Myotonic dystrophy: candidate small molecule therapeutics

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Myotonic dystrophy type 1 (DM1) is a rare multisystemic neuromuscular disorder caused by expansion of CTG trinucleotide repeats in the noncoding region of the *DMPK* gene. Mutant *DMPK* transcripts are toxic and alter gene expression at several levels. Chiefly, the secondary structure formed by CUGs has a strong propensity to capture and retain proteins, like those of the muscleblind-like (MBNL) family. Sequestered MBNL proteins cannot then fulfill their normal functions. Many therapeutic approaches have been explored to reverse these pathological consequences. Here, we review the myriad of small molecules that have been proposed for DM1, including examples obtained from computational rational design, HTS, drug repurposing and therapeutic gene modulation.

Introduction

Myotonic dystrophy type 1 (DM1; OMIM #160900) is a multisystemic disorder that is caused by an excessive number of CTG triplet repeats in the 3′-untranslated region (3′-UTR) of the *dystrophia myotonica* protein kinase (*DMPK;* Entrez ID: 1760). Expanded *DMPK* transcripts are toxic (reviewed in Ref. [1]) and have a wide range of pathological consequences. The repeat RNA's main effect is muscle atrophy and weakness of skeletal and respiratory muscles, which ultimately leads to respiratory distress and death. Cardiac conduction defects also arise as the second-most-common cause of mortality in DM1. Less studied symptoms include endocrine system, gastrointestinal tract and brain disturbances, with characteristic symptoms like daytime drowsiness, attention deficit and dysexecutive syndrome [2]. The molecular mechanisms that cause DM1's pathogenic phenotype include a variety of factors (reviewed in Ref. [3]). Three muscleblind-like splicing factor paralogs: MBNL1, 2 and 3, are sequestered by CUG hairpin structures in characteristic ribonuclear foci, which leads to depletion of the MBNL alternative splicing factors and 'spliceopathy'. In its normal functioning, the most investigated family member, MBNL1 (UniProtKB: Q9NR56) binds to the consensus sequence YGCY (Y = pyrimidine) [4] through evolutionarily conserved tandem pairs of zinc fingers, and antagonizes the splicing pattern of multiple transcripts. MBNL collaborates with other splicing factors such as CUG-BP and ETR-3-like factor (CELF) proteins, which are in patients and in DM1 disease models [5]. The combined effect of low MBNL1 and high CELF1 levels affects splice site choice in the cardiac troponin T (*cTNT*; Entrez ID: 7139), insulin receptor (*INSR*; Entrez ID: 3643), muscle-specific chloride ion channel (*CLCN1*; Entrez ID: 1180) and sarcoplasmic/endoplasmic reticulum calcium ATPase 1 (*ATP2A1*; Entrez ID: 487) transcripts, among others [6]. Mutant *DMPK* RNA also triggers gene misregulation at the level of transcription [7], translation [8], gene silencing [9] and polyadenylation [10].

Pathogenic CTG repeats have been expressed in well-established cell lines to model the molecular hallmarks of DM1. In HeLa cells the repeats were co-expressed with *ATP2A1*, *cTNT* or *INSR* splice-minigenes to provide a fast and informative screen readout

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[11,12]. The mouse myoblast C2C12 cell line expressing CUG expansion (CUG_{exp}) has also been characterized and used to investigate DM1 dysregulated molecular processes in the murine context [13]. However, the most informative cell models are those obtained from patients, usually their primary myoblasts or immortalized skin fibroblasts that conditionally express MyoD [14]. CUG_{exp} toxicity and MBNL sequestration have been modeled in mouse, fly, worm and zebrafish, and their phenotypes are consistent with lack of functional MBNL proteins being the reason for numerous DM1 symptoms (reviewed in Ref. [15]). Consistent with this hypothesis, overexpression of MBNL1 (UniProtKB: Q9JKP5) in a mouse model that expresses 250 CUG repeats under the human skeletal actin promoter (HSA^{LR}) rescued normal adult splicing and myotonia [16]. Cell and fly disease models have been used in conjunction with computational studies of CUG_{exp} structure, for high-throughput screens to explore potential therapeutic strategies [17–20]. Antisense oligonucleotides (ASO) could potentially liberate MBNL1, either by sterically blocking its interaction with CUG_{exp} or by RNase-H-mediated degradation of toxic RNA (reviewed in Ref. [21]). Antibodies have also been used to block *TWEAK/Fn14* signaling in DM1, with desirable effects, in a disease model [22]. However, for the purpose of this review, we will concentrate on small molecule drugs.

Computational screening methods

CUG repeat RNA can form fully and noncanonically paired stem-loop structures that resemble an A-form DNA helix but with deeper and wider major grooves [23]. The 3D structure of the interaction of MBNL1's zinc fingers with CUG_{exp} has also been resolved [24]. Subsequent structural studies of the MBNL1–hairpin interaction showed that the protein binds and loosens the RNA hairpin structure, which further facilitates multivalent binding [6]. The 1x1 U–U internal loops that form in CUG hairpins are dynamic structures, in which the uracil nucleobases take up more than one stable form. The most stable configuration of the two uracils is an *anti–anti* conformation. The less stable *syn–anti* configuration is also possible because it is stabilized by a Na^+ pocket that forms in such an RNA configuration [19]. An additional important feature of the uracil bases is the arrangement of their H-binding surfaces. The acceptor-donor-acceptor (ADA) pattern makes it difficult to accommodate a compound owing to repulsive electrostatic interactions in the ADA–DAD conformation [25]. Taken together, the U–U mismatch appears as the main target for candidate compounds where binding efficiency depends on the thermodynamics and conformation of the uracil mismatch.

