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The Role of Flexibility in the Rational Design of Modularly Assembled Ligands Targeting the RNAs that Cause the Myotonic Dystrophies

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Abstract

Modularly assembled ligands were designed to target the RNAs that cause two currently untreatable neuromuscular disorders, myotonic dystrophy types 1 (DM1) and 2 (DM2). DM1 is caused by an expanded repeating sequence of CUG, and DM2 is caused by expanded CCUG repeats. Both are present in non-coding regions and fold into hairpins with either repeating 1×1 nucleotide UU (DM1) or 2×2 nucleotide 5'CU/3'UC (DM2) internal loops separated by two GC pairs. The repeats are toxic because they sequester the RNA splicing regulator Muscleblind-like 1 protein (MBNL1). Rational design of ligands targeting these RNAs was enabled by a database of RNA motif-ligand partners compiled using Two-Dimensional Combinatorial Screening (2DCS). One 2DCS study found that the 6"-azido-kanamycin A module binds internal loops similar to those found in DM1 and DM2. In order to further enhance affinity, the ligand was assembled on a peptoid backbone to precisely control the valency and the distance between ligand modules. Designed compounds are more potent and specific binders to the toxic RNAs than MBNL1 and inhibit the formation of the RNA-protein complexes with nanomolar IC₅₀'s. This study shows that three important factors govern potent inhibition: (1) the surface area sequestered by the assembled ligands; (2) the spacing between ligand modules since a longer distance is required to target DM2 RNAs than DM1 RNAs; and, (3) flexibility in the modular assembly scaffold used to display the RNA-binding module. These results have impacts on the general design of assembled ligands targeting RNAs present in genomic sequence.

Keywords

RNA; Triplet Repeat Disorders; Drug Design; Aminoglycosides; Modular Assembly

Introduction

RNA plays central roles in many biological processes beyond simply encoding or synthesizing cellular proteins. For example, non-coding RNAs regulate gene expression in a variety of ways. Both pri- and pre-microRNAs comprise about 1% of the human genome yet

regulate expression of 10% of all genes.[1] Aberrant expression of microRNAs can cause diseases such as cancer and viral infections.[2,3] Other non-coding RNA targets are present in genomic sequence, such as untranslated regions in mRNAs. Understanding their biological roles has provided a better understanding of cellular biology and has opened up new areas in RNA research.

Despite advances in understanding the roles of RNA in biology, only relatively few advances have been made in understanding how to target RNA with small molecules. Given their potential impact on the development of therapeutics or probes of cellular function, these limited advancements have hampered the field.

In an effort to develop rational methods to target RNA with small molecules, a database of RNA motif-ligand partners is being constructed via Two-Dimensional Combinatorial Screening (2DCS) (Figure 1). [4–7] This database can then be mined against cellular RNA sequences or structures to enable a rational and modular approach to target RNA with small molecules (Figure 2). One particularly difficult problem in targeting RNA has been the identification of specific binders. However, by merging the RNA motif-ligand database with modular assembly strategies, specific RNA binders have been designed.[8,9] Specificity is programmed into designer ligands because: (1) the RNA motif-ligand database, which is the output of 2DCS, defines the privileged RNA motifs that bind small molecules; and, (2) modular assembly allows design of compounds that bind RNAs with multiple targetable motifs that are separated by defined distances, e.g., two GC base pairs. Such an approach has facilitated the design of ligands targeting the RNAs that cause myotonic muscular dystrophy types 1 and 2.[8,9]

