#### Biochimie 208 (2023) 19-30

Contents lists available at ScienceDirect

### Biochimie

journal homepage: www.elsevier.com/locate/biochi

# The role of Cannabidiol and tetrahydrocannabivarin to overcome doxorubicin resistance in MDA-MB-231 xenografts in athymic nude mice

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#### ARTICLE INFO

Article history: Received 16 August 2022 Received in revised form 9 December 2022 Accepted 14 December 2022 Available online 17 December 2022

Keywords: Cancer Cannabidiol and tetrahydrocannabivarin MDA-MB-231 resistant cancers Doxorubicin

#### ABSTRACT

The significant resistance to currently available chemotherapeutics makes treatment for TNBC a key clinical concern. Herein, we studied the anti-cancer potentials of synthetic cannabidiol (CBD) and Tet-rahydrocannabivarin (THCV) when used alone or in combination with doxorubicin (DOX) against MDA-MB-231 resistant cells. Pre-treatment with CBD and THCV significantly increased the cytotoxicity of DOX in MDA-MB-231 2D and 3D cultures that were DOX-resistant. Transcriptomics and Proteomics studies revealed that CBD and THCV, by downregulating PD-L1, TGF- $\beta$ , sp1, NLRP3, P38-MAPK, and upregulating AMPK induced apoptosis leading to improved DOX's chemosensitivity against DOX resistant MDA-MB-231 tumors in BALB/c nude mice. CBD/THCV in combination with DOX significantly inhibited H3k4 methylation and H2K5 acetylation as demonstrated by western blotting and RT-PCR. Based on these findings, CBD and THCV appear to counteract histone modifications and their subsequent effects on DOX, resulting in chemo-sensitization against MDA-MB-231 resistant cancers.

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#### 1. Introduction

Triple Negative Breast cancer is one of the deadliest cancers for caucasian and african american women in North America [1,2]. Among breast cancer patients, about 15–20% are diagnosed with TNBC, which is indicated by no expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). Trop-2 protein, a cell membrane glycoprotein is overexpressed in TNBC and its downregulation has shown to delay the growth of TNBC. TRODELVY® (sacituzumab govitecan-hziy) is a Trop-2-directed antibody and topoisomerase inhibitor conjugate and has been indicated for use against metastatic TNBC. Despite these advances, chemotherapy remains the

standard of care for TNBC. Compared with other breast cancer subtypes, TNBC is highly invasive and metastatic and characterized by aggressive clinical course, high relapse rate, and poor overall survival [3]. Given little efficacy for existing therapeutic regimens against a significant number of TNBC patients and continuously developed resistance of TNBC cells to chemotherapy, novel treatment strategies with significantly enhanced potency and safety are still urgently required to address unmet medical needs.

Several systemic chemotherapeutic agents have been used for TNBC and among those, doxorubicin (DOX) is commonly used and works through multiple mechanisms: DNA intercalation, topoisomerase II inhibition and free radical formation [4]. Further, longer exposure to chemotherapeutics generates an adaptive cellular response resulting in acquired drug resistance [5–10]. Resistance to DOX in TNBC is frequently seen and subsequently it leads to development of multi-drug resistant phenotypes in tumor cells [11–13]. Several different hypotheses have been proposed for DOX resistance mostly suggesting the altered expression of specific protein markers; NF- $\kappa$ B and small modifier proteins like ubiquitin







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(SUMO) [14]. Further, resistance which is related to epigenetic modification has also been reported. Increased levels of IL-6, IL-8, IL-1 $\beta$ , transforming growth factor beta (TGF- $\beta$ ), and the prion protein (PrPc) have been connected with DOX resistance in various tumors [15]. Further, several investigators have explored other treatment regimen for TNBC, including immunotherapy, cyclindependent kinase 4/6 (CDK4/6) inhibitors, tyrosine kinase inhibitors (TKIs), clustered regularly interspaced short palindromic repeats (CRISPR), nanoparticles based delivery systems, repurposing of existing drugs, and electrochemotherapy (ECT) [15,16]. However, chemotherapy is still being commonly used either alone or in combination with other newer therapies.

Cannabis anecdotally has been a folklore medicine for a longtime to treat a variety of disease states. In recent years, the therapeutic use of cannabis and cannabinoids has garnered more acceptance in the public domain. Several Phyto-cannabinoids are available from the plant Cannabis sativa along with terpenes and they target the endocannabinoid system and several other biological pathways [17,18]. Hence, these agents can possibly have a array of therapeutic effects on the central and peripheral nervous system, immune, cardiovascular, reproductive, and ocular systems [19,20]. Presently, cannabinoids, such as dronabinol, nabilone, epidiolex and others, are approved for the treatment of cancer-related adverse effects such as nausea and vomiting and also for epileptic seizures [21]. Cannabinoids have also been shown in our and other laboratories for their role in cancer in various preclinical cancer models [22–25]. Further, studies conducted in our laboratory with Cannabidiol (CBD) in combination with DOX demonstrated that CBD at low doses acted as a chemosensitizer against wild type MDA-MB-231 cells [25]. Further tumor xenograft studies with MDA-MB-231 cells showed that the combination (CBD+DOX) was able to reduce the tumor burden through decreased expression of proteins which were involved in inflammation, metastasis and increased expression of proteins involved in apoptosis [26]. Another study conducted by Elbaz et al., showed that CBD significantly inhibited epidermal growth factor (EGF)-induced proliferation and chemotaxis of breast cancer cells. Further, it was demonstrated that CBD inhibited EGF-induced activation of EGFR, ERK, AKT and NF-KB signaling pathways as well as MMP2 and MMP9 secretion [27]. A study conducted by Almeida et al., to evaluate the role of cannabinoids in exemestane resistant breast cancer cells showed that CBD could overcome resistance in these cells by inducing apoptosis, as observed in Giemsa/Hoechst stain suggested by the presence of morphological features of apoptosis [28]. CBD has also been demonstrated to overcome cisplatin resistance in lung tumors which was mediated partly via an ion channel receptor, TRPV2, present on lung adenocarcinoma and by modulating oxidative stress pathways [29].

