Antineoplastic Properties of THCV, HHC, HHCV and their anti-Proliferative effects on HPAF-II, MIA-paca2, Aspc-1, and PANC-1 PDAC Pancreatic Cell Lines

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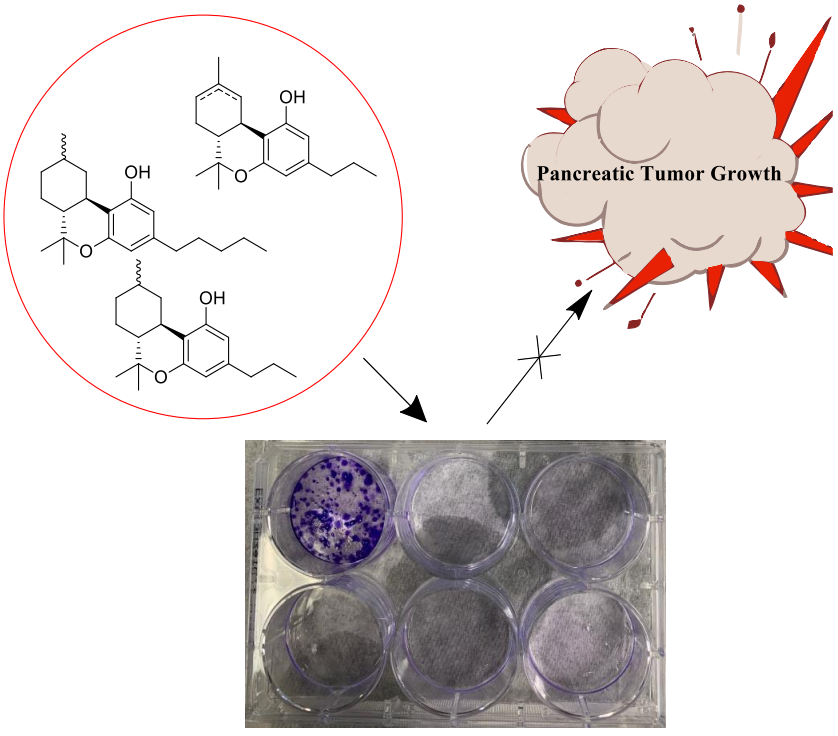


THCV IC_{50} = 9.7 µM
9R IC_{50} = 12.7 µM
HHCV IC_{50} =5.56 µM

Abstract

Cannabinoid receptors CB_1 and CB_2 are the primary endogenous receptors with which cannabinoids interact, inducing physiological and psychological effects. Although interactions with other receptors including TRPV1 and GPCR55, have been recognized in earlier studies, these interactions may play a significant role in cancer remediation through the unspecified upregulation

or downregulation of specific pathways. The main active constituents within the cannabis plant are cannabidiol (CBD) and tetrahydrocannabinol (THC), which have been categorized as either non-intoxicating with benefit or intoxicating with no benefit. These categories are constantly ignored, as cannabinoids have shown efficacy in the treatment of certain diseases and ailments as single-agent compounds. Tetrahydrocannabivarin (THCV), a rare cannabinoid, is a homologue of THC, with the C5 alkyl chain having three carbons rather than the standard five carbon length. THCV has garnered attention in a clinical setting as an anti-obesity drug treating glucose issues. Hexahydrocannabinol (HHC), a hydrogenated analogue of THC, is a rare cannabinoid like THCV. These cyclic cannabinoids are considered rare, because they are typically found in minimal to trace amounts within cannabis sativa and their given C. indica, and C. ruderalis sub species. Increased popularity of these rare cannabinoids has led to proposed experimentation leading to assessing the cytotoxicity of these cannabinoids toward, cancer cells of the pancreas (MIA-PaCa2, HPAF-II, and PANC1). The data evaluated through such studies led to the proposed idea of these rare cyclic cannabinoids towards the treatment of pancreatic cancer due to the modest efficacy as single agent antineoplastics compared to common single agent antineoplastics on the market, with evidence being strongly presented when compared to commercially available anticancer agents poly(ADP-ribose) polymerase (PARP) inhibitors.

Keywords: cannabinoids, THCV, HHC, pancreatic, cancer, *in-vitro*, PDAC.