DM1 and DM2 are caused by tandem repeating non-coding RNA sequences.[10–12] The expanded repeats cause a toxic gain-of-function in the RNA (Figure 2). DM1 is caused by an expansion of CUG repeats in the 3' untranslated region of the dystrophin myotonia protein kinase (DMPK) mRNA [13], while DM2 is caused by an expansion of a CCUG tetranucleotide repeat in intron 1 of the zinc finger 9 protein (ZNF9) pre-mRNA.[11] Both the expanded DM1 and DM2 repeats fold into hairpin structures displaying regularly repeating 1×1 nucleotide UU internal loops (DM1) or 2×2 nucleotide 5'CU/3'UC internal loops (DM2) (Figure 2).[14,15] These structures sequester Muscleblind-like 1 protein (MBNL1), an RNA splicing regulator (Figure 2).[16–18] Sequestration of MBNL1 causes DM1 and 2 through a variety of mechanisms including causing aberrant splicing of the main muscle chloride channel [19] and insulin receptor [20] pre-mRNAs. Aberrant splicing of these RNAs explains the muscle atrophy and insulin insensitivity observed in DM patients.

The disease model described above points to a potential therapeutic strategy to treat myotonic dystrophy in which a small molecule would bind to the toxic repeats and inhibit formation of the DM RNA-MBNL1 complex. If this were to occur *in vivo*, then there would be an increase in the amount of free MBNL1, which could restore normal splicing patterns. [21]. Two pieces of evidence suggest that this strategy is viable: (1) correction of splicing defects in DM1-affected cells by overexpression of MBNL1;[20] and (2) correction of splicing defects and altered muscle excitability in a DM1 mouse model by an oligonucleotide complementary to CUG repeats.[14]

In the previous studies targeting both DM1 and DM2 RNAs using modularly assembled small molecules, the RNA-binding modules displayed on a peptoid backbone were either a derivative of Hoechst 33258 [22] or 6'-N-5-hexynoate kanamycin A [8,9]. In this current study, we utilize 6"-azido-kanamycin A as the RNA-binding module, which was shown to bind DM-like internal loops by 2DCS. By using a different module, we have confirmed previously identified features in assembled ligands that provide potent DM RNA-MBNL1

inhibitors as well as uncovered new ones. These include: (1) the surface area sequestered by the assembled ligands; (2) the spacing between ligand modules since a longer distance is required to target DM2 RNAs than DM1 RNAs; and, (3) flexibility in the scaffold used to display the RNA-binding module. These results are important for the design of potent ligands targeting DM RNAs and may provide a rational basis to exploit other RNA targets present in genomic sequence.

Results and Discussion

Module Identification and Modular Assembly

In a previous 2DCS study, it was determined that the RNA internal loops that preferred to bind 6"-azido-kanamycin A (**Kaz**, Figure 1) contain pyrimidines. A subset of the selected loops is shown in Figure 1.[7] Comparison of these selected structures to toxic RNAs found commonalities with the repeating RNAs that cause myotonic dystrophy types 1 and 2 (DM1 and DM2, respectively). Because DM1 and DM2 RNAs display regularly repeating pyrimidine-rich internal loops, we assembled the **Kaz** ligands onto a peptoid scaffold (Scheme 1) to bind multiple loops simultaneously and thus inhibit formation of the RNA-MBNL1 complexes (Figure 2).

Previously, several important studies have reported small molecule ligands that bind a single 5'CUG/3'GUC repeat.[23,24] These monomeric ligands bind the RNA repeats with dissociation constants ranging from 450 nM to 10 μ M and inhibit the RNA-MBNL1 interaction with K_I 's in the low micromolar range.[23,24] Modularly assembled ligands have been utilized by our group to target the DM1 and the DM2 RNAs.[8,22] The assembled compounds that were used displayed multiple copies of either 6'-N-5-hexynoate kanamycin A[8] or a bis-benzimidazole[22] module conjugated onto a peptoid backbone. These ligands bind toxic RNAs with nanomolar affinities and inhibit the formation of the toxic RNA-protein interactions with IC_{50} 's in low nanomolar range. The enhanced potency of the assembled ligands can be attributed to the larger surface area coverage of modularly assembled ligands than monomeric ones.[8,22] In this study, **Kaz** modules, which have been shown to prefer DM-like RNA internal loops via 2DCS (Figure 1)[7], were displayed on a peptoid backbone to study how the identity of the ligand, ligand spacing, and flexibility of the scaffold affect RNA binding affinity and potency. Such studies are critical to further understand how to develop highly potent and specific ligands that target the DM RNAs as well as others in genomic sequence.

