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Reducing levels of toxic RNA with small molecules

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Abstract

Myotonic dystrophy (DM) is one of the most common forms of muscular dystrophy. DM is an autosomal dominant disease caused by a toxic gain of function RNA. The toxic RNA is produced from expanded non-coding CTG/CCTG repeats, and these CUG/CCUG repeats sequester the Muscleblind-like (MBNL) family of RNA binding proteins. The MBNL proteins are regulators of alternative splicing, and their sequestration has been linked with mis-splicing events in DM. A previously reported screen for small molecules found that pentamidine was able to improve splicing defects associated with DM. Biochemical experiments and cell and mouse model studies of the disease indicate that pentamidine and related compounds may work through binding the CTG*CAG repeat DNA to inhibit transcription. Analysis of a series of methylene linker analogs of pentamidine revealed that heptamidine reverses splicing defects and rescues myotonia in a DM1 mouse model.

Microsatellite expansion disorders are a class of disease that result from the aberrant expansion of short DNA repeats (1-10 bases). The repeats can be located in either the coding or non-coding portions of the genome (1). Microsatellite repeat expansions located in the coding region of the genome can result in protein gain- or loss-of-function, resulting in disease symptoms (2), whereas repeats found within either non-coding or coding regions, upon transcription, can act via an RNA gain-of-function mechanism to result in disease (3-5). One of the first disorders to be identified as an RNA gain-of-function disease was myotonic dystrophy (DM). DM is characterized by many different symptoms, including but not limited to, cataracts, cardiac conduction defects, cognitive dysfunction, muscle wasting, and myotonia, where muscles have delayed relaxation (reviewed in (3, 6)). There are two types of DM: type 1 (DM1) and type 2 (DM2). DM1 is caused by the aberrant expansion of CTG repeats in the 3' UTR of the *DMPK* gene (7, 8). Unaffected individuals have between 5 and 37 repeats, whereas DM1 patients have more than 50 repeats. Upon transcription, these

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repeats sequester RNA binding proteins in nuclear foci. Similar circumstances cause DM2, where CCTG repeats in the first intron of the *ZNF9* gene expand and lead to production of a toxic CCUG repeat RNA (9).

One of the protein families that bind the CUG/CCUG repeats is the Muscleblind-like (MBNL) family of proteins (10). In humans, there are three paralogues: MBNL1, MBNL2, and MBNL3. The MBNL proteins are zinc-finger RNA binding proteins that regulate alternative splicing events (11) and play a role in RNA localization (12, 13). When expanded CUG/CCUG repeats are present, MBNL proteins are sequestered to the repeat RNA and are no longer able to properly regulate alternative splicing of their target pre-mRNA transcripts (13-19). Many of the mis-splicing events observed in DM are MBNL dependent, and some of these events have been correlated with disease symptoms. One target of the MBNL proteins is the *Clcn1* transcript, which encodes a chloride ion channel. The MBNL proteins are negative regulators of exon 7a in *Clcn1* (when MBNL proteins are present, exon 7a is excluded). In the presence of CUG/CCUG repeats, the levels of free MBNL are not sufficient to properly regulate splicing of exon 7a. This leads to chloride channelopathy and the characteristic DM symptom myotonia (delayed muscle relaxation) (20).

Although there are currently no treatments available to patients with DM, many different approaches are under development. Several methods focus on displacing MBNL proteins from the CUG/CCUG repeat structures with small molecules (21-24), multivalent compounds (25-27), small peptides (28-30), or antisense oligonucleotide analogs (31) in DM model systems. Other techniques seek to cleave the repeats (32-34) or trigger their degradation (35-39) in DM model systems.

