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Hypericin repairs misregulated splicing events associated with myotonic dystrophy in type 1 myoblast lines

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Abstract

Myotonic dystrophy is associated with the molecular event of misregulated splicing of a subset of RNAs, which contribute to some of the disease phenotypes. One of the misregulated splicings that leads to one of the disorder's phenotypes is insulin resistance (IR). Other misregulated splicing events considered in this study included Sarcoplasmic Endoplasmic Reticulum ATPase 1 (SERCA1), and muscleblind protein isoforms 1 and 2 (MBNL1 and MBNL2). Assays based on the above splicing events were designed for reverse transcription (RT-PCR) analysis and applied to myotonic dystrophy type 1 (DM1) myoblasts derived from differentiated fibroblasts to test for a compound that could reverse misregulated splicing in the cultures. Reverse Transcribed PCR methods quantified by the Genescan genetic analyser were utilised for the study. In this study, hypericin, a protein kinase C inhibitor, was observed to affect a slight reversal of misregulated splicing associated with the disorder in the insulin receptor (IR) by enhancing the relative proportion of the muscle signalling type (IR-B) in myotonic dystrophy type 1 myoblasts. Additionally, hypericin increased the proportion of the skeletal muscle isoform (SERCA1a) in DM1 myoblast cultures compared with mock control (DMSO-treated) ($p=0.002$). Furthermore, hypericin treatment was also observed to correct missplicing in *MBNL1* and *MBNL2* by reducing the proportion of isoforms containing exon 7. In this study, hypericin treatment corrected splicing abnormalities in all splicing assays performed in DM1 myoblasts. Taken together, the results indicate that hypericin may have therapeutic potential in the DM1 cell model used in this study.

Keywords

Misregulated splicing, molecular target, muscleblind proteins, Protein Kinase C inhibitor, therapeutic screening

Introduction

Myotonic dystrophy (DM) is the most common form of muscular dystrophy in adults and is inherited as an autosomal dominant neuromuscular disease (Harper, 2001). The disorder manifests in two forms, namely: myotonic dystrophy type 1 (DM1) and myotonic dystrophy type 2 (DM2) (Harper, 2001). The length of expanded repeats associated with DM is due to gene mutations, which also affect predisposition of individuals to infection (Udosen and Nya, 2017; Udosen, 2019)

Splicing misregulation is a molecular feature in both forms of myotonic dystrophy (Harper, 2001). The type 1 form of myotonic dystrophy (DM1) is more common and is mainly defined molecularly by the presence of expanded CTG repeats ranging from 50 to 3000 (Machuca-Tzili et al., 2005). Another molecular event implicated in myotonic dystrophy involves proteins that interact with RNA, which have been suggested to associate with expanded-repeat RNA to form nuclear foci, one of which is the muscleblind protein. The muscleblind proteins (MBNL) have been shown to colocalise with expanded CUG or CCUG repeats in nuclear foci (Miller et al., 2000; Fardaei et al., 2001; Fardaei et al., 2002).

MBNL1, MBNL2 and MBNL3 proteins were found to colocalise with RNA foci in DM1 and DM2 cells (Miller et al., 2000; Mankodi et al., 200; Fardaei et al., 2002). A rational approach to a possible therapeutic intervention in DM might involve blocking the entire expanded-repeat RNA from interacting with the muscleblind protein, thereby making the protein available to perform its splicing regulatory functions.

Several studies have been carried out to identify inhibitors that bind to expanded CUG or CCUG repeat RNAs to prevent their interaction with muscleblind-like proteins (Gareiss et al., 2008; Arambula et al., 2009; Pushechnikov et al., 2009; Warf et al., 2009).

As previously stated, one of the main molecular characteristics of the disorder is the loss of splicing regulation in the patient's cells. It has been reported that MBNL1 depletion leads to misregulation of splicing in many genes and, in some cases, results in myotonic dystrophy (DM) phenotypes (Ranum and Cooper, 2006).