One avenue of pursuit of compounds with therapeutic potential against DM1 is rational chemical selection of new scaffolds with improved binding to CUG_{exp}. The basic idea is that active molecules have common features that allow them to interact specifically with the target RNA and release the sequestered proteins [26] (Fig. 1a). The selectivity of the molecules is of great importance because they need to have higher affinity for RNA than for DNA. To achieve this selectivity, a scanning method was used in combination with molecular dynamics simulations, which led to new predictions about modifications that increase selectivity for RNA over DNA [27]. Extraction of sublibraries with specific RNA-focused targets from big libraries of compounds has proved to be useful [28]. An additional way of acquiring desirable drugs is to search these RNA motif–ligand databases by a chemical similarity searching approach, like in the case of pentamidine and Hoechst 33528 [29]. Potential drugs can be validated by a variety of functional assays, from *in vitro* studies of the MBNL1–CUG_{exp} interaction [30] to utilization of DM1 animal models [31].

Molecules that target toxic RNA

Compounds obtained from rational design and from similarity searches

In recent years a series of compounds with desirable activity against CUG repeats has been rationally designed, or searched for, using similarity scans (Table 1). For example a compound described simply as 'Ligand 1' [32] has a specific and high affinity toward U–U mismatches in CUG hairpins and it was shown to destabilize the MBNL1–CUG_{exp} complex. Ligand 1 was designed with a rational approach, in which the DNA intercalator acridine was conjugated with a triaminotriazine unit that recognizes U–U. Even though it had very little potential to become a drug itself, its scaffold was a starting point for further molecules to be designed [32]. Ligand 1 was improved by adding a polyamine side-chain linked to another top lead compound. This helped to overcome poor water solubility and failure to penetrate the cellular or nuclear membrane. The 'New Ligand 1' that emerged [33] was evaluated for its ability to liberate MBNL1 from CUG_{exp} in surface plasmon resonance (SPR) experiments. The UV melting experiments showed that New Ligand 1 does indeed inhibit the MBNL1–CUG_{12} interaction by stabilizing the hairpin. New Ligand 1 reduced the fraction of cells with CUG_{exp} foci in a HeLa cell DM1 model by 86%. This desequestration of MBNL1 corrected *INSR* missplicing [33]. The affinity of New Ligand 1 was then increased through the multivalent approach [34]. This strategy focuses on tethering two active anti-DM1 molecules using a linker, with favorable rigidity, polarity and conformation, to increase the compound's affinity for CUG_{exp}. For New Ligand 1 the best results were obtained with polyamines, placed between the acridine rings. The activity of the resulting compound: 'Ligand 9', was evaluated by SPR chip, and in DM1 cells by time-lapse confocal microscopy, where it dispersed foci well [35]. Another compound: Ligand 3, was rationally designed from two triaminotriazine units linked by a bisamidinium component. This combination let Ligand 3 bind three consecutive CUG units instead of only one. *In vitro* and *in vivo* experiments confirmed its ability to reduce foci and to partially rescue *cTNT* and *INSR* splicing. At high concentrations, Ligand 3 partially rescued the degenerative phenotypes of DM1 flies [11]. Thanks to its additional chemical groups, Ligand 3 was later combined into a new bivalent ligand (Ligand 2a), which inhibited the MBNL1–CUG_{exp} interaction *in vitro*, dispersed foci in DM1 cells and corrected splicing of *INSR* and ameliorated DM1-like phenotypes in *Drosophila* [36]. Finally, 'New Ligand 9' emerged that binds CTG_{exp} and CUG_{exp}, inhibiting the transcription of toxic RNA and the formation of MBNL1–CUG_{exp} aggregates. Additionally, New Ligand 9 has an RNase-A-like activity, which acts selectively against the hairpin structure [37]. A strategy that combines ligand- and structure-based drug design techniques allowed identifying new scaffolds with potential activity against DM1. Subsequent analysis of intrinsic RNA dynamics and molecular docking results led to identification of two compounds: a