In a recent study, we employed a competitive electrophoretic mobility shift assay (EMSA) to screen for compounds that could disrupt the MBNL1/(CUG)₄ interaction. The most promising compound in this screen was pentamidine (Figure 1), an FDA approved drug for the treatment of *Pneumocystis carinii* infections. Pentamidine showed therapeutic promise *in vivo*, rescuing mis-splicing of transiently transfected minigene reporters in a HeLa DM1 cell model expressing 960 interrupted CUG repeats and, most encouragingly, improving mis-splicing of endogenous transcripts in a DM1 mouse model expressing 220 CUG repeats (21). Because pentamidine was discovered due to its ability to disrupt the protein/RNA interaction in our competitive EMSA, we hypothesized that it acts *in vivo* by binding CUG repeat RNA and displacing MBNL, freeing MBNL to regulate alternative splicing. However, during subsequent work we found that pentamidine does not effectively function in the competitive EMSA without the presence of the dye bromophenol blue (BPB). Other biophysical experiments were also unable to demonstrate significant affinity between pentamidine and CUG repeat RNA. These results prompted us to investigate other mechanisms by which pentamidine might rescue mis-spliced transcripts in our DM model systems. We found that, upon treatment with pentamidine, CUG transcript levels drop significantly in both the cell and mouse models, suggesting that pentamidine is not directly blocking MBNL binding as previously proposed, but either decreases transcription of the CUG RNA or increases the rate of degradation.

We also undertook a structure activity relationship (SAR) study in an effort to develop a pentamidine analog with improved physiochemical properties. Analogs containing between three to nine methylene carbons (Figure 1) were synthesized and tested for their ability to rescue the mis-splicing of MBNL regulated minigenes in our HeLa cell splicing assay. In general, analogs with more methylenes more efficiently rescued splicing. The most promising compound, heptamidine, was tested for its ability to rescue mis-splicing and myotonia in a transgenic mouse model of DM1.

RESULTS AND DISCUSSION

Pentamidine treatment reduces CUG RNA levels in cells

During our studies with pentamidine, we discovered that a dye, bromophenol blue (BPB), used to aid in gel loading/tracking, was necessary for pentamidine to robustly inhibit the MBNL1/(CUG)₄ interaction (Supplemental Figure 1, Supplemental Table 1). Interestingly, despite the fact that pentamidine required BPB *in vitro*, in our cell and mouse models of the disease, pentamidine was capable of modulating splicing without any added dye (21). This suggested that either another molecule acted synergistically with pentamidine in these models, or pentamidine was acting through some other mechanism.

This prompted us to re-examine our model of pentamidine's mode of action. To this end, we probed CUG levels in HeLa cells transiently transfected with a plasmid containing 960 CUG repeats using northern blotting analysis (Figure 2). As cells were treated with increasing pentamidine concentrations, we observed a dose-dependent decrease in CUG levels (Figure 2b). We also treated cells with propamidine (methylene linker length of 3) and heptamidine (methylene linker length of 7) (Figure 2a,c). Propamidine also reduced CUG repeat transcript levels in a dose-dependent manner, but was not as effective as pentamidine. Interestingly, at low doses of propamidine (20 μ M), an increase in CUG repeat levels is observed. We were unable to observe significant reduction with heptamidine treatment before significant cell death occurred. In this system, heptamidine was cytotoxic at concentrations above 17.5 μ M.

There are two likely mechanisms to account for the reduction in repeat transcript: either pentamidine destabilizes the RNA or inhibits its transcription. We favored the hypothesis that pentamidine is affecting transcription of the repeats because CTG*CAG repeats are known to cause RNA polymerase II stalling (40, 41), perhaps creating a sensitivity to transcriptional inhibition. Secondly, pentamidine has been shown to stabilize DNA in thermal melting studies (42, 43) probably by inter-strand hydrogen bonding in the minor groove (44). If pentamidine bound and stabilized the CTG*CAG repeats, it could reduce or inhibit RNA polymerase II activity through the repeat region, resulting in reduced CUG repeat levels in cells.

We suspect that all three compounds would have similar mechanisms, but increasing CUG repeat levels at low concentrations of propamidine, and a statistically insignificant change in repeat levels confound interpretation. Pentamidine has been shown to increase transcription through GAA repeats through an unknown mechanism (45); propamidine may be able to function similarly. Propamidine may also act to up-regulate proteins that would aid in the

transcription of long repeat transcripts (such as Supt4h (46)), but at high levels bind to the CTG DNA directly and inhibit transcription. Heptamidine is much more toxic to these cells, and we suspect that if we were able to treat at higher concentrations, we would observe reductions in CUG repeat levels.

