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Influencing uptake and localization of aminoglycoside-functionalized peptoids†

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Abstract

The development of small-molecule therapeutics that target RNA remains a promising field but one hampered with considerable challenges that include programming high affinity, specificity, cell permeability, and favorable pharmacokinetic profiles. Previously, we employed the use of peptoids to modularly display RNA-binding modules to enhance binding affinity and specificity by altering valency and the distance between ligand modules. Herein, factors that affect uptake, localization, and toxicity of peptoids that display a kanamycin derivative into a variety of mammalian cells lines are reported. A series of peptoids that display various spacing modules was synthesized to determine if the spacing module affects permeability and localization. The spacing module does affect cellular permeability into C2C12, A549, HeLa, and MCF7 cell lines but not into Jurkat cells. Moreover, the modularly assembled peptoids carrying the kanamycin cargo localize in the cytoplasm and perinuclear region of C2C12 and A549 cells and throughout HeLa cells, including the nucleus. These studies could contribute to the development of general strategies to afford cell permeable, modularly assembled small molecules that specifically target RNAs present in a variety of cell types.

Introduction

Peptoids are N-substituted glycine oligomers that were developed as peptidomimetics.¹ Their syntheses are modular, straightforward, and high yielding. Protecting groups are generally not required. A variety of side chains have been incorporated into peptoids including azides, thiols, hydrazines, aldehydes, and heterocycles. Side chains have also been used for chemoselective conjugation reactions.^{2–4} Peptoids also display favorable pharmacokinetic profiles, are generally more cell permeable than their peptide counterparts,⁵ and are protease-resistant. They have been used as intracellular transporters of drugs and nucleic acids,^{6–8} to target proteins,^{9–12} and as diagnostics for Alzheimer's disease¹³ and multiple sclerosis.¹⁴

We recently reported the use of the peptoid scaffold to target a mutant RNAs that cause myotonic muscular dystrophy (DM).^{15,16} In our case, the peptoid scaffold was used to modularly assemble ligand modules that bind RNA in order to increase affinity and specificity. The compounds, which displayed derivatives of the aminoglycoside kanamycin

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A, are also potent inhibitors of the mutant RNA-protein interactions that cause DM. The RNAs sequester the protein muscleblind-like 1 (MBNL1), a splicing regulator, in the nucleus causing its inactivation. Like peptoids displaying other side chains, the compounds are cell permeable. However, they localize mainly in the cytoplasm and the perinuclear region.

The peptoid scaffold used in our previous studies was synthesized using standard chemistry and monomers with amino groups. In order to display the aminoglycoside derivative, 6'-*N*-5-hexynoate kanamycin A, 3-azidopropylamine was used. After synthesis of the peptoid backbone, a Cu-catalyzed Huisgen dipolar cycloaddition reaction is used to conjugate the kanamycin. In order to determine the optimal distance between kanamycin ligand modules, different numbers of propylamines were conjugated between 3-azidopropylamines. The optimal peptoid was used as a lead molecule for optimization of pharmacokinetic properties, including nuclear localization, improved uptake, and low toxicity. It has been previously reported that the spacing module used can influence cellular permeability and localization.¹⁷ Therefore, a series of peptoids were synthesized with various spacing modules. The effects of the spacing module on *in vitro* potency, uptake, localization, and toxicity were investigated.

Materials & methods

Instrumentation

NMR spectra were recorded on a Varian NMR operating at 400 or 500 MHz on proton. Chemical shifts were referenced to residual solvent or an internal tetramethylsilane standard. Mass spectra were recorded on a LCQ Advantage Ion Trap LC/MS equipped with a Surveyor HPLC system or on a Bruker Biflex IV MALDI-TOF spectrometer.

HPLC separations were completed on a Waters 1525 Binary HPLC Pump equipped with a Waters 2487 Dual Absorbance Detector system monitoring absorbance at 220 and 254 nm. Analytical HPLC separations were completed using a Waters Symmetry C8 or C18 column (5 μ m, 4.6 $\times$ 150 mm), and preparative HPLC separations were completed using a Waters Symmetry C8 column (7 μ m, 19 $\times$ 150 mm).

Sonication was performed using a Branson Branson[®] 5210, 140 watts, 47 kHz sonicator. Resin was agitated by shaking on a Thermolyne Maxi-Mix III[™] shaker. All pH measurements were performed at room temperature using a Mettler Toledo SG2 pH meter that was standardized at pH 4.0, 7.0, and 10.0 prior to recording measurements.

Chemicals

Fmoc-Rink resin and *N,N'*-diisopropylcarbodiimide (DIC) were from AnaSpec; *N,N*-dimethylformamide (DMF; 99.8% anhydrous) was from Acros or Baker and was used without further purification. All other reagents were from Acros or Alfa Aesar and were used without further purification with the exception of piperidine, which was distilled prior to use. The tris-(benzyltriazolylmethyl)amine (TBTA) catalyst was synthesized as described.^{18,19} The HPLC solvents used were HPLC grade methanol from Burdick & Jackson or Honeywell and water obtained from a Barnstead NANOpure Diamond Water Purification System operating at 18.2 m Ω cm. NANOpure water was used to prepare buffers in all biological assays.

HPLC Purification

Preparative HPLC: a linear gradient of 10% to 90% B in A over 60 min was applied using a flow rate of 5 mL min⁻¹ (A: water + 0.1% trifluoroacetic acid (TFA; (v/v)), B: methanol +

0.1% TFA (v/v)). Analytical HPLC: a linear gradient of 0% to 100% B in A over 30 min using a flow rate of 2.4 mL/min.