Another minor cannabinoid  $\Delta$ 9-tetrahydrocannabivarin (THCV) has recently shown interest from various investigators for its role in diabetes, pain, weight loss and as an anticancer agent [30,31]. THCV is a homologue of THC, but it contributes to a variety of pharmacological effects which are different from THC. One of the biggest advantages of THCV over THC is the lack of psychoactive effects. In rodent studies, it was observed that THCV decreased appetite, increased satiety, and up-regulated energy metabolism, making it a clinically useful remedy for weight loss and management of obesity in type 2 diabetic patients. Compared to other minor cannabinoids, not many publications are available demonstrating the effects of THCV in cancer [30]. However, there are some reports where cytotoxicity studies were conducted for THCV in prostate cancer cell lines DU-145 and LNCaP, and the IC50 values were observed to be 17.5  $\mu$ M [32]. However, no reports are available for the role of THCV in overcoming resistance in TNBC.

231 tumor xenografts, the role and mechanism of action of CBD and THCV in overcoming DOX resistance in TNBC at the molecular level, using RNA seq analysis and proteomics. Further, the role of various epigenetic markers with cannabinoids in TNBC was also investigated in this study.

#### 2. Materials and methods

#### 2.1. Materials

CBD (GLP and GMP grade) was purchased from Purisys TM (Athens, GA). THCV was purchased from Open book extracts (Roxboro, NC). DOX was purchased from AK Scientific, Inc (Union City, CA). Dulbecco's Modified Eagle Medium (DMEM) and DMEM/ Ham's F12 (1:1 Mixture) media were acquired from Millipore Sigma (St. Louis, MO). Triple Negative Breast Cancer (TNBC) cells (MDA-MB-231) and immortalized human non-tumorigenic breast epithelial (MCF-10A) cells were purchased from ATCC (Rockville, MD, USA). MDA-MB-231 cells were made resistant to doxorubicin by using serial amounts of doxorubicin starting from 0.1  $\mu$ M to 25 µM over a period of 6–8 months. Fetal bovine serum (FBS) was purchased from Biotechne (Minneapolis, MN). MDA-MB-231 cells were cultured in DMEM medium supplemented with 10% FBS and antibiotics (5000 units penicillin, 5 mg streptomycin and 10 mg neomycin/mL; GIBCO). Immortalized human non-tumorigenic breast epithelial (MCF-10A) cells were grown in DMEM medium supplemented with 10% horse serum, EGF (10 ng/mL), Hydrocortisone (500 ng/mL), Insulin (10  $\mu$ g/mL), Cholera toxin (100 ng/mL) and antibiotics (5000 units penicillin, 5 mg streptomycin and 10 mg neomycin/mL; GIBCO). All these cells were maintained under standard conditions at 37 °C with 5% CO2 in a control humidified incubator and were used with passage number below 15.

#### 2.2. Methods

#### 2.2.1. Cell viability assay

MDA-MB-231 (DOX resistant) and MDA-MB-231 (wild typecontrol) cell line was seeded in 96-well plates at a density of 5000 cells/well and MTT assay was performed according to standardized protocols [16,33]. Before initiating treatment, cells were incubated overnight at 37 °C and 5% CO<sub>2</sub>. Cells were then treated with CBD, THCV and DOX (2.5-30 µM) range for 48 h. Treatment was terminated by removing the media containing CBD, THCV and DOX. Further, 0.5 mg/mL of MTT labeling reagent (100 µL/well) was added in each well. Cells were then incubated for 4 h in normal cell culture conditions. At the end of 4 h, excessive MTT solvent was removed and 100  $\mu$ l of DMSO (cell culture grade) was added in each well in the remaining reduced MTT crystals. Further, the 96 wellwell plate was kept on a shaker until crystals were visibly dissolved. Finally, absorbance was measured at 562 nm wavelength using a plate reader. Cell viability then was calculated as a ratio of the control untreated cells.

#### 2.2.2. Cell viability assay in magnetic 3D cell culturing

Unlike 2D assay, in magnetic 3D assay, cell suspension was first mixed with nanoshuttle solution. Briefly, the nanoshuttle solution (1  $\mu$ l for 10,000 cells) was added in cell suspension and volume was made up to 1 ml using DMEM F12 media. The cell suspension was then gently mixed and centrifuged thrice at 800 rpm for 5–7 min. After each centrifugation, the cell suspension containing nanoshuttle solution was gently mixed and centrifuged again. After centrifugation, required quantity of media (sufficient for 96 wells) was added in the cell suspension containing nanoshuttle solution. About 12,000–15,000 cells were then plated in 96 well microplate (cell-repellent surface). Plate was placed on top of Spheroid drive

while seeding the cells. The plate with Spheroid drive was then incubated at 37 °C and 5% CO<sub>2</sub> for 3 days in the incubator to form spheroids. Media was changed on 3rd day and fresh media was added. While changing the media, the plate was kept on top of the holding drive. The old media was then carefully discarded and fresh media was added without disturbing the spheroid. Finally, the cells were treated with DOX, CBD, THCV, DOX+CBD and DOX+THCV for 48 h on 5th day. Termination of treatment involved aspiration of media was followed by the addition of 0.5 mg/mL MTT labeling reagent (100 µL/well). Cells were incubated in normal cell culture conditions for 4 h to allow for the mitochondria of viable cells to drive the reduction of visible blue crystal-like structures. After 4 h, the excess MTT solvent was removed, and the remaining reduced MTT crystals were dissolved in cell culture grade MTT. This was done through the addition of 100 µL/well DMSO and slightly rocking the plate until crystals were visibly dissolved. Absorbance was then measured using a plate reader at 562 nm wavelength. Cell viability was calculated as a ratio of the control untreated cells [34].

#### 2.2.3. Anti-tumor study in athymic nude mice

BALB/c-nu/nu mice were procured from Envigo (Indianapolis, IN) and were acclimatized for 7–10 days. All the animal studies were reviewed and approved by the Institutional Animal Use and Care Committee of Florida Agricultural and Mechanical University (protocol number: 020-06) and complied with the NIH guidelines (Guide for the care and use of laboratory animals). All animal experiments were performed by following ARRIVE guidelines. MDA-MB-231 cells (DOX resistant) (4.0 million in 100 uL of Matrigel) were injected subcutaneously to BALB/c-nu/nu mice and allowed to reach the tumor volume of 300–600 mm<sup>3</sup>. Animals were then subjected to randomized grouping (4 animals per each group) before treatment. The various groups were: a) control, b) DOX, c) CBD, d) THCV, e) DOX+CBD, and f) DOX+THCV. Animals were administered with DOX (5 mg/kg) by i.v. route, and CBD (10 mg/kg) and THCV (15 mg/kg) by i.p route. In group e and f, DOX, CBD and THCV were administered separately to the animals. Animals were treated twice a week for 2 weeks. Further, tumor volume of each animal was measured periodically using vernier calipers and the tumor volumes were calculated by the formula: Tumor volume = 1/ $2 \text{ xy}^2$  (where 'x' and 'y' represent the length and width of the tumors) [16,35]. At the end of the study, the animals were euthanized, tumors were collected and processed for further analysis.

