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Myotonic dystrophy: Approach to therapy

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Abstract

Myotonic dystrophy (DM) is a dominantly-inherited genetic disorder affecting skeletal muscle, heart, brain, and other organs. DM type 1 is caused by expansion of a CTG triplet repeat in *DMPK*, whereas DM type 2 is caused by expansion of a CCTG tetramer repeat in *CNBP*. In both cases the DM mutations lead to expression of dominant-acting RNAs. Studies of RNA toxicity have now revealed novel mechanisms and new therapeutic targets. Preclinical data have suggested that RNA dominance is responsive to therapeutic intervention and that DM therapy can be approached at several different levels. Here we review recent efforts to alleviate RNA toxicity in DM.

Introduction

Myotonic dystrophy (dystrophia myotonica, DM) is an important genetic cause of progressive neuromuscular disability. The cardinal features include muscle weakness, myotonia (slow muscle relaxation), and early cataracts. In addition, affected individuals often experience cardiac arrhythmias and changes in neuropsychological function. Genetic testing shows an expanded CTG repeat in the 3' untranslated region of *DM Protein Kinase* (*DMPK*) in DM type 1 (DM1) [1] or an expanded CCTG repeat in intron 1 of *Cellular Nucleic Acid Binding Protein* (*CNBP*) in DM type 2 (DM2) [2]. Currently there are no disease-modifying treatments for either disorder.

In DM, mutant transcripts containing expanded CUG- or CCUG-repeats (CUG^{exp} or CCUG^{exp} RNA) exert a toxic gain-of-function (reviewed by Todd and Paulson[3]). Arguably, the best characterized mechanism for RNA toxicity is through sequestration of splicing factors in the Muscleblind-like (MBNL) family. Two members of the family, MBNL1 and MBNL2, are widely expressed, whereas MBNL3 is expressed mainly in placenta [4]. All MBNL proteins share a similar arrangement of four RNA-binding domains, an overlapping set of target binding sites in the transcriptome, and high affinity for CUG-

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repeats (K_D 's of 3 to 15 nM) [5–8]. When the cellular mass of CUG^{exp} or CCUG^{exp} RNA rises above a disease-causing threshold, the resulting titration of MBNL proteins affects alternative splicing, polyadenylation, or expression level for hundreds of genes. Expression of CUG^{exp} RNA also affects cell signaling, causing downstream effects on muscle metabolism and RNA processing [9–11]. Finally, expanded RNA repeats are sometimes recognized by translational machinery, in a manner that causes translation to initiate within the repeat tract, despite the absence of a canonical start codon [12]. The phenomenon of repeat-associated non-ATG (RAN) translation leads to production of neurotoxic peptides (reviewed by Ranum and colleagues in this issue).

Two additional points of DM biology deserve emphasis in the therapeutic context. The first is that toxic RNAs are retained in nuclei and form nuclear foci [13, 14]. The formation of foci is probably nucleated by CUG^{exp}/CCUG^{exp} – MBNL interactions, through a process that also depends on other factors and signaling pathways [15–18]. The molecular crowding in foci produces a high local concentration of MBNL binding sites. Under conditions of protein excess, a fraction of MBNL is recruited into foci, but is exchangeable with free MBNL in the nucleoplasm, and activity is retained [7, 19]. However, under conditions of CUG^{exp} excess, when binding sites are not fully occupied, the escape of MBNL is reduced, producing loss of MBNL activity. These two conditions, protein versus RNA excess, may roughly correspond to the pre-symptomatic and full-blown stages of DM1, wherein the transition from one to the other depends on length of the CUG^{exp} tract. Notably, the latter quantity is not fixed because expanded CTG repeats grow larger in somatic cells over time. Consistent with this model, splicing dysregulation is mild in early DM1 but in later stages it becomes quite severe, resembling mice with combinatorial loss of both *Mbnl1* and *Mbnl2* [20, 21].

This model, if substantially correct, has important therapeutic implications. (1) Agents acting preferentially in the nucleus, such as, antisense oligonucleotides, may have greater impact than those acting predominantly in the cytoplasm, such as, siRNAs [22, 23]. (2) Early stabilization of CTG expansions may prevent the emergence of DM symptoms. (3) Foci are not insoluble collections of denatured material. Instead, they are dynamic structures that are accessible to drugs, and they can be dispersed [7, 16, 19, 24, 25]. (4) Even a modest release of MBNL, whether by decreasing levels of toxic RNA or blocking CUG^{exp}-interactions, may produce disproportionate reversal of RNA processing defects, and potentially improve the symptoms. (5) Early-stages of DM1 may largely reflect titration of MBNL proteins, the highest affinity poly(CUG) binding proteins [26], whereas in later stages the exposure of unoccupied CUG^{exp} binding sites may drive a broader pathogenic cascade, which again can be corrected by reverting to conditions of MBNL excess [7].