Synthesis of kanamycin A derivatives

The 6'-*N*-5-hexynoate kanamycin A²⁰ and 6''-azido kanamycin A²¹ compounds were synthesized as previously described.

Spacing modules used for peptoid syntheses

Eleven different amines were used as spacing modules (Fig. 1A). All amines were used directly in the corresponding peptoids except for **Gly**, **Lys**, and **Arg**. The amine submonomer for the synthesis of **2 K-4-Gly** was glycine *tert*-butyl ester (CSPS Pharmaceuticals). The Lys-like submonomer for synthesis of **2 K-4-Lys** was afforded by coupling *N*-(*tert*-Butoxycarbonyl)-1,3-propanediamine²² to the peptoid followed by deprotection. The **2 K-4-Arg** peptoid was synthesized by guanidinylation of the free amines in **2 K-4-Lys** using Goodman's reagent.²³

Synthesis of *N*-(*tert*-Butoxycarbonyl)-1,3-propanediamine (lys submonomer)²²

The *N*-(*tert*-butoxycarbonyl)-1,3-propanediamine was prepared as follows: 22 mmoles of the diamine was stirred in 100 mL of chloroform. Then, 0.2 equivalents of (Boc)₂O was dissolved in 50 mL of chloroform and added dropwise at 0 °C. The reaction was stirred for 2 h at room temperature. The solution was filtered, and the filtrate was concentrated. The oily residue was diluted with ethyl acetate and washed with brine. The aqueous layer was extracted again with ethyl acetate, and the organic layers were combined. They were dried over MgSO₄, and the solvent was evaporated. Yield 71%, ¹H NMR (CDCl₃) δ 5.14 (br, 1H), 3.23 (br, 2H), 3.16 (m, 2H), 2.75 (t, 2H, *J*=6.7 Hz), 1.61 (t, 2H, *J*=6.7 and 6.7 Hz), 1.39 (s, 9H); MS (ES⁺) *m/z* 175 ([MH]⁺, 100).

General protocol for peptoid synthesis

Deprotection of resin—Fmoc-protected Rink amide polystyrene resin with a substitution level of 0.67 mmol g⁻¹ (0.15 g, 100 μmol) was swollen in dichloromethane (DCM; 1 mL) for 20 min. The column was drained, and the resin was deprotected with 1 mL of 20% piperidine in DMF for 40 min with shaking at 800 rpm. After draining the column, the resin was rinsed with six 3 mL portions of DMF and then six 3 mL portions of dry DMF (abbreviated DMF/dDMF (6 × 3/6 × 3 mL)).

Coupling of chloroacetic acid—Chloroacetic acid (1 mL, 2M in DMF) and DIC (1 mL, 2M in DMF) were added to the resin-bound amine, and the resin was shaken at 1000 rpm for 30 min. The column was drained, and then the resin was rinsed with DMF/dDMF (6 × 3/6 × 3 mL).

Alkylation of corresponding amine—The corresponding amine, either 3-azidopropylamine or the spacing submonomer (1 mL, 2 M in *N*-methyl-2-pyrrolidone (NMP)), and *N*-*N*-diisopropylethylamine (DIPEA; 1 mL, 2 M in NMP) were added to the column, and the resin was shaken for 3 h at 1000 rpm. After draining the column, the resin was rinsed with DMF/dDMF (6 × 3/6 × 3 mL).

Fluorescein-labeling of peptoid—The resin-bound peptoid was washed with DCM (3 × 2 mL), and dried under stream of air. The peptoid from a small portion of the resin was cleaved and analyzed by MS-ESI prior to fluorescein-labeling. Fmoc-6-aminoheptanoic acid (3 equivalents), PyBOP (3 equivalents), HBTU (3 equivalents) and DIPEA (10 equivalents) were prepared in 2 mL of NMP and added to resin-bound peptoid. The resin was shaken for

5 h at room temperature. The Fmoc protecting group was removed by adding 2 mL of 20% piperidine in DMF for 30 min with shaking at 800 rpm. This step was repeated. The solution was then drained, and resin was rinsed with DMF/dDMF ($6 \times 3/6 \times 3$ mL). Fluorescein was then coupled by adding 5(6)-carboxyfluorescein (5 equivalents), HOBt (20 equivalents) and DIC (20 equivalents). The reaction was incubated with shaking for 14 h in the dark. The solution was then drained, and resin was washed with DMF/dDMF ($6 \times 3/6 \times 3$ mL).

Synthesis of peptoids containing arg spacing modules²³—The peptoid backbone (15 μ mol) was dissolved in 125 μ L of water (0.12 M). Then, *N,N*-diBoc-*N*''-triflylguanidine (prepared as previously described;²⁴ 3 equivalents per amine; dissolved in 625 μ L of 1,4-dioxane) was added in small portions such that all components were dissolved. After 5 min, 3 equivalents of triethylamine per amine were added. The reaction was stirred for 24 h at room temperature. A portion of the reaction was deprotected with 50 : 50 TFA/DCM, dried and analyzed *via* ESI-MS.

Conjugation of 6'-*N*-5-hexynoate kanamycin A on the solid phase—All peptoids were conjugated to 6'-*N*-5 hexynoate kanamycin A *via* a solid phase reaction unless noted otherwise. To the peptoid (10 μ mol, two conjugation sites) were added 6 equivalents of solid 1,3,3''-tri-*N*-(*tert*-butoxycarbonyl)-kanamycin- 6'-*N*-hexynoate (3 equivalents per conjugation site). A solution of 0.1 M copper (II) acetate, 1 M DIPEA and 0.01 M TBTA in 3 : 7 pyridine/DMF (5 mL) was prepared and added to the resin. The click reaction was completed at 50 °C for 36 h. The kanamycin-functionalized peptoid was cleaved from the resin by adding 2×2 mL of 30 : 20 : 1 TFA/DCM/water and purified by HPLC as described above.

