Intrinsically cell-penetrating multivalent and multitargeting ligands for myotonic dystrophy type 1

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Developing highly active, multivalent ligands as therapeutic agents is challenging because of delivery issues, limited cell permeability, and toxicity. Here, we report intrinsically cell-penetrating multivalent ligands that target the trinucleotide repeat DNA and RNA in myotonic dystrophy type 1 (DM1), interrupting the disease progression in two ways. The oligomeric ligands are designed based on the repetitive structure of the target with recognition moieties alternating with bisamidinium groove binders to provide an amphiphilic and polycationic structure, mimicking cell-penetrating peptides. Multiple biological studies suggested the success of our multivalency strategy. The designed oligomers maintained cell permeability and exhibited no apparent toxicity both in cells and in mice at working concentrations. Furthermore, the oligomers showed important activities in DM1 cells and in a DM1 liver mouse model, reducing or eliminating prominent DM1 features. Phenotypic recovery of the climbing defect in adult DM1 *Drosophila* was also observed. This design strategy should be applicable to other repeat expansion diseases and more generally to DNA/RNA-targeted therapeutics.

multivalent ligand | DNA/RNA-targeting therapeutics | cell-penetrating peptide mimic | myotonic dystrophy type 1 | trinucleotide repeat expansion diseases

Multivalency is an effective strategy for the development of potent and selective ligands, especially at cell surfaces. The thermodynamic and kinetic advantage of multiple binding moieties interacting with many receptor sites is attributed to several possible factors, including steric stabilization, the chelation effect, and concentration effects (1–3). For the maximum benefit of multivalency to accrue, there must be a suitable spatial arrangement of the ligands and a strain-free, ligand-receptor “fit” upon complexation. Dimeric ligands can be viewed as simplified versions of multivalent ligands, and there is ample evidence that optimization of the linkage between two binding motifs is necessary to obtain significant affinity improvement (4–6). However, rather than using an optimized dimeric linkage, multivalent ligands are often prepared by covalent attachment of ligands to readily available extrinsic scaffolds, such as a PNA or dendrimer. In these cases, the ease of synthesis often comes with loss of tunability and spatial optimization (7–9). A separate consideration comes into play when the target is intracellular. Namely, the increase in mass can reduce cell permeability, although the use of a cell-penetrating peptide (CPP) as the scaffold can help (9).

Trinucleotide repeat expansion diseases (TREDs) are outstanding targets for exploring intracellular multivalent approaches, because the pathogenic targets involve regular and repeating sequences of DNA and RNA. For example, myotonic dystrophy type 1 (DM1), an incurable TRED, originates in expanded CTG repeats [d(CTG)^{exp}] in the 3'-UTR of the *DMPK* gene on chromosome 19 (Fig. 1). An RNA gain-of-function model for DM1 involves r(CUG)^{exp} transcripts altering the level of splicing regulators, such as muscleblind-like 1 (MBNL1) and CUG binding

protein 1 (CUGBP1), leading to misregulation of pre-mRNA splicing (10–13).

Recent reports shed light on additional pathobiology of DM1, such as microRNA dysregulation (14) and repeat-associated non-ATG (RAN) translation (15, 16). Nonetheless, the sequestration of MBNL1 by r(CUG)^{exp}, and their coaggregation into distinctive nuclear foci is considered as a hallmark of DM1 (17). Importantly, MBNL1 knockout mouse model studies have reported that >80% of DM1 missplicing defects and related symptoms are directly associated with the loss of MBNL1 by r(CUG)^{exp} (18, 19). Thus, therapeutic efforts focus on inhibiting the transcription of d(CTG)^{exp} to reduce r(CUG)^{exp} levels (20–22) or on inhibiting the interaction of r(CUG)^{exp} with MBNL1 (23–27). Here, we report an oligomeric ligand that targets both d(CTG)^{exp} and r(CUG)^{exp} in a multivalent fashion and contains a structure designed to mimic CPPs for inherent cell permeability (Fig. 1). The mixture of oligomers reduces the disease foci and toxic r(CUG)^{exp} level in cellular assay and shows promising in vivo activity using a known adult DM1 *Drosophila* phenotypic assay and a liver-specific DM1 mouse model.