Pentamidine reduces amount of transcript in T7 transcription assays

Based on pentamidine's ability to reduce CUG repeat transcript levels, we hypothesized that pentamidine might generally affect transcription of the CTG*CAG repeat DNA. To test this hypothesis, we tested for inhibition of transcription in a simplified system. Using T7 (or SP6, in the case of (CAG)₅₄) RNA polymerase, we measured the amount of transcript produced in *in vitro* transcription reactions of several different linearized plasmids with different concentrations of pentamidine. Pentamidine has long been known to bind AT regions of DNA (preferring four or more consecutive AT pairs) (44, 47-49), so several plasmids with varying AT content were analyzed along with CTG and CAG repeats (Figure 3, Supplemental table 1).

With 72% AT content, pTRIEX was the most AT rich transcript, and, as expected, transcription of this template was the most inhibited by addition of pentamidine ($IC_{50} = 5.6 \pm 2.3 \mu M$). Transcription of two other plasmids without repeats, APP3 and APP5, with AT contents of 42% and 50% respectively, showed moderate inhibition by pentamidine with IC_{50} values of $36.1 \pm 3.8 \mu M$ and $19.1 \pm 4.6 \mu M$ respectively. Thus, inhibition of non-repeat containing plasmids correlated to AT content with pentamidine robustly inhibiting transcription of AT-rich templates. However, when pentamidine was used to inhibit the transcription of 54 CAG or CTG repeats, this trend did not hold (Figure 3c). AT content of both (CTG)₅₄ and (CAG)₅₄ is 36% and pentamidine inhibited transcription of these templates with IC_{50} values of $14.2 \pm 4.7 \mu M$ and $13.2 \pm 2.3 \mu M$ respectively. If AT content is the sole determining factor of transcription inhibition, we would expect these IC_{50} values to correlate with the non-repeat plasmid results and be closer to 50 μM . This AT-independent transcription inhibition was observed with different polymerases and promoter regions (T7 and SP6), and we attributed it to the DNA repeat sequences.

Pentamidine binds CTG*CAG repeat DNA

To further test the hypothesis that pentamidine inhibits transcription through direct interactions with the DNA itself, rather than RNA polymerase, we synthesized fluorescent analogs of pentamidine. N¹,N³-bis(4-amidinophenyl)pentane-1,5-diamine (pent-BAPPA) (Figure 4a, n=5), and N¹,N³-bis(4-amidinophenyl)heptane-1,7-diamine (hept-BAPHA) (Figure 4a, n=7) are based upon the originally reported N¹,N³-bis(4-amidinophenyl)propane-1,3-diamine (prop-BAPPA) (Figure 4a, n=3) (50) which fluoresces at ~390 nm when protected from aqueous solvent by binding the minor groove of DNA (50).

In order to test the fluorescent analogs' binding to CTG*CAG repeats, we titrated a long hairpin DNA with 11 CTG*CAG repeats and a loop composed of 5 thymines: 5' GGCC (CTG)₁₁ GCGC TTTT GCGC (CAG)₁₁ GGCC. As CTG*CAG hairpin DNA was added, we observed an increase in fluorescence in all three analogs (Figure 4b-d). Prop-BAPPA had

an apparent K_d of $2.7 \pm 0.4 \mu\text{M}$ compared to pent-BAPPA and hept-BAPHA which had apparent K_d values of $4.3 \pm 1.0 \mu\text{M}$ and $3.9 \pm 0.3 \mu\text{M}$ respectively (Figure 4e).

The fluorescent analogs demonstrate that prop-BAPPA, pent-BAPPA and hept-BAPHA (and by extension propamidine, pentamidine and heptamidine) can interact with CTG*CAG repeat DNA, albeit more weakly compared to AT rich DNA (50). However, if polymerases are susceptible to inhibition in the repeat regions (perhaps due to repeat induced stalling (40, 41)), a modest interaction may be all that is necessary to inhibit transcription and observe a reduction in CUG or CAG repeat levels.

Pentamidine analogs rescue two mis-spliced minigenes in tissue culture

A common tactic to improve the physiochemical properties of a drug candidate is to vary the hydrophobicity of the molecule, and we utilized this strategy by varying the length of pentamidine's methylene linker. The most polar analog was propamidine, with a three-carbon linker, and the least polar was nonamidine, with a linker of nine carbons (Figure 1). Due to the BPB complication in the EMSA, we decided to use our DM1 cell model for screening the activity of the methylene linker analogs.

