Changes in Alternative Splicing as Pharmacodynamic Markers for Sudemycin D6

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ABSTRACT:

OBJECTIVE: The aim of the study was to define pharmacodynamic markers for sudemycin D6, an experimental cancer drug that changes alternative splicing in human blood.

METHODS: Blood samples from 12 donors were incubated with sudemycin D6 for up to 24 hours, and at several time points total RNA from lymphocytes was prepared and the pre-messenger RNA (mRNA) splicing patterns were analyzed with reverse transcription-polymerase chain reaction.

RESULTS: Similar to immortalized cells, blood lymphocytes change alternative splicing due to sudemycin D6 treatment. However, lymphocytes in blood respond slower than immortalized cultured cells.

CONCLUSIONS: Exon skipping in the *DUSP11* and *SRRM1* pre-mRNAs are pharmacodynamic markers for sudemycin D6 treatment and show effects beginning at 9 hours after treatment.

KEYWORDS: Alternative splicing, sudemycin, splicing inhibition, lymphocytes RNA

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Introduction

All human cells generate messenger RNA (mRNA) through pre-mRNA splicing, a process that removes intervening sequences (introns) and splices exonic sequences together prior to their export into the cytosol.[1] This process is conserved in all eukaryotic cells and performed by a multienzyme complex, the spliceosome.[2,3] Changes in alternative splicing are a hallmark of cancer and targeting the spliceosome has been proposed as a possible treatment for cancer.[4,5]

Sudemycins

Bacteria generate natural products that bind to components of the spliceosome; 2 of the best-known examples are FR901464 and pladienolide, which causes selective regression of tumors in in vivo cancer models,[6,7] making them promising anticancer agents. FR901464 is chemically unstable, and thus, more stable compounds were designed and optimized through focused medicinal chemistry; these compounds are collectively called sudemycins.[7,8] Sudemycins selectively stop the growth of tumors in mice and preferably target cancer cells, sparing nonneoplastic cells through an unknown mechanism.[7] Similar to FR901464, sudemycins bind to the U2 component SF3B1, which is part of the spliceosome.[9] In cell culture, sudemycin D6 does not inhibit splicing but change certain alternative splicing patterns within 3 to 6 hours in immortalized cells, possibly by causing a dissociation of the U2 complex.[9]

The aim of the study was to characterize RNA splicing biomarkers in primary human cells using an ex vivo assay we previously developed.[10] We identified human RNA targets of sudemycin D6 in human ex vivo blood samples that can be used in future human clinical trials.

Methods

An overview of the assay is shown in Figure 1.

Human subjects

Healthy volunteer blood donors were recruited in accordance with the institutional review board protocol #15-0077, approved by the University of Kentucky. To be included, subjects must be more than 18 years old, not on current chronic medication, and free from hepatitis. Blood was taken from healthy subjects in the morning after an overnight fast to limit lipids that possibly interfered with the RNA isolation. The intake of liquids was permitted.

Blood collection

About 30 mL of venous blood was collected in BD Vacutainer vials (Becton, Dickinson, Franklin Lakes, NJ, USA) containing

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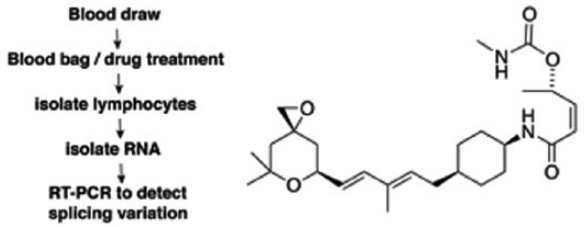

Figure 1. Overview of the assay. (A) Overall experimental design: patient blood was drawn and citric acid and glucose were added. The treatment with sudemycins is performed in blood storage bags for up to 24 hours, followed by isolation of lymphocytes in Ficoll gradients, isolation of RNA, and RT-PCR detection. (B) Chemical structure of sudemycin D6. RT-PCR indicates reverse transcription-polymerase chain reaction.

acid citrate dextrose ("yellow cap") solution A (trisodium citrate [22.0 g/L], citric acid [8.0 g/L], and dextrose [24.5 g/L]). The blood samples were transferred from the Vacutainer vials into 2 separate platelet storage bags (Blood Cell Storage, Inc., Seattle, WA, USA): 1 containing 18 mL of blood and the other containing 12 mL of blood.