Peptoids that display propargylamine were synthesized and conjugated to **Kaz** via a Huisgen dipolar cycloaddition reaction [25] (HDCR, Scheme 1), which is the same anchoring chemistry used to immobilize **Kaz** onto microarrays used in 2DCS (Figure 1).[7] Valency and spacing were controlled by the number of propargylamine couplings (**a** + 1, Scheme 1) and the number of propylamine spacers (**b**, Scheme 1) inserted between the propargylamine submonomers, respectively. The nomenclature used for the assembled compounds has the general format (**a** + 1)**M**-**b** where (**a** + 1) is the valency or number of ligand modules displayed, **M** is the ligand module displayed, and **b** is the number of propylamine spacing modules (Scheme 1). The ligand modules are **Kaz** which was conjugated to alkyne-displaying peptoids, and 6'-N-5-hexynoate neamine (**N**) or propargylamine (**P**) which were conjugated to azide-displaying peptoids.[8] Thus, **3Kaz-2** is a peptoid in which three **Kaz** modules are displayed on a peptoid, each separated by two propylamine spacing modules. Modularly assembled compounds that display **N** or **P** were used to control for non-specific inhibition of the RNA-MBNL1 complexes; **3N-4** and **3P-4** are weak inhibitors of the DM1 RNA- and DM2 RNA-MBNL1 complexes with IC_{50} 's > 1 μ M (Table 1).

Potency of Assembled Ligands for Inhibition of the DM1 RNA and DM2 RNA-MBNL1 complexes

A series of dimeric ligands with different spacings between the RNA-binding modules (the value of **b**, Scheme 1) were synthesized. Their potencies for inhibiting the DM1 RNA- and the DM2 RNA-MBNL1 complexes were determined using a microtiter plate assay as previously described.[8] Model RNA constructs (Figure 3) were used to mimic the DM1 RNA (**RNA2**) and the DM2 RNA (**RNA3**) by inserting 12 copies of 5'CUG/3'GUC or 5'CCUG/3'GUCC, respectively, into a hairpin cassette (**RNA1**). Results show that the IC₅₀'s of the most potent dimers were 500 nM for DM2 **RNA3** and 3 μM for DM1 **RNA2** (Table 1).[26] The optimal dimer for the DM1 RNA has **Kaz** ligand modules separated by two propylamines while the optimal dimer for the DM2 RNA has four propylamines (Table 1). [26] These were the same optimal spacings identified for the 6'-N-5-hexynoate kanamycin A ligand module.[8] Surprisingly, modularly assembled compounds with higher valencies were less potent inhibitors. For example, **2Kaz-2**, **3Kaz-2**, and, **4Kaz-2** had IC₅₀'s of 3, 19, and >25 μM for inhibition of the DM1 **RNA2**-MBNL1 complex while **2Kaz-4**, **3Kaz-4**, and, **4Kaz-4** had IC₅₀'s of 0.5, 1.7, and 4.0 μM for inhibition of the DM2 **RNA3**-MBNL1 complex (Table 1). Since previous studies showed that potency increased as a function of valency, we set out to determine the cause of the decreased potency observed with the **Kaz** modules.[8,22]