Background

Cannabinoid History

Cannabinoid receptors CB_1 and CB_2 are known to be expressed during the mediation of certain cancer growth [1]. Including but not limited to the TRPV channel (Transient receptor potential cation channel subfamily V member 1) [2] and GPCR55 (G-Protein Coupled receptor 55) [3], these other receptors are also expressed during the genesis of certain cancers and may play a role in the remediation of cancer [4,5]. Several studies have shown anti-proliferative and pro-apoptotic properties of cannabinoids towards certain cancers *in-vitro*. Limited clinical studies on the treatment of pancreatic cancer with cannabinoids as antineoplastics have been conducted [6]. Synergistically, CBD and THC have shown to treat various ailments and diseases due to their non-specific modulation of CBD/THC targets [7]. As rare cannabinoids are being reintroduced to the spotlight, Tetrahydrocannabivarin (THCV) and Hexahydrocannabinol (HHC) are of interest due to the rarity in nature, as these cannabinoids are found in minimal to trace amounts [8,9] within the *C. sativa* plant.

THCV is a cyclic cannabinoid found in minimal amounts within the *cannabis sativa* plant. THCV is a homologue of Δ9-THC, with the primary difference located in the alkyl chain on the C5 carbon with an alkyl chain of three carbon lengths rather than a five-carbon length alkyl chain similar to THC. THCV has been explored as an anti-obesity drug in conjunction with metformin for reducing blood sugar set in early stage clinical , as well in murine models, reduced appetite has been shown [10,11]. HHC (Hexahydrocannabinol) is an analogue of Δ9-THC, the difference lying

in the cyclohexene ring being hydrogenated to form the cyclohexyl ring, the lack of the double bond would assume the binding affinity to the cannabinoid receptors to be lessened [12]. HHC is determined to have novel status as a cannabinoid due to its prevalence being found in trace amounts in nature with limited to no data being accounted for. Although the cannabinoid was elucidated and synthesized in the 1940's by Adams, no clinical research or pre-clinical research has been done on the efficacy or effects on HHC. Several safety studies and articles have been written for the safety on consumption [13] and possible SAR for cannabinoid research [14], aside from the limited studies, nothing has been of status that contributes to the field of cannabinoid chemistry. Cannabinoids for decades have been proposed for years with *in-vivo* and *in-vitro* modelling studies providing evidence towards the treatment of certain cancers and ailments [15]. Cannabinoids such as CBD and THC have been revealed to treat insomnia, anxiety, PTSD, cachexia, appetite disorders, and other common ailments, derived from diseases [16]. The structures of the cannabinoids are shown in figure 1 below, depicts the shared pharmacophore with differences in the alkyl chain and the double bond.

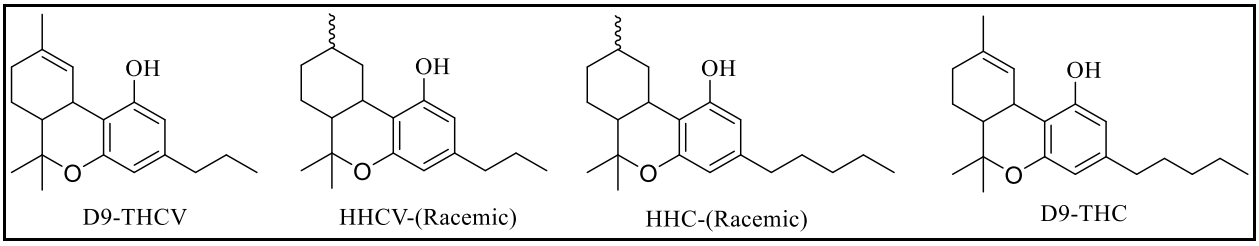


Figure 1. Pharmacophore of THCV, HHCV, and HHCV (Left), depicting the differences from the parent scaffold Δ9-THC (Right).