In the disease situation, abnormal regulation of splicing is observed for some of the splicing processes, which are usually regulated in a developmental manner, as well as in disorders where they cannot express the normal adult splice form mRNA occurring in adult-based tissues. These observed developmental control of splicing might be definitive, such as *MBNL1* exon 7, *SERCA1* exon 22, as well as *ZASP* exon 11, and *cTNT* exon 5, or manifest as partial, as in *IR* exon 11 and in *MBNL2* exon 7. This results in the abnormal fetal mRNA form and the expression of a functionally irrelevant protein, leading to disease symptoms (Ranum and Cooper, 2006). The main symptoms of myotonic dystrophy are myotonia and insulin resistance, which result from splicing abnormalities in the muscle-specific chloride channel (*CLCN-1*) and the insulin receptor (*IR*), respectively (Ranum and Cooper, 2006).

The insulin resistance phenotype observed in the disease is due to a defective ability to express the normal wild-type splice isoform of *IR* (IR-B), which is usually expressed in adult skeletal muscle. Alternatively, myotonic dystrophy patients exhibit the nonmuscle IR isoform (IR-A), which has been reported to have a lower signalling ability than that found in adult skeletal muscle, and the expression of IR-A in skeletal muscle cells of DM was associated with reduced response to insulin

compared to control cells from unaffected individuals (Savkur et al., 2001; Savkur et al., 2004). The other events of misregulated splicing observed in DM include SERCA1 and SERCA2 (Kimura et al., 2005), cardiac troponin T (Philips et al., 1998), myotubularin-related protein 1 (Buj-Bello et al., 2002), tau (Sergeant et al., 2001), and fast skeletal troponin T (Kanadia et al., 2003). Reversal and rescue of splicing defects have now provided a molecular target for the development of potential therapy for the disorder.

Protein Kinase Cs are serine and threonine kinases that are stimulated by phorbol esters (Mellor & Parker, 1998). A Protein Kinase C inhibitor, Ro-31-8220, was used by Wang et al (2009) to demonstrate the ability to relieve cardiac conduction defects and cardiac abnormalities in an inducible cardiac-specific mouse DM model of the disease. Additionally, the inhibitor (Ro-31-8220) was observed to reverse misregulated splicing events in exon 21 of Ank2 as well as exon 16 of Mtmr3, which are regulated exclusively by CUG-BP1 (Wang et al, 2009). This justified the inclusion of the protein kinase C inhibitor, hypericin, in this study. In this report, hypericin, a Protein Kinase C inhibitor previously identified in a therapeutic screening study to inhibit interactions between MBNL proteins and expanded repeat units to forestall foci formation in DM cell lines (Ketley et al., 2013; Udosen et al., 2023), was utilised to assess its outcome on the rescue of splicing misregulation of *IR*, *SERCA1*, *MBNL1* and *MBNL2* in DM1 myoblast cell lines.

Material and methods

Cell Culture

The cells utilised in this report were DM1 fibroblast cells and a wild-type cell line from David Brook's Laboratory at the University of Nottingham. Cell lines used in this study were the patient cell line (designated as KBTeloMyoD) and a wild-type cell line (designated as SBTeloMyoD); they were already maintained as standardised cells from a standard laboratory at the University of Nottingham (Udosen et al., 2023). Ethical approval was not required for this study because the experiments involved previously established immortalised cell lines and did not involve direct human or animal participation.

The fibroblast cells used for this work were already telomerised (Udosen et al., 2023; Udosen et al., 2026). The DM cells obtained were grown in Dulbecco's Modified Eagle's Medium made up of L-glutamine, 4,500mg/L glucose and pyruvate (GIBCO, United Kingdom); 10% Foetal Bovine Serum (FBS) from GIBCO, United Kingdom, along with the antibiotic Penicillin/Streptomycin, comprising of 10,000µg/mL streptomycin sulphate suspended in 0.85% saline and 10,000 units of Penicillin G, all obtained from GIBCO, United Kingdom. The DM1 fibroblasts were treated with 1mg/ml doxycycline from Clontech, United States, which induced the fibroblasts cells expressing inducible MyoD to differentiate into myoblasts. After differentiation, the myoblasts were subsequently used for the various treatments. The Dimethyl Sulphoxide and hypericin used for treatment were obtained from Sigma-Aldrich, United Kingdom.

For treatments with hypericin and Dimethyl Sulphoxide (DMSO), the cells were subjected to different concentrations of the test compound at 100 µM, 48 µM, 8 µM, and 1.6 µM. The treatments were optimised at 48µM. The compounds used for this study were a protein kinase C inhibitor called hypericin, while Dimethyl Sulphoxide (DMSO) was utilised as a

mock control. The treatments were performed in triplicate to assess the effect of the test molecule on the reversal of splicing abnormality in DM1 myoblasts.