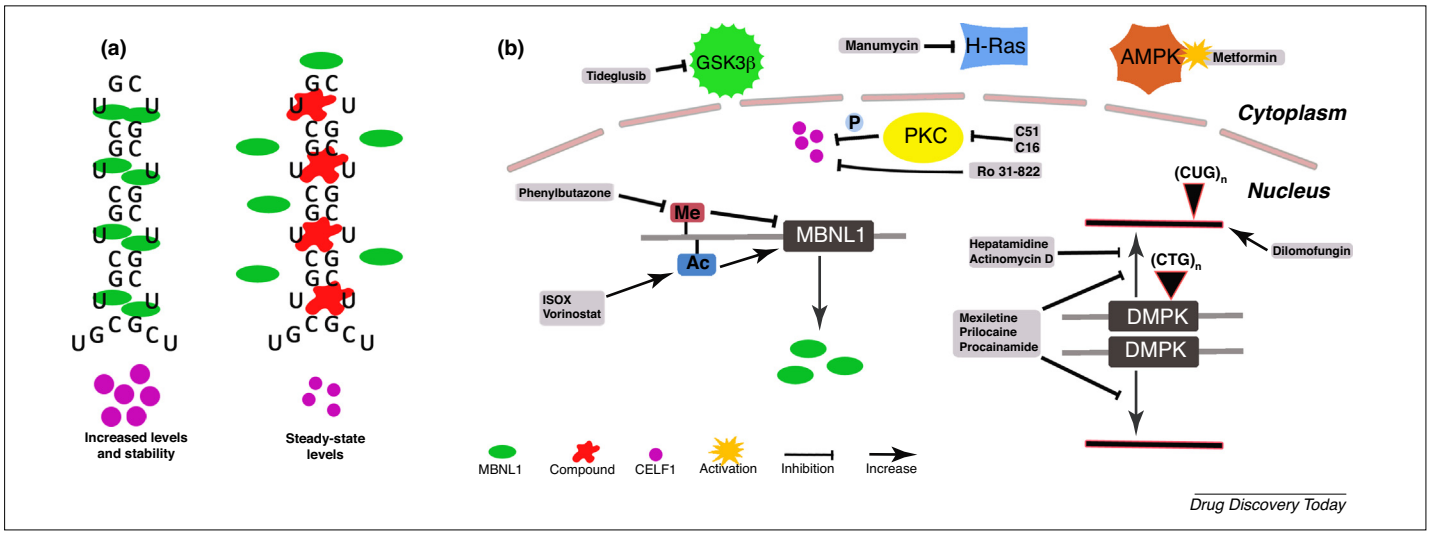

FIGURE 1

Q9 Mechanisms of action of selected anti-DM1 compounds. (a) In myotonic dystrophy type 1 (DM1) the 3'-untranslated region of the *dystrophia myotonica* protein kinase (*DMPK*) gene contains an abnormally expanded number of CTG repeats. Once transcribed, this CUG expansion (CUG_{exp}) RNA forms a hairpin structure that sequesters muscleblind-like protein (MBNL1) and activates CUG-BP and ETR-3-like factor 1 (CELF1) through a not-well-established pathway (left). Most candidate compounds against DM1 compete with MBNL1 for binding to CUG_{exp} and liberate the sequestered protein (right), whereas other molecules unfold or tighten the hairpin structure (not shown). **(b)** Examples of known anti-DM1 compounds' mechanisms-of-action. In some cases, the drugs work by modulating endogenous gene expression. Mexiletine, prilocaine and procainamide inhibit normal and mutant *DMPK* transcription, whereas actinomycin D and heptamidine specifically decrease the level of CUG_{exp}-containing mRNA. Dilomofungin, conversely, increases mutant *DMPK* mRNA levels by stabilizing the transcript. Phenylbutazone has two mechanisms. In addition to competing for MBNL1 binding to CUG_{exp}, it acts at the epigenetic level and increases transcription of *MBNL1* by reducing methylation of a given enhancer. ISOX and vorinostat inhibit histone deacetylases, which repress *MBNL1* expression, thus available MBNL1 protein levels are increased. Other compounds affect cell signaling pathways and have an indirect, beneficial effect on DM1. Ro 31-8220, C51 and C16 are protein kinase C (PKC) inhibitors that impede phosphorylation (P) of CELF1, which is known to be hyperphosphorylated in DM1, however Ro 31-8220 seems to have a PKC-independent mechanism-of-action. It is unclear whether PKC inactivation occurs in the cytoplasm or nucleus but in the DM1 context it affects splicing events so we represent it in the nuclear compartment. Tideglusib inhibits glycogen synthase kinase 3 (GSK3)β which indirectly affects phosphorylation of CELF1, probably via the cyclin D3/CD4 pathway. Metformin acts via activation of AMP-activated protein kinase (AMPK) and manumycin inhibits Ras farnesyltransferase in the H-Ras pathway, although the exact relationship between these pathways and DM1 remains poorly understood.

substituted pyrido[2,3-d]pyrimidine 'Compound 1–3' and pentamidine-like 'Compound 2–5'. Neither compound decreased the number of foci in DM1 fibroblasts. Indeed, Compound 2–5 tends to increase it. However, both compounds increased the amount of free MBNL1 in the nucleus and cytoplasm, and increased the climbing speed of DM1 flies [38]. Searching for new scaffolds from the motif–ligand database led to discovery that the *bis*-benzimidazole (H), a compound designed using a computational approach, binds to 1x1 nucleotide U–U internal loops of the CUG_{exp} structure [39]. Application of the multivalent concept allowed designing new molecules with 2–5 H units linked by 4 units of spacers (nH-4). These molecules displaced MBNL1 from CUG_{exp} *in vitro* at nanomolar concentrations, and improved the *cTNT* splicing defect in the micromolar range. All the nH-4 compounds disrupted the nuclear foci, but 2H-4 rescued the splicing defects best. 3H-4 improved the nucleocytoplasmic transport of *DMPK* mRNA (UniProtKB: Q09013), which is impaired in DM1. The conclusion was that the higher the number of monomers the better the DM1 phenotype recovery was but the worse the water solubility of the compounds was [40], which is crucial for druggability. To allow recognition of two adjacent U–U loops the 2H-K4NMeS was generated, which is a dimeric display of H units on an *N*-methyl peptide backbone [41]. Treatment with 2H-K4NMeS improved the hallmarks of DM1 patient-derived cells by altering MBNL1-dependent splicing events and reducing the number of nuclear foci. Crosslinking of chlorambucil and biotin to the compound gave 2H-K4NMeS-CA-biotin, which had higher selectivity toward pathogenic lengths of CUG repeats and potently inhibited MBNL1–CUG_{exp} complexes *in vitro*. To improve the allele selectivity, the authors used *in situ* click chemistry. They appended 2H-K4NMeS with bioorthogonal azide and alkaline moieties and determined the optimal distance between them *in vitro*. Finally, they synthesized the dual functionalized derivative N_3-2H-K4NMeS-Aak. This derivative was far more potent at rescuing MBNL1-dependent pre-mRNA splicing defects than the morpholino ASO CAG that recognizes the RNA structures [41,42].

Pentamidine is an FDA-approved diamide, which is composed of two phenymamidine groups that are joined by a five-carbon methylene linker. Pentamidine was originally thought to bind CUG repeats to dissolve foci [43], but it was later reported that its true mechanism-of-action (MoA) is inhibition of CTG repeat transcription [44]. Pentamidine and heptamidine (a derivative with seven-carbon methylene linker) were studied by SAR analysis to find their anti-DM1 features. Testing pentamidine and heptamidine analogs, which have different planarity, linker length and amidine substitutions, led to the discovery of 'Compound 13', which reduced foci and rescued missplicing of *Clcn1* (Entrez ID: 12723) and *Atp2a1* (Entrez ID: 11937) in the HSA^{LR} mouse model. Compound 13 was less

TABLE 1

Hit compounds that target toxic RNA.

