FD-895 and Pladienolide B Modulate RNA Splicing Associated With Wnt Signaling Pathway

Deepak Kumar
University of California San Diego

Manoj K. Kashyap (manojkkashyap@gmail.com)
Stem Cell Institute https://orcid.org/0000-0002-3064-8452

Zhe Yu
UC San Diego Health System: University of California Los Angeles Health System

Ide Spaanderman
University of California San Diego Health System

Reymundo Villa
UCSD: University of California San Diego

Thomas J. Kipps
UCSD: University of California San Diego

James J. La Clair
UCSD: University of California San Diego

Michael D. Burkart
UCSD: University of California San Diego

Januario E. Castro
UCSD: University of California San Diego

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Abstract

Background
Alterations in RNA splicing are associated with different malignancies including leukemia, lymphoma, and solid tumors. The RNA splicing modulators such as FD-895 and pladienolide B have been investigated in different malignancies to target/modulate spliceosome for therapeutic purpose.

Methods
The different cell lines were screened using RNA splicing modulator to test in vitro cytotoxicity as well as ability to modulate RNA splicing capability via induction of intron retention (using RT-PCR and qPCR) was assessed. The Cignal Finder Reporter Array was used to screen the HeLa cell line to know the pathway affected the most by the splice modulators. Further the candidates associated with the pathways were validated at protein levels using western blot assay and gene-gene interaction study were carried out using GeneMANIA.

Results
We show that FD-895 and pladienolide B have more apoptotic activities than conventional chemotherapy in different solid tumors. We found FD-895 or pladienolide B modulate Wnt signaling pathways and mRNA splicing. We showed that FD-895 or pladienolide B significantly down regulates not only Wnt signaling pathway-associated transcripts (GSK3β and LRP5), but also both transcript and proteins including LEF1, CCND1, LRP6, and pLRP6.

Conclusion
These results indicate FD-895 and pladienolide B inhibit Wnt signaling by impeding with phosphorylation of LRP6 and modulates mRNA splicing through induction of intron retention in cervical cancer cells.

Introduction
Clinical and patient data indicate that splicing and the machinery that guides it offers tremendous potential as a chemotherapeutic target[1]. Here, small molecule leads have been discovered that modulate the complex macromolecular machine that guides this process, the spliceosome[1; 2]. It is comprised of two types: wherein the major spliceosome is comprised of U1, U2, U4, U5, U6 snRNAs, and the minor spliceosome comprised of U11, U12 together with U5 [3; 4]. Within the major component, the SF3B core unit is comprised of spliceosome associated proteins including SF3B1 (Splicing Factor 3b Subunit 1), U2AF1 (U2 Small Nuclear RNA Auxiliary Factor 1), and SRSF2 (Serine And Arginine Rich Splicing Factor 2) that have been implicated in large a number of malignancies[5] including chronic lymphocytic leukemia (CLL)[6], Uveal melanoma[7], and myelodysplastic syndrome[8]. Recent structural studies have shown that pladienolide B and related FD-895 polyketides and related analogs bind to a
specific pocket within this SF3B core comprised of SF3B1, SF3B3 (Splicing factor 3B subunit 3) and PHF5A (PHD Finger Protein 5A)[9; 10].

To date, a panel of splicing modulators (SPLMs) have been screened using in vitro and in vivo models for their anti-cancer properties including FR901464[11][12], spliceostatin A (a derivative of FR901464)[13], thailanstatin A[14], meayamycin[15], isoginkgetin[16], sudemycin (analogos of FR901464) [17; 18], and herboxidiene[19] to target the spliceosome[5]. SPLMs including spliceostatin A[20], and sudemycin analogs (sudemycin C1, sudemycin D6) shown to prominently induce intron retention and exon skipping (types of alternative splicing events) in different cell lines[21; 22; 23]. While many of these agents showed splice modulatory activity, but their primary mechanisms of inducing tumor cell death remains unknown.

E7107 (a synthetic analog of pladienolide B) entered Phase 1 clinical trials by groups at MD Anderson in Houston[24], and the Erasmus University Medical Center in Rotterdam [25]. A total of 26 patients with solid tumors were enrolled in the US-based Phase I and treated at escalating doses beginning at 0.6 mg/m². E7107 was received as a 30-minute intravenous infusion on days 1 and 8 and repeat this cycle every 21 days. The selection of subsequent dose levels was performed according to accelerated design. The MTD for E7107 observed was E7107 was, therefore, 4.3-mg/m². Among 31% of patients, the disease observed to be stable. The adverse events associated with E7107 were diarrhea, vomiting, and nausea. Blindness was observed in two patients at the 2nd and 7th cycle after receiving 3.2 mg/m² and 4.3 mg/m², respectively. The loss of vision event in patients led to the discontinuation of this study[24].

In the Dutch Phase I trial, 40 patients with solid tumors were enrolled and doses from 0.6 to 4.5 mg/m² were explored. The maximum tolerated dose (MTD) was 4.0 mg/m². At 4.5 mg/m², two patients experienced diarrhea of grade 4. At 4.0-4.5 mg/m², dose limiting toxicity (DLT) grade 3 diarrhea, nausea, vomiting, and abdominal cramps were observed. After drug discontinuation at 4.0 mg/m², one patient experienced reversible grade 4-blurred vision. The pharmacokinetic analysis revealed a plasma half-life between 5.3 to 15.1 h. There was no complete or partial response was observed in this trial. Serious issues with vision were observed in both trials. An increase in the pre-mRNA (intron retained) was observed in this study at MTD in the peripheral blood mononuclear cells[25]. Both trials were ultimately suspended.