Ex vivo sudemycin treatment

The blood was treated with 1 μM sudemycin D6, dissolved in dimethyl sulfoxide (DMSO) or an equal volume of DMSO in the control. During the incubation time, 3-mL samples were taken at 0, 3, 6, 9, and 24 hours.

Lymphocyte isolation

Lymphocytes from the samples were isolated using Sigma-Aldrich Accuspin System-Histopaque 1077 gradient tubes (Sigma, St Louis, MO, USA), according to the manufacturer's protocol. After the isolation, lymphocytes were pelleted at 5000×*g* for 3 minutes.

RNA extraction

Total RNA was isolated from the pelleted lymphocytes using TRIzol (Invitrogen, Waltham, MA, USA). About 1 mL of TRIzol was added to the lymphocyte pellet and homogenized using a 1-mL pipette and incubated for at least 5 minutes. The aqueous solution was isolated by adding 200 μL of chloroform/1 mL TRIzol, separated by centrifugation at 12000×*g* for 5 minutes. RNA was precipitated using 500 μL isopropanol/1 mL original TRIzol, washed with 75% ethanol, and resuspended in 25 μL water.

Reverse transcription-polymerase chain reaction was performed using 1 μg RNA and 1 pM reverse primer as previously described.[9] The primers (Table 1) are located in constitutive exons flanking the alternative exons (Figure 1B).

Statistical Analysis

Gene expression signals were quantified using ImageJ[11] and relative quantities were determined by band intensities within a lane. Exon inclusion was calculated by dividing the intensity of the regulated gel band by the sum of both band intensities. A Shapiro-Wilk test was used to determine the normality for validity of the analysis of variance (ANOVA), where $P < .05$ demonstrates a non normal distribution.[12] The exon inclusion proportion was analyzed with an ANOVA test and Tukey HSD (honest significant difference) post hoc[13] using IBM SPSS Statistics treatments.[14] Changes were considered significantly different with $P < .05$. Subjects were separated by age, sex, and ethnicity and analyzed with paired *t* test and considered significantly different with $P < .05$.

Results

Selection of subjects

The blood donors were 21 to 51 years of age, 4 men, 8 women, from different ethnicities (mean age = 31.75, median age = 28) years (Table 2).

Assay

To test the effect of sudemycin D6 under in vivo conditions, we treated whole blood samples ex vivo with sudemycin D6. We used treatment conditions similar to blood banking, which keeps cells intact for several days. Citric acid and glucose were added as an anticoagulant and nutrient, respectively, and the blood was stored in blood bags that allowed gas exchange during the experiments. We did not observe any hemolysis.

Changes in alternative splicing caused by sudemycin D6 treatment

Sudemycin D6 is a compound that binds to the splicing component SF3B1, which is part of the U2 small nuclear ribonucleoprotein complex. We previously performed array analyses and found that sudemycin D6 changes the usage of numerous alternative splice sites at low μM concentrations within hours in HeLa, RH19, and HEK293 cells.[9] In most cases, sudemycin causes exon skipping within 3 to 6 hours of treatment. Importantly, these changes were reversible, ie, the exon skipping was not detectable after 9 hours, which likely reflects the inactivation of sudemycin D6 in aqueous solution.[9] To identify biomarkers for possible sudemycin D6 clinical trials, we treated human blood samples ex vivo, using the sudemycin D6 concentration of 1 μM that showed an effect in cell culture. Because splice site selection can be individual specific,[15] we tested subjects from different ethnicities, both sexes and ages.

We tested 6 splicing events in the *DUSP11, SRRM1, RPp30, AURKB, MLH3*, and *PAPOLG* genes (Figure 2A to F) that showed high expression and reproducible changes in RH19 and HEK293 cells.[9] These findings were quantified by

Table 1. Primers used.

