class of RNA-mediated diseases is caused by expanded repeating RNAs, or microsatellite disorders. There are more than 20 known microsatellite disorders, including myotonic dystrophy (DM) and amyotrophic lateral sclerosis (Lou Gehrig's Disease; ALS).[5] The cellular consequences of repeats are varied and can include alterations at the protein, RNA, and DNA levels.

Previously, we designed inhibitors of the RNA that causes myotonic dystrophy type 2 (DM2) that were based on RNA motif–small molecule interactions.[6] DM2 is caused by a toxic gain of function by a r(CCUG) repeat expansion (r(CCUG)^{exp}) located in intron 1 of the zinc finger protein 9 (*ZNF9*) pre-mRNA.[7] The RNA folds into a hairpin structure that contains repeating units of 5′CCUG/3′GUCC (2×2 nucleotide internal loops) in the stem. The loops form high-affinity binding sites for muscleblind-like 1 protein (MBNL1), a regulator of alternative pre-mRNA splicing, which is inactivated upon binding (Figure 1a). Our designed inhibitors of r(CCUG)^{exp} are based on a kanamycin A derivative, which is acylated at the 6′ position and binds 5′CCUG/3′GUCC with high affinity. Notably, acylation of the amine at the 6′ position ablates binding to rRNA.[8] Indeed, the kanamycin derivative and modularly assembled (or multivalent) compounds thereof improve DM2-associated defects in a cellular model.[6a] As observed for other repeat expansions, modularly assembled compounds are more potent inhibitors of cellular dysfunction, presumably because of their high affinities, selectivities, and the larger surface areas that they sequester on the target.[6a] Even though multivalent compounds are more potent and selective in vitro, in some cases in cellulo potency decreases as a function of valency because they are less cell-permeable.[9] Thus, a careful balance must be struck between ligand size, potency, and permeability.

Given the above considerations, we sought to develop a strategy to target RNA repeat expansions that could exploit the cellular permeability of the small molecule "modules" and the potency and selectivity of higher molecular weight multivalent compounds. In situ click chemistry, by a Huisgen 1,3-dipolar cycloaddition reaction (HDCR), could provide such a strategy. That is, two modules would bind adjacent sites in the target, bringing otherwise unreactive groups into close proximity to form a covalent bond; in particular, azide and alkyne moieties react to form stable triazoles.[10] Indeed, this approach has been used in vitro to target acetylcholine esterase and the DNA minor groove.[10,11] Translating such an approach to cellular systems could be of high impact, enabling the development of highly selective chemical biology probes that catalyze the synthesis of inhibitors only in cells expressing a disease-causing biomolecule, but this has not been demonstrated thus far. Expanded repeating RNAs are perhaps ideal targets for this approach because they are modular like the compounds that they will template (Figure 1a).

The development of an in cellulo, in situ click approach for r(CCUG)^{exp} was enabled by using a model of the binding of dimeric 6′-*N*-acylated kanamycin A (2K-4) to r(CCUG) repeats.[6a] Analysis of this model showed that an azido group at the 6″ position (N_3-K) and an alkyne group at 6′ position (K-Ak) could be within close enough proximity to react upon binding to adjacent 2×2 nucleotide internal loops in r(CCUG)^{exp} (Figure 1b). Therefore, when N_3-K and K-Ak are mixed in equal amounts, a dimer can be formed; likewise, a derivative that has both a 6″ azide and a 6′ alkyne (N_3-K-Ak; Figure 1b) can form an oligomer. We also synthesized a compound with an activated, electron-deficient alkyne, N_3-

[*] Dr. S. G. Rzuczek, Dr. H. Park, Prof. Dr. M. D. Disney
The Scripps Research Institute
Department of Chemistry
130 Scripps Way, #3A1, Jupiter, FL (USA)
E-mail: disney@scripps.edu