Conjugation of 6'-*N*-5-hexynoate kanamycin A in solution—Conjugation of 6'-*N*-5-hexynoate kanamycin A to the peptoid backbone for **2 K-4-Lys** and **2 K-4-Arg** was completed in solution instead of the solid phase as follows: After cleaving the fluorescein-labeled peptoid backbone from the resin, the peptoid was purified by HPLC as described above. To the peptoid backbone (8 μ mol; dissolved in 4 mL of 50 : 50 MeOH/H₂O) were added 6 equivalents of 6'-*N*-5-hexynoate kanamycin A (3 equivalents per conjugation site; dissolved in water), 1 equivalent of ascorbic acid (8 μ L of 1 M in H₂O; 0.5 equivalents per conjugation site), and 1 equivalent of CuSO₄ (8 μ L of 1 M in H₂O; 0.5 equivalents per conjugation site). The reaction was performed in a microwave reactor at 110 °C, with a pre-stirring time of 30 s and a reaction time of 3 h.²⁵ The kanamycin-functionalized peptoid was purified by HPLC as describe above.

Purification and characterization of compounds—The peptoids labeled with fluorescein were purified *via* preparative HPLC as described above. All peptoids were analyzed by analytical HPLC and are >90% pure. Concentrations of the stock solutions were determined in 0.1 M PBS buffer by measuring the absorption of fluorescein using an extinction coefficient 45 000 M⁻¹cm⁻¹ at 496 nm.²⁶

MBNL displacement assays—Displacement assays were completed as described¹⁶ in black 384-well plates coated with Streptavidin (Nunc). 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 IC₅₀ (eqn (1));

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

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. The number of moles of RNA immobilized in each well was determined using SYBR Green II as described.²⁷ Approximately ~20% of the moles of RNA delivered to a well are immobilized.

Cell culture—Five cell lines were studied: C2C12 (murine myoblasts), A549 (adenocarcinomic human alveolar basal epithelial cells), HeLa (human cervical adenocarcinoma cells), MCF7 (human breast adenocarcinoma cells) and Jurkat (human leukemic T lymphocyte cells). The C2C12, A549, HeLa and MCF7 cell lines were grown as a monolayer at 37 °C and 5% CO₂ in 1X High Glucose DMEM (Invitrogen) supplemented with 10% FBS (Invitrogen) and 0.5% penicillin/streptomycin. The Jurkat cell line was grown at 37 °C and 5% CO₂ in 1X RPMI Media (Invitrogen) supplemented with 10% FBS (Invitrogen) and 0.5% penicillin/streptomycin.

Fluorescence microscopy—The cellular localization of the modularly assembled compounds was determined for C2C12, A549, HeLa, and MCF7 cell lines. Cells were grown in 35 mm poly-D-lysine coated glass-bottomed culture dishes (MatTek Corporation). After 24 h of growth, the medium was removed and replaced with fresh medium containing 5 μM of the compound of interest. The compound-containing medium was removed after 24 h, and the cells were washed at least three times with 3 mL of 1X DPBS (Invitrogen). The cells were then imaged using Zeiss Axio Observer inverted fluorescence microscope.

Quantification of uptake and toxicity of ligands—Cellular uptake and toxicity of modularly assembled ligands were quantified using flow cytometry. Uptake assays were completed as described above for microscopy. For adherent cell lines (C2C12, A549, MCF7, and HeLa), the compound-containing medium was removed, and the cells were washed with 3 mL of 1X DPBS (Invitrogen). The cells were then trypsinized from the plate surface and pelleted. They were washed with 1X DPBS, and then resuspended in 1 mL of ice-cold 1X DPBS.

Jurkat cells, which are non-adherent, were pelleted in the compound-containing medium. After pelleting, the cells were washed twice with 1X DPBS and then resuspended in 1 mL of ice-cold 1X DPBS.

In order to assess toxicity, 1 μL of 1.5 mM propidium iodide or Sytox Red (Invitrogen) per the manufacturer's protocol 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 (positive uptake).

Results & discussion

We previously reported that a peptoid backbone is a useful scaffold to multivalently display RNA-binding modules.^{15,16,27} Specifically, a kanamycin derivative was displayed on a peptoid backbone to bind the regularly repeating internal loops present in the RNAs that cause myotonic dystrophy. The compound displaying kanamycin ligand modules at

optimized distances is high affinity and disrupts the RNA-protein interaction that causes DM with a nanomolar IC₅₀. In order to control the distance between the kanamycin binding modules, the number of propylamine couplings between ligand couplings was systematically changed. The optimal distance to target the RNA that causes myotonic dystrophy type 2 (DM 2) is achieved by coupling four propylamine submonomers between ligand-displaying submonomers. These compounds are denoted **nK-4** where **n** is the number of ligand modules displayed or valency, **K** indicates the kanamycin derivative binding modules, and **4** denotes the coupling of four spacing submonomers between ligand modules.

In order to optimize the bioactivity of this lead compound, the identity of the spacing module was varied in order to determine its effect on potency, cellular uptake, toxicity, and cellular localization (Fig. 1A). Since the toxic RNA-protein interaction that causes DM occurs in the nucleus, we were interested in determining if the spacing module could direct the compound to the nucleus.

