Abbreviated title: hPSCs based drug screening for DM1

**Stem cell-based drug screening reveals therapeutic potential of repurposable cardiac glycosides in myotonic dystrophy type 1**

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## Abstract

Myotonic dystrophy type 1 (DM1), the most frequent myopathy of genetic origin in the adult, has currently no treatment. Cells differentiated from stem cell lines derived from preimplantation genetically-diagnosed DM1 embryos recapitulate major pathological mechanisms. Here, a phenotypic drug screening based on those cell models reveals that cardiac glycosides reduce both ribonucleoprotein intranuclear inclusions and associated abnormal RNA alternate splicing. These effects occurred, however, at different concentrations. The drugs decreased the volume of inclusions only in the micromolar range that was associated to cytotoxicity, whereas they corrected several gene isoform ratios in a harmless nanomolar range. This calcium-dependent effect involved AKT and GSK3 and was additive or synergistic with inhibition of the ERK signaling pathway. Similar biological effects were recorded in a DM1 mouse model treated with low doses of digoxin. These results open the path for a clinical trial repurposing a cardiac glycoside in DM1 patients and further underscore the value of stem cell-based assays for drug discovery in monogenic diseases.

## Introduction

Myotonic dystrophy type 1 (DM1), the most common form of adult muscular dystrophy of genetic origin with a prevalence of 1/8,000 worldwide, remains a fully unmet medical need. Its multisystemic symptoms, which include myotonia, muscle wasting, cardiac conduction defects, insulin resistance, cataracts and cognitive dysfunction, are linked to dysregulated RNA alternate splicing that affects hundreds of genes[1-3](#_ENREF_1). The origin of that dysregulation lies in changes of the bioavailability of RNA-binding proteins, in particular MBNL1 which is sequestered in ribonucleoprotein intranuclear inclusions (called foci) triggered by a CTG repeat expansion in the 3’ untranslated region of the DMPK gene *(*dystrophic myotonia protein kinase)[4](#_ENREF_4), [5](#_ENREF_5). Accordingly, a major effort is currently been made in order to generate therapeutic tools specifically designed in order to target the molecular substrate of that pathological binding between the mutant RNA and proteins[6](#_ENREF_6).

A complementary approach for drug discovery has more recently been made possible by the demonstration that cells differentiated from pluripotent stem cell lines carrying the mutant DMPK gene - obtained as ES cell lines from preimplantation genetically-diagnosed (PGD) embryos or via reprogramming iPS cell lines from patients - recapitulate the major cellular and molecular hallmarks of the disease[7-11](#_ENREF_7). These cell lines provide a never-ending supply of well-characterized biological resources that are amenable to unbiased phenotypic high throughput drug screening. Over the past years, such an approach has revealed instrumental for identifying candidate therapeutic compounds for other monogenic diseases, such as familial dysautonomia, Huntington disease, the Phelan McDermid syndrome and progeria[12-14](#_ENREF_12).

Here we have used cells differentiated from an ES cell line derived from a human embryo carrying a DM1 mutation in order to seek compounds that would affect foci and altered ratios of alternate transcripts. This approach has revealed beneficial effects of FDA-approved cardiac glycosides on both DM1 biological markers and an associated functional defect in myogenesis.

## Results

### High throughput drug screening on DM1 cells

The drug screening workflow involved two sequential steps: i) a primary assay in which foci were quantified by automated cell imaging, ii) a dose-response exploration of hit compounds for their effects on both foci and selected genes with altered ratios of alternate transcripts using q-RTPCR. Mesodermal stem cells (DM1\_MSCs) were successfully differentiated from an ES cell line derived from a PGD embryo that carries over 1000 CUG repeats, and compared to MSCs differentiated from a wild-type ES cell line[9](#_ENREF_9). Results were secondarily checked on other cell and animal models of the disease.

Nuclear foci were identified in DM1\_MSCs using an automated RNA fluorescence *in situ* hybridization (FISH) assay developed in order to detect expanded CUG repeats (**Supplementary Figure 1**). DM1\_MSCs contained an average of 2.5 foci whereas none was observed in control cells (**Figure 1A**). Those results were robust as confirmed by a high 0.71 Z’factor calculated for the assay (**Figure 1B**).

The primary drug screening was carried out considering the number of intranuclear foci per cell using 12,089 compounds assayed at a 10 micromolar concentration, including a set of 1,120 FDA-approved drugs. Compounds were to be retained as candidates when the number of foci reached 1.5 standard deviations below mean value, in the absence of unacceptable cytotoxicity (cut-off of cell survival set at 20% of control). That target actually revealed non-informative, since none of the compounds tested demonstrated a capacity at decreasing the number of foci in the absence of unacceptable cell toxicity (Data not shown). It is only when these results were secondarily filtered by taking into account the overall intranuclear area covered by the foci, that four hit compounds emerged. Their effects were characterized by the combination of an increase in number of foci and a decrease of the intranuclear area that they occupied, which suggested a partial disaggregation of the ribonucleoprotein inclusions. These compounds were Cycloheximide and three members of the cardiac glycoside family, namely Strophantidine, Ouabain and Digoxigenin (**Figure 1C, Supplementary Figure 1**). The first compound was excluded from further analysis due to its well-known activity as a protein synthesis inhibitor. Supporting the hypothesis that the apparent reduction in size of the foci was due to a general decrease in cell protein content, we observed a decreased expression of MBNL1 and CUGBP1 as well as an exacerbation of defective alternate splicing associated to DM1 after treatment with Cycloheximide (**Supplementary Figure 2**). As concerns cardiac glycosides, the existence of a common biological mechanism was validated by the recording of similar effects for 4 other members of the same chemical family (**Figure 1D-E**).