Once conjugated, the 6'-N-5-hexynoate kanamycin A ligand module used previously has ten atoms between the aminoglycoside and the peptoid backbone.[8] In contrast, when **Kaz** is conjugated, there are only four atoms between the ligand and the peptoid. Therefore, second-generation peptoids were synthesized to increase the distance between the peptoid backbone and the aminoglycoside (Scheme 1). In order to do so, a flexible ethylene glycol linker was used to conjugate **Kaz** to the peptoid. These compounds have the general format (**a** + 1)**KazL-b** where **KazL** denotes conjugation of **Kaz** to the flexible linker, **L**. These peptoids showed enhanced potency as valency increased. For example, **2KazL-2** and **4KazL-2** had IC₅₀'s of 1.8 and 0.05 μM, respectively, for inhibition of the DM1 **RNA2**-MBNL1 complex while **2KazL-4** and **4KazL-4** had IC₅₀'s of 1.0 and 0.02 μM for inhibition of the DM2 **RNA3**-MBNL1 complex (Table 1). Thus, potencies for the tetrameric ligands are enhanced by >500-fold for the DM1 model system and 200-fold for the DM2 system by increasing the flexibility of the ligand module.

Potencies for each assembled compound were used to calculate the multivalent effect (MV), which quantifies the bonus in potency that an assembled ligand provides over a monomeric one (Table 1). The multivalent effect is calculated by first multiplying the IC₅₀ value by the valency, affording the normalized IC₅₀ or NIC₅₀. The IC₅₀ for the **Kaz** monomer is then divided by the NIC₅₀ of the compound of interest to afford the multivalent effect. The MV values range from 1 for **Kaz** to 2,500-fold for **4KazL-4**. Not surprisingly, the increased flexibility of the **KazL** compounds provides enhancements in the magnitude of the multivalent effect. For example, **4Kaz-2** has a MV of <1.9-fold for inhibition of the DM1-MBNL1 complex while **4KazL-2** has a MV of 950-fold. Analogously, **4Kaz-4** has a MV of 12.5-fold for inhibition of the DM2-MBNL1 complex while **4KazL-4** has a MV of 2,500-fold.

In order to investigate the selectivity of the optimal modularly assembled compounds, the potencies of the ligands were determined in the presence of about 15-fold molar excess in nucleotides of yeast bulk tRNAs (Table 1). The IC₅₀'s for the **4KazL-2** and **4KazL-4** ligands inhibiting the DM1- and DM2 RNA-MBNL1 complexes are mid nanomolar (~200 nM) in the presence of these competitor nucleic acids.

Affinities and Specificities of Modularly Assembled Ligands

The affinities and specificities of the RNA-ligand complexes were also determined as previously described.[8,27,28] Six different RNAs were tested (Figure 3) including DM1 **RNA2**, DM2 **RNA3**, and **RNA4** which contains 12 copies of the 5'CAG/3'GAC motif present in RNAs that cause spinocerebellar ataxia type 3 (SCA3). Other control RNAs include **RNA5** and **RNA6** that have 12 copies of 2×2 nucleotide internal loops, 5'UU/3'UU and 5'CC/3'CC, respectively, and fully base-paired **RNA7**. Results show that the optimally spaced ligands are all higher affinity DM1 and DM2 binders than MBNL1. The ligands with two propylamine spacing modules are DM1-specific while the ligands with four spacing modules are DM2-specific. Importantly, ligand flexibility can drastically affect RNA binding affinity (Table 2).

For the DM1 **RNA2**, the **Kaz** monomeric ligand binds with a dissociation constant of 920 nM while it binds to **RNA1** (the cassette in which the RNAs were embedded), **RNA4**, **RNA6**, and **RNA7** with dissociation constants >5 μ M. The **2Kaz-2** dimer binds weakly to **RNA2** while the corresponding compound with the ethylene glycol linker, **2KazL-2**, is higher affinity with a K_d of 75 nM. A similar trend is observed with the tetramers, **4Kaz-2** and **4KazL-2**, which have dissociation constants of >500 nM and 20 nM, respectively. The **4KazL-2** ligand binds **RNA2** at least ~12.5 times more tightly than **RNA3** (the DM2 RNA), **RNA4** (the SCA3 RNA), **RNA6**, and fully paired **RNA7**. It should be noted that RNAs with long stretches of periodically repeating internal loops are rare in genomic sequences while stretches of base pairs are more common.[29] Fortunately, the **4KazL-2** ligand binds fully paired RNA very weakly. In addition, each ligand binds to the DM1 RNA with the expected stoichiometry (Table 2).[28]