Significance

Multivalent ligands have tremendous potential as therapeutic agents; however, their efficacy is limited by delivery issues, poor cell permeability, and toxicity. We report here a strategy wherein multivalent ligands are designed to be intrinsically cell-penetrating, allowing them to target the expanded trinucleotide repeat sequences of DNA and RNA that cause myotonic dystrophy type 1 (DM1). The multivalent ligand studied shows cell permeability and low toxicity both in cells and in mice. Importantly, the ligand reduced or eliminated DM1 defects in DM1 cells and in vivo, validating the multivalent strategy. The approach should be broadly applicable to other repeat expansion diseases and to any multivalent oligomeric therapeutic agent whose activity can accommodate structural elements that mimic cell-penetrating peptides.

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Fig. 1. DM1 pathogenesis and therapeutic strategy. CTG expanded repeats in 3'-UTR of the *DMPK* gene is transcribed to $r(\text{CUG})^{\text{exp}}$, forming a hairpin secondary structure with U–U mismatches. The structured RNA sequesters MBNL1 and causes splicing misregulation and other DM1 defects. A designed multivalent ligand has a multitargeting ability toward $d(\text{CTG})^{\text{exp}}$ and $r(\text{CUG})^{\text{exp}}$. The oligomeric inhibitor blocks transcription of $d(\text{CTG})^{\text{exp}}$ by $d(\text{CTG})^{\text{exp}}$ binding and intervenes the $r(\text{CUG})^{\text{exp}}$ –MBNL1 interaction. These strategies are expected to decrease toxic $r(\text{CUG})^{\text{exp}}$ levels and restore MBNL1 levels, respectively, thereby recovering DM1 defects.

Results

Rational Design of a Multivalent Ligand. We recently reported a small-molecule inhibitor of the $r(\text{CUG})^{\text{exp}}$ –MBNL1 interaction (26) whose structure (**1** in Fig. 2) features a bisamidinium core as a general RNA groove binder and two triaminotriazine units designed for selective recognition of U–U mismatches. Although the synthesis was not trivial, we were able to prepare dimeric ligand **2** using the alkyne-azide copper-assisted (CuAAC) click reaction and thus a triazole linker between two monomer units (Fig. 2A) (5). Dimer **2** inhibited formation of the MBNL1– $r(\text{CUG})_{16}$ complex with a $K_i = 25 \pm 8$ nM that was nearly 1,000-fold lower than that measured for monomer **1** and $r(\text{CUG})_{12}$ ($K_i = 16 \pm 6$ μM). However, **2** did not perform well in DM1 cellular assays compared with 1,000-fold enhanced activity in vitro.

Several lines of evidence suggest that the structure of **2** is particularly unsuitable for cell-membrane penetration. First, its large size (molecular mass = 1,166.4 Da) (5) places it well outside of the Lipinski rule of 5 (Ro5), where compounds with molecular mass < 500 are most likely to show excellent passive diffusion across the cellular membrane. Although some clinically useful and orally bioavailable drugs have molecular masses between 500 and 1,000, a range known as “beyond the Ro5” (bRo5), these agents are usually cyclic peptides, often with *N*-methyl groups. Invariably, a steep drop-off in membrane permeability occurs at molecular mass > 1,000, and an upper size limit appears to be molecular mass $\sim 1,000$ for passive diffusion of small molecules across cell membranes (28–30). Beyond the high molecular mass, dimer **2** violates at least two other Ro5s

with a large number of N–H and hydrogen-bond acceptor groups. The final challenge for **2** to enter the cell comes from the need to move four cations (amidinium groups) across the membrane. Interestingly, previous studies with oligoarginines and aromatic amide foldamers found that four positive charges were not enough to efficiently translocate into cells, whereas eight cationic groups were found to be the most efficient (31, 32). These CPPs and their mimics frequently have molecular masses >1,000, yet readily enter cells through either energy-dependent or -independent mechanisms.