The potential clinical relevance of those results was dampened, however, by the fact that all those compounds induced a reduction of approximatively 50% in DM1\_MSC viability, when tested in the micromolar range used for the primary testing. The second step of the workflow explored, therefore, dose-response curves in order to determine whether cardiac glycosides at lower concentrations could reduce the intranuclear area occupied by foci in the absence of cytotoxicity. In parallel, a concomitant rescue of dysregulated alternate splicing was sought using the defective inclusion of exon 11 in the insulin receptor gene (IR) as a representative (**Figure 2A**). The results of those parallel dose-response analyses were paradoxical. Cardiac glycosides were efficient on the total intranuclear area occupied by foci only in the micromolar range, but this was associated not only to a decreased cell viability but also to an exacerbation of the DM1-related alteration in the ratio of IR transcripts. Conversely, all cardiac glycosides tested normalized the DM1-altered ratio of IR transcripts at concentrations ranging from 10 nM to 500 nM, depending upon the chemical structure, in the absence of overt cytotoxicity and of any effect on foci (**Figure 2B-C**).

**Digoxin effect on RNA alternate splicing**

Digoxin, the best documented and prescribed medication in the cardiac glycoside family, was used as a representative for further characterizing a therapeutic potential in DM1.

The long-term effect of the drug was demonstrated by chronic treatment of DM1\_MSCs with 50nM of digoxin for 4 weeks. This treatment normalized the ratio of IR transcripts up to control values, without modulating either the number or the size of foci (**Figure 3A**). Other splice defects associated to DM1 were also shown to be normalized, including SERCA1 inclusion of exon 22, cTNT exon 5, CLCN-1 exon 7a in DM1\_hES derived MSCs and NMDAR1 exon 5 in DM1\_hES derived Neurons (**Figure 3B, Supplementary Figure 3**).

The lack of correlation between the effects of cardiac glycosides on foci and dysregulated RNA processing was confirmed by treating wild type MSCs with digoxin in the nanomolar range. This also led to an increased inclusion of exon 11 in IR and exon 22 in SERCA1, although in those cases above control values (**Figure 3B-C**). Similar results were obtained with other cellular systems, namely, primary cultures of myoblasts and hES-derived neurons (**Figure 3D, Supplementary Figure 3**). In addition, there was no effect of digoxin either on the level of expression or on the localization in DM1 cells of MBNL1 and CUGBP1, the mislocalization and change in bioavailability of which are deemed responsible for the impairment of RNA alternate splicing (**Supplementary Figure 4**).

The consequences of digoxin treatment on RNA alternate splicing was explored more comprehensively using RNA deep sequencing, comparing DM1\_MSCs treated or not for two days with 50nM of the drug. Analysis of annotated splice junctions identified 43 modified splices with Delta ψ≥ 20 and 223 with 10 ≤Delta ψ≤ 20 (**Figure 4A, Supplementary Table 1**). The comparison of this data set with a recently published list of genes, which exhibited dysregulated RNA alternate splicing in muscle biopsies from DM1 patients[3](#_ENREF_3), revealed 10 additional events on which a beneficial effect of a 50nM digoxin treatment (**Figure 4B**).

### Molecular mechanisms that underlie the effects of cardiac glycosides on RNA alternate splicing

In a search for the molecular mechanisms underlying the therapeutic effects of cardiac glycosides in DM1\_MSCs, a series of hypotheses were sequentially explored.

We first checked that these effects were associated to a change in the intracellular concentration of calcium, which is the common outcome of treatments by cardiac glycosides. Treatment with the calcium ionophore A23187, which increases intracellular calcium content, led to the normalization of IR exon 11 inclusion. Combination of digoxin and A23187 led to neither an additive nor a synergistic effect (**Figure 5A**), supporting the hypothesis of a calcium-dependent mechanism for digoxin effects on RNA alternate splicing. This was further validated by the abolition of the drug effect on IR and SERCA1 RNA alternate splicing when calcium was withdrawn from the cell culture medium (**Figure 5B-C**).