A second point is that DM mutations, although located in noncoding regions, may reduce the expression of mutant alleles, raising questions whether loss-of-function may contribute to the phenotype, or possibly impose a safety limit on knockdown therapies that create or aggravate a DMPK or CNBP deficiency state. CNBP is a ubiquitously expressed DNA- and RNA-binding protein that functions to regulate transcription and translation [27]. One study showed that the CCTG expansion in intron 1 does not affect transcription or processing of the mutant *CNBP*RNA, and that CCUG^{exp} RNA in DM2 foci is a decay intermediate of the

excised intron [28]. In fact, basal expression of CNBP protein was normal even in rare examples of homozygous DM2 muscle cells. Other studies, however, showed significant reduction of CNBP protein [29, 30]. In this regard, it is important to note that disruption of *Cnbp* causes severe brain deformity in homozygous mice and craniofacial malformations and muscle abnormalities in heterozygotes [31]. These developmental effects do not necessarily predict that postnatal knockdown would be unsafe, but they do raise the possibility that *CNBP* silencing strategies for DM2 will need to be highly selective for the mutant allele.

In the case of DM1, the basal expression of DM kinase protein is reduced by half due to nuclear retention of the mutant *DMPK* (mut*DMPK*) mRNA [13]. It is unclear, however, how this may affect function of skeletal muscle and heart, the two tissues with highest levels of *DMPK* expression. Initial reports indicated that *Dmpk* deletion in mice causes cardiac conduction defects in heterozygotes [32] and skeletal myopathy in homozygotes [33]. However, a recent study found no effect of *DMPK* knockdown or knockout on growth, survival, cardiac conduction, ventricular function, or muscle contraction in mice [34]. In addition, *DMPK* knockdown was well tolerated in monkeys [35]. Taken together, these data suggest that collateral knockdown of wild-type *DMPK* may be tolerable, at least to some extent. With these findings in mind, numerous therapeutic approaches have been designed with the goal of specifically targeting the mutant allele or its RNA product. Additional approaches have been developed to target signaling pathways downstream from CUG^{exp}/CCUG^{exp} expression. These strategies are discussed below and summarized in Figure 1.

Reducing RNA toxicity by silencing transcription

In general, transcription inhibition is not considered a robust generic strategy for treating gain-of-function mutations. However, for repeat expansion mutations there are indications that transcription elongation is a promising therapeutic target. The transcription elongation factor *SPT4* was isolated in a screen for factors that modulate polyglutamine toxicity in yeast [36]. Subsequently, it was found that *SPT4* is required for transcription of expanded CAG repeats in yeast, and that knockdown of the human paralog *SUPT4H* inhibits the expression of expanded CAG- or GGGGCC-repeats in patient-derived cells [36–38]. While it has not yet been determined whether SUPT4H is required for transcription of expanded repeats at the DM1 or DM2 loci, Berglund and colleagues have shown that pentamidine and related antibiotics can bind to CTG•CAG repeats and reduce the expression of CUG^{exp} RNA [39]. Additionally, they showed that actinomycin D, a DNA intercalator that inserts preferentially at GpC dinucleotides, can reduce the expression of CUG^{exp} RNA at doses below those necessary for general transcription inhibition [40]. It also appears that *DMPK* expression is upregulated by muscle regeneration [41], suggesting a feed-forward cycle in which initiation of muscle damage may drive higher expression of toxic RNA. If correct, any treatment that breaks the cycle of damage and regeneration may have the added benefit of reducing transcription of CUG^{exp} RNA.

Post-transcriptional silencing of *DMPK* or *CNBP*

Various nucleic acid-based technologies have been used to obtain post-transcriptional knockdown of toxic RNA (Table). For example, Wansink and colleagues showed that antisense oligonucleotides (ASOs) comprised of CAG-repeats, targeted directly against the CUG-repeat, were highly effective for reducing mut*DMPK* mRNA in DM1 cells [42, 43]. This result was surprising since the ASOs were incapable of activating RNase H1, the usual co-factor for antisense knockdown. Although the mechanism remains a mystery, the data suggest a novel pathway for antisense knockdown that may specifically apply to expanded repeats. Lentiviral vectors expressing (CAG)₁₅ antisense RNA were also quite effective for *DMPK* silencing, and again showed high selectivity for the mutant allele in cells [44]. To obtain this effect, it was necessary to drive high accumulation of therapeutic RNA in the nucleus by fusing the (CAG)₁₅ sequence to a fragment of U7 snRNA.