RNA Extraction and Reverse Transcription PCR

Cell lines for total RNA extraction were processed in accordance with the Purescript-Gentra purification kit protocols. Flasks of cells were dislodged with trypsin, and about 1,000,000 cells were pelleted at 16,000g for 5 seconds in Eppendorf tubes. The contents of the tube were removed, and the cell pellets were retained. About 20µL of DEPC H2O was added to inhibit RNase, and 300µL of cell lysis solution was added to lyse the pelleted cells. This was followed by the addition of 100µL of protein-DNA precipitation solution, and the mixture was inverted 10 times to ensure uniform mixing prior to ice incubation for 5 minutes. Subsequently, centrifugation was performed for 5 minutes at 16,000g to pellet proteins and DNA, and the supernatant containing RNA was transferred to a fresh tube with 300µL of 100% isopropanol. The mixture was inverted several times to ensure uniformity and then centrifuged to pellet the RNA. The supernatant was removed, and the RNA pellet was washed with 75% ethanol before centrifugation. The clear fluid portion was removed, and the RNA pellet was air-dried for 20-30 minutes. The pellets were subsequently dissolved in 50 µl of DEPC-treated water and incubated on ice for 30 minutes. The nanodrop was used to quantify RNA before reverse transcription.

The total RNA was obtained from treated and untreated myoblasts, which was subsequently utilised for the synthesis of cDNA involving reverse transcriptase and random hexamers from New England Biolabs. An aliquot of the above cDNA (1µl) was then subjected to PCR as stated below:

For analysis of *IR* splicing, PCR was performed utilising 1 µl of cDNA, involving forward oligonucleotide IRfor along with reverse primer IRrev. Amplification of thirty cycles was undertaken, made up of 95°C for 5 minutes, along with repeated cycles for 30 seconds at 95°C; binding of primers to cDNA template at 60°C for 30 seconds; with extension of amplicon at 72°C for a 30-second duration. The final extension step was performed for 5 minutes at 72°C. PCR products were electrophoretically resolved on 3% agarose gels and the bands were viewed under UV light. The relative proportions of insulin receptor splice forms were determined using a fluorescently labelled forward oligonucleotide, and their amplification products were assessed using the Genescan genetic analyser after 26 cycles of PCR.

For the analysis of *SERCA1* splice variants, 1/20th of cDNA was subjected to amplification using forward primer SERCA1for along with reverse primer SERCA1rev at thirty-four cycles comprising of 95°C for a period of 5 minutes, followed by cycles of 95°C for a 30-seconds duration; annealing of primers for a period of 1 minute at 60°C with extension for 1 minute at 72°C. Final extension of amplicon was carried out at 72°C for a 5-minute duration. The estimation analysis of *SERCA1* splice variants was carried out after 27-28 cycles using a fluorescently-labelled reverse primer.

For the analysis of *MBNL1* splice variants, 1 µl of cDNA was used for PCR with forward primer MBNL1for along with the reverse primer MBNL1rev (Table 1) using 30 cycles made up of 5 minutes

duration at 95°C with repeated cycles of 30 seconds period at 95°C; primer annealing for 30 seconds at 62°C; with extension of 45 seconds duration at 72°C. A final extension step was carried out for a period of 5 minutes at 72°C. Estimation analysis of different *MBNL1* splice forms was performed in cycles of 22-24 using a fluorescently labelled forward oligonucleotide.

For the analysis of *MBNL2* splice variants, 1/20th of cDNA was subjected to PCR with forward primer MBNL2for with reverse primer MBNL2rev using 30 cycles comprising of 5 minutes duration at 95°C for denaturation, along with repeated cycles of 30 seconds at 95°C; primer annealing for a 30 seconds duration at 60°C with an extension for a 45 seconds duration at 72°C. A final extension was performed for 5 minutes at 72°C. Quantification analysis of *MBNL2* splice variants was performed using a forward fluorescent primer at 22-24 cycles (Table 1).

Normalisation of RT-PCR was performed using *GAPDH* as control, having forward primer GAPDHfor along with the reverse primer sequence GAPDHrev (Table 1).