Cannabinoid Receptors

CB_1 and CB_2 receptors are coupled to the GPCR (G-Protein Coupled Receptor) family of proteins [17]. CB_1 is the prominent subtype located within the CNS (Central Nervous System) and are as well expressed within the PNS (Peripheral Nervous System) [18] below in figure 2 is the receptors. The discovery of the CB_1 and its prominence within the CNS and PNS has garnered attention for possible treatment of neurodegenerative and neuropsychological disorders that can be treated through this avenue [19]. Although acitvation of CB_1 receptors are also indicated with the psychotropic effects negatively assocated with use of psychoactive cannabinoids [20]. The CB_2 receptor plays an integrative role within the brain, G.I (Gastro-Intestinal), PNS (Peripheral Nervous System), and the immune system [21]. Unlike the CB_1 receptors, the activation of the CB_2 Receptors with cannabinoids, do not provide the psychotropic "high" that is associated with agonists of the CB_1 receptor, which would be the more likely place to design compounds for better treatment [22]. CB_2 plays a significant role in anti-inflammation and remediation in cancer growth [22]. CB_2 receptors are implicated in a variety of modulatory functions, including immune suppression, induction of apoptosis, and induction of cell migration [23a]. The CB_1 receptors like the CB_2 receptors can be allosterically modulated by synthetic ligands, in a positive and negative fashion [24]. THCV is shown to act as an antagonist of the CB_1 receptor in small concentrations

but can act as a partial agonist at larger concentrations [25]. HHC in animal models are known to bind to the CB_1 receptor producing similar effects to THC [14].

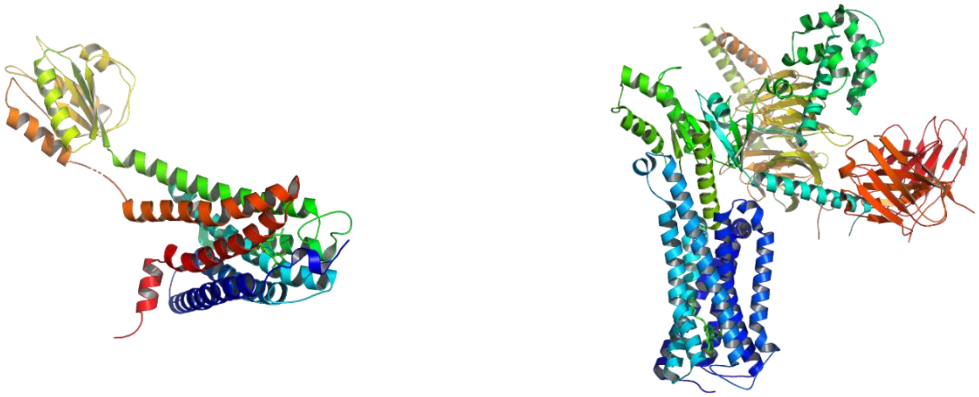


Figure 2. Represented on the left is the CB1 receptor, represented on the right is Cryo-EM structure of human cannabinoid receptor 2-G_i protein [23b].

The GPCR receptor

GPCR (G protein-coupled receptors) are cell surface receptors that can detect molecules on the cellular membranes and activate cellular responses [26]. The GPCR activation is mediated through bound agonists. Estimated 34% of approved drugs target the GPCR complex [27] in various target organs. The GPCR complex, is implicated in a variety of physiological processes, not limited to but including, regulation of immune system activity and inflammation, autonomic nervous system transmission, homeostasis modulation, and implicated in growth and metastasis of certain types of tumors [28]. The CB_1 and CB_2 receptor are class A serpentine G protein-coupled receptor (GPCR) that signals primarily through the adenylyl cyclase-inhibiting heterotrimeric G protein G_i and the ERK1/2 (extracellular signal-regulated kinases 1/2) pathways [26]. Although they are not limited to these specific pathways, they are the most common pathways. Over 100 GPCR's have been expressed at the mRNA level within pancreatic adenocarcinoma tumors [28,29]. Several GPCRs GPRC5A, F2R and F2RL1 are expressed in multiple PDAC cell types while other GPCRs are expressed in a specific setting within microenvironments of the cell [29,30]. Although the research of targeting GPCRs in pancreatic cancers are relatively new [31], increased the relevance of targeting this complex using agonists and antagonists of GPCR becomes pertinent.