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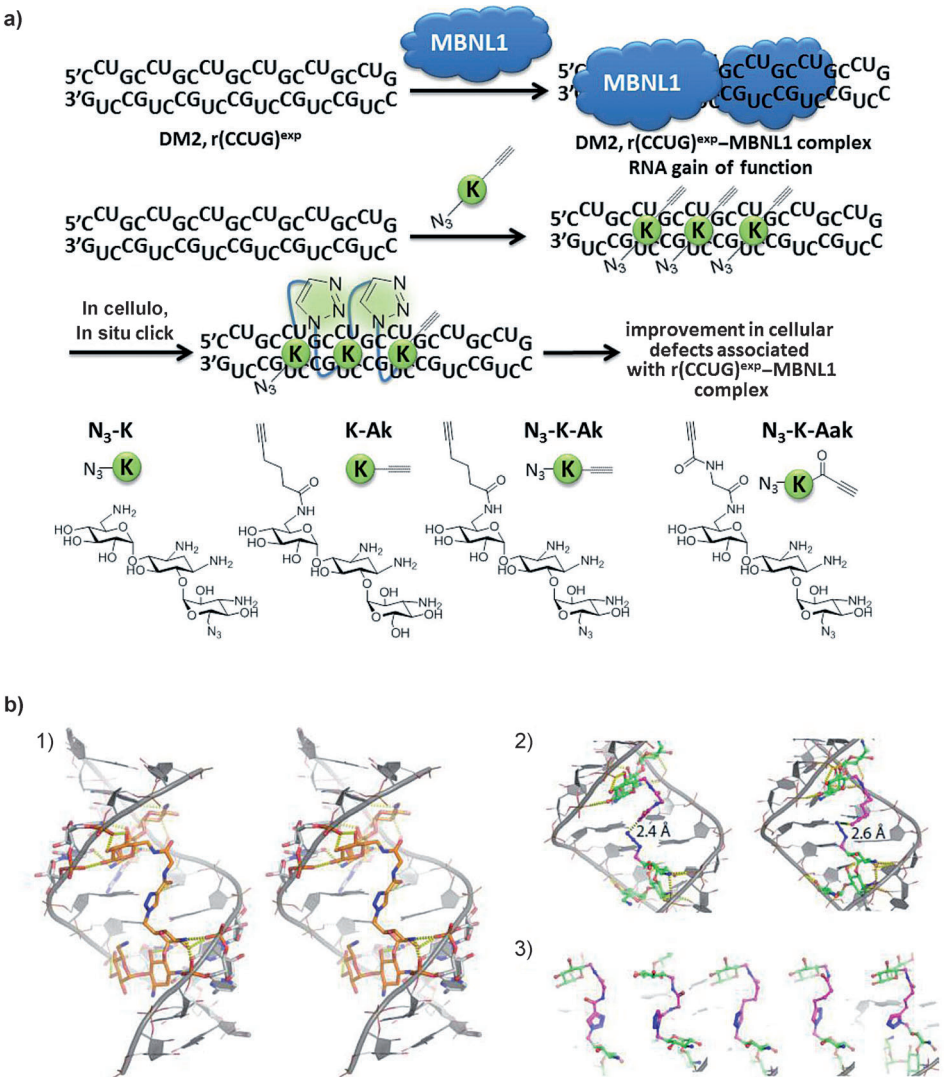

Figure 1. In cellulo, in situ click chemistry to synthesize potent inhibitors of the RNA that causes DM2. a) DM2 is caused by a r(CCUG) repeat that binds and sequesters the protein muscleblind-like 1 (MBNL1). Small molecules that contain azide and alkyne functional groups (N_3-K, K-Ak, N_3-K-Ak, and N_3-K-AaK) bind adjacent sites in r(CCUG)^{exp} and undergo a Huisgen dipolar cycloaddition reaction. b) Molecular dynamics (MD) simulation models of clickable modules binding to a mimic of r(CCUG)^{exp}. 1) A low-energy state in the MD simulation of the 1,4-triazole adduct from N_3-K and K-Aak is shown in stereoview. The RNA is shown as a grey cartoon, and the K dimer is shown as an orange stick model. Hydrogen bonds between the RNA and the K dimer are shown as yellow dashed lines. 2) Conformational searching reveals a close proximity between azide and alkyne groups of the K modules bound to adjacent sites. The linkers are highlighted as magenta sticks, and K moieties are shown as green sticks. 3) Low-energy snapshot of MD simulations showing other linker models. The coloring scheme is the same as in (2).