Statistical analysis of data was performed using a t-test, which was performed using Graphpad Prism Software with the test of significant difference at 95% level (p=0.05).

Table 1. List of oligonucleotide primers used in Reverse Transcription PCR

Primer	Sequence
IRfor	5'CCAAAGACAGACTCTCAGAT3'
IRrev	5'AACATCGCCAAGGGACCTGC3'
SERCA1for	5'TTCGTTGCTCGGAACTACC3'
SERCA1rev	5'GGTTGGGAAGGGGAATTTAC3'
MBNL1for	5'GCTGCCCAATACCAGGTCAAC3'
MBNL1rev	5'TGGTGGGAGAAATGCTGTATGC3'
MBNL2for	5'ACAAGTGACAACACCGTAACCG3'
MBNL2rev	5' TTTGGTAAAGGATGAAGAGCACC3'
GAPDHfor	5'GATGACAAGCTTCCCGTTCTCAGCC3'
GAPDHrev	5'TGAAGGTCGGAGTCAACGGATTTGGT3'

Results

To test the validity of the splicing concept in DM1, we selected four splicing events that are associated with DM1. The splicing events utilised in this study included insulin resistance (*IR*), *SERCA1*, muscleblind protein 1 and 2, which were used to determine the compound's efficacy in altering the spliceopathy in DM1 cells. The assay for splicing in *IR* was based on splicing variants of exon 11, which are reduced in myotonic patient muscle cell cultures but increased in wild-type cultures. The oligonucleotides employed were domiciled on exons 10 and 12, which flanked the alternatively spliced exon, which is exon 11. It produced RT-PCR amplicons of 167 bp and 131 bp corresponding to the included (*IR-B*) or excluded

exon 11 (*IR-A*), respectively, which were subsequently utilised in the treatment of DM1 and wild-type myoblasts (Figure 1).

Hypericin treatment partially reverse abnormal splicing of the insulin receptor in DM1 myoblasts compared with controls (p=0.001; p=0.008). Statistical analysis of data showed that hypericin treatment was significant with mock treatment (DMSO) (p=0.001) and showed a significant difference compared to untreated controls (p=0.008), as shown in Figure 2. In addition, hypericin treatment showed that the insulin receptor splice variant (IR-B) was significantly different from that in wild-type cells (p=0.006).

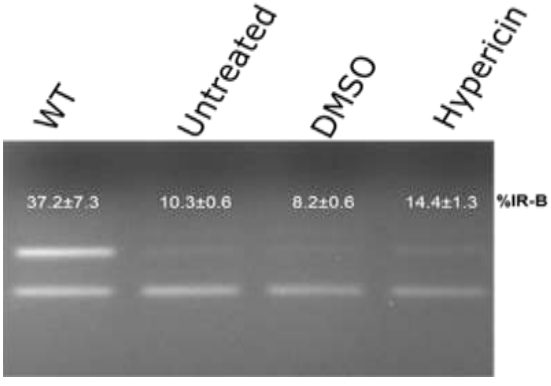


Figure 1. Hypericin treatment induces a slight increase in the normal *IR-B* variant (167bp) relative to the abnormal splice form *IR-A* (131bp) in DM1 myoblasts, in contrast with controls (DMSO and untreated) on 3% agarose gel.

Another splicing abnormality observed in DM is SERCA1, which was used to measure the effect of compound treatment in this study. Alternative splicing of exon 22 generates two variants: SERCA1a, containing exon 22, and SERCA1b, which lacks exon 22. Hypericin treatment showed a slight increase in the SERCA1a variant compared with mock and untreated controls (Figure 3).

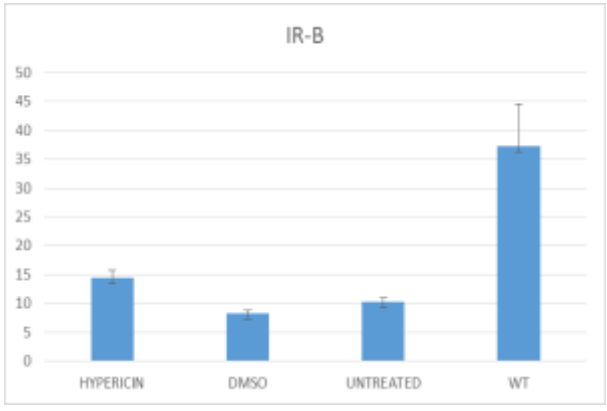


Figure 2. The proportions of adult signalling form of insulin receptor (IR-B) across the different treatment groups.