In our previous studies, we explored the activity of SPLMs on normal PBMCs (including T and B lymphocytes) and found that SPLMs spare these cells as compared with leukemic B cells. We also reported that FD-895 and pladienolide B exhibited modulation of mRNA spicing and induced apoptosis in patient-derived CLL-B cells [26]. Here we found that FD-895 and related synthetic analogs block the G2/M phase of the cell cycle, down regulating the cyclin b1, phospho-CDC2, CDC2, and modulate PLK-1 splicing[27].

In this study, we evaluated FD-895 and pladienolide B for their efficacy against solid tumors by conducting activity analyses and pathway analyses. Our data indicate that both FD-895 and Pladienolide B possess highly selective potent anticancer activity in HCT116, MCF-7, MDA-MB-468, HS578T, SKOV3,
and HeLa cell lines. Some of the cell lines such as OV-2008, it is resistant variant C13, A2780, and 786-O were partially attributable to mRNA splicing and Wnt signaling pathway inhibition. Here, we report on a further investigation on the role of SPLM activity in inducing tumor cell death by splice modulation of Wnt signaling activity.

Here, we report the effect of splice modulators on different pathways and selected Wnt signaling pathway, and evaluated its participation in the cellular response to splice modulators.

**Materials And Methods**

**Ethical statement**

Informed consent was taken of the healthy donor prior to collection of blood samples from San Diego Blood Bank according to the regulation of the Institutional Review Board and Ethics committee at UC San Diego and maintained strict compliance with the Helsinki Declaration.

**Compounds**

FD-895 was prepared through total synthesis\[28\]. Pladienolide B (sc-391691, Santa Cruz Biotechnology, Santa Cruz, CA), etoposide (E1383, Sigma-Aldrich, St Louis, MO) and cisplatin (479306, Sigma-Aldrich, St Louis, MO) were obtained commercially (Fig. 1). Oligonucleotides were purchased via custom synthesis (Integrated DNA Technologies).

**Cell culture methods**

The MCF-7 (RRID:CVCL_0031), MDA-MB-468 (RRID:CVCL_0419), HS578T (breast cancer, RRID:CVCL_0332), A2780 (RRID:CVCL_0134), SKOV3 (ovarian cancer, RRID:CVCL_0532), 786-O (renal adenocarcinoma, RRID:CVCL_1051), HeLa (Cervical cancer, RRID:CVCL_0030), and HEK-293 cell lines were obtained from ATCC. MCF7 was cultured in DMEM + 10% FBS + 2mM L-glutamine and Pen/Strep supplemented with 0.01 mg/mL human recombinant insulin. Other cell lines were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 100 U/mL of penicillin and 100 µg/mL of streptomycin. Additionally, two ovarian cancer cell lines with differential cisplatin sensitivity, OV-2008 (sensitive, RRID:CVCL_0473) and it’s resistant variant C13 were obtained from Prof. Stephen Howell (UC San Diego). To complete the set, a final colon cancer cell line HCT116 (RRID:CVCL_0291) was obtained from the Johns Hopkins School of Medicine, Baltimore, MD. The suspension cell lines including Jeko-1, JVM2, and Mino cell lines were cultured in RPMI-1640 supplemented with 10% FBS along with 1% Pen-Strep. All cell lines were incubated 37°C in an atmosphere of 5% CO₂ and routinely monitored for Mycoplasma infections by PCR analyses.

**Flow cytometry analyses**

Normal primary peripheral blood mononuclear cells (PBMCs) were treated with FD-895 (100 nM to 2.0 µM), and pladienolide B (100 nM to 2.0 µM), for 48 h. Cell viability was determined by flow cytometry
after staining with conventional live staining with 40 µM 3,3’dihexyloxacarbocyanine iodide (DiOC<sub>6</sub>; Life Technologies, Carlsbad, CA) and 15 µM (10 µg/mL) of propidium iodide (PI; Sigma-Aldrich, St Louis, MO). Data were analyzed by using FlowJo software (version 6.4.7; Tree Star). Using this assay, viable cells excluded PI and stained brightly positive for DiOC<sub>6</sub> as it targets metabolically active mitochondria of alive cells [26][29].

**Cell proliferation assays**

The cell proliferation assays were conducted in adherent cell lines (HCT116, MCF-7, MDA-MB-468, HS578T, OV-2008, A2780, SKOV3, 786-O, HEK-293, and HeLa) by using CellTiter 96 AQueous non-radioactive colorimetric method (G5421, Promega, Madison, WI). Briefly, a total of 3000 cells/well were seeded in a 96-well flat-well plate followed by treatment with FD-895 (100 nM to 2.0 µM), pladienolide B (100 nM to 2.0 µM), cisplatin (1 µM to 30 µM) or etoposide (1 µM to 30 µM) in triplicate for 48 h at 37°C. Following the incubation, 20 µL of CellTiter 96 AQueous solution (Promega, Madison, WI) was added directly to each well. Non-treated cells were considered as the control. After staining, the plates were incubated for an additional 2 h and then read on a 96-well plate reader (Molecular Devices, Sunnyvale, CA). Absorbance readings were record absorbance at 490 nm using empty wells (air) for background collection.