Optimal ligands that bind the DM2 **RNA3** are also high affinity and specific. The **Kaz** monomer binds more tightly to the DM2 RNA than the DM1 RNA with a K_d of 680 nM. The dimers **2Kaz-4** and **2KazL-4** bind **RNA3** with similar affinities (145 nM and 80 nM, respectively) in good agreement with their similar potencies for disrupting the **RNA3**-MBNL1 complex (Table 1). Consistent with the observed potencies, **4Kaz-4** binds weakly to the DM2 RNA (K_d >250 nM) while the corresponding ligand with the extended, flexible linker, **4KazL-4**, binds with a dissociation constant of 30 nM. The compound also binds weakly to **RNA4**-**RNA7**. As was the case with the optimal DM1 ligands, the optimal DM2 ligands bind **RNA3** with the expected stoichiometries (Table 2).[28]

The modularly assembled **Kaz** compounds are slightly less potent and bind with slightly less affinity than the modularly assembled compounds that display 6'-N-5-hexynoate kanamycin A.[8] This indicates that the manner in which the ligand is displayed can affect affinity and potency. Interestingly, the position of functionalization does not appear to affect cellular uptake or toxicity.[8,26] However, the distance between the ligand module and the peptoid backbone, and hence flexibility of the ligand modules, is an important consideration when modularly assembling compounds, at least for the aminoglycoside derivative **Kaz**.

Uptake of Assembled Ligands into Mouse Myoblasts

The modularly assembled ligands were tested for uptake into mouse myoblasts using fluorescence microscopy. Results show that all modularly assembled compounds permeate cells when 5 μ M of the ligand is added to a cell culture. Comparison of the uptake patterns of the dimeric and tetrameric ligands show that there is no significant difference in their uptake or cellular localization (Figure 4). This is interesting because one would expect that as the size of the assembled ligand increases that uptake would decrease. In fact, earlier studies have shown that a monomeric aminoglycoside is taken up by C2C12 cells only in small amounts compared to the modularly assembled ligands.[8,9]

Uptake and toxicity of the assembled ligands were quantified using flow cytometry (Table 3). After incubating the cells with the ligand of interest for 14 h, they were treated with propidium iodide, which stains cells with compromised cell membranes. All modularly assembled ligands are more cell permeable than **Kaz-FITC**, the monomeric ligand. At least five times more cells are positive for uptake. By comparing the normalized fluorescence intensities for the ligands, the modularly assembled ligands are present in ~2- to ~10-fold higher concentrations. The results also show that the ligands are non-toxic; at worst, only ~5% of the cell population that has taken up fluorescently labeled modularly assembled ligand are stained with propidium iodide after a 14 h incubation. Similar results were also obtained after the cells were incubated with the ligand of interest for 24 or 48 h. These data are available in the Supporting Information.

Conclusion

In summary, it has been shown that the flexibility of the RNA-binding modules displayed on a peptoid backbone is an important determinant for binding affinity and potency of ligands. Such studies are critical to better understand factors that affect high affinity binding of assembled ligands to RNA or other biomolecules, such as proteins targeted by modularly assembled carbohydrates.[30,31] In the future, it would be beneficial to design more rigid backbones that pre-organize ligands for binding RNA. This could increase the potency and affinity of modularly assembled ligands that target RNA.

Experimental Section

Chemical Synthesis

Details of chemical syntheses and analytical data are in the Supporting Information.