The strategy that is furthest in clinical development employs RNase H1-active ASOs. In this case the targeting sequence is located outside of the repeat tract, thus preserving the chief advantage of antisense technology, its remarkable target specificity. Experiments in transgenic mice showed that ASOs targeting 5' or 3' of the repeat tract caused marked reduction of CUG^{exp} RNA in skeletal muscle, accompanied by release of MBNL protein from foci, correction of splicing errors, elimination of myotonia, and improvement of muscle architecture [45]. These results were surprising given that ASO uptake in muscle is relatively low, causing failure of previous attempts to silence muscle-expressed targets. These findings prompted speculation that toxic RNA was particularly susceptible to ASOs due to prolonged dwell time in the nucleus, where RNase H1 is localized [46]. These findings spurred efforts to develop an optimized *DMPK*-targeting ASO [35], which recently entered clinical trials in patients with DM1.

Few studies have examined post-transcriptional silencing for DM2, which may in part reflect a concern that CNBP reduction would be deleterious. In addition, it is unclear whether targeting outside of the repeat tract would actually accelerate the clearance of CCUG^{exp} RNA, if indeed it is already undergoing exonuclease decay [28]. Finally, there are technical limitations for monitoring knockdown efficiency, considering that the CCUG-repeats in foci are devoid of flanking sequences and therefore difficult to quantify using conventional RNA assays [28].

Small molecules to inhibit MBNL:(C)CUG-repeat interactions or disperse RNA foci

As discussed above, delivery of ASOs and siRNAs to muscle and heart poses a significant challenge, and CNS penetration is minimal unless ASOs are directly injected into CNS tissue or cerebrospinal fluid (see references [47, 48] for exceptions). A number of groups have therefore pursued the alternative approach of using rational design or high-throughput screens to identify small molecules that upregulate MBNL1, inhibit MBNL:CUG^{exp} interaction, or disperse RNA foci, and phenotypic screens for compounds that improve function in *Drosophila* models have also been conducted [16, 49–54]. In this respect, the biophysical properties of expanded CUG- or CCUG-repeats are favorable for drug

development (reviewed by Bernat and Disney [55]). These sequences form extended hairpins that are quite stable, despite the presence of a periodic U:U or U:C/C:U mismatch in the stem, forming a well-defined yet distinctive surface for drug binding. Furthermore, the reiterated binding motif and molecular crowding in foci may tend to drive strong RNA-ligand interactions. Several groups exploited these features by identifying CUG-binding monomers and then forming multimers using linkers of defined length so that the inter-subunit distance was tuned to the periodicity of the CUG- or CCUG-repeat duplex [56–58]. By this approach, compounds with higher specificity and affinity were obtained. More recently, Disney and colleagues achieved CUG repeat-catalyzed assembly of these molecules *in situ* by incorporating azide-alkyne click chemistry into the linker [53]. Others have used dynamic combinatorial libraries to achieve similar results *in vitro* [50]. Common across some of these molecules are aromatic groups that are predicted to intercalate between the U-U mismatches or occupy the grooves of CUG-repeat hairpins. Indeed, alterations of linker length between amidine groups was shown to modulate the potency of diamidines for displacing MBNL from CUG-repeats and rescuing mis-splicing in DM1 cell models [54]. Surprisingly, erythromycin, a commonly used rRNA-binding antibiotic, was also observed to displace MBNL from CUG repeats, and provide partial splicing rescue in a mouse model of DM1 [52].

Drug-induced release of MBNL protein from CUG repeats has been shown to restore MBNL activity and rescue splicing defects in DM1 model systems. However, unless such compounds also inhibit RAN translation, they pose a theoretical risk of promoting nuclear export, thereby increasing production of RAN peptides [59]. Another potential risk is that high-affinity poly(CUG)-binding compounds may slow the degradation of toxic RNA. For example, in a screen for compounds that inhibited binding of (CUG)₁₂ to recombinant MBNL1 *in vitro*, the compound with highest activity caused slower turnover and increased accumulation of CUG^{exp} RNA in cells [60].