When analysed statistically, a significant difference was observed between hypericin treatment and mock (p=0.02) and between hypericin treatment and untreated controls (p=0.027), as seen in Figure 4. In addition, there was no difference between the controls (p=0.39). The percentage of the splice variant *SERCA1a* was significantly different between hypericin treatment and the wild-type cell line (p=0.009). Another misregulated splicing observed in DM is the muscleblind protein 1 (MBNL1), which is misregulated in DM by inclusion of exon 7 in DM cell cultures (Lin et al., 2006).

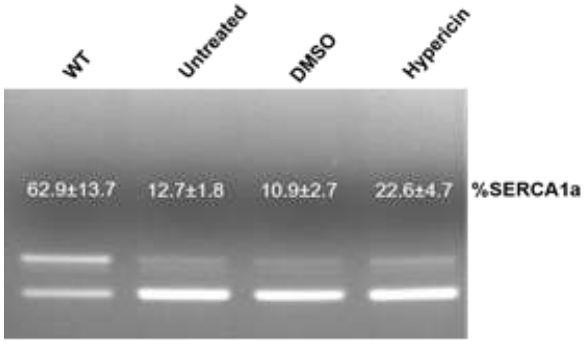


Figure 3. Hypericin-treated DM1 myoblasts demonstrated an increase in *SERCA1* exon 22 variant (277bp) relative to *SERCA1b* variant (235bp) in contrast with controls.

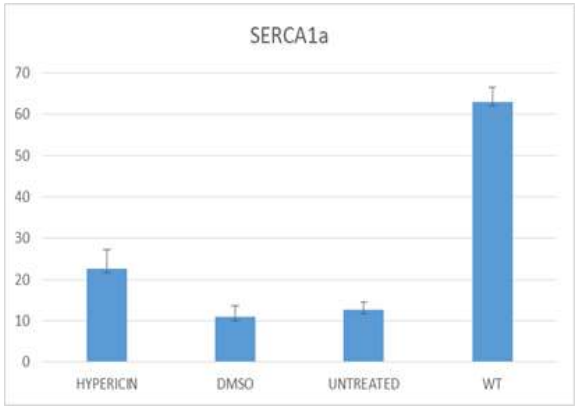


Figure 4. Chart showing the relative proportion of SERCA1a among the different treatment groups in DM1 myoblasts.

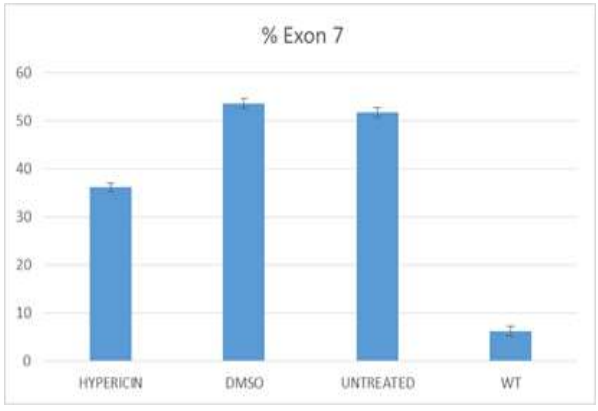


Figure 6. Chart showing the proportion of inclusion of exon 7 of *MBNL1* across the different treatment groups in DM1 myoblasts.

This was applied to test the efficacy of the PKC inhibitor, hypericin, in rescuing the splicing abnormality of *MBNL1* in DM, and was carried out in compound-treated DM1 myoblasts and control cells. In this study, hypericin significantly decreased *MBNL1* + exon 7 variant inclusion compared with DMSO treatment and the untreated control (Figure 5). Statistical analysis showed that hypericin treatment had significant differences in exon 7 inclusion when compared with controls-DMSO (p=0.0003) and untreated (p=0.0012) as shown in Figure 6. Also, there was no significant difference between the controls (p=0.21).