Selection of spacing modules

The spacing modules that were used to investigate their influence on the peptoid's properties are shown in Fig. 1A. Spacers **Tyr** and **Bzn** were chosen based on investigations of peptide-RNA complexes that determined RNA binding preferences for amino acids.²⁸ **Tyr** mimics tyrosine, which stacks on uracil; **Bzn** is similar to phenylalanine, which stacks on adenine and uracil bases in RNA.²⁹ **Pyr** binds to mimics of the bacterial rRNA A-site.³⁰ Thus, it is likely that peptoids synthesized with these spacing modules will have enhanced binding to RNA. Other spacing modules have functional groups that facilitate uptake of ligands into cells in addition to forming favorable interactions with RNA. For example, oligomers of **Lys** and **Arg** are molecular transporters that facilitate uptake of dyes or drugs into several cell types.^{28,31–33} **Eth**, **Gly**, **Arg** and **Tyr** all contain hydrogen bond donors that could possibly form interactions within the RNA. **Flb** is fluorinated; fluorination can enhance biological activity.³⁴ **Bzn** and **Dip** contain a phenyl group, which is a common functionality in known drugs.^{35–37} The nomenclature to describe peptoids in which different spacing modules were coupled is **nK-4-XYZ** where **n** is the valency, **K** denotes conjugation of the kanamycin derivative, **4** denotes the four spacing modules present between the binding modules, and **XYZ** denotes the identity of the spacing module (Fig. 1A).

Potency of second generation peptoids

The potencies of the second generation peptoids for inhibiting the DM2 RNA-MBNL1 interaction were determined using an *in vitro* displacement assay (Table 1 and Fig. 2).¹⁶ Briefly, r(CCUG)²⁴ with a 3' single stranded region is immobilized into a well of a 384-well streptavidin-coated plate *via* hybridization to a biotinylated DNA. The compound of interest and then MBNL1-β-galactosidase fusion protein are added. After washing, the amount of MBNL1 present is determined by β-galactosidase activity.

The first generation peptoid with propylamine spacing modules, **2 K-4-Ppl**, has an IC₅₀ of 0.089 μM.¹⁶ Perhaps not surprisingly, peptoids with lysine- and arginine-like spacing modules, **2 K-4-Lys** and **2 K-4-Arg**, were more potent than **2 K-4-Ppl** with IC₅₀'s of 0.040 and 0.045 μM, respectively. The improved potency is likely due to electrostatic interactions between the phosphodiester backbone and the positively charged spacing modules. Other peptoids have IC₅₀'s with 3-to 7-fold decreases in potency including **2 K-4-Ibt** (0.26 μM), **2 K-4-Tyr** (0.27 μM), **2 K-4-Bzn** (0.29 μM), **2 K-4-Met** (0.30 μM), **2 K-4-Eth** (0.57 μM), and **2 K-4-Pyr** (0.60 μM). **2 K-4-Flb** and **2 K-4-Gly** showed significant decreases in DM2-MBNL1 inhibition with IC₅₀'s of 2.8 and 3.0 μM, respectively. **2 K-4-Dip** showed no inhibition of the DM2 RNA-MBNL1 interaction up to 25 μM.

The peptoid scaffold leads to efficient uptake of kanamycin derivatives into murine myoblasts (C2C12 cell line)

The cellular permeability and toxicity of the peptoids were then determined using flow cytometry. Each peptoid and monomeric kanamycin derivatives were fluorescently labeled with fluorescein. Thus, the distribution of the relative fluorescence intensity in the fluorescein channel for cells treated with compound can be compared to untreated cells as determined by flow cytometry. After treating the cells for 24 h with 5 μ M compound, the cells are harvested by trypsinization or centrifugation. In order to determine if the compound is toxic, the cells are then treated with propidium iodide or SYTOX Red. The resulting flow cytometry histograms are divided into quadrants: cells that have no associated fluorescence; cells that only have fluorescence in the fluorescein channel (indicates uptake of the fluorescein-labeled peptoid); cells that only have fluorescence in the propidium iodide or SYTOX Red channel (background control for dead cells); and cells that have fluorescence in both the fluorescein and the propidium iodide or SYTOX red channels (indicates uptake of the fluorescein-labeled peptoid and toxicity). The percentages reported below are the percentage of cells that have fluorescence in the fluorescein channel only. The relative amount of peptoid taken up by the cells can be determined by comparing the normalized geometric means of relative fluorescence intensity, or the mean fluorescence intensity of the population of cells that are positive for uptake of the peptoid.

Two different kanamycin A derivatives were also synthesized and fluorescently labeled in order to study their uptake and toxicity into murine myoblasts (C2C12 cell line). **FITC-K** (Fig. 1B) was synthesized by coupling fluorescein isothiocyanate (FITC) to 6'-N-5-hexynoate kanamycin A after installation of an amine-containing linker.²⁰ Analogously, **FITC-Kaz** was synthesized from 6"-azido kanamycin A by reacting first with propargylamine and then FITC.²⁰ Both **FITC-K** and **FITC-Kaz** show poor cellular permeability (~10% of cells are fluorescent) into the C2C12 cell line as assessed by flow cytometry. Neither compound is toxic under the conditions tested.