MBNL-1 Displacement Assays

Displacement assays were completed as previously described[8] in black 384-well plates coated with Streptavidin (Nunc). In these experiments, 2.5 pmoles of **RNA2** or **RNA3** was delivered to each well. Resorufin- β -D-galactopyranoside was used as a substrate to quantify the amount of β -galactosidase, and hence MBNL1, present. Fluorescence intensity was measured using a BioTek FLX-800 plate reader. By comparing the fluorescence intensities to wells containing no inhibitor (maximum response) and no RNA (minimum response), the percentage of MBNL1 bound can be determined. The percentage of MBNL1 bound was plotted versus ligand concentration and the resulting curve fit to SigmaPlot's 4-parameter logistic function in order to determine the IC_{50} (Equation 1):

$$y = D + \frac{A - D}{1 + \left(\frac{x}{IC_{50}}\right)^{Hill\ slope}} \quad 1$$

where y is the percentage of MBNL1 bound, D is the minimum response plateau, A is the maximum response plateau, and x is the concentration of ligand. A and D are typically 100% and 0%, respectively. Each IC_{50} is the average of at least two measurements.

In order to determine the multivalent effect, the IC_{50} 's were normalized for the number of ligand modules conjugated to the peptoid backbone. These normalized IC_{50} 's, or NIC_{50} 's, were calculated by multiplying the IC_{50} by the number of ligand modules displayed on the peptoid. Multivalent effects were calculated by dividing the IC_{50} for **Kaz** (monomer) by the NIC_{50} of the compound of interest. The number of moles of RNA immobilized in each well

was determined using SYBR Green II as described.[22] Approximately 20% of the moles of RNA delivered to a well are immobilized.

RNA Binding Assays

The affinities of RNA-ligand complexes were determined as described using a fluorescence emission-based assay.[8] Briefly, RNA was folded in 1X MBNL Buffer (50 mM Tris HCl, pH 8.0, 50 mM NaCl, 50 mM KCl, 1 mM MgCl₂) without MgCl₂ by incubating at 60 °C for 5 min followed by slowly cooling to room temperature. Then, MgCl₂, BSA, and ligand of interest were added to final concentrations of 1 mM, 40 µg/mL, and 75 nM, respectively. The RNA was serially diluted in 1X MBNL buffer containing 40 µg/mL BSA and 75 nM ligand and incubated for 1 h at room temperature. Fluorescence intensity was determined using a BioTek FLX-800 plate reader. Stoichiometries were determined by constructing plots of [Ligand]/[RNA] vs. change in fluorescence. The resulting curves were fit to two lines and the equations solved simultaneously to afford the stoichiometry as previously described.[27,32] Scatchard analyses were completed to determine dissociation constants, accounting for statistical effects by using a functional form of the Scatchard equation for large ligands binding to a lattice (Equation 2) [28,33]:

$$\frac{v}{[L]} = \frac{N(1 - lv/N)}{k} \left(\frac{1 - \frac{lv}{N}}{1 - \frac{(l-1)v}{N}} \right)^{l-1} \quad 2$$

where v is the moles of ligand per moles of RNA lattice, $[L]$ is the concentration of ligand, N is the number of repeating units on the RNA, l is the number of consecutive lattice units occupied by the ligand, and k is the microscopic dissociation constant. This equation simplifies to the commonly used form of the Scatchard equation for simple systems.[28,33]

Cell Uptake and Flow Cytometry

Cell Culture and Microscopy: The C2C12 (mouse myoblast) cell line was grown as a monolayer at 37 °C and 5% CO₂ in 1X High Glucose DMEM (Invitrogen) supplemented with FBS (10%, Invitrogen) and penicillin/streptomycin (0.5%). For uptake experiments, cells were grown in 6-well plates containing sterile glass cover slips. After 14 h of growth, the medium was removed and replaced with fresh medium containing 5 µM compound of interest. The medium was removed after 14 h, and the cells were washed with 1X DMEM without phenol red (Invitrogen). The cover slip was mounted in 1X DMEM without phenol red and the edges sealed with Valap (1:1:1 of Vaseline/lanolin/paraffin). The cells were then imaged using Zeiss Axio-Imager Z1 Axiophot wide-field fluorescence microscope.