Hits[a]	Model[b]	Active concentration[c]	Biological readout[d]	Refs
Ligand 1	(CUG)4–MBNL1	IC_{50}[e] = 52 ± 20 μM	CUG–MBNL1 complex formation inhibition	[32]
	$(CUG)_{12}$–MBNL1	IC_{50} = 46 ± 7 μM		
New Ligand 1	$(CUG)_{12}$–MBNL1	IC_{50} = 15 ± 2 μM	CUG–MBNL1 complex formation inhibition	[32]
	HeLa DM1 cell model	50–100 μM	Foci reduction	
			Splicing rescue (*INSR*)	
Ligand 9	$(CUG)_{12}$–MBNL1	IC_{50} = 1.1 ± 0.1 μM	CUG–MBNL1 complex formation inhibition	[35]
	HeLa DM1 cell model	20–50 μM	Foci reduction	
Ligand 3	$(CUG)_{12}$–MBNL1	IC_{50} = 115 ± 14 μM	CUG–MBNL1 complex formation inhibition	[11]
	HeLa DM1 cell model	100 μM	Foci reduction	
		75–100 μM	Splicing rescue (*cTNT* minigene)	
		50–100 μM	Splicing rescue (*INSR* minigene)	
	Drosophila DM1 model	200–400 μM	Suppression of CUG-induced neurodegeneration	
Compound 2a	$(CUG)_{16}$–MBNL1	IC_{50} = 290 ± 20 nM	CUG–MBNL1 complex formation inhibition	[36]
	HeLa DM1 cell model	1–100 μM	Foci reduction	
		100 μM	Splicing rescue (*INSR* minigene)	
	Drosophila DM1 model	50 μM	Suppression of CUG-induced toxicity	
		20–100 μM	Rescue of larval mobility defect	
New Ligand 9	$(CTG{\cdot}CAG)_{74}$ transcription assay	50–100 μM	Transcription inhibition	[37]
	$(CUG)_{16}$	5–100 μM	Cleavage of hairpin structure	
	HeLa DM1 cell model	50 μM	Foci reduction	
		100 μM	Splicing rescue (*INSR* minigene)	
		25–150 μM	Reduction of $(CUG)_{exp}$ level	
	Drosophila DM1 model	200 μM	Suppression of CUG-induced toxicity	
		100–400 μM	Rescue of larval mobility defect	
		400 μM	Reductionof $(CUG)_{exp}$ level	
2H-4	HeLa DM1 cell model	5–25 μM	Splicing rescue (*cTNT* minigene)	[40]
		25 μM	Foci reduction	
	C2C12 DM1 cell model		Rescue of *DMPK* mRNA nucleocytoplasmic transport	
3H-4	HeLa DM1 cell model	50 μM	Splicing rescue (*cTNT* minigene)	[40]
		25 μM	Foci reduction	
	C2C12 DM1 cell model	2.5–10.0 μM	Rescue of *DMPK* mRNA nucleocytoplasmic transport	
4H-4	HeLa DM1 cell model	10–50 μM	Splicing rescue (*cTNT* minigene)	[40]
		50 μM	Foci reduction	
	C2C12 DM1 cell model	2.5–10.0 μM	Rescue of *DMPK* mRNA nucleocytoplasmic transport	
2H-K4NMeS	$(CUG)_{12}$–MBNL1	–	CUG–MBNL1 complex formation inhibition	[41]
	DM1 myoblasts	10 nM	Foci reduction	
			Splicing rescue (*MBNL1*, *CAMK2G*, *NCOR2*)	
2H-K4NMeS-CA-biotin	$(CUG)_{12}$–MBNL1	–	CUG–MBNL1 complex formation inhibition	[41]
	DM1 myoblasts	10 nM	Foci reduction	
			Splicing rescue (*MBNL1*, *CAMK2G*, *NCOR2*)	
N_3-2H-K4NMeS-Aak	DM1 myoblasts	100 pM	Splicing rescue (*MBNL1, NCOR2, NFIX, CAMK2G*)	[41]
Compound 13	HeLa DM1 cell model	80 μM	Splicing rescue (*cTNT* minigene)	[45]
			Foci reduction	
	HSA^{LR} mice	10–20 mg/kg	Splicing rescue (*Clcn1, Atp2a1*)	
Compound 1 [(E)-4-phenyl-2-(3-(thiophen-2-yl)acrylamido) thiophene-3-carboxylic acid]	$(CUG)_{12}$–MBNL1	IC_{50} = 52 ±12 μM	CUG–MBNL1 binding inhibition	[48]
Compound 2 [1,8-diamino-3,6-di(pyrrolidin-1-yl)-2,7-naphthyridine-4-carboxilic acid]	$(CUG)_{12}$–MBNL1	IC_{50} = 2 ± 0.4 μM	CUG–MBNL1 binding inhibition	[48]
	HeLa DM1 cell model	300 μM	Foci reduction	
			Splicing rescue (*INSR* minigene)	
			Splicing rescue (*cTNT* minigene)	
Compound 1–3	DM1 myoblasts	100 μM	Increase levels of free MBNL1	[38]
	Drosophila DM1 model		Rescue of impaired climbing capacity	
Compound 2–5	DM1 fibroblasts	40 μM	Increase foci number	[38]
	DM1 myoblasts		Increase levels of free MBNL1	
	Drosophila DM1 model		Rescue of impaired climbing capacity	

[a] Names of the synthetic compounds.
[b] *In vitro* or *in vivo* disease models used to discover the indicated compounds' activity.
[c] This column includes concentrations, range of concentrations and doses of compounds that were effective in a particular DM1 model.
[d] Evidence for an effect on pathological features of DM1 in biological and *in vitro* models.
[e] IC_{50} is the half-maximal inhibitory concentration at which the compound hinders the MBNL1–CUG complex formation.