**Reverse transcriptase PCR (RT-PCR) analyses**

HCT116, MCF-7, MDA-MB-468, HeLa, Jeko-1, JVM-2 and Mino cells (10<sup>6</sup> cells/well) were treated with 100 nM FD-895, 100 nM pladienolide B, 30 µM cisplatin or 30 µM etoposide for 4 h. RNA isolation was done using mirVana miRNA Isolation Kit (Ambion, Austin, Texas). The 200 ng of RNA was subjected to DNase I (Life Technologies, Carlsbad, CA). The cDNA was prepared by using SuperScript III Reverse Transcriptase Kit (Life Technologies, Carlsbad, CA), and PCR reactions were performed in 20 µL of reaction volume. PCR conditions were 95°C for 3 min; 35 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 45 s; followed by 72°C for 5 min. PCR products were separated on a 2% agarose gel and stained with ethidium bromide. Details of the primers used for RT-PCR are described in Table-1.

**Quantitative reverse transcriptase-PCR (qRT-PCR) analyses**

The HeLa cells treated with 100 nM FD-895 or 100 nM pladienolide B for 6 h, 12 h, or 24 h and the RNA isolation and cDNA preparation were done as described above. The amounts of mRNA of LEF1 (Lymphoid enhancer-binding factor-1), FN1 (fibronectin 1), and CCND1 (cyclin D1) genes were determined using Power SYBR Green PCR master mix (Applied Biosystems, Foster City, CA) real-time qRT-PCR using specific primers[30]. PCR was conducted using 5 picomole of each primer and 20 ng of the obtained cDNA. PCR conditions were 50°C for 2 min; 95°C for 10 min; 40 cycles of 94°C for 15 s, and 60°C for 1 min. The mRNA levels were calculated using the 2<sup>-ΔΔCT</sup> method[31]. GAPDH was used as a control for normalization.

**Pathway reporter arrays**
Cignal Finder Reporter Array (336821, Qiagen/SABiosciences, Frederick, MD) was used to assess 45 different signaling pathways. HeLa cells were seeded into wells (50,000 cells/well) of the Cignal Finder 96-well plates (CCA-901L, Qiagen, SABiosciences, Frederick, MD) for introducing pathway reporters into cells by reverse transfection according to the manufacturer's protocol. Briefly, reporter DNA constructs in each plate well were re-suspended with 50 µL Opti-MEM and then mixed with 50 µL diluted Lipofectamine 2000 transfection (Life Technologies, Carlsbad, CA) reagent. Cells were suspended in Opti-MEM (Life Technologies, Carlsbad, CA) supplemented with 10% of FBS and 0.1 mM non-essential amino acids at a density of $6 \times 10^5$ cells/ml, and then 50 µL of the cell suspension was added into each plate well and mixed with DNA resident in the plate and added transfection reagent. The cells were incubated for 3 h. Following transfection; the cells were treated with vehicle (Opti-MEM) or 100 nM FD-895 for 3 h in Opti-MEM media. Luciferase and renilla expression were determined (Qiagen/SABiosciences corp., Frederick, MD).

**Western blot analyses**

HeLa cells were treated with 100 nM FD-895 or 100 nM pladienolide B for 12 h, 24 h, and 48 h for β-catenin, LEF1, LRP6 (LDL Receptor Related Protein 6), and phospho-LRP6. The cells were then washed with PBS (2 × 5 mL) and lysed with modified RIPA buffer at 4°C. Untreated cells were used as a control. The whole-cell protein was quantified according to the Bradford method[32]. Lysates in sample buffer (2% Sodium dodecyl sulfate (SDS), 10% glycerol, 80 mM Tris•HCl (pH 6.8), 720 mM β-mercaptoethanol and 0.001% bromophenol blue) were denatured at 95°C for 5 min. Total cellular proteins (30 µg via Bradford analyses) were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) using a 4–20% Criterion Precast Gel (Bio-Rad, Hercules, CA), and the proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). After blocking with 5% bovine serum albumin (BSA) for 1 h in Tris-buffered saline, 0.1% Tween 20 (TBST, 20 mM Tris•HCl, 137 mM NaCl, 0.1% Tween-20 pH 7.6), the membrane was incubated with the following primary antibody overnight at 4°C. The primary antibodies including rabbit mAb anti-LEF1, rabbit anti-Phospho-LRP6 (Ser1490), rabbit anti-LRP6, rabbit mAb anti-β-catenin, and mouse Ab anti-β-actin were obtained from Cell Signaling Technology (Beverly, MA) and used at a dilution of 1:1000. After primary mAb staining and washing thrice with TBST, the membranes were incubated with HRP-labeled anti-rabbit (sc-2030, Santa Cruz Biotechnology) or HRP-labeled anti-mouse (sc-2031, Santa Cruz Biotechnology) secondary antibodies with a dilution of 1:5000 dilutions for 40 min at rt. After incubation, the membrane was washed thrice times with TBST and developed using an enhanced chemiluminescence (ECL) kit (Pierce Thermo Scientific Inc., Rockford, IL).

**Bioinformatics analysis**

Gene-gene interaction networks were predicted and generated with GeneMANIA (Gene Multiple Association Network Integration Algorithm) available at http://genemania.org[33].

**Statistical analysis**
The data presented as mean ± standard deviation (SD). The data was analyzed using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA). Multiple groups were compared using Bonferroni correction and \( p < 0.05 \) was considered statistically significant.