It was shown in our previous studies that kanamycin-functionalized peptoids (dimers, trimers, and tetramers) are cell permeable in the C2C12 cell line. Therefore, two monomeric peptoids were synthesized that display **K** and **Kaz**, **1 K-0** and **1Kaz-0**, respectively. Conjugation of the cationic ligand onto the peptoid improved the percentage of cells that are positive for uptake by 2-fold and 8-fold for **K** (23%) and **Kaz** (86%), respectively (Table 2). **1Kaz-0** has an ~5-fold increase in toxicity (11%), likely due to the presence of an additional amino group at the 6' position. The presence of amino groups has been showed to contribute to aminoglycoside toxicity.³⁸ Alkylation of amino groups, such as in the clinically used amakacin, decreases toxicity.³⁸ Approximately the same amount of compound is taken up by the cells, independent of the type of kanamycin derivative (**FITC-K** or **FITC-Kaz**) or conjugation to a peptoid scaffold (**1 K-0** or **1Kaz-0**) as assessed by the normalized geometric mean in the fluorescein channel (Table 2). Taken together, the peptoid scaffold improves cellular permeability.

Uptake and toxicity of peptoids into the C2C12 cell line

The uptake and toxicity properties of the second generation peptoids are summarized in Table 2 and Fig. 3. The compounds can be classified into three groups: those with decreased cellular permeability as compared to the parent compound, **2 K-4-Ppl**; those with similar permeabilities as **2 K-4-Ppl**, and those with increased cellular permeability. (The reported percentages are the percentage of cells that have fluorescence in the fluorescein channel only.) **2 K-4-Eth** (39%), **2 K-4-Dip** (42%), and **2 K-4-Arg** (55%) were less permeable than the parent peptoid **2 K-4-Ppl** (78%). Similar permeabilities were observed for **2 K-4-Gly** (64%), **2 K-4-Flb** (65%), **2 K-4-Ibt** (66%), and **2 K-4-Tyr** (67%) while improved uptake

was observed for **2 K-4-Lys** (83%), **2 K-4-Bzn** (87%), **2 K-4-Pyr** (92%), and **2 K-4-Met** (94%).

The relative amount of compound taken up by cells can be estimated by the normalized geometric mean of the histograms. Many of the peptoids had values between 4–11 relative fluorescence units (RFU). However, the presence of **Flb** and **Dip** increased the amount of compound taken up by the cell to ~20 RFU (Table 2).

Fortuitously, toxicity of the compounds was generally low, with less than 10% of the cells showing uptake of compound and propidium iodide or SYTOX red. Interestingly, the two spacing modules that significantly increased the amount of compound within the cell also increased toxicity. **2 K-4-Flb** and **2 K-4-Dip** had toxicity rates of 34% and 58% respectively (Table 2).

Localization of peptoids in C2C12 murine myoblasts

Despite displaying a wide range in cellular permeability, cellular localization is independent of the identity of the spacing module. Ligands were localized mainly in the perinuclear and cytoplasmic region (Fig. 4 and Supporting Information).

Uptake & toxicity of peptoids in other mammalian cell lines

In order to determine if the patterns of uptake, toxicity, and cellular localization were cell line independent, a subset of compounds (**1 K-0**, **1Kaz-0**, **2 K-4-Met**, **2 K-4-Eth**, **2 K-4-Lys**, **2 K-4-Gly**, **2 K-4-Arg**, **2 K-4-Pyr** and **2 K-4-Tyr**) were tested for uptake, toxicity and localization in A549, HeLa, MCF7 and Jurkat cell lines. The subset of compounds was chosen as it represents a broad spectrum of functional groups and lipophilic properties of the side chains (Fig. 1A). The cell lines chosen are model systems for different types of cancer: A549 cells were obtained from human lung carcinomas; HeLa is a cervical adenocarcinoma cell line; MCF7 is a breast carcinoma cell line; and Jurkat cells are acute leukemic T-lymphocytes. Any variations observed in uptake and/or localization could prove advantageous as different organs could be preferentially targeted by changing the spacing module used in the synthesis of the peptoid. Toxicity for this subset of compounds in all cell lines was low with <10% of cells showing toxicity as screened by propidium iodide or Sytox Red staining (dosed at 5 μ M concentration for 24 h).

A549 cell line—As observed with the C2C12 cell line, uptake of **FITC-K** and **FITC-Kaz** ligand modules is poor (Table 3 and Fig. 3). Conjugation of **K** to the peptoid scaffold (**1 K-0**), however, does not improve cellular permeability. In contrast, an ~3-fold increase in uptake is observed with **Kaz** is conjugated to a peptoid scaffold. Low toxicity is observed for all four compounds.

The most variation in cellular uptake as a function of spacing module was observed with the A549 cell line (Table 3 and Fig. 3). The lowest uptake was observed for peptoids with the **Eth**, **Pyr**, and **Met** spacing modules. Moderate uptake was observed for peptoids containing **Gly**, and **Tyr** while improved uptake was observed for peptoids containing **Lys** and **Arg**.

By analyzing the geometric means of the flow cytometry histograms, the relative amounts of compound inside the cells can be compared. In general, the peptoids that were in the largest percentage of cells were also present in the highest concentrations (Table 3 and Fig. 3). For example, **2 K-4-Arg** and **2 K-4-Lys** are present in 83% and 75% of cells respectively, and are in at least 2-fold higher concentration as compared to peptoids with other spacing submonomers that are moderately cell permeable.