Each analog was tested for the ability to rescue splicing of transiently transfected minigenes in a HeLa cell model of DM1. Two different minigenes containing exons that are mis-spliced in DM were tested: *TNNT2* (also known as cTNT), containing the alternatively spliced exon 5, and *INSR*, containing the alternatively spliced exon 11. MBNL proteins facilitate exclusion (negative regulation) of exon 5 of *TNNT2*: when MBNL proteins are present, exon 5 is excluded. The typical level of exon 5 inclusion when the *TNNT2* minigene is expressed in HeLa cells is $64 \pm 2\%$. The inclusion level increased to $82 \pm 3\%$ when HeLa cells co-expressed a DMPK plasmid containing 960 CUG repeats (Supplemental Figure 2a). Presumably, this change in *TNNT2* exon 5 inclusion is due to the sequestration of endogenous MBNL proteins to the CUG repeats. Propamidine did not affect *TNNT2* mis-splicing at concentrations up to $80 \mu\text{M}$ (higher concentrations were toxic). Analysis of octamidine and nonamidine was limited by toxicity as well. The remaining linker analogs rescued *TNNT2* mis-splicing to varying degrees. The concentration necessary to observe 50% rescue (EC_{50}) for butamidine was $23 \pm 5 \mu\text{M}$, which was similar to pentamidine ($\text{EC}_{50} = 20 \pm 4 \mu\text{M}$). Interestingly, at higher concentrations, butamidine and pentamidine lowered exon 5 inclusion levels below that of wild type. Hexamidine and heptamidine showed improvements over pentamidine with EC_{50} values of $12 \pm 3 \mu\text{M}$ and $15 \pm 6 \mu\text{M}$, respectively. Finally, octamidine showed a slight rescue of *TNNT2* mis-splicing; however, because of toxicity, it was not possible to treat the cells with high enough concentrations to obtain an accurate EC_{50} value (Figure 5a). Thus, hexamidine is able to rescue *TNNT2* mis-splicing at the lowest dose compared to other linker analogs.

We also tested the ability of the linker analogs to rescue the mis-splicing of an exon that is positively regulated by MBNL proteins: exon 11 of the *INSR* gene. When wild type HeLa cells expressed the *INSR* reporter minigene, exon 11 inclusion was $71 \pm 1\%$. When 960 CUG repeats were co-expressed with the *INSR* reporter, exon 11 inclusion dropped to $48 \pm 7\%$ (Supplemental Figure 2b). Unlike the *TNNT2* study, all linker analogs were able to partially or fully rescue the mis-splicing of exon 11 of *INSR* when CUG repeats were

expressed (Figure 5b). Propamidine rescued mis-splicing with an EC_{50} of $42 \pm 19 \mu\text{M}$. Butamidine and pentamidine were similar with EC_{50} values of $37 \pm 7 \mu\text{M}$ and $31 \pm 2 \mu\text{M}$, respectively. Hexamidine also rescued *INSR* mis-splicing ($EC_{50} = 15 \pm 1 \mu\text{M}$), as did heptamidine ($EC_{50} = 9 \pm 1 \mu\text{M}$). Unlike with the *TNNT2* minigene, both octamidine and nonamidine rescued *INSR* mis-splicing with EC_{50} values of $7 \pm 1 \mu\text{M}$ and $6 \pm 1 \mu\text{M}$.