PDAC and Pancreatic Cancer

PDAC (Pancreatic ductal adenocarcinoma) due to the lack of early detection and the limited response to designed treatments, is considered to have a terrible prognosis. PDC is highly aggressive with lethal malignancy. PDAC is the most common type of pancreatic neoplasm, and

accounts for more than 90% of pancreatic cancer cases [32]. PDAC has an average 5-year survival rate of less than 10% [33]. The need for new treatments for pancreatic cancer is pertinent, as many of the on-market compounds are limited for the direct treatment of pancreatic cancer. The poor genomic and proteomic analysis of various tumors fails to distinguish the proper target and treatment plan. Aside from poor prognosis, TME (tumor microenvironment) is characterized by dense desmoplasia and extensive immunosuppression. Extensive desmoplasia results in various cell infiltration, vascularization, and hypoxia, preventing drugs to target such areas specifically [32,34]. PDAC target through this experiment, is through GPCR ligand targeting, as the membrane protein is present it also accounts for 20% of all cancers that contain a mutated GPCR or g-alpha subunits [35]. Targeting this receptor using cannabinoids might prove to be a possible target of choice as GPCRs mediate a broad range of autocrine and paracrine responses in cancer cells. They bind to a diverse group of ligands, including small peptides, lipids, and proteins (e.g., chemokines) [36]. The density of GPCRs on the cell surface is typically 10^3–10^4 receptors/cell, which should be adequate to ensure ample uptake of the targeted drug cargo [37].

Methods

CBD was used as the building block for the synthesis of HHC. CBD was purchased in bulk from GVB Biopharma and converted to delta-8 THC. Although THC synthesis is accessible according to known synthesis [38]. For Industry purposes, THC bulk creates a facile process. Treating THC with hydrogen gas will afford the racemic mix of HHC, a light to dark yellow oil. Purification of the completed reaction crude afforded the desired product. Cannabidivarin (CBDV) was used as the building block for D9-THCV. CBDV was purchased in bulk from BayMedica. Although the CBDV synthesis is accessible [39], for industry purpose the CBDV bulk creates a facile process. Starting from clean CBDV isolate, treating CBDV with triisobutylaluminum (TiBa), creates the desired product, after purification.

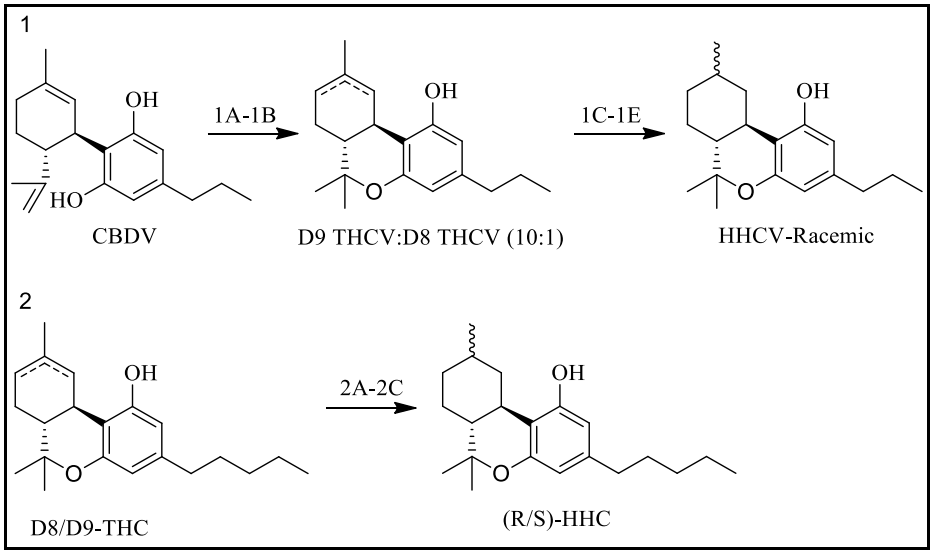


Figure 3. Cannabinoids THCV, HHC and HHCV; Reagents and conditions:(1A) CBDV, DCM, Argon purge 1hr, rt, (1B) Triisobutylaluminum, rt, overnight. (1C) THCV D8/D9, EtOH, Argon Purge 1hr,rt., (1D) Pd/C, 1-5 bar, (1E) H2, 25°C-50°C, 3-72 hr., (2A) THC, EtOH, Argon purge 1hr, rt., (2B) Pd/C, 1-5 bar, (2C) H_2, 25°C-50°C, 3-72 hr.