**Results**

**In vitro cytotoxicity evaluation of FD-895 and Pladienolide B in colon, breast, cervical cancer cell lines**

In 2016, we reported the apoptotic activity of FD-895 and pladienolide B in CLL-B, mantle cell lymphoma (MCL) and other B and T lymphoma cell lines[27] Further, we were interested to explore the apoptotic activity of FD-895 and pladienolide B in different solid tumor cell lines. Using an expanded panel of cell lines, we found that FD-895 and pladienolide B demonstrated a range from 30.7 ± 2.2 to 415.0 ± 5.3 nM (Table-2) across breast, colon, and cervix tumor cell lines (Fig. 1A-D). Upon treatment of normal PBMCs with FD-895 and pladienolide B, the lack of activity (IC\(_{50}\) values ≤ 450 nM) was not achieved in normal PBMCs, an observation that suggests that both the FD-895 and pladienolide B spare the normal PBMCs, but not the leukemic B cells (Fig. 1E) [26]. We also tested the normal cell line HEK-293 in response to FD-895 (100 nM to 2 µM) and found that there was non-significant cell death induced with varying concentrations of FD-895 when compared with control (\( p > 0.05 \)), but cisplatin (30 µM) induced significant cell death in HEK-293 cells (\( p < 0.05 \), Supplementary Figure-1).

**Cytotoxic evaluation of FD-895 and Pladienolide B in ovarian cancer cells regardless differential cisplatin sensitivity**

Next, we turned our attention to explore the activity of splice modulation on cell lines displaying sensitivity or resistance to cisplatin, as the latter is an issue in the treatment of solid tumors including ovarian[34], cervical cancer[35], gastric adenocarcinoma[36], prostate cancer[37], colorectal[38], and head and neck squamous cell carcinoma[39]. Here, we used two human ovarian cancer cell lines, one consisting of a cisplatin-sensitive parental line, OV2008 and the other stably cisplatin-resistant subline, OV2008/C13 derived by *in vitro* selection with cisplatin. We began by screening these cell lines for their induction of apoptosis when treated with FD-895, pladienolide B, cisplatin, or etoposide. FD-895 and pladienolide B induced significant apoptosis in both parental and cisplatin-resistant OV2008 cells (Fig. 2A-B). We also observed that FD-895 and pladienolide B demonstrated significant apoptosis in A2780 and SKOV3 ovarian cancer cell lines as compared to cisplatin and etoposide (Fig. 2C-D). These findings suggest that nanomolar concentrations of SPLMs have the potential to overcome cisplatin resistance. We also evaluated the apoptotic activity of FD-895 and pladienolide B in 786-O (renal) cancer cells. We found that both splice modulators were ten-fold more efficient at induced apoptosis in than cisplatin and etoposide (Fig. 2E).

**FD-895 and Pladienolide B induced spliceosome modulation marked by intron retention in cancer cells**

In previous studies, we found that FD-895 and pladienolide B induced intron retention (IR) in CLL and MCL cells[26; 27; 28]. Here, we expand our understanding of their ability to induce IR across an expanded
A panel of cancer cell lines, including Jeko-1, Mino, JVM2, HeLa, HCT116, MCF-7, and MDA-MB-468 cell lines. In brief, we incubated $10^6$ cells/well from each cell line with a 100 nM FD-895, 100 nM pladienolide B, 30 µM cisplatin or 30 µM etoposide for 4 h. After treatment, the levels of spliced and unspliced gene expression were evaluated by RT-PCR. We observed that cells treated with FD-895 or pladienolide B demonstrated IR, which was not observed with non-splice modulatory controls, cisplatin, or etoposide (Fig. 3) using DNAJB1 (DnaJ Heat Shock Protein Family (Hsp40) Member B1) as a surrogate marker for spliceosome modulation[26]. IR was observed in cells treated with FD-895 or pladienolide B for DNAJB1 when compared to the intronless gene RNU6A used as a loading control RNA (Fig. 3A-G).

**FD-895 down regulates and modulates splicing of proteins involved in Wnt signaling**

To investigate the intracellular signaling pathways affected by FD-895, we applied the Cignal 45-Pathway Reporter Array to simultaneously analyze FD-895 effect on 45 different signaling pathways[40]. HeLa cells, selected due to their SPLM sensitivity, were treated with FD-895 over 12 h and baseline-signaling profile was compared to vehicle control (Fig. 4A). Interestingly, treatment with 100 nM FD-895 modulated a number of pathways including notch signaling, octamer-binding transcription factor 4 (OCT4), activating transcription factor 6 (ATF6), NANOG, and Wnt signaling pathway as early as within 1 h but decreased as the time progressed (Fig. 4A).

Among these pathways, we found that the Wnt signaling pathway was activated at an early time point but down regulated as the duration of FD-895 prolonged, as monitored by the TCF/LEF reporter in cervical cancer cell line HeLa (Fig. 4A). From this data, we selected Wnt signaling pathway to study further because as Wnt5A overexpression has been correlated with cervical carcinoma[41]. We began by exploring its effect on the TCF/LEF reporter system. As shown in Fig. 4A, we observed that TCF/LEF showed downregulation in 100 nM FD-895 treated HeLa cells after 12 h (Fig. 4A).