All the compounds were able to partially or fully rescue *INSR* mis-splicing, while only butamidine, pentamidine, hexamidine, and heptamidine rescued *TNNT2* (Figure 5). One possible explanation for this observation is that different MBNL targets require different concentrations of MBNL proteins in the cell to be properly regulated (51). For example, nonamidine is toxic to HeLa cells at concentrations above $4 \mu\text{M}$. *TNNT2* is not rescued; however, *INSR* shows 30% rescue by $4 \mu\text{M}$. Presumably the same amount of MBNL proteins are freed inside the cell, so the difference in rescue observed between the two minigenes is likely at least partially due to the amount of MBNL required for proper splicing regulation. Interestingly, when rescue was observed with *TNNT2*, those compounds had slightly lower EC_{50} values as compared to *INSR* (Figure 6). Additionally, *TNNT2* exon 5 inclusion was often decreased to inclusion levels that are lower than wild type (Figure 5a), suggesting that these compounds may function to block exon 5 inclusion of the *TNNT2* pre-mRNA, either by interacting with the *TNNT2* pre-mRNA (21) or affecting other regulators of alternative splicing, perhaps through altering their transcription or translation (52, 53). The effect of pentamidine and its analogues on the splicing of the *TNNT2* pre-mRNA does not appear to be a general phenomenon, because splicing of other pre-mRNAs tested were not altered by pentamidine in the absence of CUG repeats (54).

As linker length increases, we observed an increase in efficacy, but we also observe an increase in toxicity to HeLa cells. This could be due to the increased lipophilicity of the molecule as the carbon chain becomes longer. While pentamidine satisfies some of Lipinski's "rule of 5" requirements for orally available drug-like compounds (55), its constitutively charged amidines hinder its ability to diffuse through membranes. As linker length increases, the hydrophobicity of the molecule also increases. Thus, the compounds with longer linkers may cross the membrane more effectively, resulting in increased intracellular concentrations, leading to increased efficacy and toxicity as observed in the HeLa cell splicing assay.

Heptamidine rescues mis-splicing in a DM1 mouse model and reduces myotonia

Because heptamidine rescued mis-splicing of both minigenes in HeLa cells while retaining water solubility, it was tested in the *HSA*^{LR} transgenic DM1 mouse model. The *HSA*^{LR} DM1 mouse model expresses 220 CUG repeats under the skeletal promoter (56). Two different endogenous pre-mRNAs were observed: *Clcn1*, the mis-splicing of which has been shown to cause myotonia (20), and *Atp2a1* (also called *Serca1*), which is a robust marker of mis-splicing in DM (57). MBNL proteins have been shown to promote exclusion of exon 7a of the *Clcn1* gene. Wild type adult mice included exon 7a of the *Clcn1* pre-mRNA at a level of $4 \pm 1\%$ while *HSA*^{LR} mice included exon 7a at steady state levels of $47 \pm 1\%$ (Figure 7a, note that exon 7a inclusion isoforms are subject to nonsense mediated decay). Treatment with heptamidine caused a dose-dependent reduction of exon 7a inclusion in *HSA*^{LR} mice,

returning to wild type levels ($6 \pm 1\%$) at the dose of 20 mg kg^{-1} per day heptamidine for 7 days (Figure 7a).

The MBNL regulated exon of *Atp2a1* (exon 22) is included in the presence of MBNL proteins (positively regulated). Wild type adult mice included this exon at $100 \pm 1\%$ (Figure 7a). When MBNL proteins are sequestered by the 220 CUG repeats present in *HSA*^{LR} mice, exon 22 inclusion dropped to $23 \pm 3\%$. Although full rescue with heptamidine could not be reached, under a treatment regimen of 30 mg kg^{-1} per day for 7 d, exon 22 inclusion levels returned to $62 \pm 4\%$ (Figure 7a). Additionally, after being treated with 30 mg kg^{-1} heptamidine for 7 d, mice went untreated for 10 d, and splicing of *Clcn1* and *Atp2a1* was examined. In both mRNAs, exon inclusion levels returned to pre-treatment *HSA*^{LA} levels (Figure 7a).

In addition to splicing defects, *HSA*^{LR} mice exhibit myotonia, manifested by runs of repetitive action potentials. We graded the severity of myotonia by insertion of extracellular recording electrodes into muscle tissue (electromyography) under general anesthesia. In glucose-treated controls, we observed grade 3 myotonia in vastus muscle, which indicates abundant repetitive discharges with nearly all electrode insertions. When treated with 20 or 30 mg kg^{-1} heptamidine, the myotonia was reduced from grade 3 to grade 1 (occasional myotonic discharge) or grade 0 (no myotonia) (Figure 7b). These results are consistent with *Clcn1* splicing rescue observed under high heptamidine dosages, and show that by correcting DM1 mis-splicing events with a small molecule, myotonia can be alleviated.