Given the considerations above, it appears that ligand **2** is too large and too charged to passively penetrate the cell membrane as a small molecule and yet too small and insufficiently cationic to enter the cells through CPP-type mechanisms. Thus, we sought to connect four to six monomeric units of **1** to create a CPP mimic with 8–12 cationic groups. Ligand **1** is designed to recognize $(\text{CUG})_3$ with the bisamidinium linker, designated linker A in Fig. 2A, binding the groove of the central CUG and the triaminotriazine units recognizing the terminal U–U mismatches. In linking monomeric units of **1**, it can be critical to select the appropriate linker. The monomeric units of dimer **2** are linked by a triazole group (linker B, Fig. 2A). In this study, we considered using the optimized linker A, as seen in ligand **3**. The central bisamidinium in **3** provides two more positive charges, as well as additional groove binding, potentially improving cell permeability and affinity for the target $r(\text{CUG})^{\text{exp}}$. The regular alternating structure of **3** further suggested oligomeric or polymeric ligand **4** with multivalent binding capability (Fig. 2A).

To validate our molecular design, we performed a preliminary molecular modeling study using molecular dynamics (MD) simulations. Following the same approach we reported (26) for the MD simulation of ligand **1** bound to $[\text{r}(\text{CUG})_6]_2$, the oligomeric ligand **4** was manually docked into $[\text{r}(\text{CUG})_{15}]_2$. The ligand used had three bisamidinium moieties and four triaminotriazine units, corresponding to a degree of polymerization (DP; i.e., number of repeat units) of 3.5. A 10-ns MD simulation suggested stable major groove binding with the triaminotriazine units recognizing every other U–U mismatch along the sequence (SI Appendix, Fig. S1). The triaminotriazine units formed multiple hydrogen bonds to and were positioned roughly coplanar with one of the uracil groups, whereas the other was flipped out. This simple computational study supported our rational design wherein oligomeric ligand **4** can selectively recognize $r(\text{CUG})^{\text{exp}}$ in a multivalent fashion.

Preparation of Oligomer 4 by Polycondensation and Interaction with $r(\text{CUG})_{16}$. The alternation of triaminotriazine and bisamidinium moieties in **4** suggested a single-step synthesis involving the polycondensation of diamine **5** and bisimide **6** (Fig. 2B), precursors that can be prepared in one or two steps from commercially available compounds (SI Appendix, SI Materials and Methods). The polycondensation reaction was performed in DMF at 35 °C, reliably

Fig. 2. Rational design and synthesis of multivalent ligands. (A) Structure of ligand **1** with two triaminotriazine U–U or T–T recognition moieties (green hexagons) connected through a groove-binding bisamidinium linker (optimized linker A). Dimeric ligand **2** was previously prepared by click chemistry (triazole linker B). Ligand **3** shows an alternative design for a dimer, linking two monomers using a groove binder linker A. This design provides a more regular structure, enabling further elongation to an oligomeric ligand **4**. (B) Synthesis of oligomeric ligand **4** by polycondensation reaction. (C) MALDI-TOF characterization of oligomer **4** shows the formation of an oligomeric mixture ranging from 4 to 8 mer (average molecular mass of ~ 2 kDa). See text for explanation of R end groups. DHB was used as the matrix. The spectrum was collected by using reflective mode. Intens., intensity.

Fig. 3. Ligand **4** as an inhibitor of $r(\text{CUG})^{\text{exp}}$ -MBNL1 and $d(\text{CTG-CAG})^{\text{exp}}$ transcription. (A) Reduction of disease foci in DM1 patient-derived myoblasts upon the treatment of oligomer **4** at 200 nM. Foci areas are indicated with white arrows. (Scale bars, 5 μm .) (B) Schematic description of splicing pattern of IR pre-mRNA and the result of splicing assay in a presence of oligomer **4**. Full recovery of the IR splicing defect was obtained at 200 nM. P values were calculated against data points of the diseased sample. $*P < 0.05$. No significant differences between normal cells and each of all the treated samples ($P > 0.05$, Student's t test, two-tailed). (C) A plot of normalized RNA transcript level against the oligomer **4** concentration. RNA transcript level was normalized to the level of a loading reference RNA, HIV fs RNA, for quantitative comparison between the loading samples. Oligomer **4** inhibited $d(\text{CTG})^{\text{exp}}$ transcription in a dose-dependent manner ($\text{IC}_{50} = 100$ nM), but no significant inhibition was detected with the control DNA template throughout all the tested concentrations. Error bars indicate standard error of the mean from three independent experiments.

dCTG-Targeting by Oligomer 4 and Inhibition of Toxic RNA Synthesis.