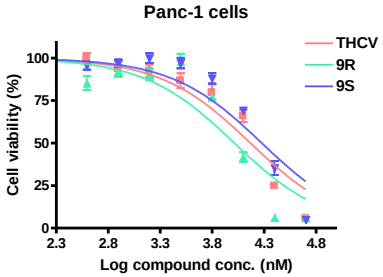
In-vitro screening of THCV, HHC, and HHCV

Cell viability using MTT assay. A total of 3000-8000 PDAC cells were seeded per well in 96-well plates. Following attachment, cells were treated with different compounds at varying doses as indicated for 72 h. Growth inhibition was determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma-Aldrich, St. Louis, MO, USA; catalog No. M2128) assay. The MTT solution was added to the media at a final concentration of 0.8 mg/mL and cells were incubated at 37 °C for 2 h. After aspiration of media, formazan crystals were dissolved in DMSO. Optical densities were measured at 570 nm using SynergyHT plate reader (BioTek, Winooski, WI, USA). To calculate IC_{50} values for all drugs, GraphPad Prism Software were used (GraphPad Software, San Diego, CA, USA).

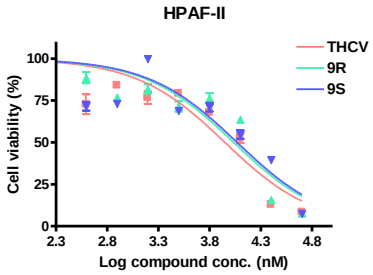
Compounds synthesized that have suitable physicochemical properties will be screened for target cell toxicity at the Karmanos Cancer Institute, Wayne State University, against pancreatic cancer cell lines and normal, healthy pancreatic cells. Further analyses will be performed to elucidate the effects of the compounds on the cells, using the standard techniques of the Institute [40] and to investigate the proposed mechanisms of action. The testing regimen will be flexible to accommodate findings as the project progresses. Different activity profiles of compounds against the cell lines may suggest testing combinations.

Results

The use of THCV and HHC as rare cannabinoids in the treatment of pancreatic cancer has been revealed through *in-vitro* studies. Use of certain pancreatic cell lines from PDAC has been tested. PANC-1, HPAF-II, AsPc-1, and MIA-PaCa2 cell line were tested against THCV and HHC to generate IC_{50} values to determine efficacy as possible antineoplastics using the PANC-1, HPAF-II, and AsPC-1 cell lines as shown in figure 4. Against the PANC-1 and HPAF-II cell lines THCV and HHC have generally low IC_{50} values. The S-isomer of HHC compared to the R-isomer of HHC on the PANC-1 cell line have a difference of almost 2 times the micromolar IC_{50} values, which could be based off the conformation of S-Isomer and how the compound binds and interacts with the cells compared to the R-Isomer following the properties of cell binding and receptor conformation.



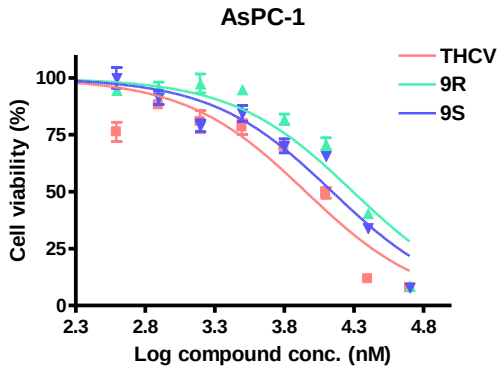
Compound	IC50 (µM)
THCV	14.712
9R	10.312
9S	18.933



Compound	IC50 (µM)
THCV	8.892
9R	10.835
9S	11.68

Figure 4. Effect of cannabinoid compounds on the proliferation of Panc-1 and HPAF-II pancreatic cancer cells

Shown below in figure 5 is THCV and HHC tested against the AsPC-1 cell line, and the HHC compound has slight variations within the IC_{50} values between the R/S-Isomers, but THCV shows a lower IC_{50} values across all three cell lines. Although the values are in micromolar concentration, the values are still low enough for viable data.