Similar to localization in C2C12 cells, cytoplasmic and perinuclear localization was observed for all compounds in the A549 cell line. The uptake of unfunctionalized peptoids with amino and guanidylated modules in A549 cell line has been previously reported.⁸ The amine-containing peptoids localize in the cytosol while the guanidynyl-containing peptoids localize in the nucleus.⁸

HeLa & MCF7 cell lines—Conjugation of the kanamycin ligand modules **K** and **Kaz** to afford **1 K-0** and **1Kaz-0**, respectively, does not improve cellular permeability (within error) in HeLa or MCF7 cell lines (Table 3 and Fig. 3). This trend was also observed for the **K** dimers as uptake was generally low for all compounds. The percentage of cells that were positive for uptake of the dimeric peptoids ranged from 11–55% for HeLa and 14–37% for MCF7. Approximately 20% of cells were fluorescent independent of the spacing module with the exception of the peptoid with the **Arg** spacing modules. A previous study found that the uptake of polyproline helices was enhanced by the presence of guanidinium groups in MCF7 cells.³⁹ The normalized geometric means, indicating relative concentrations, are also similar for all peptoids ranging from 3.2–6.6 RFU for HeLa cells and 3.3–6.4 RFU for MCF7 cells. Interestingly, the most cell permeable peptoid for both cell lines is **2 K-4-Arg** (Table 3).

A previous study investigated the uptake of peptoids with different spacing modules into HeLa cells.¹⁷ Peptoids with no net charge were ~3-fold more cell permeable than peptoids with a net charge of –1 or +1. Even larger effects (~8-fold decrease) were observed for peptoids with net charges of –4 or +4.¹⁷ We observed an approximately 5-fold range of cellular permeabilities, although ~20% of the cells were fluorescent when treated with most peptoids. This is likely because the highly cationic kanamycin ligands dominate the peptoids' properties.

Despite having similar uptake profiles, cellular localization was different for HeLa and MCF7 cells. Similar to localization in C2C12 and A549 cells, cytoplasmic and perinuclear localization was observed for all compounds in the MCF7 cell line. Polyproline helices that are conjugated to lysine-containing peptides or that are guanidynlated are located throughout MCF7 cells.³⁹ Interestingly, peptoids with Lys and Arg spacing modules were not present in the nucleus of MCF7 cells in our studies. In contrast, fluorescence was observed throughout HeLa cells, including the nucleus. In a previously reported study, the uptake and cellular localization of peptoids containing 1–9 **Lys** spacing modules was investigated in three cell lines including HeLa.⁴⁰ In good agreement with the studies reported here, the peptoids were distributed throughout the cells including the nucleus.⁴⁰

Jurkat cell line—In contrast to the HeLa and MCF7 cell lines, the Jurkat cell line is much more cell permeable even to the unconjugated ligand modules (Table 3). This could be due to its nonadherent properties of the Jurkat cell line, although larger differences in the permeabilities of **Arg**- and **Lys**-containing peptoids were reported in previous studies.⁶ Approximately 85% of the cells are fluorescent when treated with **K**, **Kaz**, **1 K-0**, or **1Kaz-0**. Increasing the length of the peptoid to a dimer or changing the spacing module does not significantly alter cellular permeability as the percentage of cells that were fluorescent is ~90% for all compounds. The amount of compound inside the cells is also increased by about 2-fold as compared to the other cell lines (Table 2 and 3), independent of the spacing module. Taken together, the spacing module and the peptoid backbone are not important determinants for uptake into Jurkat cells.

Comparison of cellular permeabilities in all cell lines—A range of permeabilities was observed for the C2C12 and A549 cell lines. However, the spacing modules that led to enhanced permeability are different: **Pyr**, **Bzn**, and **Lys** spacing modules improve uptake

into the C2C12 cell line while **Arg** and **Lys** increase uptake into A549. The **Arg** spacing module also improved uptake into HeLa and MCF7 cells, which were generally less permeable to the peptoids than the C2C12, A549, and Jurkat cell lines. For all cell lines with the exception for C2C12, **2 K-4-Arg** enters the cells in the highest concentration (Table 2 and 3). Excluding Jurkat cells, the presence of the **Eth** spacing module is inversely related to cellular permeability. **Dip**-containing peptoids also show poor permeability into C2C12 cells while the permeabilities of the **Pyr**- and **Met**-containing peptoids were within error of **Eth** permeability into A549. Similar to A549 cells, the peptoid containing the **Met** spacing module also exhibits poor cellular uptake into MCF7 cells.

Comparison to other studies

It is interesting that the **Arg** and **Lys** modules lead to improved cellular uptake in the C2C12, A549, HeLa, and MCF7 (**Arg** only) cell lines (Table 2 and 3). Polyarginines and polylysines are known molecular transporters and their cellular uptake likely receptor-mediated (nonendocytotic).^{41,42} Polyarginines have been used to facilitate uptake of peptides,^{41,43} peptoids,⁶ and peptidomimetics.⁴⁴ They are generally more efficient than their polylysine, polyhistidine, and polyornithine counterparts.^{6,45,46} Based on these results, guanidinoaminoglycosides were synthesized and studied for cellular permeability. Guanidynlation significantly improved cellular uptake into two cell lines.⁴⁷ Previously, a high throughput method was developed in order to assess the permeability of peptoid libraries.^{17,48} From that study, it was determined that the more highly charged (negatively or positively) peptoid octamers were less cell permeable.¹⁷ ENREF_44 Despite the highly cationic kanamycin cargo,⁴⁹ our peptoids are cell permeable.

Implications

The peptoids with Lys- and Arg- spacing modules enhance potency by about 2-fold (Table 1) and enhance cellular permeability in C2C12, A549, and HeLa cell lines. These positively charged side chains could be enhancing potency and affinity by forming additional interactions with the phosphodiester backbone, sugar, or bases. They could, however, decrease specificity for the DM2 RNA.