K-Aak (Figure 1 a), which was employed by the Dervan group to assemble polyamides using DNA as a template.[11b] All compounds were synthesized by using variations of known routes (Figure 1; see also the Supporting Information, Figure S1–S5).[6b,12]

We first tested the ability of r(CCUG)$_{12}$ (Figure S6) to template assembly of K oligomers in vitro. After incubation, reaction products were analyzed by mass spectrometry. Indeed, higher-valency compounds were formed in the presence of r(CCUG)$_{12}$, but not in its absence (Figure S7). To determine if this templated reaction was specific to r(CCUG)$_{12}$, we studied the abilities of other RNAs to catalyze oligomer formation, including r(CUG)$_{12}$, r(AUUCU)$_{12}$, r(CGG)$_{12}$, r(CAG)$_{12}$, an RNA hairpin with a fully paired stem, and tRNA (Figure S6). Importantly, no significant reaction products were observed for all RNAs, except for r(CUG)$_{12}$, for which a small amount of dimer was observed (<10%; Figure S8). It is not surprising that r(CUG)$_{12}$ templated a small amount of the dimer as it binds K, albeit with a much lower affinity than r(CCUG)$_{12}$.[13] Furthermore, the optimal distance that separates K RNA-binding modules in a multivalent compound is much shorter for r(CUG)$_{12}$ than for r(CCUG)$_{12}$.[13] Taken together, these studies illustrate that the templated reaction is selective for r(CCUG)$_{12}$ and controlled by the positioning of the functional groups, the RNA-binding module, and the RNA target (Figure S8). These studies provided impetus for testing this approach in cellular systems and to study the biological impact of the templated compounds on DM2-associated defects, which include alternative pre-mRNA splicing defects and the formation of nuclear foci.[3a]

To confirm that multivalent compounds are indeed templated in cellulo, a cellular model system in which r(CCUG)$_{300}$ is expressed was employed.[14] Cells were co-treated with N_3-K and N_3-K-Ak or N_3-K-Aak. N_3-K was used to poison the reaction to limit the molecular weights of the products, allowing for mass spectral analysis. After treatment, reaction products were partially purified from cell lysates by precipitating cellular material and proteins with organic solvent. Mass spectral analysis of purified fractions showed that oligomerization occurred in cells expressing r(CCUG)$_{300}$, as both dimeric and trimeric reaction products were observed when cells were treated with N_3-K-Ak or N_3-K-Aak (Figure S9–S11). Importantly, oligomerization was not observed in cells that did not express the RNA (Figure S9–S11). Thus, templated synthesis only oc-

curred in disease-affected cells, suggesting that r(CCUG)$_{300}$ catalyzes the synthesis of its own inhibitor.

To confirm the extent of the templated reaction and that r(CCUG)$_{300}$ was indeed the catalyst for oligomerization, we developed and implemented an approach termed ChemReactBIP (Chemical Reactivity and Binding Isolated by Pull-down; Figure 2a). Cells were treated with K-Ak, N_3-K-Ak, or N_3-K-Aak in the presence of a kanamycin analogue that contains a biotin moiety at the 6′ position (N_3-K-Biotin; Figures S5 and S12–S14). N_3-K-Biotin terminates the polymerization reaction in cellulo and allows oligomerized compounds and their bound cellular targets to be captured with streptavidin beads after gentle lysis (Figure 2b,c). In agreement with studies completed with N_3-K as described above, oligomerized products were only formed in cells that expressed r(CCUG)$_{300}$, and the conversion of monomer into products was greater for N_3-K-Aak than for N_3-K-Ak, as expected (Figure 2b).[11b] ChemReactBIP also allowed us to identify the cellular targets of the templated reaction. A qRT-PCR analysis of the pulled-down fraction confirmed that r(CCUG)$_{300}$ is a major target of the products of N_3-K-Biotin and K-Ak, N_3-K-Ak, or N_3-K-Aak (Figure 2c; qRT-PCR = quantitative real-time polymerase chain reaction). The amount of r(CCUG)$_{300}$ target pulled down increased as a function of potency: N_3-K ≈ K-Ak < N_3-K-Ak < N_3-K-Aak (see below). As observed in our other experiments, multivalent compounds were only observed when cells expressed r(CCUG)$_{300}$ (Figure 2b).