Since we observed both splicing rescue and myotonia reduction in the *HSA*^{LR} mice, we wanted to know if transcript levels were lower in the mice, similar to the reduced transcript levels observed in the HeLa DM1 cell model treated with pentamidine. Using qRT-PCR, we observed reduction of both the mRNA and pre-mRNA levels of the HSA transcript in mice treated with 15 mg kg^{-1} heptamidine per day for 7 d (Figure 7c). Interestingly, using *HSA*^{SR} mice, which express a transgene that is identical except for having only 5 CTG repeats in the 3' UTR, we did not observe significant reduction of transcript levels either in the mRNA or the pre-mRNA (Figure 7d). This suggests that the effect is dependent upon the presence of extended repeats, rather than the HSA promoter, gene, or short repeats.

It is interesting to note that while heptamidine was able to significantly reduce the *HSA*^{LR} transcript, the same effect was not observed in the HeLa cell model. This may be due to the relative amounts of repeats in the mouse versus HeLa cell model (220 repeats in a transgene compared to 960 repeats in a transfected plasmid, with the potential for multiple plasmids per cell).

In the *HSA*^{LR} mouse model, heptamidine completely reverses *Clcn1* mis-splicing and partially reverses *Atp2a1* mis-splicing at lower concentrations than pentamidine (21). Heptamidine also was able to rapidly (one week) and significantly reduce the myotonia phenotype that has been associated with *Clcn1* mis-splicing, as well as reduce the levels of *HSA*^{LR} transcript. It is encouraging that varying the linker length resulted in significant improvements over our lead compound pentamidine, but additional optimization is required. There is still toxicity in both the HeLa cell and mouse models (some mice treated with a

dose of 40 mg kg⁻¹ heptamidine daily did not survive for one week). We are currently working on analogues that will aid in identifying the pharmacophore of pentamidine-like compounds in order to guide our design of next generation compounds.

When pentamidine was initially identified as a potential therapeutic agent for treating myotonic dystrophy, we postulated that it did so by disrupting the complex between MBNL and CUG repeat RNA (21). We subsequently found that pentamidine does not effectively disrupt the MBNL1/(CUG)₄ complex in our EMSA without the addition of the dye BPB. It is possible that pentamidine and its analogs bind the expanded CUG repeat RNA and displace MBNL proteins as we originally proposed; however, due to the requirement for BPB to obtain significant binding inhibition *in vitro*, and the lack of robust interaction between pentamidine and CUG repeat RNA, this mode of action appears increasingly unlikely. Our experiments show that pentamidine (and related compounds) most likely function by inhibiting transcription of CTG*CAG repeat DNA. This was observed in CUG transcript reduction in both the HeLa cell and mouse DM1 models, as well as in *in vitro* transcription assays. While these experiments do not rule out other possible mechanisms, repression of repeat transcription is consistent with our previous observation that pentamidine significantly reduced nuclear foci formation in HeLa cells (21). It is also probable that pentamidine is acting through more than a single mechanism especially considering the “delay” between significant reduction of CUG transcript and splicing rescue in the HeLa cell model. Because pentamidine is a promiscuous DNA binding molecule, it is likely that it affects the transcription of other genes, especially genes that contain AT rich tracts. It is also easy to imagine some of these affected genes are themselves regulators of alternative splicing and may enhance or inhibit pentamidine’s apparent rescue of DM induced missplicing. Additional experiments are warranted to determine the mode (or modes) of action of pentamidine and its analogs in order to guide further small molecule drug development for DM1.

METHODS

MBNL purification, RNA labeling, and EMSA

See supporting information text.

Synthesis of pentamidine analogs

Pentamidine isethionate was purchased from Sigma-Aldrich and the HCl salt was accessed by recrystallization from hot 10% (w/v) aqueous HCl. Propamidine, butamidine and hexamidine were synthesized as described by Tidwell and coworkers (58). Heptamidine, octamidine and nonamidine were prepared in an analogous fashion.

Prop-BAPPA was synthesized as described by Vazquez and coworkers (50). Pent-BAPPA and hept-BAPHA were prepared in an analogous fashion.