Targeting signaling pathways downstream of CUG/CCUG expression

Another therapeutic strategy is to target signaling pathways activated downstream of CUG^{exp} expression, including protein kinase C (PKC), glycogen synthase kinase 3 beta (GSK3beta), and AMP-activated protein kinase (AMPK)/mammalian target of rapamycin (mTOR). In some DM1 mouse models and in human DM1 heart and muscle, inappropriate PKC activation leads to hyperphosphorylation of CELF family members, including CELF1 and CELF2, to cause downstream changes in CELF-dependent regulation of RNA splicing, RNA stability, and translation [9]. Inhibition of PKC by small molecules leads to normalization of CELF protein levels, rescuing muscle wasting in mouse models [61]. While a number of PKC inhibitors are in the clinic or trials, it is unclear which PKC isozyme is activated in DM1, and which symptoms would be rescued by PKC inhibition.

The stability and activity of GSK3beta in skeletal muscle is increased in transgenic mouse models and DM1 patients [10]. It has been proposed that phosphorylation of CELF1 also lies downstream of GSK3beta, in a cyclin 3-dependent manner. Inhibitors of GSK3beta normalize cyclin 3 protein levels, normalize CELF1 protein levels, and rescue muscle weakness in a transgenic mouse model. GSK3beta has been suggested as a therapeutic target

for DM1, and GSK3beta inhibitors are in clinical trials for diabetes, cancer, and Alzheimer's disease.

Deregulation of the AMPK/mTOR pathway has also been shown to occur in a mouse model of DM and DM1 cells [11]. Normalization of this pathway by administration of 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR), an AMPK activator, or rapamycin, an inhibitor of mTOR, reduced muscle relaxation time following tetanic stimulation, albeit by different mechanisms. AICAR is on a list of substances banned in athletes due to performance enhancing capabilities. Rapamycin is an FDA-approved immunosuppressant medication.

Concluding remarks

Great strides have been made in identifying molecular targets for treating DM. Agents that reduce the production, accelerate the degradation, or alleviate the toxicity of repetitive RNA have shown encouraging results in preclinical models. It remains to be seen however, if any of these substances are safe and effective in DM patients. As therapies advance to human trials, it will be necessary to consider the possibility that targeting one mechanism may fail to address or even exacerbate others. This could occur, for example, if partial removal or detoxification of CUG^{exp} RNA repeat permits ongoing growth of the expanded repeat in the genome, or if, as discussed above, the release of MBNL protein may promote increased RAN translation. On the other hand, it appears that this disease process can be simultaneously targeted at several sequential steps (Figure 1), with reasonable expectations for additive or synergistic effects. This argues for continued pursuit of all strategies discussed above and suggests that the optimal regimen may require several drugs in combination.

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Transcriptional silencing

- ① Inhibition of RNA polymerase co-factors
- ② Small molecules that bind to GC-rich repeats

Post-transcriptional silencing

- ③ Antisense oligonucleotides
- ④ Small RNAs targeting CUG repeats

Inhibiting interactions between MBNL and toxic RNA

- ⑤ Small molecules - monomers and polymers
- ⑥ Peptides

Targeting pathways downstream of RNA toxicity

Figure 1.
Strategies for treating myotonic dystrophy

Table

Agents used for post-transcriptional silencing of toxic RNA in DM1.

Agent	Target sequence	Comment
antisense oligonucleotide (RNase H1 active)	flanking sequence that lies 5' or 3' of the CUG-repeat tract	Successful knockdown and correction of phenotype with systemic administration in transgenic mice[45], now in clinical trials
Antisense oligonucleotide (RNase H1 active)	CUG-repeat tract	Local activity in transgenic mice with direct intramuscular injection[62, 63]
Antisense oligonucleotide (RNase H1 inactive)	CUG-repeat tract	Highly efficient knockdown in DM1 cells, and local activity in transgenic mice with direct intramuscular injection[24, 42]
siRNA	flanking sequence 5' of the CUG-repeat tract	Preferential knockdown of wild-type <i>DMPK</i> in patient-derived cells when delivered using transfection reagent[23]
siRNA	CUG-repeat tract	Local activity in muscle of transgenic mice with direct intramuscular injection[64]
shRNA	flanking sequence 5' of the CUG-repeat tract	Preferential knockdown of mut <i>DMPK</i> mRNA in patient-derived cells with expression from lentiviral construct [23].
antisense RNA	CUG-repeat tract + adjacent 3' flanking sequence	Preferential knockdown of mut <i>DMPK</i> mRNA in patient-derived cells using retroviral vector[65]
CAG-repeat antisense RNA fused to hU7-snRNA	CUG-repeat tract	Preferential knockdown of mut <i>DMPK</i> mRNA in patient-derived cells using lentiviral vector[44]