From this data, we selected Wnt signaling pathway to study further because as Wnt5A overexpressed and has been correlated with cervical carcinoma [41]. We began by exploring its effect on the TCF/LEF reporter system. As shown in Fig. 4A, we observed that TCF/LEF showed down regulation in 100 nM FD-895 treated HeLa cells after 12 h. We also investigated the effect of FD-895 and pladienolide B on mRNA expression of selected genes LEF1, FN1, and CCND1[42][43][44] which involved in the Wnt/β-catenin signaling pathway. The HeLa cells were exposed to 100 nM of FD-895 or pladienolide B for different times and LEF1, CCND1, and FN1 mRNA levels measured by qRT-PCR. The expression of LEF1, CCND1, and FN1 was significantly decreased by 8.33, 6.25, and 5.26 fold respectively (Fig. 4B-D) as the incubation period increased with the maximum level appearing at 20% at 24 h. Further, we performed the RT-PCR to detect splicing in genes involved in Wnt signaling pathway like GSK3β and LRP5. HeLa cells treated with 100 nM FD-895 showed intron retention in GSK3β and LRP5, an observation that was not observed in non-splicing controls (30 µM cisplatin) (Fig. 4E). We then conducted Western blot analyses to study the correlation between mRNA and protein. Treatment with 100 nM FD-895 or 100 nM pladienolide B treatment resulted in the down regulation of β-catenin, LEF1, total LRP6, and phospho-LRP6 protein.
levels (Fig. 4F). Altogether, the results from qRT-PCR and Western blot data demonstrate down regulation of key Wnt signaling pathway molecules in the HeLa cell line.

**Bioinformatics based Gene-Gene Interaction**

Our next studies explored the effects of FD-895 and pladienolide B on select gene-gene interaction networks using GeneMANIA. We used *DNAJB1*, *LEF1*, *CTNNB1* (Catenin, beta-1), *LRP6*, and *SF3B1* as “Input” genes for gene-gene interaction analysis and included *SF3B1* in this analysis because *SF3B1* is a component of the spliceosome-binding pocket of pladienolide B [45; 46]. As shown in Fig. 5, we found the selected genes were part of the network directly or indirectly associated with *SF3B1*.

We obtained total twenty genes from GeneMANIA analysis and among those top ten interactor molecule emerge in the network were based on the size of the circle including *NELFE* (negative elongation factor complex member E, 1), *SF3B6* (splicing factor 3b subunit 6, rank-2), *Wnt3A* (Wnt family member 3A, 3), *CTNNBIP1* (catenin beta interacting protein 1, rank-4), *CDX1* (caudal type homeobox 1, rank-5), *DKK1* (dickkopf Wnt signaling pathway inhibitor 1, rank-6), *CTNNA1* (catenin alpha 1, rank-7), *PIAS4* (protein inhibitor of activated STAT 4, rank-8), *DKK4* (dickkopf Wnt signaling pathway inhibitor 4, rank-9), and *TCF7L2* (transcription factor 7 like 2, rank-10). Among these genes few genes are involved in Wnt signaling such as *Wnt3A*, *CTNNA1*, *DKK4*, and *TCF7L2* suggest that there is an important role of RNA splicing machinery in regulation of Wnt signaling pathway.

**Discussion**

Splice modulation offers a unique opportunity to selectively modulate ongoing rapid cellular growth, and has shown early promise as a therapeutic target [47][48]. Leveraging methods developed in prior studies in CLL, we tested *in vitro* activities of FD-895 and pladienolide B in solid tumor and mantle cell lymphoma cells. Here, our studies show that FD-895 and pladienolide B show potent apoptotic activity at nanomolar concentrations across the majority of cell lines screened. Both splice modulators demonstrated significant induction of apoptosis in human ovarian cancer cells, OV2008 and C13 (cisplatin resistance), and renal carcinoma. Interestingly, FD-895 and pladienolide B spares and normal PBMCs (include T cells, B cells, and NK cells, monocytes, and dendritic cells), suggesting their ability to induce *in vitro* cytotoxicity preferentially in cancer cells in a significant manner suggesting a therapeutic window for treatment options. These findings were in concordance with our previous findings in normal B cells[26]. We also tested the normal cell line HEK-293 in response to FD-895 and found that non-significant cell death in contrasts with cisplatin. This data further supported the observation that SPLMs demonstrate profound enhancement of activity in tumor cell lines, but not in normal cells[49; 50].

In other studies as well, HEK-293 cell line was also tested *in vitro* in response to isoginkgetin (splicing modulator control), a bioflavonoid or other SPLMs and found that the effects on cell viability induced by SPLMs at nanomolar concentrations were significantly lower than that obtained by treatment with SPLM control like isoginkgetin [18; 51; 52].
Following *in vitro* cytotoxicity assays, we assessed if RNA splicing modulators induce IR in the different solid tumor as well as in mantle cell lymphoma cells. Using *DNAJB1* has been used as a surrogate marker of splicing modulation; we found that FD-895 as well as pladienolide B induced IR in *DNAJB1* in all the cell lines at 100 nM[26; 28].

Next, we turned our attention to identify the specific pathways regulated by these splice modulatory events. In HeLa cells, we observed that treatment with 100 nM of FD-895 resulted in modulation of ATF6-C/EBP-β-signaling (ATF6 and C/EBP response elements), Wnt signaling (TCF/LEF reporter), type 1 interferon-induced signal transduction (ISRE reporter), CREB signaling (cAMP response element, CRE is the response element), NANOG, and OCT4 pathways. The following discussion provides a brief overview of the significance of these pathways in their response to splice modulation by FD-895.