As explained, recent studies showed that $r(\text{CUG})^{\text{exp}}$ can produce additional toxicity, which adds to the complexity of the DM1 disease pathobiology. For example, the $r(\text{CUG})^{\text{exp}}$ transcript can disrupt the translation of MEF2 protein, leading to microRNA dysregulation in DM1 heart tissue (14). Additionally, $r(\text{CUG})^{\text{exp}}$ undergoes RAN translation, producing homopeptides that may be toxic (15, 16). For these reasons, we tested the potential of oligomer **4** to regulate the levels of toxic $r(\text{CUG})^{\text{exp}}$. Although in our previous studies, we did not see $d(\text{CTG})^{\text{exp}}$ binding by monomeric ligand **1**, N -substituted analogs that add additional cationic groups are able to target both the DM1 DNA and RNA expansions (22).

We first checked whether oligomer **4** can inhibit the transcription of $d(\text{CTG})^{\text{exp}}$ in vitro using a $(\text{CTG})_{74}$ -containing DNA template. A strong dose-dependent inhibition effect of **4** was observed toward the $(\text{CTG})_{74}$ template ($\text{IC}_{50} = 100$ nM), whereas no inhibition was apparent for the transcription of a non-repeat-containing control template (Fig. 3C and *SI Appendix, Fig. S84*). This finding is consistent with oligomer **4** selectively and potently targeting $d(\text{CTG})^{\text{exp}}$ transcription, possibly by stabilizing an analogous hairpin secondary structure formed in transcription bubble (38). The potential of **4** to act as a transcription inhibitor and down-regulate the toxic RNA levels in cells was tested. After treatment of the DM1 model cell culture with **4** for 72 h, the level of $r(\text{CUG})_{960}$ was found to be reduced by 20–50%, depending on the oligomer concentration. These results indicate that oligomer **4**, by inhibiting $d(\text{CTG})^{\text{exp}}$ transcription, can suppress the level of $r(\text{CUG})^{\text{exp}}$, the DM1 causative agent (*SI Appendix, Fig. S8B*).

Recovery of Phenotypic Defect in DM1 *Drosophila* Adult Climbing Assay.

The promising cell-permeability and multitargeting features of **4** produced significant improvement of cellular DM1 phenotypes, which motivated us to further study its activity in vivo. We used a phenotypic assay with adult DM1 *Drosophila* that takes advantage of their innate climbing behavior (39). Because DM1 is a neuromuscular disease affecting both the central nervous system and the motor system, DM1 flies show age-dependent progressive climbing defects (39, 40). By aging DM1 adult flies for several days or even months, more $r(\text{CUG})^{\text{exp}}$ is accumulated, allowing for disease symptoms that range from a mild to a severe climbing defect. In turn, this tunability allows screening of therapeutic ligands with widely different activities. Moreover, the climbing assay allows fast screening using a larger number of adult flies, generating statistically important data faster and more conveniently.

With the aged adult DM1 flies created with $(\text{CTG})_{480}$ as reported, but with an *Elav-GAL4*-inducing system (40), we

evaluated head to head the ability of monomeric ligand **1** and oligomer **4** to reverse the climbing failure (*SI Appendix, Fig. S9*). For normal flies with CTG_{60} , treatment with **4** (80 μM) and monomer **1** (400 μM) did not affect their climbing ability, suggesting minimal toxicity of compounds. When it came to DM1 flies (CTG_{480}), almost 80% of the flies failed to climb, but treatment with oligomer **4** at a concentration of 20–80 μM rescued their climbing ability in a dose-dependent fashion. At the highest dose of 80 μM , the failure rate was dropped to 37%, representing a 54% recovery. In contrast, monomer **1** decreased the failure rate to 55%, a 31% recovery, at a fivefold higher concentration (400 μM). The phenotypic improvement in DM1 adult flies demonstrated the in vivo activity of **4**, although the molecular mechanism of action was not investigated.