PRIMER	SEQUENCE	AMPLICON SIZE
DUSP 11 forward	5′-GAC ATC AAG TGC CTG ATG ATG A-3′	212, 151
DUSP11 reverse	5′-ATG TCC CCG GCA CCT ATT-3′	
RPp30 forward	5′-TAT ATC TAG TGC TGC AGA AAG G-3′	193 (retained intron)
RPp30 reverse	5′-GCC TAA AGA AAG TGG GGA TAA-3′	
SRRM1 forward	5′-GAC TCT GGC TCC TCC TCC TC-3′	209, 167
SRRM1 reverse	5′-GGA CTT CTC CTC CGT CTA CCA-3′	
MLH3 forward	5′-TTA TTG CCT GTT TGA TGA GCA C-3′	220, 150
MLH3 reverse	5′-TCC TTT GTT CCT CTG TCA CTG TT-3′	
PAPOLG forward	5′-AAG AGA TCC CAT TCC CCA TC-3′	178, 112
PAPOLG reverse	5′-TGC GTG ATG TAT CAA TAG TTG GA-3′	
AURKB forward	5′-ATG ACC GGA GGA GGA TCT AC-3′	182 (retained intron)
AURKB reverse	5′-GAT GGA CCT CCA GCT ACA AG-3′	

Table 2. Age and ethnicity of subjects.

SAMPLE NO.	SEX, SELF-IDENTIFIED ETHNICITY	AGE
380	Female, white	21
657	Female, white	21
346	Female, Multi: African American and white	21
M.T.	Female, white	23
559	Female, white	24
278	Male, Hispanic/Latino	27
296	Female, white	29
786	Male, Asian	31
767	Male, white	44
944	Female, white	44
902	Female, African American	45
S.S.	Male, white	51

calculating the percent exon inclusion as the intensity of the band containing the alternative exon divided by the intensity of all bands (Figure 3A to F).

Our assay amplifies mRNA isoforms containing or skipping an alternative exon using the same set of polymerase chain reaction primers and is thus internally controlled.

DUSP11 and *SRRM1* splicing patterns were changed in all subjects beginning at 9 hours of treatment and did not revert to the original splicing patterns after 24 hours, which is in contrast to the splicing patterns in transformed cells that revert to the pretreatment ratio at this time point. Despite the small sample number, these changes were highly significant (P values in a 1-way ANOVA: *DUSP11*: 9 hours: $P = 1.11 \times 10^{-8}$; 24 hours: $P = 6.27 \times 10^{-13}$; *SRRM1*: 9 hours: $P = .00031$; 24 hours: $P = 1.47 \times 10^{-9}$).

Although the splicing patterns for *MLH3* and *PAPOLG* showed a similar trend, the patterns varied between the various individuals. *AURKB* (12/12 cases) and *RPp30* (11/12 cases) showed no changes in overall expression or alternative splicing, respectively, in human blood samples, which is in contrast to previous results in HEK293 and RH19 cells that exhibit changes.