The next step of the analysis aimed at determining intracellular signaling pathways that may be triggered by the drug and affect RNA alternate splicing. Following treatment of DM1\_MSCs with 50 or 100 nM of digoxin for 48h, western blot analyses indicated increased phosphorylation of AKT, GSK3 and S6 and decreased activation of ERK (**Figure 5D**). Combination of digoxin with either LY294002 or CHIR99021, which inhibit specifically AKT and GSK3, respectively, attenuated the beneficial effect of digoxin on IR exon 11 inclusion (**Figure 5A**), indicating that those pathways contribute to the effects of the drug. Conversely, inhibition of ERK phosphorylation by PD0325901 in DM1\_MSC normalized IR exon 11 inclusion in the absence of digoxin, and co-treatment with digoxin resulted in an additive or synergistic effect, suggesting a cooperative mode of action (**Figure 5A**). Downstream of those signaling pathways, two RNA-binding proteins that were previously shown to be modulated by cardiac glycosides[15](#_ENREF_15), namely, SRSF3 and TRA2B, were not affected in treated DM1\_MSCs (**Supplementary Figure 4**).

Last, the effect of digoxin was evaluated in vivo, in the HSALR transgenic DM1 mouse model, which expresses 220 CUG repeats in skeletal muscle and exhibits dysregulated alternate splicing. Digoxin was administrated daily by intraperitoneal injection for 7 days in two dosage regimens: 0.02mg/kg per day and 2 mg/kg, respectively. Rescue of Mbnl1, Ldb3 and Clcn1 dysregulated RNA splicing was observed after digoxin treatment only at the lower dosage (**Figure 6A**), in the absence of a change in expression of the mutant DMPK (**Figure 6B**). Mbnl1 exon 5 inclusion, for instance, was recorded at 17.44 ±2.7% after digoxin treatment at 0.02mg/kg per day, down from 35.13 ±2.2% in untreated HSALR mice. This recovery was only partial, however, as the isoform ratio in wild-type mice is below 1% (**Supplementary Figure 5**).

### Functional effects of digoxin

In an attempt to identify a functional correlate for those biological results, digoxin effect was challenged on the altered in vitro myotube differentiation observed with DM1 primary human myoblasts[16](#_ENREF_16), [17](#_ENREF_17). Digoxin treatment of those cells at 50 nM for 7 days increased the number of formed myotubes, as quantified by both the myosin heavy chain immunostaining (number of MF20-positive cells), and the number of nuclei per MF20-positive cell (**Figure 7A-B and Supplementary Figure 6**). Similar positive results on the myogenic process were obtained after just 2 days of treatment with digoxin as well as other cardiac glycosides (**Supplementary Figure7**). Deprivation of calcium from the culture medium during myotube differentiation fully abolished the functional benefit of digoxin (**Figure 7C**).

## Discussion

The main result of this study is the identification of a therapeutic potential for members of the cardiac glycosides family of drugs in Myotonic Dystrophy type 1 (DM1). These compounds were able to promote changes towards normalization in RNA alternate splicing of genes affected by DM1. This encouraging result for a disease which is yet beyond curative therapy deserves, however, to be qualified as the effects were partial and non-specific. Combinatorial approaches with other treatments should, therefore, be envisaged. These results have been obtained through high throughput drug screening based on human pluripotent stem cell line derivatives that carried the mutant gene and recapitulated the cardinal cellular features of the disease. Similar experimental paradigms may be proposed for a vast array of monogenic diseases, provided relevant read-outs are identified.

Myotonic dystrophy type 1 is characterized by the accumulation of the DMPK mRNA in cell nuclei, due to the presence of an abnormally long stretch of CTG triplet repeats in the 3’UTR of the gene. In part through the formation of intranuclear ribonucleoprotein inclusions, that mutation provokes a major alteration in the bioavailability of RNA-binding proteins, most particularly MBNL1 and CUGBP1[18](#_ENREF_18). This, in turn, induces changes in RNA alternate splicing in a number of genes that are physiological targets of those proteins. In the present study, cardiac glycosides were showed to affect those pathological mechanisms in different manners, depending upon their concentration. At high, cytotoxic concentration –in the micromolar range- they provoked a partial disaggregation of the inclusions. This, however, did not correlate with a recovery of the RNA alternate splicing activity, which rather deteriorated further. Such a phenomenon was reminiscent of that what observed in a previous study in which the expression of MBNL1 was decreased in primary fibroblasts derived from DM1 patient using a specific siRNA[19](#_ENREF_19) . Cardiac glycosides may thus have induced the disaggregation of the ribonucleoprotein inclusions by decreasing altogether the synthesis of proteins, including MBNL1. This hypothesis is supported by the fact that cycloheximide, a well-known protein synthesis inhibitor, elicited a similar combination of results. At lower, non-cytotoxic concentration –in the nanomolar range- cardiac glycosides affected neither intranuclear inclusions nor the concentration of MBNL1 and CUGBP1. In contrast, they elicited the recovery of DM1-affected ratios of transcripts for several genes. Molecular mechanisms triggered by the drugs were observed in wild-type cells and, therefore, were not specific to DM1.