In Vivo Activities of Multivalent Ligands in a Liver-Specific DM1 Mouse Model.

As a result of the multisystemic nature of DM1, symptoms other than muscle wasting and myotonia are often exhibited by patients. These symptoms include cardiac conduction defects, cataracts, testicular atrophies, dysphagic symptoms, and other gastrointestinal-tract-related symptoms (41, 42). Abnormal elevation of liver enzymes such as aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, and lactate dehydrogenase along with nonalcoholic fatty liver disease and gallbladder dysfunction are also common in DM1 patients (43–45), and even though such liver-associated pathologies may be the first symptom observed, they remain understudied. Therefore, we developed a liver-specific DM1 mouse model by expressing 960 CUG repeat-containing *DMPK* RNAs in a tetracycline-inducible (46) and hepatocyte-specific manner (TRE-960I; ApoE-rTA mouse model; *SI Appendix, Fig. S104*). The liver-specific CUG_{960} mouse model served as an attractive model for screening in vivo efficacy of oligomer **4**, considering the ease of delivery of compounds to the liver via i.p. injections (47). An 8-d pathogenic $r(\text{CUG})_{960}$ RNA induction and treatment scheme was used in this study (Fig. 4A). Efficient $r(\text{CUG})_{960}$ RNA expression was observed in the mouse livers upon feeding a 6 g/kg Dox-containing diet, as seen by quantitative RT-PCR (qRT-PCR) (Fig. 4B). Continued injections with oligomer **4** led to a significant reduction in the level of $r(\text{CUG})_{960}$ RNA, likely due to its inhibitory effect on $d(\text{CTG})^{\text{exp}}$ transcription, as observed with in vitro and DM1 cell studies. Dox diet and oligomer **4** treatment as mentioned above had no significant impact on the body weight or liver-to-body-weight ratios (*SI Appendix, Fig. S11A and B*). Histological analysis showed no overt signs of liver toxicity—such as inflammation, injury, or fibrosis—in oligomer **4**-treated mice (*SI Appendix, Fig. S11F*). Furthermore, the effects of oligomer **4** in reducing $r(\text{CUG})_{960}$ RNA load were specific, as the mRNA levels of three other nontargeted genes,

More interestingly, the same activities of correcting DM1 molecular defects were also observed in vivo in a liver-specific CUG₉₆₀ mouse model, fully rescuing multiple MBNL1-regulated splicing defects. Our liver-specific r(CUG)₉₆₀ mouse model is expected to be a useful model to understand pathogenic mechanisms of DM1 in the liver and related symptoms, which have not been examined before. Also, considering a facile delivery of compounds to the liver via i.p. injections, our mouse model can

serve as an initial in vivo platform for potential DM1 therapeutic agents. Importantly, oligomer **4** showed no sign of toxicity at the working concentrations in HeLa cells, DM1 patient cells (GM03987), and both in vivo models. The maximum tolerated dose (MTD) was determined to be 40 mg/kg in C57BL/6 mice, which is significantly higher than that for dimeric ligand **2** (3 mg/kg) (**5**).

Our oligomeric ligand inherently mimics the structure of CPPs such as oligoarginines or Tat. In this regard, we believe that this approach represents a paradigm in multivalent ligand design, where the direct assembly of binding moieties serves as their own delivery agent. The ability to prepare oligomer **4** by a condensation polymerization streamlines the synthesis, but moving forward it will be important to prepare homogenous oligomers for testing. Nonetheless, our general design principle should be applicable to other TREDs, such as Huntington's disease and spinocerebellar ataxias, considering the similarities in their pathogenesis.

Materials and Methods

All reagents were purchased from standard suppliers and used without further purification unless otherwise noted. r(CUG)₁₆ was purchased from

Integrated DNA Technologies or synthesized on an Applied Biosystems 3400 DNA Synthesizer, followed by HPLC purification. NMR spectra and mass spectral analyses were performed by using instrumentation available in the School of Chemical Sciences, University of Illinois. Details of the synthesis, compound, and oligomer characterization, as well as additional experimental details and additional figures, can be found in *SI Appendix*. We strictly followed the National Institutes of Health guidelines for use and care of laboratory animals, and all experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Illinois, Urbana-Champaign.

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