TABLE 2

Active anti-DM1 compounds.

Natural compounds[a]	Model[b]	Active concentration[c]	Biological readout[d]	Refs
Resveratrol	HeLa DM1 cell model	100 μM	Splicing rescue (*INSR*)	[56]
	Skeletal muscle cells			
	Human normal fibroblasts			
	DM1 fibroblasts			
	HepG2 cells			
Berberine	DM1 myoblasts	20–80 μM	Splicing rescue (*cTNT*)	[59]
Harmine	DM1 fibroblasts	20–80 μM	Splicing rescue (*cTNT, INSR*)	[59]
	Human normal myoblasts		Splicing rescue (*cTNT*)	
	DM1 myoblasts			
	DM1 myoblasts	80 μM	Foci reduction	
			Increased total levels of MBNL1	
		40 μM	Increased total levels of MBNL1	
	HSA^{LR} mice	40 mg/kg	Splicing rescue (*Clcn1*)	
Dihydroberberine	HSA^{LR} mice	10 mg/kg	Splicing rescue (*Clcn1*)	[59]
			CLCN1 protein levels	
Palmatine	HSA^{LR} mice	40 mg/kg	CLCN1 protein levels	[59]
		25 mg/kg		
Lomofungin	$(CUG)_{12}$–MBNL1	717 nM	CUG–MBNL1 binding inhibition	[49]
	C2C12 DM1 cell model	10 μM	Splicing rescue (*Atp2a1*)	
Dilomofungin	$(CUG)_{12}$–MBNL1	42 nM	CUG–MBNL1 binding inhibition	[49]
	C2C12 DM1 cell model	10 μM	Splicing rescue (*Atp2a1*)	

Marketed drug	Model	Active concentration	Biological readout	Refs
Actinomycin D	HeLa DM1 cell model	10 nM, 18 h	Foci reduction	[51]
		5–20 nM, 18 h	Reduced CUG RNA levels	
	DM1 fibroblasts	1–6 nM, 18 h		
	HSA^{LR} mice	0.025 mg/kg, 5 days	HSA transcript reduction	
		0.125–1.25 mg/kg, 5 days	Splicing rescue (*Atp2a1, Clcn1, Mbnl1, Vps39, Nfix, Ldb3*)	
Erythromycin	$(CUG)_{100}$–MBNL1	10–50 μM	CUG–MBNL1 binding inhibition	[13]
	C2C12 DM1 cell model	25 μM	Foci reduction	
		50 μM	Splicing rescue (*Atp2a1*)	
	DM1 fibroblasts	100 μM	Foci reduction	
		500 μM	Splicing rescue (*MBNL1, MBNL2, NCOR2*)	
	HSA^{LR} mice	150 mg/kg per day for 8 days (daily intraperitoneal)	Splicing rescue (*Clcn1, Atp2a1, Bin1, Cacna1s, Camk2b, Ryr1, Nfix, Ldb3*)	
		50 mg/kg per day for 8 days (daily intraperitoneal)	Splicing rescue (*Clcn1, Atp2a1*)	
Metformin	DM1 mesodermal precursor cells	25 mM	Splicing rescue (*INSR, cTNT, CLCN1*)	[61]
	Wild-type mesodermal precursor cells	10 mM	Splicing change (*INSR*)	
	DM1 myoblasts	25 mM	Splicing rescue (*INSR, cTNT, ATP2A1, DMD, KIF13A*)	
	Peripheral blood lymphocytes from patients with diabetes	2.1 g/day for over a year 3 g/day for over a year	Splicing change (*INSR*)	
Mexiletine	C2C12 mouse myoblasts	50 nM	Decrease in *Dmpk* mRNA levels	[55]
Prilocaine	C2C12 myoblasts	1 μM	Decrease in *Dmpk* mRNA levels	[55]
	CD1 mice (gastrocnemius muscle)	1.25 mg/kg	Decrease in *Dmpk* RNA levels Reduction of DMPK protein levels	
Procainamide	CD1 mice (gastrocnemius muscle)	25 mg/kg	Decrease in *Dmpk* mRNA levels	[55]
Manumycin	C2C12 DM1 cell model	10–40 μM	Splicing rescue (*Clcn1* minigene)	[62]
	HSA^{LR} mice	75 ng/μl (3 μg in 40 μl)	Splicing rescue (*Clcn1*)	
Thiamine (vitamin B1)	DM1 patients	Intramuscular injection (100 mg) twice a week for 12 (patient 1) and 11 months (patient 2)	Increase of muscular strength	[57]
Modafinil	DM1 patients	–	General benefit (lower fatigue score)	[58]
Phenylbutazone	C2C12 mouse myoblasts	50–972 μM, 24h	Increase of *Mbnl1* expression	[31]
	HSA^{LR} mice	16.7 mg/kg/day for 12 weeks	Splicing rescue (*Clcn1, Nfix, Rpn2*) Improvement of wheel running activity	

TABLE 2 (*Continued*)