#### 2.2.4. RNA sequencing and data analysis

RNA Sequencing was performed according to the published reports with slight modifications [18]. TRIzol reagent (ThermoFisher; 15596018) was used to isolate RNA from MDA-MB-231 tissue to perform RNA sequencing. The NEBNext Ultra RNA Library Prep Kit and the NEBNext Poly (A) mRNA Magnetic Isolation Module were used to make the mRNA library. For quality control, the library was run on an Agilent Bioanalyzer with an HS DNA chip (5067-4626), and KAPA Library Quantification Kit (KR0405) was used for quantification. The library was then pooled and sent to the college of medicine translational laboratory at Florida State University at the appropriate equal molar concentration for Illumina NovaSeq 6000 sequencing. The RNA sequencing data was analyzed using iDEP software; all genes with a 10% count, 10% variance and unannotated were isolated and normalized using Log2 counts per million. The differentially expressed genes were discovered using DEseq2. The heatmap facilitates in the shows of differentially expressed genes and pathways done by using the iDEP software. A DESeq2 data set was used to graph the volcanoes, and log10 (False Discovery Rate corrected p-value) was applied to the log2 (fold change).

#### 2.2.5. Protein extraction

Whole cell lysates were isolated from MDA-MB-231 resistant tumors treated with CBD, THCV and their combinations with DOX at various concentrations according to the method as previously described [36–38]. Tissues were homogenized in RIPA buffer containing Protease and phosphatase inhibitor cocktail (Sigma-Aldrich). Tissues were disrupted using a homogenizer and centrifuged at 16,000 RCF at 4 °C for 30 min. Protein concentrations were determined using BCA protein estimation.

#### 2.2.6. Western blot analysis

Protein extracts (30  $\mu$ g) from each tissue were mixed with SDS sample loading buffer (Bio-Rad Laboratories, CA, USA), separated on SDS-PAGE after heating, and transferred onto PVDF membranes using the Trans-Blot turbo transfer system. Following that, the membrane was blocked for 1 h at room temperature with 3% BSA-PBST. The membrane was treated overnight at 4 °C with primary antibodies (Supplementary Table 2). The blots were washed three times with PBST for 5 min each, then incubated with suitable HRP-conjugated secondary antibodies for 1 h at room temperature before being rinsed three times with PBST for 5 min each. Immunoblots were incubated with Super Signal West Pico Chemiluminescent substrate and Western blot images were scanned by ChemiDoc Instrument (Bio-Rad). Densitometric sacnning were made with the NIH ImageJ software.

#### 2.2.7. Proteomics

Proteomics studies were performed as previously described [39]. Briefly, 100 µg of protein was diluted in 5% SDS, 50 mM TEAB (Triethylammonium bicarbonate), and 20 mM DTT and incubated for 10 min at 95 °C. The mixture was incubated with 40 mM Iodoacetamide at RT in the darkroom for 30 min. The mixture was acidified with 2.5% phosphoric acid. The material was then added to a 100 mM TEAB wash/binding buffer and thoroughly mixed. For flow-through, the mixture was added to the S-Trap, which was inserted in a tube (waste). Proteins were captured on column after 30 s of centrifugation at 4000 g and washed three times with 150  $\mu$ L of wash buffer. Before transferred to a new tube for digestion, S-Trap columns were spun at 4000 g for 1 min to thoroughly remove wash/binding buffer and incubated at 37 °C overnight with sequencing grade trypsin (trypsin: protein = 1 : 10, wt/wt) in 100 mM TEAB digestion buffer. Peptides were eluted by spinning the column at 4000 g for 1 min (Elution buffer-50 mM TEAB in H2O, formic acid (0.2%) in H2O, and 50% acetonitrile/formic acid (0.2%) in H2O. Elutions were dried in a speed vac and resuspended in formic acid (0.1%) at a of 1  $\mu$ g/ $\mu$ L conc before placed into autosampler glass vials.

Proteins (20  $\mu$ g) were isolated on S-trap micro column. Pierce high pH reverse phase peptide fractionation kit was used to separate all of the eluted peptides into three fractions for each sample. Then all the samples were vacuumed dried and submitted to FSU Translational Science Laboratory for mass spectrometry. Peptides were examined using an Exploris 480 Orbitrap mass spectrometer linked to an Easy-nLC-1200 nanoflow liquid chromatography system and load sample on a 2 cm trap column. Samples were then run on a 100C18 HPLC Analytical Column at a flow rate of 300 nL/min. Full MS scans were obtained.

Mass spectroscopic proteomic raw data was obtained using the data dependent acquisition technique and processed using Thermo Fisher Scientific Software Proteome Discoverer Version 2.5, and the Mascot tool to look against the uniport human database. To find differentially expressed proteins, the following criteria were used: a) a peptide score of  $\geq 10$ ; b) FDR confidence of < 0.01, c) unique peptides following digestion with value  $p \leq 0.05$ . Setting the threshold fold change value to  $\geq 1.5$  yielded significantly

differentially expressed proteins and further organized into different groups: biological process and molecular functions using Gene Ontology (GO) assignments and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways by DAVID software.