Additionally, the exon 7 inclusion variant was significantly different between the wild-type cell line and hypericin treatment (p= 0.0001). Furthermore, the exon 7 inclusion variant of *MBNL2* was significantly different between hypericin-treated cells and wild-type cells (p=0.0001). Closely related to MBNL1 is MBNL2, which is abnormally spliced in DM. Abnormal splicing of MBNL2 in DM results in the inclusion of exon 7. In DM1 myoblasts, hypericin treatment decreased the proportion of the +exon7 splice variant compared with mock and untreated controls (Figure 7).

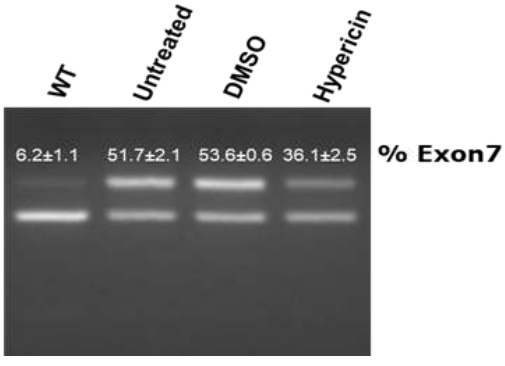


Figure 5. Hypericin treatment effects reversion of *MBNL1* +exon 7 splicing variant (270bp) in relation to the –exon7 splice variant (216bp) in DM1 myoblasts when compared with controls on 3% agarose gels.

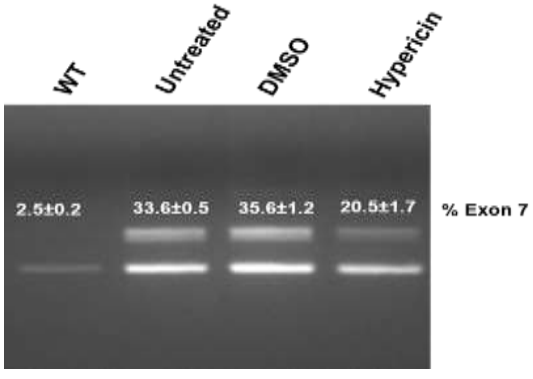


Figure 7. Hypericin induced a decreased +exon 7 splice variant (266bp) in relation to –exon 7 splice variant (212bp) of MBNL2 when compared with controls in DM1 myoblasts on 3% agarose gels.

Statistical analysis showed that hypericin treatment resulted in a significant difference in exon 7 inclusion compared with the untreated control, but not with the mock control (Figure 8).

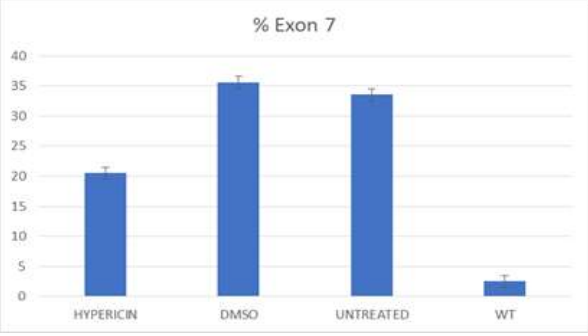


Figure 8. Chart for the proportion of percentage inclusion of exon 7 of MBNL2 across the different treatment groups in DM1 myoblasts.

Discussion

Abnormal alternative splicing of certain genes associated with DM is one of the major molecular events in the disease and could serve as a therapeutic target for screening potential therapeutic agents for the condition (Wojciechowska et al., 2018). Splicing abnormalities in some genes could serve as screening targets for therapeutic intervention. Among the prominent ones is the abnormal splicing of the insulin receptor (*IR*). One of the misregulated splicing events that contributes to the major symptoms of the disease is the insulin receptor (*IR*), which contributes to insulin resistance in DM (Savkur et al., 2001). The *IR-A* isoform lacks exon 11, which is necessary for its biochemical activity, and this was demonstrated to be due to *MBNL1* and *MBNL2* depletion and/or elevated levels of CUG-BP1 in DM1 myoblast cell line (Ho et al., 2004; Dansithong et al., 2005; Paul et al., 2006). In this study, hypericin partially reversed the misregulated splicing of the insulin receptor (*IR*) in DM1 myoblasts, increasing the *IR-B* isoform, which contains exon 11. Compared with the mock control and the untreated, the reversal was significant, indicating that hypericin could be a potential therapeutic agent in the myotonic dystrophy type 1 cell model used in this study.