Marketed drug	Model	Active concentration	Biological readout	Refs
ISOX	HeLa DM1 cell model	5 μM	Splicing rescue (*ATP2A1* minigene)	[12]
	DM1 fibroblasts	5 μM	Increase of *MBNL1* expression Splicing rescue (*ATP2A1, INSR)*	
Vorinostat	HeLa DM1 cell model	5 μM	Splicing rescue (*ATP2A1* minigene)	[12]
	DM1 fibroblasts	5 μM	Increase of *MBNL1* expression Splicing rescue (*ATP2A1, INSR)*	
Pentamidine	$(CTG{\cdot}CAG)_{54}$ transcription assay	IC_{50}[e] = 14.2 ± 4.7 and 13.2 ± 2.3, respectively	Transcription inhibition	[44]
	HeLa DM1 cell model	20 μM 31 μM	Splicing rescue (*cTNT* minigene) Splicing rescue (*INSR* minigene)	
	Drosophila DM1 model	1 μM	Release of MBNL1 from foci. Improvement of cardiac rhythmicity and contractility	[67]
Tideglusib	Clinical trial Phase II	400 and 1000 mg	Safety and efficiency	Identifier: NCT02858908
TDZD-8	HSA^{LR}	10 mg/kg for 2–7 days	Normalization of GSK3β and cyclin D3 expression Increase of muscular strength Decrease of myotonia	[63]
Lithium	CHO DM1 cell model HSA^{LR}	20 μM 0.24% for 2 weeks	Normalization of cyclin D3 expression Normalization of GSK3β/cyclin D3/CELF1 pathway Increase of muscular strength Decrease of myotonia	[63]

Hits	Model	Active concentration	Biological readout	Refs
Heptamidine	HeLa DM1 cell model	15 μM	Splicing rescue (*cTNT* minigene)	[44]
		9 μM	Splicing rescue (*INSR* minigene)	
	HSA^{LR} mice	20 mg/kg, 7 days	Splicing rescue (*Clcn1*)	
		30 mg/kg, 7 days	Splicing rescue (*Atp2a1*)	
		–30 mg/Kg	Decrease of myotonia	
		15 mg/kg, 7 days	Transcription reduction	
Imidazolo-oxindole inhibitor C16	DM1 myoblasts	1 μM	Downregulation of *CELF1*	[53]
		1 μM	Foci reduction	
		0.5 μM	Release of MBNL1 from foci	
		1 μM	Splicing rescue (*ATP2A1, LDB3, MBNL1, DMD*)	
	HepG2 cells	1 μM	Splicing rescue (*MTMR3, SORBS1, KIDINS220, CAPZB*)	
Pyrimidine-based inhibitor C51	DM1 myoblasts	30 μM	Downregulation of *CELF1*	[53]
		30 μM	Foci reduction	
		30 μM	Release of MBNL1 from foci	
		30 μM	Splicing rescue (*ATP2A1, LDB3*)	
	HepG2 cells	30 μM	Splicing rescue (*MTMR3, SORBS1*)	
Ro 31-8220	DM1 fibroblasts	4.4 μM	Foci reduction	[17]
Ro 31-8220	DM1 fibroblasts DM1 myoblasts	4.4 μM	Release of MBNL1 from foci	
	DM1 myoblasts	10 μM	Splicing rescue (*ATP2A1, INSR*)	
	Zebrafish DM1 model	5 μM for 24 h	Rescue of pathologic body length to width ratio	
	Mouse DM1 cardiac model	6 mg/kg	Decrease of mortality rate Prevents cardiac electrophysiology and contractile disfunction Splicing rescue (*Ank2, Mtmr3, Sorbs1*)	
Quinoline 53	Congenital DM1 myoblasts	0.4–5.0 μg/ml	Improves delayed myogenesis	[64]

[a] Names of the compounds.
[b] *In vitro* or *in vivo* disease models used to discover the indicated compounds' activity.
[c] This column includes concentrations, range of concentrations and duration of treatment and doses of compounds that were noticed effective in a particular DM1 model.
[d] Evidence for an effect on pathological features of DM1 in biological and *in vitro* models.
[e] IC_{50} is a half maximal inhibitory concentration at which the compound hinders the MBNL1–CUG complex formation.

toxic than pentamidine but also less potent at comparable concentrations [45].

Compounds identified from HTS

$MBNL1–CUG_{exp}$ aggregates are a histopathological hallmark of DM1 and have been widely used as a biological readout in drug discovery. The first combinatorial screen tested 11 325 molecules to reveal 'hit' compounds that disrupt this pathological binding [46]. *In vitro* and *in vivo* testing of various hits (Table 2) showed they remove MBNL1 from foci. Another high-content screen based on this kind of phenotypic assay (hit rate 3%) led to the discovery of two compounds, which reduced the number of foci: Ro 31-8220 and chromomycin A3. Further *in vitro* testing proved that both compounds rescue the missplicing of *INSR* and *ATP2A1*, probably because they de-sequester MBNL1. Ro 31-8220 was also evaluated in a zebrafish DM1 model and it partially rescued the mutant phenotype, whereas chromomycin A3 did not penetrate the embryo chorion [17]. Despite the fact that Ro 31-8220 is a protein