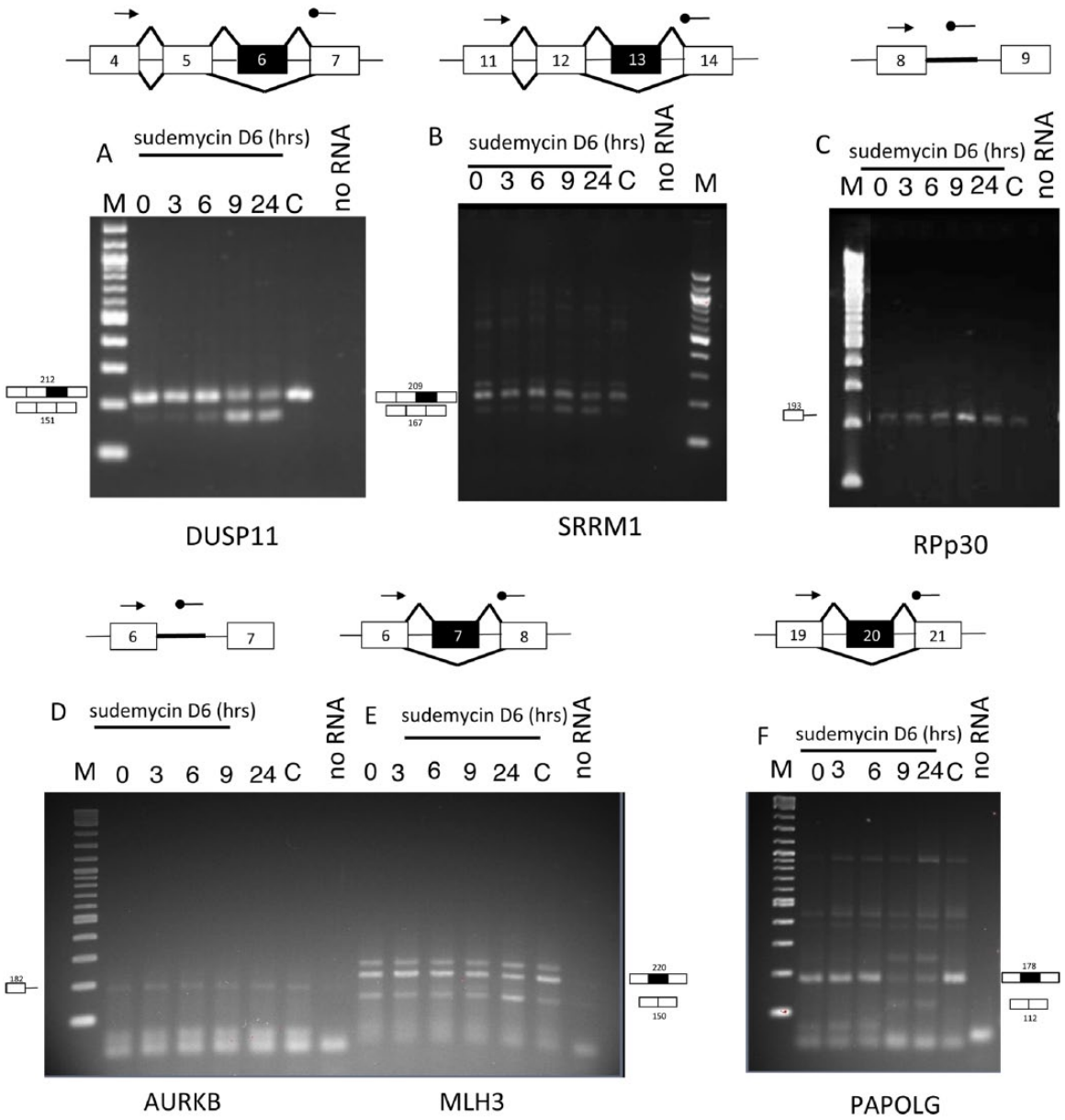

Figure 2. Representative change of splicing patterns in tested genes. Shown are ethidium bromide–stained agarose gels after reverse transcription-polymerase chain reaction analysis. Numbers indicate the time of treatment with 1 µM sudemycin D6 in hours. M: 100-base pair marker, C: blood without sudemycin but dimethyl sulfoxide for 24 hours in the blood bag. The amplicon sizes are given in Table 1, and the structure of the RNA products is schematically indicated. (A) *DUSP11*, (B) *SRRM1*, (C) *RPp30*, (D) *AURKB*, (E) *MLH3*, and (F) *PAPOLG*.

Differences between sex, age, and ethnicity

There was no difference between the sexes and ethnicities. However, unexpectedly, after 24 hours of sudemycin D6 treatment, samples from subjects older than 30 years showed a lower percentage of exon inclusion than samples from subjects younger than 30 years in the *PAPOLG* gene, suggesting that age modulates the response to sudemycin D6 (Figure 4).

Discussion

We were looking for a simple and robust assay to monitor the effect of sudemycin D6 and possible future improved sudemycins in primary human cells. Sudemycins have previously been shown to change splicing patterns in numerous cell lines, including immortalized leukemia cell lines.[16] As a model for primary cells, we choose blood because it contains a variety of cell types in a physiological environment. By adding sudemycin to blood ex vivo, we could circumvent clearance of the liver. Using the blood from 12 healthy donors, we found that sudemycin D6 changes splice site usage of the *DUSP11* and *SRRM1* pre-mRNA after 9 hours of treatment. Our tested subjects showed statistically significant changes in the splicing patterns of *DUSP11*, *SRRM1*, and *PAPOLG*. However, the degree of response was variable for *PAPOLG* where older subjects showed a stronger response to sudemycin. Alternative splicing patterns are frequently developmentally regulated[1] and it has been reported that some alternative splicing patterns are age dependent in