Another important molecular marker in DM is splicing misregulation in *SERCA1* involving exon 22 exclusion. Sarcoplasmic/endoplasmic reticulum Ca2+ -ATPase (*SERCA*) are responsible for regulating calcium homeostasis in skeletal muscle (Kimura et al., 2005). *SERCA1* produces two alternatively spliced forms: *SERCA1a*, the adult splice variant, along with *SERCA1b*, the embryonic variant, observed to be expressed in fast-twitch (type 2) skeletal muscle. DM1 skeletal muscle cultures showed a significant proportion of embryonic isoform lacking exon 22 (*SERCA1b*) compared with controls (Kimura et al., 2005). This study found that hypericin significantly reversed the splicing abnormality of *SERCA1* in DM1 myoblast cells by increasing the relative proportion of the adult splice form (*SERCA1a*) compared to mock and untreated controls. MBNL1 is implicated in the regulation of alternative splicing, especially of chloride channels and insulin resistance (*IR*). In DM cell cultures, the proportion of isoforms containing exon 7 has been observed to increase. Exon 7 has been associated with enhanced *MBNL-MBNL* dimerisation, and the abnormally high inclusion of this exon in DM1 could accelerate the potential for *MBNL1* protein to be sequestered by nuclear CUG repeat expansions (Tran et al., 2011). In this study, hypericin was observed to significantly decrease exon 7 isoforms of *MBNL1* and *MBNL2* in DM1 myoblasts compared with mock and untreated controls.

Spliceopathy of *CLCN1* and *SERCA1* have been used in screening therapy studies of DM (Mulders et al., 2009; Warf et al., 2009; Wheeler et al., 2009). Therapeutic reversal of spliceopathy in DM has been demonstrated using antisense against CUG repeats (Francois et al., 2011), antisense oligonucleotides (Mulders et al., 2009; Wheeler et al., 2009), siRNA against *MBNL* (Dansithong et al., 2005), MBNL1 overexpression (Kanadia et al., 2006; Hino et al., 2007) and a kinase inhibitor (Udosen, 2020). This makes a case for rescuing splicing abnormalities in DM to identify small molecules that could reverse the DM phenotype(s). In the treatment analysis of splicing abnormality in DM1 myoblasts in this study, hypericin treatment showed potential to reverse DM molecular signatures in cells across the four abnormal splicing situations used in this work, namely: exon 11 of *IR*, exon 22 of *SERCA*1, and exons 7 of both *MBNL1* and *MBNL2*. Based on the effect of hypericin treatments on the splicing assays for DM employed in this study, it is possible to suggest that it could cause redistribution of *MBNL1*, as it rescued the splicing misregulation in *IR* exon 11, *SERCA1* exon 22, and exons 7 of both *MBNL1* and *MBNL2*. Hypericin is a protein kinase C inhibitor, which could be responsible for such a possible effect. Moreover, it is hypothesised that hypericin could block phosphorylation of exon 7-containing isoforms of both MBNL1 and MBNL2, thus hindering its translocation to the nucleus, since exon 7 is important for its nuclear localisation and possesses affinity for entrapping MBNL1 to expanded CUG repeats (Cardani et al., 2009; Francois et al., 2011). The use of hypericin in this study to assess its effect on correcting misregulated splicing in DM1 is based on a previous study (Udosen et al., 2023), although it was not effective in myotonic dystrophy type 2 (DM2) fibroblast cell lines (Udosen, 2020). However, the study has limitation due to the fact that the toxicity levels of hypericin was not undertaken. Further study is required to establish therapeutic potential of hypericin in mouse models of the disease condition.

Conclusion

Taken together, this study suggests that hypericin is a potential therapeutic molecule based on the myotonic dystrophy type 1 cell line used.

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Authors' contributions

I. U. Udosen:
Conceptualization; Investigation; Methodology; Formal analysis; Data curation; Visualization; Writing – original draft.

M. I. Akpanabiatu:
Validation; Writing – review & editing; Supervision.

U. F. Ufot:
Software; Resources.

Conflict of interest

Authors declare no conflict of interest.

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