Flow Cytometry Analysis for Uptake and Toxicity: Cell uptake and toxicity of modularly assembled ligands were quantified using flow cytometry. Uptake assays were completed as described above except the ligand of interest was incubated with the cells for 14h, 24 h or 48 h. The medium was removed and the cells were trypsinized. After pelleting, the cells were washed with 1X DPBS, centrifuged, and the DPBS removed. The cells were resuspended in 1 mL of ice-cold 1X DPBS and placed on ice. Then, propidium iodide (1 µL of 1.5 mM) was incubated with the cells on ice in the dark for 20–30 min. Analysis of 10,000 events was completed using a BD LSR II System Flow Cytometer. Quadrants were set using cells that were not treated with ligand (but stained with propidium iodide) to control for autofluorescence in the fluorescein channel. The quadrants assigned ≤1% of the untreated cells as fluorescent. Results are summarized in the Supporting Information, Tables S1–S3.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Two-Dimensional Combinatorial Screening (2DCS) identified the RNA internal loops that are the preferred RNA-binding motifs for 6''-azido-kanamycin A. A subset of these selected motifs is shown. For example, internal loops with U across from U have a two-tailed *p*-value of 0.037. These internal loops are similar to those found multiple times in the RNAs that cause DM1 (1×1 nucleotide UU loop) and DM2 (2×2 nucleotide 5'CU/3'UC loop).

Figure 2.

Toxic DM1 and DM2 repeats, CUG or CCUG, respectively, fold into RNA hairpins that sequester the RNA splicing regulator Muscleblind-like 1 protein (MBNL1). Designed modularly assembled ligands are potent inhibitors of these pathogenic RNA-protein complexes. The spacing between RNA-binding modules and their flexibility are important factors that affect the potency, RNA binding affinity, and specificity of these compounds.

Figure 3.

The RNA constructs used to study the potencies of the assembled ligands for inhibiting the DM1 RNA- and DM2 RNA-MBNL1 complexes and the affinities of RNA ligand-complexes. **RNA2** is a mimic of the repeating RNA motif found in DM1 and **RNA3** is a mimic of the repeating motif found in DM2.

Figure 4. Uptake and localization of modularly assembled dimeric and tetrameric ligands into mouse myoblasts. All images: Left, phase contrast of C2C12 mouse myoblasts; middle, fluorescence; right overlay of the phase contrast and fluorescence images.

Scheme 1.

Synthesis of peptoids conjugated to 6''-azido-kanamycin A to control the spacing and valency of the RNA-binding module. The ethylene glycol spacer was installed to increase the flexibility of the RNA-binding module. Ligand nomenclature is indicated beneath each series of compounds. For example, **2Kaz-1** indicates conjugation of two **Kaz** modules onto propargylamine submonomers that are separated by one propylamine spacing submonomer. Likewise, **2KazL-1** indicates conjugation of two **Kaz** modules onto 2-(2-(prop-2-ynyloxy)ethoxy)(ethanamine) linkers that are separated by one propylamine spacing submonomer.

Table 1

Potencies of **Kaz** and modularly assembled compounds for inhibition of RNA-MBNL1 interactions. MV is the multivalent effect, or the fold increase in potency afforded by modularly assembled ligands.