For compound characterization, see supporting information text.

Northern blot analysis

For tissue culture methods, see supporting information text. A total of 1 µg of cellular RNA was loaded onto a 1% formaldehyde denaturing agarose gel with 1x MOPS buffer (20 mM MOPS, 8 mM NaOAc, 1 mM EDTA) in Northern Sample Buffer (67% formamide, 21% formaldehyde solution, 1x MOPS buffer). Gel was run in 1x MOPS buffer supplemented with 10% formaldehyde solution at 100 V for 4 – 4.5 h. Gel was transferred to a 0.45 micron nylon Magna membrane (GE) and crosslinked using a UV Stratalinker (Stratagene) on the optimal crosslink setting. Membranes were probed at 55°C overnight using radiolabeled oligos: 5'-(CAG)₂TCGAG(CAG)₄ for CUG repeats in DMPK960 and 5'-TCCACCACCCTGTTGCTGTAGCCAAATTCG for GAPDH. Membranes were washed in 0.2% SDS in 5x SSC and autoradiographed. Blots were quantified using ImageQuant (Molecular Dynamics). The relative levels of RNA were calculated by first normalizing lanes within the same gel using the GAPDH signal, and then gels were by normalizing levels of repeats in the untreated cells to 1. Errors were determined by calculating the standard deviation of triplicate data.

In vitro transcription assay

For methods, see supporting information text.

Fluorescent DNA binding

The CTG repeat hairpin DNA was purchased from IDT and was snap annealed. This annealed DNA was then titrated into 0.3 mL of 0.5 µM solution of fluorescent analog (either prop-BAPPA, pent-BAPPA, or hept-BAPHA) and the fluorescent spectra were recorded after each subsequent addition. The emission maxima of each DNA addition were plotted against DNA concentration, and fit with the equation

$$Y = m_2 + m_0 * \frac{m_3}{m_1 + m_0}$$

(where m_0 is the DNA concentration, m_1 is the apparent K_d , m_2 is initial fluorescence of the binding curve, and m_3 is the maximum fluorescence) in order to determine an apparent K_d .

Splicing analysis in cell culture

Splicing was performed as described previously (21). For details, see supporting information text.

Heptamidine treatment of mice

Homozygous *HSA*^{LR} transgenic mice in line 20b (FVB inbred background) were previously described (56). Gender-matched mice of 10–14 wks age were treated with heptamidine at the indicated dose by daily intraperitoneal injection for 7 d. Control mice received 5% glucose injections. Mice were sacrificed 1 d after the final injection and vastus (quadriceps) muscle was obtained for splicing analysis. Mice in the withdrawal (WD) group were treated with 30 mg kg⁻¹ heptamidine for 7 d and then left untreated for 10 d before sacrifice. Mice were sacrificed 1 d after final treatment and vastus muscle was obtained for splicing

analysis. RNA was isolated, reverse transcribed, and amplified by PCR, and analyzed on agarose gels using a fluorimager as previously described (21).

Electromyography

Electromyography was performed under general anesthesia as described previously (59). Briefly, at least 10 needle insertions were performed in vastus muscle and myotonic discharges were graded on a four point scale: 0, no myotonia; 1, occasional myotonic discharge in less than 50% of needle insertions; 2, myotonic discharge with more than 50% of insertions; and 3, myotonic discharge with nearly all insertions. **qRT-PCR.** Quantitative real-time PCR of human skeletal actin (HSA) normalized to 18S rRNA was performed using a StepOnePlus Real Time PCR system (Applied Biosystems), analyzed using the Quantitative-Comparative C_T ($-C_T$) method, and displayed as relative quantity (RQ). HSA primer sequences were 5'-GACGAGGCTCAGAGCAAGAGA and 5'-TGATGATGCCGTGCTCGATA-3'. The probe sequence was 5'-CCTGACCCTGAACTAC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Structure of pentamidine linker analogs. Methylene carbon linker highlighted by gray parenthesis (n = 5 for pentamidine).

Figure 2.