kinase C (PKC) inhibitor [47], independent studies in patient-derived cell lines proved that it is not its kinase-inhibitory effect that rescues DM1 features [17]. Another two compounds that inhibit formation of the MBNL1–CUG_{12} complex were discovered in a HTS (PubChem AID: 2675; hit rate = 0.036%) and confirmed by AlphaScreen® technology and homogeneous time-resolved fluorescence energy transfer. 'Compound 1' (a thiophene-containing small molecule) and 'Compound 2' (a substituted naphthyridine molecule) have a high affinity and selectivity for MBNL1 protein and CUG_{exp} RNA, respectively. Structural models revealed that Compound 1 binds MBNL1 in its RNA recognition domain (the zinc finger), and Compound 2 interacts with U–U loops in CUG_{exp}. Compound 1 caused a shift in the alternative splicing of several MBNL1-dependent splicing events toward a DM1-like profile whereas Compound 2 improved DM1-like splicing defects. These results demonstrated that targeting the MBNL1 RNA-recognition domains deprives the protein of its function and these domains are therefore not suitable drug targets [48]. A high-throughput homogeneous time-resolved fluorescence energy transfer assay screen (PubChem AID: 2675) identified the antimicrobial agent lomofungin as a potent inhibitor of the MBNL1–CUG_{exp} interaction [49]. Furthermore, its dimer, dilomofungin, inhibits the interaction 17-times more strongly. Competition dialysis analyses determined that lomofungin and dilomofungin preferentially bind pyrimidine mismatches. The monomer activity, when tested in a cellular model, rescued misspliced exon 22 of *Atp2a1* by 71%, whereas the dimer rescued 60% of the same missplicing. Despite dilomofungin having a stronger affinity for CUG_{exp}, it was less potent *in vivo,* probably because it has additional molecular targets. Treatment with the transcription blocker actinomycin D permitted evaluation of the mutant *DMPK* mRNA decay level, which was five-times lower after treatment with dilomofungin, which consequently resulted in an increased number of ribonuclear foci [49]. Lomofungin had some desirable properties in *in vitro* and *in vivo* assays; however, it underwent spontaneous dimerization to dilomofungin, which had even stronger *in vitro* activity but also unexpectedly stabilized mutant *DMPK* transcripts. Although the initial results obtained from the HTS were promising, the lomo/dilomofungin example demonstrates that screens can give false hopes and fail to be verified in cells. HTS is difficult *in vivo,* but constructs that fuse splice minigenes to the luciferase reporter (spliceosensors) in transgenic *Drosophila* have allowed a screening campaign of more than 16 000 compounds with a hit rate of 0.78%. These kinds of *in vivo* approaches are not only efficient but they also provide information about the ADMET parameters of the hit molecules [18]. Another *Drosophila*-based screening method relied on expressing CUG_{exp} in the 'mushroom bodies' of the brain. This resulted in sequestration of muscleblind protein (UniProtKB: O16011) and a semi-lethal pupal phenotype. This approach led to the discovery of the ABP1 lead compound, which is a d-amino acid hexapeptide, and probably works by preventing hairpin formation [50].

Therapeutic gene modulators

Gene modulators are compounds that have the ability to influence expression of endogenous genes, in a way that alleviates the pathogenic features of the disease (Table 2). Transcription itself, and the way it interacts with other processes, is an interesting target in drug discovery (Fig. 1b). The previously mentioned actinomycin D is a global transcription inhibitor, which is used as a drug in oncology. Actinomycin D also has affinity for CTG-rich sequences but it specifically decreased CUG_{exp} transcript levels in a DM1 HeLa cell model at 5 nM concentration. It reduced the number of nuclear foci without affecting overall transcription. HSA^{LR} mice treated with actinomycin D had reduced HSA transgene mRNA and partial or complete rescue of *Atp2a1*, *Mbnl1*, *Vps39* (Entrez ID: 269338), *Nfix* (Entrez ID: 18032) and *Ldb3* (Entrez ID: 24131) missplicing events [51]. Other compounds that work against DM1 at the transcriptional level are the pentamidine derivatives propamidine and heptamidine. They were tested in a HeLa DM1 cell model and in HSA^{LR} mice where they inhibited toxic RNA transcription in a dose-dependent manner. All the pentamidine derivatives, fully or partially, rescued *cTNT* and *INSR* missplicing in HeLa cells expressing CUG_{exp}. Moreover, heptamidine significantly rescued *Clcn1* and *Atp2a1* missplicing and strongly reversed the myotonia in HSA^{LR} mice [44].

The nonsteroidal anti-inflammatory drug phenylbutazone is a highly relevant anti-DM1 drug because of its proposed dual MoA. It not only attenuates the binding of MBNL to CUG_{exp} but also increases the transcription of *Mbnl1* by suppressing methylation of a defined enhancer region. Phenylbutazone increased MBNL1 expression up to 1.9-fold in a dose-dependent manner (Fig. 1b) in C2C12 cells. HSA^{LR} mice treated with phenylbutazone also had increased *MBNL1* mRNA and protein levels, which resulted in increased grip strength and mobility, and in a reduced number of muscle fibers with central nuclei. Moreover, the phenylbutazone-treated mice had more Clcn1 protein, as a result of rescued *Clcn1* splicing [31]. MBNL1 protein levels could also be increased in DM1 and normal fibroblasts by two histone deacetylase (HDAC) inhibitors: ISOX and vorinostat. These two compounds were identified in a flow-cytometry-based screen that sorted HeLa cells expressing ZsGreen-tagged endogenous *MBNL1*. The two drugs promoted inclusion of *ATP2A1* exon 22 and *INSR* exon 11 in DM1 and control cell lines, showing that their activity was not DM1-specific. The compounds had no effect on *DMPK* or *ATP2A1* transcription, and did not reduce the number of foci, suggesting that their MoA is exclusively epigenetic and on *MBNL1* [12].

CUG_{exp} RNA is not the only potential therapeutic target of candidate small molecules being developed. The PKCα inhibitor Ro 31-8220 reduces the hyperphosphorylation of CELF1 (UniProtKB: Q92879) (Fig. 1b), through which it was thought to ameliorate the cardiac phenotype of a DM1 mouse model [47]. However, this assumed MoA was recently challenged by the discovery that a PKCα and PKCβ double knockout does not modify the disease phenotype of DM1 mice [52]. Presumably some kinases do relive symptoms through their effect on phosphorylation. Two ATP-site-directed kinase inhibitors: the imidazolo-oxindole inhibitor C16 and the pyrimidine-based inhibitor C51, reduce the major molecular symptoms of DM1 in human myoblasts [53], and displace MBNL1 from foci. Filter-binding assays showed that these compounds do not directly disrupt the MBNL1–CUG_{exp} complexes. Freeing MBNL1 from toxic transcripts can thus be achieved indirectly. Finally, sodium channel blockers such as mexiletine, prilocaine and procainamide [54] probably act against DM1 by inhibiting *DMPK* transcription. These compounds had varied effects on *Dmpk* mRNA and protein

levels in C2C12 mouse myoblasts as well as in heart and muscle of wild-type mice [55].