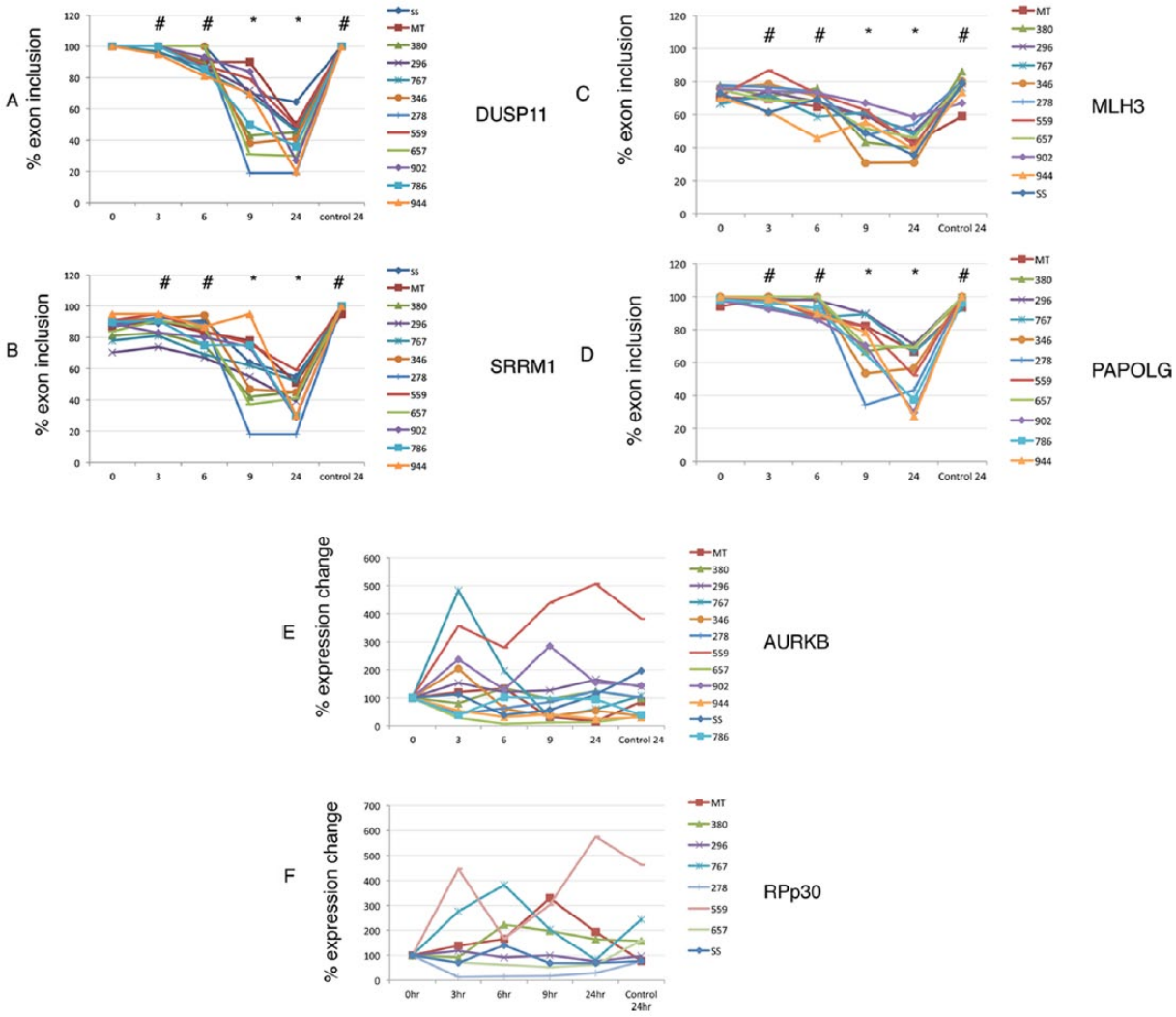

Figure 3. Quantification of the changes in splicing. The band intensities of bands after reverse transcription-polymerase chain reaction and agarose gel electrophoresis were determined by ImageJ and the percent exon inclusion was calculated as [intensity of alternative exon]/[sum of all exon intensities]. # represents nonsignificant changes compared with 0-hour control ($P > .05$) and * represents significantly different group to 0-hour control ($P \leq .05$). The individual subjects are shown by different colors and referred to in Table 2. (A) *DUSP11*, (B) *SRRM1*, (C) *RPp30*, (D) *AURKB*, (E) *MLH3*, and (F) *PAPOLG*. The changes in splicing were significant for the 9- and 24-hour time points when compared with dimethyl sulfoxide–treated controls:
DUSP11: 9 hours: $P = 1.11 \times 10^{-8}$; 24 hours: $P = 6.27 \times 10^{-13}$.
SRRM1: 9 hours: $P = .00031$; 24 hours: $P = 1.47 \times 10^{-9}$.
MLH3: 9 hours: $P = 4.39 \times 10^{-6}$; 24 hours: $P = 9.00 \times 10^{-11}$.
PAPOLG: 9 hours: $P = .000017$; 24 hours: $P = 2.15 \times 10^{-11}$.
AURKB: $P = .78$ (ns), *RPp30:* $P = .43$ (ns). ns indicates nonsignificant.

mature organisms,[17] but this is the first report that shows that age influences a splicing response to a drug.

The genes responding to sudemycin are likely merely indicators for a sudemycin D6 effect on splicing and are not causative for the death of cancer cells, as in most cases, they have no known connection with disease. For example, *DUSP11* (dual specific protein phosphatase) is a dual specificity protein phosphatase, removing phosphates from phosphoserine/threonine and phosphotyrosine residues. *DUSP11* binds directly to RNA[18] and changes in expression of the DUSP11 protein have been observed in inflammatory bowel disease.[19] *MLH3* is the MutL-Homolog 3 involved in DNA mismatch repair, and rare polymorphisms of this gene are associated with colorectal cancer.[20,21]