#### 2.2.8. Reverse transcription and RT-qPCR

To examine the expression levels of individual genes' mRNA. cDNA was synthesized from total RNA using the cDNA Synthesis Kit (Maxima H Minus First strand) as per the manufacturer's instructions (Thermo Fisher Scientific, Lithuania). Subsequently, quantitative RT-PCR was performed by using Sso Advanced Universal SYBR Green qPCR Master Mix (Bio-Rad Laboratories) on a CFX96 Touch<sup>TM</sup> Real-Time PCR Detection System (Bio-Rad Laboratories). The primers used for the reference gene were as follows. 18S rRNA, Forward 5'-GCAATTATTCCCCATGAACG-3' and Reverse 5'-AGGGCCTCACTAAACCATCC-3'; GAPDH, forward 5'-GCACCGTCA AGGCTGAGAAC-3' and reverse 5'-TGGTGAAGACG CCAGTGGA-3'. Relative mRNA expression was determined using the comparative Ct (Ct) technique, as previously reported [32], with the mean expression levels of the 18S rRNA and GAPDH genes serving as a reference for comparison. The list of forward and reverse primers is given in the Supplementary Table 1.

#### 2.2.9. Statistical analysis

For each study, three independent experiments were performed, and all measurements were performed in triplicate. GraphPad Prism 5.0 (GraphPad Software, USA) was used for performing statistical analysis. All the values were expressed as mean  $\pm$  S.E.M. Statistical comparison between two groups was carried out through *t*-test. Analysis of one-way variance (ANOVA) followed by Tukey's post hoc test was used for comparison between more than two different groups. P value less than 0.05 was considered to be statistically significant.

#### 3. Results

#### 3.1. Induction and cell viability of DOX resistant MDA-MB-231 cells

Over a period of 8-10 months, wild-type cells developed resistance to DOX at dosages ranging from 0.1 to 25 µM. In MDA-MB-231 resistant cells, the 2D cell viability assay with DOX, CBD, and THCV treatment revealed IC50 values of 31.00  $\pm$  1.63  $\mu$ M, 2.8  $\pm$  0.13  $\mu$ M and 5.66  $\pm$  0.29  $\mu$ M respectively (Table 1, Fig. 1a-c). Whereas 2D cell viability assay with DOX, CBD, and THCV treatment in wild type MDA-MB-231 cells showed IC50 values of  $2.26 \pm 0.21 \ \mu\text{M}, 3.22 \pm 0.27 \ \mu\text{M}, and 8.12 \pm 0.25 \ \mu\text{M}$  respectively. In cell viability experiments, resistant MDA-MB-231 cells exhibited an IC50 for DOX 12-14 folds times higher than those from the wild type. In addition, we investigated cell viability for combinations of CBD or THCV with DOX at different doses while holding any one drug concentration constant. DOX in combination with CBD and DOX in combination with THCV demonstrated IC50 values of 1.01  $\pm$  0.18  $\mu$ M and 1.79  $\pm$  0.12  $\mu$ M against MDA-MB-231 resistant cell line, respectively (Table 1, Fig. 1d). Using Compusyn software, we estimated the combination index, which revealed a synergistic impact with values of  $\leq$  0.93 and  $\leq$  0.79 for DOX with CBD and DOX with THCV, respectively (Table 1). Results from cell viability assay in magnetic 3D cell culture showed that IC50 for DOX alone was 194.61  $\pm$  20.64  $\mu$ M and when using a fixed concentration of DOX (97.5  $\mu$ M) and varying the concentrations of CBD and THCV, the IC50 of CBD and THCV were 12.66  $\pm$  0.45  $\mu M$ , and 20.87  $\pm$  1.73  $\mu M$ respectively. Further, the data was imported in Compusyn software, and the findings revealed synergistic effect of CBD and THCV when combined with DOX with the combination index (CI) of  $\leq$ 0.59 and  $\leq 0.91$  respectively (Table 1, Fig. 6 in supplementary file).

#### Table 1

Doxorubicin (DOX), Cannabidiol (CBD), Tetrahydrocannabivarin (THCV), and these cannabinoids combined with DOX in 2D and 3D DOX resistant MDA-MB-231 TNBC cultures are shown in Table 1 (IC50 values and combination index (CI)). The Mean  $\pm$  SEM (n = 3) was used to express the results.

Drug Solutions	IC50 (µM)		
	MDA-MB-231 wild type cells (2D assay)		
DOX alone	2.26 ± 0.21		
CBD alone	$3.22 \pm 0.27$		
THCV alone	8.12 ± 0.25		
	DOX resistant MDA-MB-231 cells (2D assay)		
DOX alone	$31.00 \pm 1.63$		
CBD alone	$2.8 \pm 0.13$		
THCV alone	$5.66 \pm 0.29$		
15μM DOX+CBD	$1.01 \pm 0.18$		
15μM DOX+THCV	$1.79 \pm 0.12$		
Combination index (CI) DOX resistant MDA-MB-231 cells (2D assay)			
DOX+CBD	≤0.93		
DOX+THCV	≤0.79		
3D DOX res MDA-MB-231 cells culture (3D assay)			
DOX alone	$194.61 \pm 20.64$		
CBD alone	$37.40 \pm 6.36$		
THCV alone	46.23 ± 7.92		
<b>97.5</b> μ <b>M DOX</b> + <b>CBD</b>	$12.66 \pm 0.45$		
97.5 $\mu$ M DOX + THCV	$20.87 \pm 1.73$		
Combination index (CI) 3D DOX res MDA-MB- 231 cells culture (3D assay)			
DOX+CBD	≤0.59		
<b>DOX</b> +THCV	≤0.91		

Increase in IC50 values of DOX, CBD, THCV, DOX+CBD and DOX+THCV treatment in magnetic 3D cell culture as compared to 2D assay may be associated with lower drug penetration due to replication of natural tumor microenvironment using 3D hydrogels. Microscopic images of 3D spheroids showed that cell morphology was altered after the treatment with DOX, CBD, THCV as compared to control (no treatment) (Fig. 7 in supplementary file). Additionally, 3D spheroid area was significantly decreased after 48 h of treatment with DOX, CBD and THCV as compared to control (no treatment) (Fig. 8 in supplementary file).

## 3.2. Chemo-sensitization effects of CBD and THCV on DOX-resistant MDA-MB-231 xenografts bearing athymic nude mice

We had previously shown that CBD inhibited the growth of wild-type MDA-MB-231 cells xeno-transplanted in athymic BALB/c nude mice, and in this study, we demonstrated that CBD (10 mg/kg)and THCV (15 mg/kg) pre-treatment, a day before the treatment with DOX (5 mg/kg), had chemo-sensitizing effects on MDA-MB-231 DOX resistant tumors bearing mice (Fig. 2). In both the CBD/ DOX and THCV/DOX combinations, tumor volumes were significantly reduced on the 14th day (p < 0.01 vs. control and p < 0.05 vs. control) as shown in Fig. 2. Though, tumor volumes were reduced by CBD and THCV therapy alone at 14th day, however this was not statistically significant as shown in Fig. 2. These findings suggest that the resistance of MDA-MB-231 breast cancers to DOX can be overcome by utilizing CBD/THCV in combination with DOX. Further, we set out to investigate the molecular alterations in tumor tissues after treatment with CBD and THCV, as well as their chemosensitization effects on DOX. So, to achieve this goal, we performed RNA sequencing and proteomics on freshly removed tissue from multiple groups of tumor-bearing mice.