CUG repeat RNA levels after treatment with pentamidine or pentamidine analog. a-c) Northern blot analysis and quantification of HeLa cells expressing 960 CUG repeats treated with a) propamidine, b) pentamidine, and c) heptamidine. Propamidine and pentamidine were observed to significantly decrease CUG levels while heptamidine did not. The CUG RNA produced from the plasmid is observed to be two different sized bands. These different bands could be the product of alternative splicing or polyadenylation of the transcript, or possibly a contraction of the repeats, as CTG repeat DNA is notoriously unstable.

Figure 3.

Inhibition of *in vitro* transcription by pentamidine. a) Representative gel images of transcription reactions. Each template was treated with increasing concentrations of pentamidine. b) IC₅₀ curves for each template, plotted on a log scale. c) AT content of each template plotted against the IC₅₀ for that template. Data for non-repeat templates (APP3, APP5 and pTRIEX) could be fit by an exponential decay curve. Pentamidine inhibited the transcription of CTG/CAG repeat templates much better than would be predicted by AT content alone. Error bars are standard deviation of three observations.

Figure 4.

Binding of fluorescent pentamidine analogs to CTG*CAG repeat hairpin DNA. a) Structure of fluorescent pentamidine analogs prop-BAPPA ($n=3$), pent-BAPPA ($n=5$), and hept-BAPPA ($n=7$). b-d) Representative CTG*CAG titrations into $0.5 \mu\text{M}$ b) prop-BAPPA, c) pent-BAPPA, and d) hept-BAPPA. e) Maximum emission of each DNA concentration curve. Data were zeroed and fit with rectangular hyperbola in order to obtain an apparent K_d . Error bars are standard deviation of three observations.

Figure 5.

Pentamidine analogs rescue mis-splicing of minigene reporters in a HeLa cell DM1 model.

a) Jitter plot representation of *TNNT2* splicing. Each point is one experiment and the line represents the average of all experiments for that condition (at least three for each concentration). Butamidine, pentamidine, hexamidine, and heptamidine rescued *TNNT2* mis-splicing in the presence of 960 CUG repeats. Octamidine had a slight effect. b) Plots of *INSR* splicing. All pentamidine linker analogs are able to fully or partially rescue *INSR* mis-splicing in the presence of 960 CUG repeats. Gray area denotes range between typical splicing and DM mis-splicing.

Figure 6.

Plot of EC₅₀ values versus methylene linker length. When *INSR* splicing is monitored, the inverse relationship between methylene linker length and EC₅₀ holds. The trend is less clear with *TNNT2*. Error bars are standard deviation of at least three separate measurements.

Figure 7.

Heptamidine rescue of endogenous mis-splicing events and myotonia in *HSA*^{LR} mice. a) *Clcn1* showed a complete rescue by 20 mg kg⁻¹ per day for 7 d. Each symbol represents the splicing outcome for vastus muscle of a single mouse. After treatment, withdrawal mice (WD) were maintained for 10 d with no additional heptamidine injections. These mice showed a complete return to the basal level of splicing impairment. *Atp2a1* showed partial rescue of mis-splicing. At 30 mg kg⁻¹ heptamidine, exon 22 inclusion is approximately 50% rescued. WD mice again reverted to the pre-treatment splicing levels. Errors are standard deviation. Gray area denotes range between typical splicing and DM mis-splicing. b) Myotonia rescue in *HSA*^{LR} mice treated with 0, 20, or 30 mg kg⁻¹ heptamidine for 7 d. Mice not treated with heptamidine showed myotonic discharge with nearly all electrode insertions (grade 3), whereas those treated with 20 or 30 mg kg⁻¹ heptamidine had occasional myotonic discharges with less than 50% of insertions (grade 1) or no myotonia (grade 0). c) qRT-PCR analysis of HSA levels in *HSA*^{LR} mice treated with a 5% glucose solution or 15 mg kg⁻¹ heptamidine for 7 d. Mice treated with heptamidine showed significant (p -value < 0.02) reduction in HSA levels as compared to glucose. Error bars are standard error. d) qRT-PCR analysis of HSA levels in *HSA*^{SR} mice treated with a 5% glucose solution or 15 mg kg⁻¹ heptamidine for 7 d. No significant difference was observed between the glucose and heptamidine treated mice with only 5 CUG repeats.