The results reported herein have implications for the therapeutic use of peptoids. Of the cell lines tested, peptoids appear to be an ideal scaffold for therapeutic targets in T-lymphocytes. The peptoids are highly cell permeable in Jurkat cells independent of the spacing module and are distributed throughout the cell. The Jurkat cell line is the only non-adherent one; whether this affects uptake and localization is unknown. If peptoids were to be used against therapeutic targets in cervical (HeLa) cells, then the **Arg** spacing module should be employed while peptoids with **Tyr**, **Arg**, and **Lys** could be used against cytoplasmic targets in lung cancer cells (A549). Because of their poor cellular permeability in MCF7 cells, peptoids may not be an ideal scaffold for treating breast cancer.

None of the spacing modules studied herein increases nuclear localization in the C2C12 cell line. Nuclear localization was of particular interest in the muscle myoblast cell line because the toxic RNA-protein complexes that cause the myotonic dystrophies occur in the nucleus. Thus, additional spacing modules or nuclear localization signals will be further investigated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.
 The chemical structures of the compounds used in this study and the spacing modules. A: Top, the general structure of the peptoids. Bottom, the spacing modules used to influence cellular uptake and localization. The **2 K-4** prefix indicates a peptoid containing **2** kanamycin (**K**) ligand modules with **4** spacing modules between the **K**'s. The **2 K-4** prefix is followed by a three letter code representing the identity of the various spacing modules. B: The structures of monomeric ligand modules, unconjugated and conjugated to a peptoid scaffold.

Fig. 2.

Inhibition of the DM2 RNA-MBNL1 interaction by peptoids with different spacing modules. **A:** IC₅₀ curves for **2 K-4-Ppl**, **2 K-4-Met**, **2 K-4-Eth** and **2 K-4-Ibt**. **B:** IC₅₀ curves for **2 K-4-Lys**, **2 K-4-Gly**, **2 K-4-Arg** and **2 K-4-Bzn**. **C:** IC₅₀ curves for **2 K-4-Pyr**, **2 K-4-Tyr**, **2 K-4-Flb** and **2 K-4-Dip**.

Fig. 3. Summary of the cellular permeability of peptoids with varying spacing modules into HeLa, MCF7, A549, C2C12 and Jurkat cell lines.

Fig. 4.

Representative fluorescence microscopy images of the cellular localization of peptoids into C2C12 cells. Left, fluorescence microscopy image of C2C12 cells treated with **2 K-4-Ppl**; Middle, fluorescence microscopy image of C2C12 cells treated with **2 K-4-Arg**; and Right, fluorescence microscopy image of C2C12 cells treated with **2 K-4-Pyr**.

Fig. 5. Representative fluorescence microscopy images of the cellular localization of **2 K-4-Pyr** into all cell lines. A, phase contrast images; B, fluorescence microscopy images of cells treated with **2 K-4-Pyr**; C, overlay of the phase contrast and fluorescence images; D, fluorescence intensity as a function of distance plots that indicate cellular localization.

Table 1

Inhibition of DM2 RNA-MBNL1 complex formation by second generation **2 K-4** peptoids displaying different spacing modules (Fig. 1A)

Ligand	IC ₅₀ (mM)	Normalized IC ₅₀ (NIC ₅₀), mM ^a	Relative potency compared to 2 K-4-Ppl ^b
2 K-4-Ppl	0.089 ^c	0.178 ^c	—
2 K-4-Lys	0.040 ± 0.031	0.080	2.2
2 K-4-Arg	0.045 ± 0.024	0.090	1.9
2 K-4-Ibt	0.26 ± 0.02	0.52	0.35
2 K-4-Tyr	0.27 ± 0.14	0.54	0.34
2 K-4-Pyr	0.60 ± 0.32	1.2	0.31
2 K-4-Met	0.30 ± 0.05	0.60	0.30
2 K-4-Bzn	0.29 ± 0.16	0.58	0.23
2 K-4-Eth	0.57 ± 0.28	1.14	0.16
2 K-4-Flb	2.8 ± 1.9	5.6	0.03
2 K-4-Gly	3.0 ± 0.6	5.6	0.03
2 K-4-Dip	>25	>50	>0.004

^aThe NIC₅₀ is determined by multiplying the IC₅₀ by the number of **K** modules displayed on the peptoid.

^bThe improved efficacy is calculated by dividing the IC₅₀ for **2 K-4-Ppl** by the IC₅₀ of the ligand.

^cPreviously determined.

Table 2

Uptake, toxicity, and geometric means for monomeric and dimeric, kanamycin-functionalized peptoids with different spacing modules in C2C12 cells