Natural compounds and drug repurposing

Many natural compounds and several plant-derived alkaloids had promising results against DM1 (Table 2). Researchers also focused on drug repurposing because of the availability of clinical and pharmacokinetic data, although the MoA against DM1 of many repurposed drugs has not been elucidated. The dietary supplement resveratrol is a polyphenolic flavonoid found in grape skin, seeds and red wine, and it enhanced inclusion of exon 11 of the *INSR* gene in different DM1 fibroblast lines as well as in skeletal muscle cells and in control fibroblasts [56]. Thiamine (vitamin B1), which is used to treat mitochondrial diseases, improved DM1 patient muscular strength and the daily life independence in a clinical trial [57], as did modafinil, which is otherwise a psychostimulant drug for managing sleep disorders. More than 90% of treated patients reported noticeable benefits from modafinil but as for resveratrol and thiamine the molecular basis of the anti-DM1 effect of these common drugs has not been elucidated [58].

Different alkaloids that inhibit MBNL–CUG_{exp} complex formation have been identified and even though their MoA it is not clear they certainly have a positive effect on DM1 pathologic features. Berberine improved the splicing of the *cTNT* in the DM1 myoblasts, although it had a negative effect on *INSR* splicing. Harmine increased the total levels of MBNL1 in DM1 myoblasts. It improved *cTNT* and *INSR* splicing and significantly reduced the number of foci [59]. Harmine, dihydroberberine and palmatine all improved the splicing of *Clcn1* but had no significant effect on *Atp2a1* in HSA^{LR} mice. Even though these alkaloids are not suited for therapeutic application owing to their toxicity and low potency, they can help to better understand the interactions of small molecules with toxic CUG_{exp} repeats [59].

As for drug repurposing, the natural antibiotic erythromycin significantly inhibited the aggregation of MBNL1 in foci. It decreased myotonia and rescued the missplicing of *Atp2a1*, *Clcn1*, *Bin1* (Entrez: 30948), *Cacna1s* (Entrez: 12292), *Camk2b* (Entrez: 12323), *Ryr1* (Entrez: 20190), *Nfix and Ldb3* in a dose-dependent manner in the HSA^{LR} mice. When administered to the C2C12 DM1 cell model, at the effective dosage used in humans, erythromycin significantly restored the *Atp2a1* exon 22 inclusion and decreased the number of foci. The erythromycin ethylsuccinate, which is another form of erythromycin that has been approved for oral usage in humans, also significantly improved missplicing of *CLCN1* and *ATP2A1* and decreased the frequency of cells with nuclear foci in DM1 fibroblasts [13]. Recently, the AMP-activated protein kinase (AMPK) pathway was discovered to be impaired in the HSA^{LR} mouse [60]. The antidiabetic drug metformin induces this pathway (Fig. 1b) and, when tested in DM1 mesodermal precursor cells (MPCs), it corrected the splicing of the *INSR*, *cTNT* and *CLCN1* genes. Metformin also had a positive effect on the splicing of *INSR*, *cTNT*, *ATP2A1*, *DMD* (Entrez: 1756) (exon 71 and 78) and *KIF13A* (Entrez: 63971) splicing in DM1 myoblasts [61]. Another pathway that might be involved in DM1 is the H-Ras pathway. Manumycin is an inhibitor of Ras farnesyltransferase, which acts via the H-Ras pathway. Manumycin also has anti-DM1 activity. The compound corrects aberrant splicing of *Clcn1* minigene in a C2C12 DM1 cell model as well as that of endogenous *Clcn1* in HSA^{LR} mice. However, manumycin does not rescue aberrant splicing of *Atp2a1* or *m-Titin* (Entrez ID: 22138) in a murine model [62]. Finally, glycogen synthase kinase 3β (GSK3β; UniProtKB: P49841) is hyperstable and hyperactive in DM1-patient skeletal muscle. High GSK3β activity changes CELF1 phosphorylation in DM1-patient muscle, which in turn inhibits the translation of various transcripts. Importantly, administration of lithium, which inhibits GSK3β, to HSA^{LR} mice restored normal phosphorylation of CELF1 and restored its translational function [63]. Lately the GSK3β allosteric modulator quinoline 53 was shown to improve myoblast differentiation in a congenital DM1 cell model without disturbing other GSK3β activities [64]. Consistent with all of the above, tideglusib is an ATP-independent GSK3β inhibitor that is currently in a Phase II clinical trial for DM1 (clinicaltrials.gov: NCT02858908).

Concluding remarks

The development of therapies against DM1 has gained momentum over the past few years because of the growing number of candidate drugs that are being proposed, the description of disease biomarkers and natural history studies that pave the way for future clinical trials. There is renewed interest from biotech companies in the disease, which could significantly accelerate the screening process and help to find specific and effective treatments for this multisystemic disorder. Among potential therapeutics for DM1, special attention is being paid to drug repurposing because it has a good balance between efficiency, safety, speed of translation to the clinical setting and cost. This can be a good interim solution before new tailored therapies targeting the root cause of the disease are developed. The discovery of reversibility of the RNA toxic effects in murine heart [65] and *Drosophila* muscle tissues [66] provides additional hope to find an effective treatment for patients.

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