Abnormal ratios of transcripts have been associated to clinical symptoms in DM1 and their normalization, if obtained under treatment, should conversely be expected to be therapeutically relevant. The effects of cardiac glycosides are not, however, specifically addressing the causative molecular defect of the disease and their effects are accordingly partial. Whether that partial recovery of ratios may be therapeutically relevant will need ultimately being explored in a clinical setting. Nevertheless, the positive effect of digoxin recorded in the present study on a myogenesis assay indicates that they do have functional consequences. Cardiac glycosides are still prescribed regularly for the management of atrial fibrillation and heart failure, although their clinical use has been steadily decreasing due to their narrow therapeutic window which makes them at risk of toxicity[20](#_ENREF_20). As cardiac conductions abnormalities are critical symptoms in DM1, careful consideration of the indication and cautious observation will be required for administration of cardiac glycosides in DM1 patients. However, several DM1 patients who tolerated digoxin or digitoxin well have already been described[21-23](#_ENREF_21),[24](#_ENREF_24). It is also notable that the narrow concentration range by which digoxin exerts a beneficial effect on DM1 phenotypes in this study is similar to tissue concentrations found in patients treated for heart failure[25](#_ENREF_25), [26](#_ENREF_26).

The ultimate goal of a therapy for DM1 is the correction of all affected genes. Targeting the causative mechanisms of the pathology, at the level of either the DMPK gene or else of its abnormal binding with proteins like MBNL1, would obviously be the most elegant therapy. This is actively pursued by a number of teams who develop various approaches based on gene transfer, antisense oligonucleotides or specifically targeted pharmacology[19](#_ENREF_19), [27-36](#_ENREF_27). The present results point to a parallel pharmacological approach aiming at impacting RNA alternate splicing of as many DM1-affected genes as possible, without affecting the causative mechanisms of the disease. Such an approach would benefit from a combination of drugs. Indeed, cardiac glycosides are not an isolated case, and a number of drugs affecting RNA alternate splicing of various sets of genes have been identified. Considering that potential therapeutic path, it is important to stress that the subtle control of the ratios of gene transcripts may be indispensable to the function, and even survival of cell populations. Altering it inappropriately may, therefore, be highly detrimental. One way forward on a safer side is to focus on drugs that are already approved for clinical use with an acceptable safety profile, as they can be proposed for repurposing. This has already been tried clinically in DM1 patients with the anti-diabetic drug metformin[36](#_ENREF_36). Interestingly, the mode of action of that drug on RNA alternate splicing involves the inhibition of mitochondrial complex 1 and the activation of AMPK, possibly leading to the modulation of autophagy. The beneficial effects of metformin on ratios of gene transcripts involve inhibition of RNA-binding proteins such as RBM3. The mode of action of cardiac glyocosides is different. They modulate the Na+/K+ ATPase protein complex upon binding and activate downstream signaling pathways leading to an increased intracellular calcium concentration[37](#_ENREF_37). Calcium is by itself responsible for changes in RNA alternate splicing patterns[38-40](#_ENREF_38). In that case, the molecular mechanisms linking calcium concentration and modulation of RNA alternate splicing may involve chromatin modifications rather than RNA-binding proteins[38](#_ENREF_38). Although calcium changes in treated cells may not be the sole mechanism underlying the effects of cardiac glycosides, their calcium-dependency that they may rely on is at least partially. Thus the effects of metformin and cardiac glycosides do not rely on fully similar intracellular pathways. They may, therefore, also concern various subsets of DM1-affected genes. Inhibition of the ERK pathway appeared, in the present study, as yet another path for regulating RNA alternate splicing, that may be triggered by other pharmacological agents and control another subset of DM1-affected genes. Therefore, it is tempting to speculate that a combination of several drugs may lead to additive or synergistic effects. From that point of view, it will be interesting to analyze the effects of other drugs known to affect RNA alternate splicing, e.g., valproate, amyloride, or kinetin[41-43](#_ENREF_41), with specific reference to DM1.