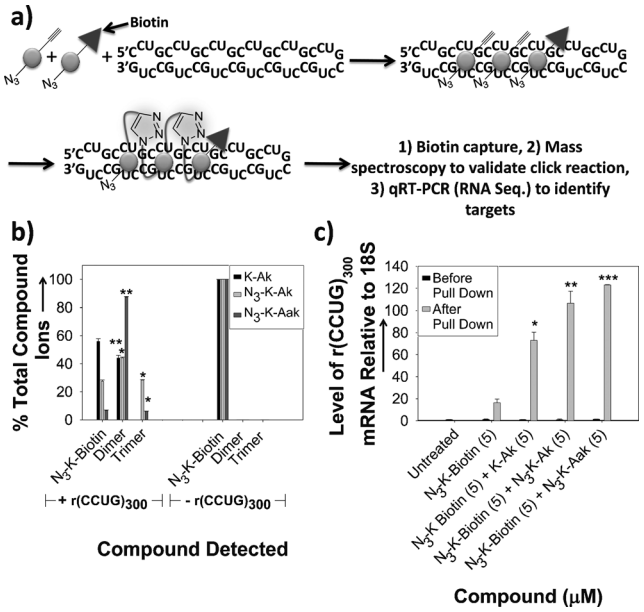

Figure 2. Identifying the extent of in cellulo click reactions and the targets of clickable small molecules. a) Schematic representation of ChemReactBIP, an approach to identify the cellular targets of small molecules. Studies were enabled by using a biotinylated monomer with a single N_3 group to participate in the click reaction, or N_3-K-Biotin, which allowed the isolation of the clicked oligomer and bound RNA targets by passing cell lysates over streptavidin resin. b) Results of the ChemReactBIP mass spectral analysis of pulled-down, templated small molecule products that were only synthesized in cells expressing r(CCUG)$_{300}$. c) Results of ChemReactBIP experiments in which the RNA targets of the clicked products were isolated and analyzed by qRT-PCR. * indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$ as determined by a two-tailed Student's t-test ($n = 3$).