## 3.3. Sequencing of RNA from tumors in athymic nude mice uncovers new molecular targets for CBD and THCV

RNA sequencing was performed on DOX-resistant MDA-MB-231 tumor tissues to examine the transcriptome changes impacted by



Fig. 1. Effect of medical marijuana on doxorubicin resistant TNBC cell viability. Bar graphs represent (a) DOX, (b) CBD, (c) THCV, (d) DOX+CBD and DOX+ THCV treatments against cell viability of 2D cultures of DOX resistant MDA-MB-231 cell lines. All the results were expressed as Mean  $\pm$  SEM (n = 3).



Fig. 2. Effect of CBD and THCV in combination with DOX reduced the tumor volumes in DOX resistant MDA-MB-231 tumor bearing athymic nude mice. Bar graph represents the tumor volume data from day 1 to day 14 after initiating the treatment with DOX, CBD, THCV, DOX+CBD and DOX+ THCV.

CBD (10 mg/kg, ip) and THCV (15 mg/kg, ip) treatment and the samples were qualified for initial screening (Suppl Figs. 1 and 2). RNA sequencing revealed that many genes were regulated differently when CBD and THCV therapy was compared to untreated control group. Fig. 3A shows a heat map image of the expression of numerous genes following CBD and THCV treatments in comparison to untreated DOX-resistant MDA-MB-231 tumors. The volcano plot (P value < 0.05 and FC > 1.0) showed that CBD (10 mg/kg) and THCV (15 mg/kg) upregulated 950 genes while downregulating 1240 genes (Fig. 3B-G). The linkage of biological pathways was determined using the KEGG pathway analysis, demonstrating differentially elevated genes. It was observed that CBD (10 mg/kg) and THCV (15 mg/kg) differentially expressed genes (after treatment) were found to be engaged in many pathways, including spliceosome, metabolic, immunological, inflammation,

mitochondrial function, apoptosis, RNA transport and signaling (Fig. 4). Among these, metabolic pathways (AMPK), immunological pathways (PD-L1), mitochondrial function (SOD), inflammation pathway (NF- $\kappa$ B, STAT3, TGF- $\beta$ ), and apoptotic pathways drew our attention because of their significance in cancer mediation as shown in Fig. 4A–C. RNA seq data revealed that CBD (Fig. 4B) and THCV (Fig. 4C) induces downregulation catalase, SP1, NLRP1, SOD2, TGF- $\beta$ , NF- $\kappa$ B, p38 MAPK and PD-L1 genes. Further, the same tissue samples were used for proteomics analysis in order to verify RNA sequencing results.

3.4. Proteomics revealed additional targets for CBD and THCV in athymic nude mice tumors, similar to RNA sequencing

Proteomics analysis was performed to describe the proteome of



Fig. 3. RNA-Seq identified differential mRNA expressions in MDA-MB-231 tumor tissues isolated from athymic nude mice. (A) Heat map illustrations of hierarchical clustering analysis of differentially expressed mRNA in tissues of control and treated MDA-MB-231 Xenograft mice. Representative volcano plots of differentially expressed genes (DEGs) B) Control Vs DOX, C) Control Vs CBD, D) Control Vs THCV. Representative scatter plots of differentially expressed genes (DEGs) E) Control Vs DOX, F) Control Vs CBD, G) Control Vs THCV.



Fig. 4. DEmRNA KEGG pathway analysis. A) Bubble plots comparing the top 10 significant DEmRNA pathways of DOX and control group mice. B) Bubble plots of the top 10 significant DEmRNA pathways of CBD and control group mice. C) Bubble plots of the top 10 significant DEmRNA pathways of THCV and control group mice.

DOX-resistant MDA-MB-231 tumor tissues and to identify the differentially regulated proteins in various treatment groups as compared to the control group. Quantitative abundance ratios for

approximately 1000 proteins were measured in the study. For CBD and THCV treated tumor tissues, Fig. 5 shows the most significantly altered proteins. In the CBD and THCV-treated groups, MTO, XRP2,

TEF, ORM1, ELP2, c-Jun, KMCP1 and AMPK were elevated, while HO-1, SOD, Bcl-xL, P-38 MAPK, TGF-β, PD-L1, CD133, NF-κB, catalase, tenascin, SP1, and NLRP3 were all downregulated (Fig. 5C and D and Table 2). Inflammation, oxidative stress, mitochondrial function, and tumor metastasis were all found to be controlled by these proteins (Suppl Figs. 3–5). Proteomics data was further validated using western blotting and RT-qPCR.