OCT4 is a homeodomain transcription factor of the POU family also known as POU5F1 (POU domain, class 5, transcription factor 1). It is involved in self-renewal of undifferentiated embryonic stem cells and therefore used as a marker for dedifferentiation [53]. OCT4 is crucial for determination of fates of the inner mass and embryonic stem cells. OCT4 is capable of maintaining pluripotency throughout the embryonic development. It is also involved in proliferation of cancer cells including pancreatic, liver, testicular and lung cancer of adult germ cells[54].

In ATF6 pathway, the central player is ATF6 that works in a concentration dependent manner. At low levels, ATF6 activates the unfolded protein response (UPR) for self-defense. At high levels, it mediates apoptosis. ATF6 is crucial for transition from self-defense to self-destruction of cells in endoplasmic reticulum (ER) stress[55].

In NANOG pathway, the NANOG transcription factor is important as it is involved in self-renewal regulation as well as maintenance of the embryonic stem cell pluripotency[56]. NANOG has been reported in number of malignancies including leukemia[57]. Both NANOG and OCT4 pathways’ are involved in regulation of pluripotency of stem cells[58].

We observed modulation of T-cell factor/lymphoid enhancer factor (TCF/LEF) in response to treatment with FD-895 in HeLa cell. Wnt alone led to an accumulation of β-catenin in the cytoplasm, but its nuclear activity is largely mediated by TCF/LEF only. TCF/LEF is an important component of Wnt or Wnt/β-catenin signaling pathway. The Wnt/β-catenin signaling has been reported in a number of malignancies including cervical cancer[59; 60]. Interestingly, we found that Wnt signaling pathway was down regulated after 12 h of FD-895 treatment. In human cells, Wnt is a secreted protein that acts as a ligand for ROR1 a receptor tyrosine kinase[61; 62; 63]. Wnt/β-catenin activation occurs upon binding of Wnt5A with membranous proteins Frizzled (FZ) receptor and lysophosphatidic acid receptors (LRP5/6) protein. This binding event leads to recruitment of the scaffolding protein Disheveled (DVL), which results in phosphorylation of LRP5/6 receptors. During the course of these studies, we found that FD-895 reduced the expression of Wnt signaling pathway-associated transcripts including LEF1, FN1, CCND1, GSK3β, and LRP5. LEF1 is a transcription factor that belong to the T cell Factor (TCF)/LEF family. LEF1 acts as
nuclear effector in the Wnt/\(\beta\)-catenin signaling pathway\[64\]. LEF1 mediates Wnt signaling pathway by through association with \(\beta\)-catenin\[41\].

Wnt/\(\beta\)-catenin pathway requires the co-receptors LRP5 and LRP6 for activity. Post-translational modifications (PTMS) such as phosphorylation, methylation, acetylation, and sumoylation play a very important role in the pathophysiology of different malignancies. \(LRP5\) is part of the signalosome complex is deregulated by cisplatin\[65\]. We also observed the same effect as we saw the non-intronic form of the \(LRP5\) vanished upon treatment with cisplatin.

Phosphorylation of LRP6 is crucial for activation of Wnt/\(\beta\)-catenin signaling as it can promote activation of Wnt signaling activation by recruiting casein kinase family proteins\[66\]. Targeting of LRP6 phosphorylation can inhibit Wnt/\(\beta\)-catenin signaling\[41\].

The co-receptors for Wnt signaling, LRP5, and LRP6, have been revealed as potential oncogenic proteins. In human breast carcinoma, the expression of LRP6 is high\[67\]. Down regulation of LRP6 inhibits breast cancer tumorigenesis, whereas overexpression of LRP6 in the mouse mammary gland induces mammary hyperplasia\[67; 68\]. We observed that FD-895 down regulated LRP6 phosphorylation and causes the degradation of the LRP6 protein, an essential component of the Wnt receptor complex, promotes \(\beta\)-catenin degradation, and down regulation of LEF1 at the protein level. Therefore, we reasoned that FD-895 might block the phosphorylation of LRP6 that is required for initial Wnt signaling.

Glycogen synthase kinase-3 (GSK3) is an intracellular component of the Wnt pathway that can directly interact and phosphorylate LRP6. Glycogen synthase kinase 3\(\beta\) (GSK3\(\beta\)) is a crucial component of insulin and Wnt signaling pathways. PTMs particularly phosphorylation of EGFR in lung adenocarcinoma, and SF3B1 in CLL has been reported/targeted\[27; 69\]. We found that treatment with FD-895, but not by cisplatin lead to induction of IR in GSK3\(\beta\). An inactivated GSK3\(\beta\) could lead to increased SNAIL activity and poor prognosis in cervical cancer\[70\].

Our results suggest that FD-895 not only modulate RNA splicing via induction of IR in genes such as DNAJB1, but also inhibit Wnt/\(\beta\)-catenin pathway via down regulation of \(\beta\)-catenin, LRP6, pLRP6, and LEF1. FD-895 can block LRP6 phosphorylation and cause degradation of LRP6 protein. Overall this suggests that LRP6 may also serve as a viable anticancer agent in these cell lines, particularly molecules that are capable of selectivity altering the phosphorylation of LRP6.