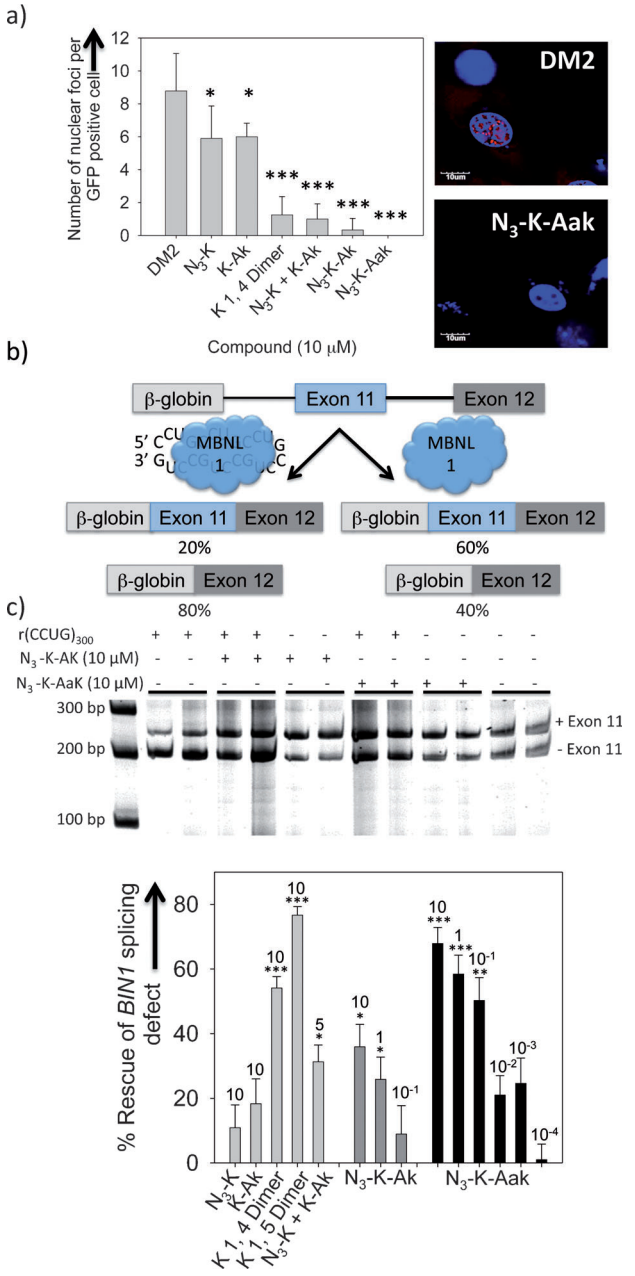

Figure 3. Results of cellular studies evaluating the effect of in cellulo, in situ click reactions on DM2-associated defects. a) Evaluation of click compounds for inhibiting the formation of nuclear foci of r(CCUG)$_{300}$. b) Schematic representation of the alternative splicing of bridging integrator 1 (*BIN1*) pre-mRNA exon 11 in healthy cells and in cells that express r(CCUG)$_{300}$. c) Rescue of the *BIN1* splicing defect by templated click reactions. Top: Representative gel image of *BIN1* splicing products in the presence and absence of r(CCUG)$_{300}$. Bottom: Quantification of *BIN1* splicing patterns in treated and untreated cells. The concentration of each compound is indicated above the corresponding bar in μM. For N_3-K and K-Ak, 5 μM indicates the concentration of each compound. * indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$, as determined by a two-tailed Student's t-test ($n \geq 3$).

As observed in other microsatellite disorders, the binding of various proteins to r(CCUG)^{exp} causes formation of nuclear foci.[3a] Fluorescence in situ hybridization (FISH) with a dye-labeled oligonucleotide was employed to determine if our compounds inhibited the formation of r(CCUG)^{exp}-containing nuclear foci (Figure 3a). In untreated cells, the average number of foci per cell was 9 ± 2. Treatment with N_3-K or K-Ak reduced the average number of foci per cell to 6 ± 2 and 6 ± 1, respectively. Treatment with an equimolar mixture of K-Ak and N_3-K (K-Ak + N_3-K) reduced the number of foci to 1 ± 1 per cell, similar to pre-synthesized dimers that mimic the reaction products of K-Ak and N_3-K (K 1,4 dimer and K 1,5 dimer; Figures S1 and S2). N_3-K-Ak and N_3-K-Aak, derivatives that click to self-oligomerize in cellulo, were even more potent, with $\ll 1$ foci per cell observed. Therefore, the extent of oligomerization correlates with bioactivity.

Disruption of nuclear foci suggests that proteins sequestered by r(CCUG)^{exp} are being freed. Thus, it is likely that the compounds also improve alternative pre-mRNA splicing defects that are caused by sequestration of MBNL1 in foci.[3a] We studied the ability of our compounds to improve dysregulation of the bridging integrator 1 (*BIN1*) pre-mRNA (Figure 3b,c; see also Figures S15, S16).[14] When r(CCUG)$_{300}$ is present in cells, exon 11 is skipped too frequently, resulting in an inclusion rate of approximately 20% in the mature mRNA. In contrast, the exon 11 inclusion rate is approximately 60% in unaffected cells. Interestingly, the trend observed for compound potency as measured by disruption of foci was mirrored by our results for improvement of the *BIN1* alternative splicing defect (Figures 2 and 3). For example, pre-synthesized dimers and N_3-K and K-Ak improved *BIN1* alternative splicing patterns to a similar extent, whereas N_3-K-Ak and N_3-K-Aak were more potent (Figure 3c). Notably, N_3-K-Aak retained its ability to improve the splicing defect at nanomolar concentrations, which corresponds to a >100-fold improvement over our first-generation modularly assembled compound[6a] and >1000-fold improvement over the monomers N_3-K and K-Ak. Importantly, these compounds did not affect *BIN1* splicing patterns in cells that did not express r(CCUG)$_{300}$ (Figure S17). Collectively, the compounds that participate in a templated click reaction are the most potent non-covalent compounds that improve DM2-associated spliceopathy known to date. Furthermore, improvements in pre-mRNA splicing are due to binding r(CCUG)$_{300}$ and not to a non-specific effect.

In summary, these studies establish that an HDCR can be used to template the synthesis of an RNA inhibitor in cellulo and only in disease-affected cells. There may be numerous applications of this method, as has been previously articulated by Sharpless and co-workers.[10] RNA repeating disorders may be a particularly attractive application. The nature of the target, which could have thousands of repeating units, could lead to high yields of templated products. Moreover, many RNA gain-of-function disorders, such as ALS, DM1, and DM2, cause brain dysfunction, making it important to develop low molecular weight compounds with the potential to cross the blood–brain barrier. Taken together, the click reaction could engender highly permeable low molecular weight monomers with potencies of multivalent compounds in both cellular and tissue models of disease.

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