The present study further emphasizes the value of pluripotent stem cell lines derived from mutant gene-carrying donors as in vitro cell models of monogenic pathologies for drug discovery. The provision of a never-ending supply of cells expressing a physiologically and pathologically relevant human genome is a first advantage over other cell models. Patients’ samples are limited in volume and lack reproducibility while immortalized cells are neither physiologically nor pathologically fully relevant. High throughput drug screening using a subtle read-out like, in the present case, the average area covered by intranuclear inclusions, would not have delivered without a large renewable supply of a relevant, reproducible and reliable cell model. The physiological relevance of the cells was of particular importance here, since ratios of gene transcripts are highly dependent upon the cell phenotypes. It is interesting to mention that a previous study had already demonstrated the capacity of cardiac glycosides to modulate RNA alternate splicing in a large number of genes[44](#_ENREF_44). However, using HEK cells as a model, that list did not comprise the 15 DM1-affected genes that were identified in the present study in mesodermal and neural derivatives of embryonic stem cells. The use of stem cells derivatives allows, in addition, comparing populations of cells displaying an exact same phenotype, which differ only by their genotype (e.g., DM1 vs WT). For instance, this was instrumental, in the present study, in determining the lack of DM1 specificity of the effects of the drugs on RNA alternate splicing. Once candidate compounds are identified, stem cell derivatives offer a platform for functional analyses. Intracellular signaling pathways could thus be defined using pharmacological manipulations, the effects of which may have been biased in less physiological cell models. Conversely, that cell model has clear limitations. Similar to other cell models, stem cell derivatives are essentially informative for cell-autonomous defects, and multi-systemic alterations cannot be readily explored. More specifically for pluripotent stem cell derivatives, one has to carefully address the issue of the stage of differentiation, which may be further biased by the developmental age of the cells. In the case of DM1, for instance, the known alteration of the Tau gene in its adult form could not be analyzed in the present study because the gene is present only in its fetal form in the neural progeny of ES cells in vitro[8](#_ENREF_8). Altogether, stem cell derivatives provide, therefore, a most interesting platform for in vitro drug discovery but their results must be confirmed in other relevant models, including in vivo as performed here, before identified compounds can be moved forward towards clinical application.

## Materials and Methods

**Cell culture**

Human embryonic stem cells (hESCs) were maintained and differentiated into Mesenchymal Stem Cells as previously described [9](#_ENREF_9) [9](#_ENREF_9). Briefly, hES were manually dissociated and plated on 0.1% gelatin-coated dishes (Sigma) in Knockout DMEM medium optimized for ES cells (Invitrogen), supplemented with 20% of Fetal Bovine Serum (FBS, Eurobio), 1mM L-glutamine (Invitrogen), 1% non-essential amino-acid (Invitrogen), and 0,1% β-mercaptoethanol (Invitrogen). Medium was changed every over two days. Confluent cells were passed with trypsin/EDTA 1X (Invitrogen) in new gelatin-coated plates and plated at a density of 8.000/cm2. After three weeks of differentiation, MSC phenotype was assessed by FACS analysis for CD44, Stro1, CD73 and endoglin expression (all from ABCAM, 1/100). MSC cells amplification was performed as previously described (Ref). Repeats size was estimated at 1.300 [9](#_ENREF_9).

Control myoblasts (CHQ) and DM1 patient myoblasts (2000 CTG repeats) were kindly provided by Dr. Denis Furling (UMR 787, Institut de Myologie, Paris, France). Myoblasts were cultured in DMEM/F-12 medium (Invitrogen) supplemented with 20% FBS (Eurobio) and 0.1% of penicillin–streptomycin (Invitrogen). For differentiation, medium was changed to 2% FBS in 75% Alpha MEM (Invitrogen) and 25% M199 medium (Invitrogen), supplemented with 1% penicillin–streptomycin and 10µg/mL insulin (Sigma). Medium was changed every 2 or 3 days.

**Chemicals**

Three different libraries were screened including 1.280 small molecules from the Prestwick library, 9.689 from ChemXinfinity and 1.280 from Lopac libray (Sigma-Aldrich). Individual small molecules, Digitoxigenin, Ouabain, Strophanditin, Digoxigenin, Oleandin, Cymarin, Digoxin, Cycloheximide, A23187, were purchased from Sigma-Aldrich, PD0325901 from Euromedex, LY294002 from OZYME, CHIR99021 from Tocris.

**Primary Screening**

hESCs derived MSC were plated at 3.000 cells per well in 384-well plates (Corning) coated with 0.1% gelatin. Cell seeding was performed using the automate Biocell 1800 (Agilent). Each compound was tested at a final concentration of 10M in 0.1% DMSO. Cells were treated with each compound in duplicate for 48h. Sequential dilution of compounds and cell treatment was performed using the automate BioCell 1800 (Agilent). Secondary validation tests were performed over a 10-point dilution range in 96 or 384 well plate format. For experiments in calcium free conditions, prior to compound addition, medium was changed to calcium free medium DMEM (Invitrogen) supplemented with 20% of Fetal Bovine Serum, 1mM L-glutamine, 1% non-essential amino-acid, 0,1% β-mercaptoethanol and 400µM EDTA (Sigma-Aldrich).

**Fluorescent In situ Hybridization (FISH)**

All steps were performed using the plate washer CW-ELX405 (BioTek Instruments) combined with a multidrop 384 (Thermofisher - Labsystem). Cells were fixed with PBS buffer containing 4% PFA (Electron Microscopy Science,) for 20 minutes at room temperature and washed with 200µL of PBS. Cells were then incubated overnight at 4 °C with 70% Ethanol. After PBS wash, cells were rehydrated with a solution of PBS containing 5mM MgCl2 for 15-30 min and then sequentially incubated with prehybridization buffer (50mM Phosphate buffer, 40% formamide, 2X SSC, 50l/ well) for 15-30 min and hybridization buffer (Prehybrydization buffer with 0,2% BSA and 7% Dextran, 50µl/well) containing 300ng/mL of the (CAG)10-Cy5 probe (5’Alexa 647- TTCTTATTCTTCAgCAgCAgCAgCAgCAgCAgCAgCAgCAg3’) (Operon) overnight at 37°C. Washing steps consisted in pre-warmed Washing buffer (Prehybrydization buffer + 0.2% BSA, 60µL/well) for 15 min at RT followed by addition of washing buffer (80µL) and incubation for 30 min at 37°C. Cells were then washed twice in PBS before nuclei counterstaining Hoechst 33258 (Invitrogen, 5µg/mL). Plates were stored at 4°C until analysis.