Gene-gene interaction is very crucial for any pathway including Wnt signaling to study the interaction between them. We used GeneMANIA analysis to study the gene-gene interaction prediction with high accuracy\[71\], identified peripheral nodes corresponding to two genes. The output of analysis led to the identification of gene interactions involved in Wnt signaling (\(Wnt3A\), \(CTNNBIP1\), \(DKK1\), and \(DKK4\)), as well as in RNA splicing (\(SF3B6\), and \(NELFE\)). Among those, there were genes such as \(SF3B6\), a gene indirectly interact with \(SF3B1\) is an integral part of the spliceosome complex. \(SF3B6\) has been reported to be associated with p53 activity in human non-small cell lung carcinoma\[72\].
Conclusion

In summary, our results suggest that both FD-895 and pladienolide B demonstrated *in vitro* toxicity in different malignancies, spared normal PBMCs, and modulates mRNA splicing. It also exerts selective toxicity to malignant cells compared with normal cells. Furthermore, we showed that FD-895 (pladienolide B was not explored) able to modulate the post-translational events as suggested by down regulation of LRP6 phosphorylation and expression of associated Wnt target genes. This data suggests that these splice modulators could be useful in targeting malignancies where Wnt/β-catenin play an important role by inhibiting mRNA splicing and LRP6 phosphorylation. These results showed that these compounds not only modulate mRNA splicing in CLL, but also in mantle cell lymphoma, and solid tumors of colon, breast, ovarian, and renal origin.

Overall this study demonstrates that FD-895 and pladienolide B modulate splicing machinery and result in downstream regulation of signaling pathways, including the Wnt/β-catenin pathway. Furthermore, the *in vitro* efficacy of FD-895 and pladienolide B was found to be superior to conventional chemotherapy as indicated in a wide range of malignant cell lines of colon, breast, ovarian, renal, and cervical origin. Further, there is a need for extensive research not only at *in vitro* but *in vivo* levels to assess synergistically the ability of splice modulators with conventional chemotherapy agents like cisplatin and etoposide to assess their potential as a combination or synergistic treatments.

Abbreviations

CCND1 Cyclin D1

CLL Chronic lymphocytic leukemia

DLT Dose limiting toxicity

FN1 Fibronectin 1

GeneMANIA Gene Multiple Association Network Integration Algorithm

GSK3 Glycogen synthase kinase-3

IR Intron retention

LEF1 Lymphoid enhancer-binding factor-1

LRP6 LDL Receptor Related Protein 6

MCL Mantle cell lymphoma

MTD Maximum tolerated dose
PBMCs Peripheral blood mononuclear cells

RT-PCR Reverse transcriptase PCR

SF3B1 Splicing Factor 3b Subunit 1

SPLMs Splicing modulators

SRSF2 Serine And Arginine Rich Splicing Factor 2

U2AF1 U2 Small Nuclear RNA Auxiliary Factor 1

Declarations

Ethics approval and consent to participate

The study involved human, animal or cell lines as a material for experimental purpose and the ethical clearance was conducted before starting the study.

Consent for publication

All authors consent to the publication of the manuscript in Journal of Experimental & Clinical Cancer Research. Further, figures or tables are original, so there was no requirement of taking permission or consent from anyone.

Availability of data and material

All data generated and analyzed during our study are included in the published article.

Competing Financial Interests statement

There is no competing financial interest associated with this manuscript.

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Authorship contributions

DK performed cell culture, cell proliferation, RT-PCR, qRT-PCR with assistance of ZH, and IS. DK performed Western blot and Flow cytometer with assistance of MKK. MKK carried out the bioinformatics analysis. DK, and MKK analyzed and interpreted the data, and wrote the manuscript. DK wrote the first draft of the
manuscript and prepared draft of the figures. DK, MKK, J JL, MDB, and JEC edited the manuscript. J JL, and RV, and MDB prepared the samples of FD-895 used within this program. TJK and JEC provided regents, chemotherapeutic agents and cell lines data. JEC, and DK conceived and guided the research. All authors read and approved the final manuscript.

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The other authors declared no potential conflicts of interests.

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Tables
Table 1
Sequences of primers used in the RT-PCR

| Primer    | Sequence                                      |
|-----------|-----------------------------------------------|
| DNAJB1- FW | 5' GAACCAAAATCAGTTCCCAAGGAAGG 3'               |
| DNAJB1 - RV | 5' AATGAGGTCCCGTTCTCGGTTG 3'               |
| RNU6A- FW  | 5' CGCTTCGGGACAGACATAC 3'                   |
| RNU6A- RV  | 5' GAATTTGCAGTGTCATCCTT 3'                  |
| LEF1-FP    | 5'-AGGAACATCCCACACTGAC-3'                   |
| LEF1-RP    | 5'-AGGTCTTTTTGGCTCCTGCT-3'                  |
| CCND1-FP   | 5'-AATGACCCCGACAGATTTC-3'                   |
| CCND1-RP   | 5'-TCAGGTCAGGCCTGCAC-3'                     |
| FN1-FP     | 5'-ACCTACGGATGACTCGTCTTT-3'                 |
| FN1-RP     | 5'-TTCAGACATTCGTTCCACTCA-3'                 |
| GSK3β-FP   | 5' ATCAAGGCACATCTGCTGGAC 3'                  |
| GSK3β-RP   | 5' CAATTGCTCTTGGGAAGTT 3'                   |
| LRP5FP      | 5' GCCTGCAACAGTGACA 3'                      |
| LRP5-RP    | 5' CCTGCAGACTATGTCTGTGA 3'                  |