#### 3.5. Validation of RNA seq and proteomics by Western blot and RTqPCR analysis

RT-qPCR and western blotting were performed on prepared histone samples of tumor lysates from DOX resistant MDA-MB-231 cancers bearing mice in order to better understand the role of transcription dysregulation, which was partially revealed in RNA sequencing of tumor tissue lysates from MDA-MB-231 tumors. As demonstrated in Fig. 6b-d, the mRNA expression of H2A, H2B, and H3 histones in CBD and THCV treated tumor tissue was substantially reduced (p < 0.01) compared to the control group, and when the same CBD or THCV combined with DOX, the same histone proteins were more significantly (p < 0.001) reduced. Although when compared to an age-matched control group, no significant changes in the expression of these histone genes were identified in the DOX alone treated group, indicating that these histone genes were playing an important role in making MDA-MB-231 cells resistant to conventional DOX treatment. As a follow-up to these intriguing findings, we looked at how post-transcriptional modifications to the same histone proteins affected the development of this particular form of cancer. Triple-negative breast tumors were found to be among the most aggressive malignancies in terms of histone acetylation and methylation at the H2 and H3 proteins. These findings led us to isolate H2 and H3 histone proteins in tumor tissue from different groups of mice, and to do Western Blot analysis on these proteins using numerous sites of acetylation or methylation. However, H3-K4 methylation and H2B-K5 acetylation in MDA-MB-231 tumors have been identified as possible markers of resistance to DOX. Fig. 6A shows that CBD or THCV combined with DOX significantly (p < 0.001) reduced these two histone alterations when compared to the control group. The RNA sequencing and proteomics findings have been further confirmed by looking at the protein and gene expression levels of a few abundantly changed proteins/genes. NF- $\kappa$ B, Bcl-xL and SOD2 protein and gene expression levels in CBD or THCV combined with DOX were considerably (p < 0.001) lowered than in the control group (Fig. 6e-h). A number of proteins, such as JNK2, PD-L1, NLRP3, SP1, P-38 Map Kinase, HO1, Catalase, and TGF- $\beta$ 1 were also considerably reduced in CBD+DOX and THCV+DOX combo treatment groups, when compared to the control group (Fig. 7 a-b). These findings show that CBD and THCV's pharmacological mechanisms of action in MDA-MB-231 tumors were limited to a few proteins and genes.

#### 4. Discussion

Current treatment options for breast cancer mainly rely on hormone therapies and antibodies targeting estrogen, progesterone, and Her2 receptors. However, TNBC is negative for each of these receptors; thus, it does not currently possess effective treatment options, especially as it responds poorly to standard chemotherapies, is susceptible to relapse, and has a tendency towards early metastatic spread to the brain, bones, and lungs [6,49]. Further, literature clearly shows that this disease develops resistance to chemotherapy at a faster rate than other types of cancer [50,51]. Because of a lack of knowledge about the disease's molecular targets and appropriate treatments, there is an unmet need to develop new therapies for TNBC. In our studies, using 2D and 3D cultures as well as tumor xenotransplanted athymic nude mice, it has been observed that the primary non-psychoactive synthetic cannabinoids have an excellent role in protecting against DOX resistant MDA-MB-231 tumors. Transcriptomics and Proteomics were carried out in tumor tissues to identify particular molecular targets and pathways that protect against this disease. Additionally, we used RT-PCR and western blotting techniques to verify a few of the most abundantly expressed gene and protein markers in our study. To our knowledge, this would be the first study to report the



Fig. 5. Proteomic identified differentially expressed proteins (DEPs) in Breast cancer after treatment. A) Representative Volcano plots of DEPs in between (a) Control and DOX groups, (b) Control and CBD, (c) Control and THCV, (d) CBD and THCV. (B) Representing illustrations of hierarchical clustering analysis of differentially expressed proteins in control and treatment groups. (C) Bar graph representation showing proteins with the largest overall increase in expression and (D) the proteins with the greatest reduction in expression upon treatment are represented.

#### Table 2

Proteomics study of MDA-MB-231 DOX resistant tumor tissue lysates reveals proteins with high	gh abundance values and their role in cancer.
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Protein Name	Role in Cancer	Expression in treatment group	Ref.
PD-L1	Programmed Cell Death Protein 1 (PD-L1) plays a vital role in inhibiting immune responses and promoting self-tolerance through modulating the activity of T-cells, activating apoptosis of antigen-specific T cells and inhibiting apoptosis of regulatory T cells.	Downregulated	[40]
Haem oxygenase- 1	Haem oxygenase-1, is an enzyme that catalyzes the degradation of haem to produce biliverdin, ferrous ion, and carbon monoxide	Downregulated	[41]
SP1	Sp1 is a transcriptional activator of a variety of genes including house-keeping genes, cell cycle regulators and tissue-restricted genes	Downregulated	[42]
SOD2	High superoxide dismutase 2 (SOD2) expression is associated with a poor prognosis at many cancer sites, the presence of metastases, and more advanced cancer.	Downregulated	[43]
NF-κB	The oncogenesis process is influenced by the pleiotropic transcription factor NF- $\kappa$ B, which upregulates genes involved in cell proliferation, metastasis, apoptosis suppression and angiogenesis	Downregulated	[44]
TGF-β	$TGF-\beta$ is the most potent inducer of epithelial-mesenchymal transition in breast cells, and it is essential for the establishment of a tumor-promoting microenvironment in breast cancer tissue.	Downregulated	[45]
STAT3 CD133	Many malignancies have constitutively active STAT3, which plays a key role in tumor development and metastasis. CD133 (Prominin 1, PROM1) is a transmembrane protein whose mRNA and glycosylated forms are highly expressed in many human cancer cell types	Downregulated Downregulated	[46] [47]
АМРК	In cancer, AMPK plays a tumor suppressor role. Activation of AMPK reduces tumor growth by targeting several tumorigenesis- related signaling pathways at various phases of tumor formation.	Upregulated	[48]

chemo-sensitization effects of CBD and THCV on DOX-resistant TNBC tumors.

We and others have shown that CBD has anti-cancer effects against various wild-type TNBC cell lines [25,52]. However, this is the first attempt to show that CBD has anti-cancer effects against DOX resistant TNBC. Further, this study is also the first to report THCV either alone or in combination with DOX in attenuating DOX resistant xenotransplanted tumors. THCV's anti-cancer activities against breast malignancies are not well-studied, and only a few disease models have been used to evaluate this compound's effects on other diseases. Our results revealed that the IC50 values of DOX, CBD, THCV, DOX+CBD and DOX+THCV against 2D cultures of DOX resistant MDA-MB-231 cell lines were significantly lower as compared to IC50 values of DOX, CBD, THCV, DOX+CBD and DOX+THCV in magnetic 3D cell culture assay. Three-dimensional culture geometry restricts the drug's ability to reach the spheroid, which may account for the higher IC50 values for DOX, CBD and THCV in these three-dimensional spheroidal cultures. In xenograft mice with head and neck squamous cell carcinoma, Yoon et al., observed that combining CBD with other anti-cancer medications such as cisplatin, Taxol, and 5-FU improved the anti-tumor efficacy of those drugs [53]. The chemo-sensitizing effects of CBD on DOX anti-cancer potential were similarly revealed in our prior research in wild type MDA-MB-231 breast cancer cell lines [24]. We further examined the same combo effects on DOX resistant MDA-MB-231 tumors in the present study. An interesting finding was that the anticancer activity of CBD or THCV when given with DOX was greater than that of either compound alone. In light of these findings, we further investigated the molecular alterations in tumors following therapy with CBD/THCV and DOX.