For experiments in which FISH was combined with immunostaining for MBNL1, FISH was performed as previously described with the exception of the hybridization buffer (50mM Phosphate buffer, 40% formamide, 2X SSC, 0,2% BSA, 1mg/ml Salmon Sperm (Invitrogen)). Immediately following FISH, cells were incubated in blocking buffer (PBS containing 3% BSA) for 30min and with primary antibody against MBNL1 (Santa Cruz Biotechnology, 1:100) diluted in blocking buffer for 1h at RT. Alexa488-goat anti mouse secondary antibody (Invitrogen, 1:1000) was used. Nuclei were stained with Hoechst 33258 (Invitrogen, 5µg/mL).

**Immunostaining**

After fixation with PFA4% for 10’, Cells were incubated over night at 4°C with the following list of primary antibodies : Mf20 (DSHB, 1/200), desmin (R&D system, 1/25), MBNL1 (Santa cruz, 1/1000), Mb1a(4A8) (Provided by Dr. Glenn Morris). Appropriated Alexa fluorescent secondary antibodies (1/1000, Invitrogen) and Hoechst (5µg/mL) were added for 1h30.

**Image acquisition and analysis**

Nuclear foci detection was performed using the Cellomics Array Scan VTI high content imaging system (Thermofisher Scientist) with the 20X objective to automatically focus cell preparation. Images were acquired in High Resolution camera mode on two channels: nuclei (channel 1, HOECHST XF93) and DMPK foci (channel 2, Cy5 Sensitive XF110). Based on the Hoechst channels, a minimum of 250 nuclei were identified using intensity thresholding, background correction and segmentation parameters. Each nuclear region was then reported on the second channel (Cy5) to limit the foci detection to the nuclear area. Foci were identified using a fixed intensity threshold, background correction and segmentation parameters. To avoid unspecific signal quantification, foci detection was gated with minimum and maximum area (0.1 µm² to 40µm²) or intensity (average intensity min 70, max 1300).

The effect of digoxin treatment on myogenic differentiation was automatically processed as previously described [45](#_ENREF_45). Using 5X magnification, Hoechst staining was used for autofocus and nuclei were identified applying intensity threshold, segmentation and background correction parameters. In the same way, Mf20 were detected. The percent of the total detected nuclear area within MF20 myotubes was used as the percent of positive cells and the total Mf20 area per field was calculated.

**Splicing analysis by RT-PCR**

Total RNA was extracted using the RNeasy Micro/Mini kit (Qiagen) and reverse transcribed using random hexamers and Superscript III Reverse Transcriptase kit (Invitrogen) according to the manufacter’s protocol. For splicing analysis, PCR amplification of endogenous or exogenous genes was carried out with recombinant Taq DNA polymerase (Invitrogen) and the primers listed in Table S1. The amplification was performed using a first step at 94°C for 3 min followed by 35 cycles of 45 s at 94 °C, 30 s at 55 °C, 30 s at 72 °C, and with a final 10 min extension at 72 °C. The PCR products were analyzed using DNA 1000 LabChip kit (Agilent) and quantified with the Bioanalyzer 2100.

**Gene expression analysis by quantitative PCR**

Quantitative PCR reactions were carried out using a Chromo4 Real-Time System (Bio-Rad) with Syber Green PCR Master Mix (Applied Biosystems). Detailed information on the primers sequences is provided in TableS1. The 2−ΔΔCt method was used to determine the relative expression level of each gene. Relative titration of INSR transcripts +/- exon 11, NMDAR1 transcripts +/- exon 5 and house keeping gene 18S was monitored with TaqMan gene expression assays using the primers and MGB probes described in TableS1 and TaqMan Gene Expression Master Mix (Applied Biosystem). This was carried out using the Lightcycler 480 (Roche, France).

**Protein extraction and western blot analyses**

Western blots analyses were carried out as previously described[7](#_ENREF_7), [8](#_ENREF_8). Briefly, cells were lyzed in RIPA 1X buffer (Sigma) containing protease inhibitors (Sigma) and phosphatases inhibitors (Roche). Nuclear and cytoplasmic fractions were obtained using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific) according to manufacturer’s protocol. Proteins were quantified by Pierce BCA Protein Assay kit (Pierce). Protein extracts (10 to 20µg) were loaded on a 4–12% SDS-PAGE gradient (Nupage® Bis–Tris gels, Invitrogen) and transferred onto Gel Transfer Stacks Nitrocellulose membranes (Invitrogen) using the iBlot Gel Transfer System (Invitrogen). Membranes were then incubated overnight at 4°C with primary antibodies listed in Supplementary Table 2. After hybridization of the peroxidase-conjugated secondary antibody, immunoreactive bands were revealedby using Amersham ECL Plus™ Western Blotting Detection Reagents (GE Healthcare). Equal protein loading was verifiedby the detection of Actin.