SRRM1 (serine and arginine repetitive matrix 1) promotes exon enhancer formation by interacting with serine-arginine–rich proteins and has no known connection to a disease,[22] similar to *RPp30*[23] that works in transfer RNA maturation and *PAPOLG* (poly(A) polymerase gamma), which is a poly(A) polymerase.[24]

There are differences between the response of cultured cells and blood lymphocytes and cultured cells, as HeLa cells change their splicing patterns after 2 to 4 hours in response to sudemycin. Furthermore, in lymphocytes, there was no reversal of splicing up to 48 of treatment, whereas we saw the pretreatment splicing patterns in cultured cells after 24 hours. It is likely that the transformation of the cells or the artificial culture conditions cause this difference.

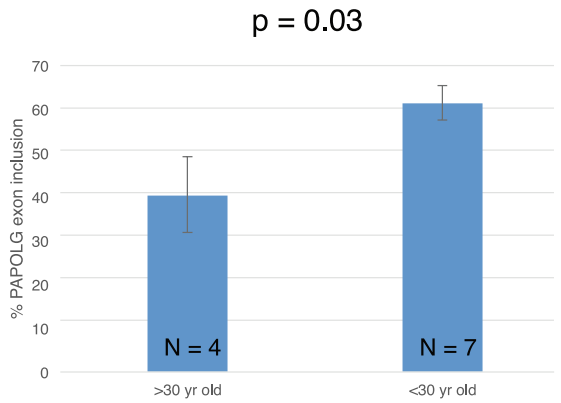

Figure 4. Comparison of sudemycin D6 response for PAPOLG in different age groups. The relative splicing changes for PAPOLG determined in Figure 3 were separated into subjects younger or older than 30 years. Both groups show a statistically significant ($P = .03$) difference in their response to sudemycin D6 treatment.

Limitations

Our study used ex vivo analysis in blood samples. Any treatment in humans will have to account for liver clearance of sudemycins. Despite highly significant changes ($P = 6.27 \times 10^{-13}$ and $P = 1.47 \times 10^{-9}$ for DUSP11 and SRRM1, respectively), the number of subjects was only n = 12.

Conclusions

Changes in alternative splicing of *DUSP11* and *SRRM1* can be used as biomarkers for sudemycin D6 treatment in human blood.

Author Contributions

MT and BD performed the experiments; JD performed statistical analysis TRB and SS devised the experiments and wrote the manuscript.

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