Tomko et al. demonstrated that atypical cannabinoids have antitumor effects on Taxol-resistant MDA-MB-231 and MCF-7 breast cancer cell lines, potentially by increasing apoptosis [54]. Several studies have suggested that cannabinoids could be used as an adjuvant to overcome chemotherapy resistance in various malignancies [55]. CBD, a phytocannabinoid, increased the sensitivity of carmustine, temozolomide, doxorubicin, and cisplatin against glioblastoma cells [56]. In consistent with earlier findings, we discovered that CBD and THCV have chemo-sensitizing effects on DOX against resistant triple negative breast cancer cell lines. Not many studies have been reported with THCV as an anticancer agent in the literature. Romano et al. demonstrated that a CO2 extracted cannabis extract, with a high content (64.8%) in  $\Delta$ 9-tetrahydrocannabivarin (THCV), inhibited nitrite production induced by lipopolysaccharides (LPS) in murine peritoneal macrophages, and thus may have a potential to modulate the inflammatory response in different disease conditions like cancer [57].

Furthermore, we discovered that combining these synthetic cannabinoids with DOX had synergistic effects in killing resistant MDA-MB-231 cells in 2D cultures as ascertained by isobolographic analysis. These findings suggest that cannabinoids have a significant potential as a complementary approach to overcoming TNBC resistance to regular chemotherapeutic treatment. Our proteomic and Western blot investigations in resistant MDA-MB-231 tumors showed that treatment with CBD and THCV upregulated AMPK, Methanethiol oxidase (MTO), XRP2, TEF, ORM1, ELP2 and others and downregulated HO-1, SOD, Bcl-xL, P-38 MAPK, TGF-B, PD-L1, CD133, NF-KB, catalase, tenascin, SP1 and NLRP3 inflammasome. Interestingly, all of these gene/protein expression levels were unaffected by DOX treatment in resistant MDA-MB-231 TNBC cells. In several studies, some of these upregulated and downregulated proteins have already been identified as indicators for overcoming resistance. For example, corosolic acid treatment in the presence of 5 FU generated an increase in the number of apoptotic cells, however this rise was reduced by compound c (AMPK inhibitor), indicating that corosolic acid may have therapeutic potential to activate AMPK and sensitize gastric cancer resistance to 5 FU [58]. The overexpression of MTO [59] and TEF [60,61] proteins were associated with tumor shrinkage and linked to multiple tumor suppressor proteins in the regulation of various cancers in many preclinical and clinical cancer studies, including those on breast, pancreatic, gastric, head and neck, gastric, and prostate cancers. Hsueh et al. reported that SOD2 enhancement by long-term inhibition of P13K pathway confers multi-drug resistance and enhanced tumorigenesis in head and neck cancers [62]. Also, HO-1 expression is usually increased in tumors, as shown in lymphosarcoma, adenocarcinoma, hepatoma, gynecologic cancers, melanocytic cancers, prostate cancers, pancreatic cancers, and other brain tumors; the increase of this HO-1 was observed in response to chemotherapy, radiation, or photodynamic therapy [63,64]. Cancer growth and metastasis indicators such as TGF-β, PD-L1, CD133, NF-κB, catalase and NLRP3 inflammasome development have been predicted for several solid organ cancers [65–67]. Based on past research and our current findings; we hypothesize that these proteins in MDA-MB-231



**Fig. 6. Effect of Cannabidiol (CBD), tetrahydrocannabivarin (THCV) in combination with DOX on Histone modifications, Inflammatory and Antioxidant enzymes:** (a) Western blots of MDA-MB-231 tumor lysates from xenotransplanted BALB/c nu/nu mice demonstrate CBD, THCV, and their combination with DOX on the expressions of H2BK5 acetylation and H3K4 methylation and respective Western blot quantification is represented by bar graphs. Bar graphs of (b), (c) and (d) represents the mRNA expressions of Histones H2A and H2B in different groups. (e) western blots represent the immuno-expressions of SOD2, Bcl-xL, NF- $\kappa$ B and JNK2 and their respective quantification. Bar graphs of (f), (g) and (h) represents the mRNA expressions of NF- $\kappa$ B, Bcl-xL and SOD2 in different groups. The Values are expressed as mean  $\pm$  SEM (n = 3). \*p < 0.05, \*\*p < 0.01 and \*\*\*p < 0.001 Vs control.

breast malignancies contribute significantly to the development of DOX resistance in MDA-MB-231 tumors. We observed that CBD and THCV were able to modulate these tumor-resistant indicators and sensitize the DOX in a novel way, but more in-depth molecular investigations are needed to fully understand the anticancer mechanisms of these synthetic cannabinoids.

Additionally, RNA seq (KEGG) analysis revealed that the altered genes in CBD and THCV treatment were known to regulate endo cannabinoid signaling, JAK STAT signaling, TGF- $\beta$  signaling, Pl3K-AKT signaling, metabolic pathways, p-53 signaling, calcium signaling, and Transcription misregulation in cancer, all of which were linked to regulating apoptosis, inflammation, autophagy process, oxidative stress, and mitochondrial function. One of the

observations from proteomics and RNA seq studies was that there was limited correlation between them. However, Tenascin/TNXB shown in Fig. 5 was also found in our RNA-seq data, but no other proteins in Table 2 were found significantly changed in transcriptome. The low correlation between our RNA-seq and proteomic data may be due to low coverage, delayed protein expression or other post-transcriptional modification. This is not surprising and similar results were also observed by other researchers [68].