**Statistic analysis**

Screening data were analyzed using SpotFire software (Discngine). Data were processed using Graph pad PRISM. Student’s t-test (ANOVA) was used to analyze two groups of data. For comparisons of more than two groups, ‘Bonferroni’s multiple comparison test’ (one-way ANOVA) was used.

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**Figure Legends**

**Figure 1: Development of a high-content assay for mutant DMPK foci.**

**A**- Average number of detected foci per cell in untreated DM1 -MSC (red dots) and WT hESC-MSC (bleu dots). Each dot represents one well among the 90 screening plates. **B**- Determination of the Z’ factor by using number of detected foci per cell between DM1 and WT\_MSC for each plate with an average value around 0.7 for the whole screening campaign. **C**- Scatter plot representation of the primary screening analysis based on the number of detected foci per cell and foci average area after normalization on non-treated DM1\_MSC. Validated compounds are shown with colored dots. **D**- Dose response analysis for foci number, foci area and cell density on DM1\_MSC after 48h treatment with seven cardiac glycosides. Average values ± S.D are given (10 points doses response curve in triplicate). **E**- Representative images of mutant DMPK mRNA foci detected by FISH on DM1\_MSC after treatment with three different cardiac glycosides for 48h.

**Figure 2: Digoxin treatment in the nanomolar range rescues IR and SERCA1 alternative splicing in DM1 cells**

**A**- Dose response analysis for IR alternate splicing on DM1\_MSC treated with digoxin for 48h. Relative expression level of the two isoforms of IR (IRA (-exon 11) and IRB (+ exon 11)) were measured by quantitative real time RT-PCR. Data represented the ratio of IRB/IRA and are indicated as mean ± S.D (n=3). **B**- Superposition of the concentration-dependent effect of digoxin on the number of foci per cell (solid lines, left axis) and IR alternate splicing (black bars, right axis) on DM1\_MSC treated for 48h. **C-** Dose-response analysis of six different cardiac glycosides on mutant DMPK foci (solid lines ; n=2 ; mean ± SEM) and IR alternate splicing (bares ; n=2, 1 experiment; mean ± SD) in DM1\_MSC treated for 48h. **C**- Effect of digoxin treatment at 50 nM for 48h on the inclusion of exon 22 of SERCA1 in WT and DM1\_MSC. The percentage of inclusion of exon 22 of SERCA1 was determined by RT-PCR followed by analysis with 2100 Bioanalyzer (Agilent). Quantification of each band was performed by using 2100 Expert software (n=3 independent experiments – 3 replicates per experiments). **D**- Concentration-dependent effect of digoxin on IR alternate splicing in WT and DM1\_MSC. Relative expression level of the two isoforms of IR (IRA (-exon 11) and IRB (+ exon 11)) were measured by quantitative real time RT-PCR. Data represented the ratio of IRB/IRA and are indicated as mean ± S.D (n=3 independent experiments – 3 replicates per experiments). **E**- Effect of digoxin treatment on IR and SERCA1 alternate splicing in primary myoblasts isolated from healthy and DM1 patients. For all graphs, data are presented as mean ± SD (n=3 independent experiments – 3 replicates per experiments) and analyzed with one way ANOVA followed by a Bonferroni post hoc test. \*: p-value < 0.05. \*\*: p-value < 0.01. \*\*\*: p-value < 0.001.

**Figure 3: Digoxin treatment rescues IR and SERCA1 alternative splicing in DM1 cells**

**A-** Effect of chronic digoxin treatment on mutant DMPK foci and alternate splicing of IR and SERCA1. DM1\_MSC were continuously treated with 50 nM of digoxin during 4 weeks. Mutant DMPK foci and alternate splicing of IR and SERCA1 were analyzed every 7 days. Untreated WT\_MSC were used as control. IR alternate splicing was analyzed by real-time quantitative RT-PCR and SERCA1 alternate splicing was quantified by RT-PCR followed by quantification using agilent electrophoresis gel. For mutant DMPK foci, data represent mean ± SD (n=4 replicates, 1 experiment). For IR and SERCA1 alternate splicing, one sample was analyzed at each time point. Relative expression level of the two isoforms of IR (IRA (-exon 11) and IRB (+ exon 11)) were measured by quantitative real time RT-PCR. Data represented the ratio of IRB/IRA and are indicated as mean ± S.D (n=1 at each time point, 1 experiment). **B**- Effect of digoxin treatment at 50 nM for 48h on the inclusion of exon 22 of SERCA1 in WT and DM1\_MSC. **C**- Concentration-dependent effect of digoxin on IR alternate splicing in WT and DM1\_MSC. Relative expression level of the two isoforms of IRA (-exon 11) and IRB (+ exon 11)) were measured by quantitative real time RT-PCR. Data represented the ratio of IRB/IRA. **D**- Effect of digoxin treatment on IR and SERCA1 alternate splicing in primary myoblasts isolated from healthy and DM1 patients. For all graphs, data are presented as mean ± SD (n=3 independent experiments – 3 replicates per experiments) and analyzed with one way ANOVA followed by a Bonferroni post hoc test. \*: p-value < 0.05. \*\*: p-value < 0.01. \*\*\*: p-value < 0.001.