Inhibitor	IC ₅₀ (μM)	NIC ₅₀ (μM)	MV	IC ₅₀ (μM)	NIC ₅₀ (μM)	MV
DMI RNA			DMI RNA			
Kaz	190±34	190	1	200 ± 5	200	1
Dimers						
2Kaz-2	3±0.4	6	32	–	–	–
2Kaz-4	4±0.2	8	24	0.5±0.23	1	200
2KazL-2	0.9±0.25	1.8	106	8±0.7	16	12.5
2KazL-2^a	1.8±0.49 ^a	3.6 ^a	53 ^a	–	–	–
2KazL-4	10±2.3	10	19	1±0.14	2	100
2KazL-4^a	–	–	–	1.7±0.35 ^a	3.4 ^a	59 ^a
Trimers						
3Kaz-2	19±2.3	57	3.3	–	–	–
3Kaz-4	>25	>75	< 2.5	1.7±0.58	5.1	39
3N-4	–	–	–	>1	>3	N/A
3P-4	–	–	–	>1	>3	N/A
Tetramers						
4Kaz-2	>25	>100	< 1.9	–	–	–
4Kaz-4	>25	>100	< 1.9	4±0.1	16	12.5
4KazL-2	0.05±0.01	0.2	950	3.6±0.68	14.4	13.9
4KazL-2^a	0.2±0.03 ^a	0.8 ^a	238 ^a	–	–	–
4KazL-2^b	0.2±0.12 ^b	0.8 ^b	238 ^b	–	–	–
4KazL-4	2±0.5	8	24	0.02±0.01	0.08	2,500
4KazL-4^a	–	–	–	0.13±0.07 ^a	0.52 ^a	385 ^a
4KazL-4^b	–	–	–	0.2±0.09 ^b	0.8 ^b	250 ^b
4N-2	>2	>8	N/A	–	–	–

^aThese inhibition assays were completed by adding the ligand and MBNL1 simultaneously to a well containing **RNA2** or **RNA3**, and incubating them for 2 h at room temperature.

^b These experiments were completed by incubating **RNA2** or **RNA3** with the ligand for 1 h at room temperature. Then, MBNL 1 and 15-fold excess in nucleotides of yeast bulk tRNAs were added for 1 h. For **RNA2**, the concentration of tRNAs in nucleotides was 16.7 μ M while for **RNA3**, the concentration of tRNAs was 20 μ M nucleotides.

Table 2

Affinities and stoichiometries of RNA-ligand interactions; stoichiometries are in parentheses.

Ligand	K _d (nM) (Stoichiometry)					
	RNA2	RNA3	RNA4	RNA5	RNA6	RNA7
Kaz	920±67 (11±3)	680±83 (11±1)	>5000	300±52 (11 ± 2)	>5000	>5000
2Kaz-2	>500	—	—	—	—	—
2Kaz-4	—	145±34 (4.0±0.6)	—	—	—	—
2KazL-2	75±8 (5.2±0.3)	>300	>500	95±20; (5.0±0.6)	>250	>500
2KazL-4	>500	80±15 (5.7±1.0)	>500	—	>500	>500
4Kaz-2	>250	—	—	—	—	—
4Kaz-4	—	>250	—	—	—	—
4KazL-2	20±2 (2.4±0.1)	~250	>500	35±7; (2.7±0.6)	>250	>500
4KazL-4	>500	30±8 (3.2±0.8)	>500	>250	>250	>250
MBNLI	250	120	630	>2500	920	1000

Table 3

Flow cytometry analysis of **Kaz** and modularly assembled **Kaz** compounds after a 14 h incubation period.

Ligand	% of Cells with Postiive Uptake	Normalized Fluorescence Intensity for Ligand	% of Apoptotic Cells	Normalized Fluorescence Intensity for Propidium Iodide
None	0.05±0.04	1.00	0.06±0.03	1.00
Kaz-FITC	10±1	3.8±0.6	1±0.3	1.2±0.3
2Kaz-2	65±7	7.7±2.8	4±2	1.5±0.3
2KazL-2	70±11	7.9±1.5	4±3	1.8±0.4
2Kaz-4	72±5	7.6±4.3	5±2	1.8±0.5
2KazL-4	73±1	8.9±1.3	3±1	1.6±0.2
4Kaz-2	83±6	32±3.8	2±0.2	2.8±1.4
4KazL-2	69±6	7.2±2.2	2±0.2	1.8±0.5
4Kaz-4	≈75	5.9±2.1	≈5	1.4±0.2
4KazL-4	53±2	6.9±1.0	3±1	1.5±0.2