CBD has been extensively studied in various cancers both in vivo and in vitro, and it has shown a greater ability in causing cancer cell death, inhibiting cell migration and invasion in vitro, decreasing tumor size, vascularization, growth, and weight, and increasing survival and inducing tumor regression in vivo of different cancer



**Fig. 7.** Western blot Validation of Proteomics Analysis in Fresh MDA-MD-231 Resistant Tumor Lysates (a) Western blots of MDA-MB-231 tumor lysates from xenotransplanted mice demonstrate CBD, THCV, and their combination with DOX on the expressions of PD-L1, NLRP3, SP2, P38 MAPK, HO-1, Catalase, and TGF- $\beta$  and (b) respective Western blot quantification is represented by bar graphs. The Values are expressed as mean  $\pm$  SEM (n = 3). \*p < 0.05, \*\*p < 0.01 and \*\*\*p < 0.01 Vs control.

models, for example, in glioblastoma cancer cells, CBD inhibited the PI3K/AKT survival pathway and also activated pro-apoptotic MAP-Kinases in U87 MG cells to inhibit brain tumors [69,70]. CBD inhibited the growth of breast cancer cells, including MDA-MB-231 cells, MCF-7 cells, MDA-MB-468 cells, and SK-BR3 cells, through regulating apoptosis, autophagy, and cell cycle arrest [71]. CBD increased apoptosis by activating caspase 3, BAX, mTOR, AKT, 4EBP1, and cyclin D while upregulating PPAR-γ expression and nuclear localization and decreasing inflammation by downregulating JAK-STAT and TGF- $\beta$  signaling and Matrix metalloproteinases (MMPs) in several breast and lung cancer studies [72–74]. Despite this, very little is known about the impact of THCV on cancer. THCV showed some lethal effects in prostate cancer cell lines DU-145 and LNCaP, with IC50 values above 17.5  $\mu$ M, and the pharmacological mechanisms underlying this activity were hypothesized by triggering caspases 3/7, p-53 protein, cell cycle arrest, apoptosis, and DNA fragmentation [32]. Together with the literature support, our findings would suggest that these two cannabinoids may play a role in regulating resistant TNBC by maintaining various pathways. One of the intriguing aspects of this study is the dysregulation of transcription; to investigate, we examined the gene expression of histone proteins (H1-H4) and found that treatment with CBD or THCV significantly altered H2-(H2BK5 acetylation), and H3- (H3K4 methylation) histones in tumor tissue lysates. Intriguingly, the downregulation of these histone changes was significantly affected by the combination of CBD/ THCV and DOX therapy in MDA-MB-231 tumor lysates that were DOX resistant. H2 and H3 histones were previously discovered to be substantially elevated and involved in the advancement of several breast malignancies [75,76]. It was also noticed that histone acetylation and methylation at various lysine sites were involved in the rapid proliferation of Breast cancers [75]. When it comes to TNBC, Messier et al., found that H3K4 methylated and H3K4 acetylated histones were enriched in MCF10A, MCF7, and MDA-MB-231 cells using a genome-wide ChIP method, and they concluded that the enrichment of this acetylated and methylated histones with late-

#### A.K. Kalvala, R. Nimma, A. Bagde et al.

stage malignancies [77]. Our findings in MDA-MB-231 TNBC cells were consistent with this study's finding that H3K4 methylation was prevalent, but the fact that these histone modifications did not respond to DOX alone treatment is intriguing, as it suggests that this modification may be contributing to the resistance to DOX. According to a review by the Mobley et al. group, the epithelial to mesenchymal transition of epithelial human colorectal tumors. myeloma cells. lymphoblastoid cells, and trophoblast stem cells is heavily dependent on H2BK5 acetylation. This study found that H2BK5 acetylation was increased in DOX resistant MDA-MB-231 tumor lysates [78]. Furthermore, these histone alterations have been associated to increased stemness in a variety of cancer types [79], but this study is the first to show that the same histone modifications are involved in the development of resistance against DOX in MDA-MB-231 tumors. Besides this, CBD and THCV treatment in combination with DOX significantly downregulated these modified histones by regulating a number of genes and proteins that are involved in tumor formation and metastasis. It has been reported that cannabinoids regulate histone modifications, such as anandamide (cannabinoid) protecting neurons from inflammation, CBD attenuating experimental autoimmune encephalomyelitis and THC (9-tetrahydrocannabinol) mediating potent anti-inflammatory properties in lymph node cells of mice by modulating H3-K4 phosphorylation and methylation [80,81]. In addition, PI3K/AKT signaling regulated H3K4 methylation in breast malignancies, and inhibiting PI3K/AKT signaling decreased promotor-associated H3-K4 methylation in human breast cancers [82]. P53 mutations and the role of TNF- $\alpha$  signaling in the advancement of colorectal tumors were both linked to H3K4 methylation [83]. It has also been shown that H3K4 methylation helps prostate tumors avoid anti-PD-L1/PD-1 immunotherapy through modulating PTEN/P13K/AKT signaling in the course of the disease [84]. Oncologists now know that H2BK5 acetylation is a critical factor in the upregulation of numerous proteins implicated in the EMT transition [78].

#### 5. Conclusion

Our findings show that CBD and THCV were found to overcome resistance against MDA-MB-231 resistant cell line in vitro in 2D and 3D cultures by several folds. Further, both these agents in combination with DOX showed synergism as determined by the isobolographic method. Our molecular studies showed that CBD/THCV in combination with DOX regulates the PI3K-AKT, p-38 MAPKinase, AMPK, TGF- $\beta$ , PD-L1 and NF $\kappa$ -B pathways in reducing resistant MDA-MB-231 tumor volumes in athymic nude mice. Our studies also suggest that these pathways regulation might be linked with H3K4 histone methylation and H2K5 histone acetylation down-regulation, which has to be further investigated in our future studies.

#### Funding

This work was supported by Consortium for Medical Marijuana Clinical Outcomes Research, Grant/Award number: SUB00002097, National Institute on Minority Health and Health disparities, (National Institutes of Health), Grant/Award Number: U54 MD007582 and NSF-CREST Center for Complex Materials Design for Multidimensional Additive Processing (CoManD), Grant/Award Number: 1735968 for providing the funding for this research work.

#### Author contributions

All authors contributed to the study conception and design. Designing experiments and analysing the results and writing the manuscript were done by AKK, RN and AB. NP, SKS, LS, AB, NK, EN, RS, PA and AN performed part of the experimental work and/or analyzed the results. Prof. MSS conceptualized the study, wrote, and revised the manuscript. All authors read and approved the final manuscript.

#### **Ethics approval**

All the animal studies were reviewed and approved by the Institutional Animal Use and Care Committee of Florida Agricultural and Mechanical University (protocol number: 020–06) and complied with the NIH guidelines (Guide for the care and use of laboratory animals). All animal experiments were performed by following ARRIVE guidelines.

#### **Declaration of competing interest**

The authors have no relevant financial or non-financial interests to disclose.

#### Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biochi.2022.12.008.

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