**Figure 4 : Beneficial effect of digoxin treatment in the nanomolar range on other alternate splicing associated to DM1**

**A**- Number of alternate splicing modified by digoxin treatment (50nM for 48h) in DM1\_MSC as identified by RNA sequencing analysis. The list of modified alternate splicing is providing in supplementary table 1. **B**- RT-PCR analysis of 10 alternate splicing described to be affected in DM1 patients and rescued by digoxin treatment (50nM for 48h) on WT and MSC\_MSC. Data represent mean ± S.D (n=3 independent experiments – 2 replicates per experiments). Data were analyzed with one way ANOVA followed by a Dunnett post hoc test. \*: p-value < 0.05. \*\*: p-value < 0.01. \*\*\*: p-value < 0.001.

**Figure 5: Calcium-dependent effect of digoxin on alternate splicing of IR and SERCA1**

**A.** Pharmacological approaches to analyze the combinatorial effect of AKT, GSK3, ERK and calcium pathways with digoxin on IR alternate splicing. DM1\_MSC were treated for 48h with or without 50nM of digoxin, in combination with different concentrations of AKT inhibitor (LY29002), GSK3 inhibitor (CHIR99021), ERK inhibitor (PD0325901) or calcium ionophore (A23187). WT\_MSC cells were used as a positive control. IR alternate splicing was quantified by RT-qPCR. Data represent mean ± S.D (n=3). **B-C.** Influence of calcium on digoxin-mediated effect on alternate splicing of IR and SERCA1 in DM1\_MSC. Cells were maintained in medium with or without calcium and treated with digoxin (50 or 100 nM) for 48h. Medium without calcium was added 30 minutes prior to the digoxin (n=3). **D.** Effect of different concentrations of digoxin on MAPK and mTOR signaling pathways. Expression level of key modulators of Akt/mTOR signaling in DM1\_MSC treated with different concentrations of digoxin for 48h including:Phospho(Ser473)-Akt, Phospho(Ser21/9)-GSK3α/β, and Phospho(Ser235-236)-Ribosomal protein S6 (rpS6), Phospho (Thr202/Tyr204) ERK and the corresponding total forms. Western blot analysis were quantified by using ImageJ software. Data represent mean ± S.D (n=3).

All data were analyzed with one way ANOVA followed by a Bonferroni post hoc test. \*: p-value < 0.05. \*\*: p-value < 0.01. \*\*\*: p-value < 0.001.

**Figure 6: Digoxin treatment partially restores in vivo splicing defects in a DM1 mouse model.**

**A.** Analysis of Mbnl1, Ldb3, Clcn1 alternative splicing by RT-PCR in HSALR mice treated with intraperitoneal injection of digoxin for 7 days (n=3 for wild type, n=4 for mock-treated and n=5 for treated). RT-PCR was followed by analysis with 2100 Bioanalyzer (Agilent). Quantification of each band was performed by using 2100 Expert software. Data are represented as mean± S.E.M. P-value was determined using the Mann–Whitney U test; \*P < 0.05 **B.** Quantitative real-time RT-qPCR analysis of HAS transgene in function of digoxin treatment. Data were normalized with the housekeeping gene 18S.

**Figure 7: Digoxin treatment restores in vitro myogenic defect in DM1**

**A.** Representative immunostaining for the myogenic marker Mf20 in WT and DM1 myoblasts differentiated for 6 days. DM1 myoblasts were treated with 50 nM of Digoxin at day 1 of differentiation. **B.** Automated quantification of myogenic differentiation as determined by the measurement of the total area stained for Mf20 per field in WT differentiated myoblasts and in DM1 differentiated myoblasts treated or not with 50 nM of digoxin, as well as the quantification of nuclei area per Mf20 positive myotube. **C.** Calcium-dependent effect of digoxin on the in vitro myogenic impairment observed with DM1 myoblasts. Cells were differentiated in presence or absence of calcium. The effect of digoxin treatment (50nM for 7 days) on myogenesis was evaluated by automated quantification according to the total area stained for Mf20 per field (D) and as the percent of the total area of nuclei identified within Mf20 positives myotubes (E). Data are presented as mean ± SD (n=3 independent experiments- 3 replicates) and were analyzed with one way ANOVA followed by a "Bonferroni's Multiple Comparison Test" \*\* p<0.05, \*\*\*p<0.001.