FP and RP denote forward and reverse primers respectively.
Table 2
IC\textsubscript{50} values for FD-895 and pladienolide B in selected tumor cell lines

| Cell lines       | Type of Cancer | FD-895 (nM) | Pladienolide B (nM) |
|------------------|----------------|-------------|---------------------|
| HCT116 (TP53, +/+)| Colon          | 34.1 ± 2.4  | 42.3 ± 3.1          |
| HCT116 (TP53, +/-)| Colon          | 75.2 ± 2.5  | 65.6 ± 2.9          |
| HCT116 (TP53, -/-)| Colon          | 100.7 ± 1.2 | 88.6 ± 2.0          |
| MCF-7 (TP53, +/+)| Breast         | 51.7 ± 1.9  | 38.7 ± 3.8          |
| MDA-MB-468 (TP53, +/-)| Breast     | 30.7 ± 2.1  | 38.5 ± 3.8          |
| HS578T (TP53, -) | Breast         | 139.7 ± 1.1 | 112.0 ± 3.1         |
| OV-2008          | Ovarian        | 311.6 ± 2.2 | 344.5 ± 1.3         |
| A2780            | Ovarian        | 415.0 ± 5.2 | 337.0 ± 7.0         |
| SKOV3            | Ovarian        | 143.4 ± 2.1 | 128.7 ± 3.2         |
| 786-O            | Renal          | 412.5 ± 2.4 | 293.6 ± 3.1         |
| HeLa             | Cervical       | 131.0 ± 3.3 | 118.4 ± 4.4         |

Figures
In vitro cytotoxicity induced by FD-895 and pladienolide B in different cancer cell lines, and normal human primary, Cancer Cells were exposed to FD-895 (100 nM to 2 uM), pladienolide B (100 nM to 2 uM), etoposide (1 µM to 30 µM), and cisplatin (1 µM to 30 µM) for 48 h. Apoptosis were measured in MCF-7 (A), MDA-MB-468 (B), HCT-116 (C) and HeLa (D) cells using MTS assay. The absorbance of the control (cells without treatment) was subtracted from the treated cells of each cell line. (E) Normal PBMC cells were exposed to FD-895, and pladienolide B. Cells were stained with propidium iodide and DiOC6 to differentiate dead and viable cells by using flow cytometer. Data presented in form of % specific induced apoptosis (% SIA). To assess the compound specific induced apoptosis vs. background spontaneous cell death from in vitro culture conditions, we calculated the percentage of % SIA using the following formula: % SIA was calculated using the following formula: % SIA = [(compound induced apoptosis – media only spontaneous apoptosis) / (100- media only spontaneous apoptosis)] × 100. The data shows the results of samples analyzed in duplicate with the mean and its respective SD. (F) Structures of pladienolide-B and FD-895.
FD-895 or by pladienolide B induced apoptosis in ovarian and renal cancer cells. Ovarian cancer cells (A) OV-2008, cisplatin sensitive (B) OV-2008 C13 mut, cisplatin resistant, (C) A2780, (D) SKOV3 or (E) renal cancer cells (786-O) were incubated with FD-895 (100 nM to 2 µM), pladienolide B (100 nM to 2 µM), etoposide (1 µM to 30 µM), or of cisplatin (1 µM to 30 µM) for 48 h. Cells viability were measured as using MTS assay. This experiment was repeated in triplicate independently. Data presented in form of % SIA. The data shows the results of samples analyzed in duplicate with the mean and its respective SD.
Figure 3

Intron retention of DNAJB1 gene in different cancer cell lines. Tumor cell lines were treated with 100 nM of FD-895, 100 nM pladienolide B, 30 µM cisplatin or 30 µM etoposide for 4 h. Analysis of IR of DNAJB1 mRNAs was evaluated by RT-PCR in (A-C) Mantle cell lymphoma cells, (Jeko-1, Mino and JVM-2), (D) HeLa, (E) MDA-MB-468 (F) HCT116 and (G) MCF-7 cells. RNU6A, an intronless gene was used as RNA quality and loading control.
Figure 4

Effect of FD-895 on different pathways and effect on Wnt signaling in HeLa cells. (A) HeLa cells were used for introducing pathway reporters into cells via reverse transfection. Post-transfection, the cells were treated with vehicle or 100 nM FD-895 for 3 h. Luciferase and renilla expression was evaluated. HeLa cells were exposed to 100 nM of FD-895 or 100 nM pladienolide B for 6 h, 12 h or 24 h and expression of (B) LEF1, (C) FN1, and (D) CCND1 were determined by qRT-PCR. (E) HeLa cells were treated with 100 nM
of FD-895 or 30 µM cisplatin for 4 h. Analysis of IR for GSK3β and LRP5 mRNAs was evaluated by using RT-PCR. (F) HeLa cells were treated with 100 nM of FD-895 or 100 nM pladienolide B for 6 h, 12 h or 24 h. Protein extracts were immunoblotted for β-catenin, phohspho-LRP6, LRP6, and β-actin.

**Figure-5.**

![Gene-gene interaction network](image)

**Figure 5**

Gene-gene interaction networks among selected genes constructed by GeneMANIA. A gene-gene network was constructed with the search tool for the retrieval of interacting genes available in GeneMANIA annotation information for selected 5 genes, including physical interaction, genetic interaction, co-expression and shared pathways and protein structure domain. The central black nodes denote 5 selected genes used as an “INPUT”, and peripheral nodes denote gene interactions with the black nodes. The network also contains 20 normal human genes. The size of the circles indicates the degree of interaction.

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.
• SupplementaryFigure1.tif