Ligand	% Cells showing uptake	% Cells showing toxicity	Normalized geometric mean, fluorescein	Normalized geometric mean, propidium iodide
FITC-K	11 ± 5.7	2 ± 0.6	3.6 ± 0.2	1.3 ± 0.4
FITC-Kaz	10 ± 1.0	1 ± 0.3	5.0 ± 0.1	1.2 ± 0.6
1 K-0	23 ± 3.3	2 ± 0.5	3.2 ± 0.1	1.1 ± 0.1
1Kaz-0	86 ± 1.5	11 ± 1.5	10.1 ± 0.2	2.2 ± 0.5
2 K-4-Eth	39 ± 6.4	3 ± 0.1	3.7 ± 0.2	1.2 ± 0.1
2 K-4-Dip	42 ± 16	58 ± 16	23 ± 4.1	5.9 ± 1.6
2 K-4-Arg	55 ± 1.7	3 ± 0.9	4.5 ± 0.1	1.2 ± 0.06
2 K-4-Gly	64 ± 9.0	4 ± 0.5	5.1 ± 0.6	1.3 ± 0.04
2 K-4-Flb	65 ± 8.9	34 ± 9.0	24 ± 4.4	4.0 ± 0.5
2 K-4-Ibt	66 ± 5.6	4 ± 1.1	5.8 ± 0.6	1.3 ± 0.1
2 K-4-Tyr	67 ± 7.0	6 ± 2.7	6.3 ± 0.5	1.4 ± 0.3
2 K-4-Ppl	78 ± 1.8	6 ± 1.7	7.5 ± 0.3	1.5 ± 0.1
2 K-4-Lys	83 ± 1.6	9 ± 3.5	9.8 ± 1.1	1.8 ± 0.2
2 K-4-Bzn	87 ± 0.4	9 ± 0.1	13 ± 0.3	1.9 ± 0.08
2 K-4-Pyr	92 ± 6.0	6 ± 6.2	11 ± 8.0	1.4 ± 0.7
2 K-4-Met	94 ± 1.2	2 ± 0.5	6.1 ± 0.3	1.0 ± 0.1

Table 3
Cellular uptake, toxicity, and geometric mean of fluorescein for monomeric and dimeric compounds in A549, HeLa, MCF7, and Jurkat cell lines

Ligand	A549			HeLa			MCF7			Jurkat		
	% Uptake	% Toxicity	Geometric mean (Fluorescein)	% Uptake	% Toxicity	Geometric mean (Fluorescein)	% Uptake	% Toxicity	Geometric mean (Fluorescein)	% Uptake	% Toxicity	Geometric mean (Fluorescein)
FTTC-K	9 ± 2.6	2 ± 0.5	2.0 ± 0.6	18 ± 5.0	2 ± 0.2	3.7 ± 0.4	27 ± 2.1	5 ± 0.4	4.3 ± 0.3	84 ± 4.3	11 ± 1.2	10.8 ± 2.0
FTTC-Kaz	11 ± 1.6	2 ± 0.7	3.6 ± 0.2	24 ± 10.6	2 ± 0.3	4.1 ± 0.8	17 ± 4.4	5 ± 0.8	3.4 ± 0.3	87 ± 1.6	12 ± 1.5	23.9 ± 7.7
1 K-0	13 ± 2.2	3 ± 0.3	3.9 ± 0.3	8 ± 1.2	1 ± 0.2	2.8 ± 0.1	18 ± 5.7	6 ± 0.6	3.6 ± 0.4	86 ± 2.0	8 ± 0.5	9.6 ± 1.3
1 Kaz-0	31 ± 8.9	3 ± 0.3	5.4 ± 0.6	21 ± 5.1	3 ± 0.3	4.2 ± 0.4	27 ± 6.1	8 ± 1.6	4.8 ± 0.8	87 ± 0.9	10 ± 0.8	13.0 ± 0.4
2 K-4-Eth	33 ± 10.8	5 ± 1.2	4.3 ± 0.6	22 ± 1.4	3 ± 0.5	4.1 ± 0.1	15 ± 6.1	5 ± 0.4	3.5 ± 0.5	88 ± 2.8	6 ± 1.0	12.3 ± 0.7
2 K-4-Arg	83 ± 1.2	11 ± 1.4	11.2 ± 0.6	55 ± 2.9	6 ± 0.3	6.6 ± 0.3	37 ± 5.8	11 ± 1.8	6.4 ± 0.3	92 ± 0.9	8 ± 1.0	34.0 ± 3.3
2 K-4-Gly	50 ± 5.4	6 ± 1.1	5.2 ± 0.4	11 ± 1.5	2 ± 0.5	3.3 ± 0.2	24 ± 2.3	5 ± 0.7	4.0 ± 0.2	89 ± 2.8	5 ± 0.6	11.3 ± 0.8
2 K-4-Tyr	63 ± 1.6	10 ± 1.2	6.6 ± 0.1	13 ± 7.5	2 ± 0.6	3.2 ± 0.7	30 ± 2.1	11 ± 1.2	5.5 ± 0.3	93 ± 0.7	7 ± 0.7	28.0 ± 3.1
2 K-4-Ppl	18 ± 4.3	9 ± 1.5	4.9 ± 0.2	13 ± 3.2	2 ± 0.5	3.6 ± 0.3	28 ± 3.5	9 ± 0.4	4.9 ± 0.3	85 ± 3.3	10 ± 1.1	9.6 ± 0.9
2 K-4-Lys	75 ± 0.4	9 ± 1.1	8.0 ± 0.2	33 ± 2.4	4 ± 1.2	4.7 ± 0.3	21 ± 3.7	8 ± 0.3	4.3 ± 0.3	92 ± 1.3	7 ± 1.3	20.4±0.4
2 K-4-Pyr	37 ± 9.1	7 ± 1.0	4.5 ± 0.5	23 ± 5.3	3 ± 1.1	4.1 ± 0.5	24 ± 5.4	9 ± 2.7	4.6 ± 0.9	91 ± 1.4	6 ± 1.1	14.1 ± 1.4
2 K-4-Met	45 ± 2.6	6 ± 0.1	4.2 ± 0.1	22 ± 3.1	4 ± 0.3	4.1 ± 0.2	14 ± 11.8	6 ± 1.0	3.3 ± 0.2	89 ± 2.3	6 ± 0.8	13.4 ± 0.8