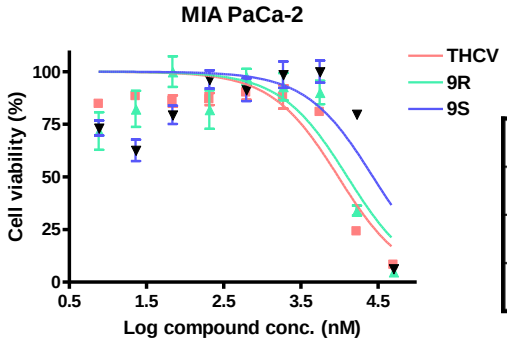


Compound	IC50 (µM)
THCV	8.975
9R	19.613
9S	13.902

Figure 5. Effect of cannabinoid compounds on the proliferation of AsPC-1 pancreatic cancer cells

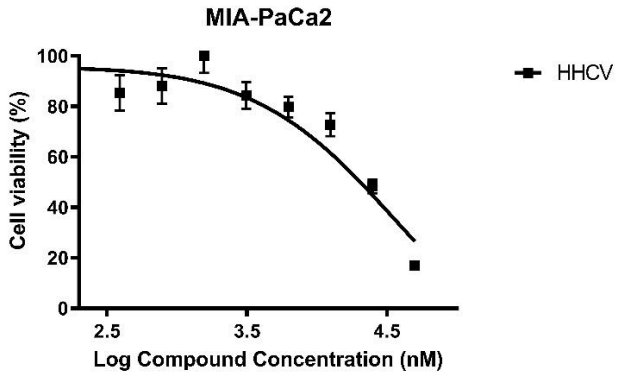
MIA-PaCa2 cell line data shown below in figure 6a, depicts the comparison of the various cannabinoids with the planar THCV providing a lower IC_{50} value compared to the flexible hydrogenated derivatives. In figure 6b-c the isolated graphs depict HHCV compared to PANC-1 and MIA-PaCa2 cells. HHCV is the hydrogenated derivative to THCV with the modification occurring in the cyclohexene to cyclohexyl ring. A pseudo-SAR was conducted on these compounds to determine the IC_{50} value and whether the hydrogenation played a role in the increase or decrease in IC_{50} value. In comparison of the compounds THCV had a lower IC_{50} than the other compounds that were tested.

Figure 6a



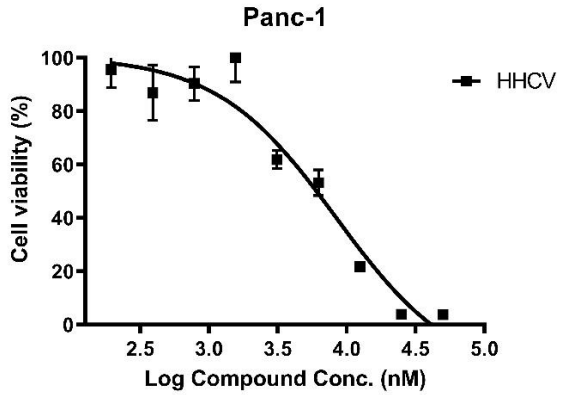
Compound	IC50 (µM)
THCV	9.675
9R	12.698
9S	27.243

Figure 6b.



Cell line	IC50 (µM)
Panc-1	5.567
MIA PaCa-2	21.228

Figure 6c.

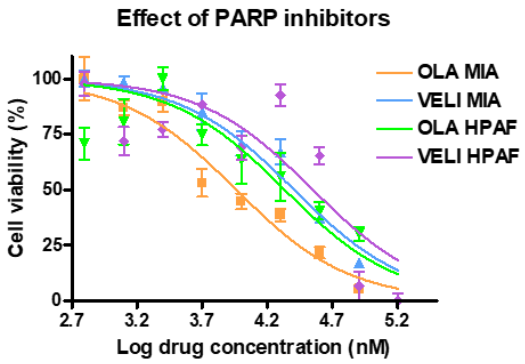


Cell line	IC50 (µM)
Panc-1	5.567
MIA PaCa-2	21.228

Figure 6a-c. Effect of cannabinoid compounds on the proliferation of MIA PaCa-2 pancreatic cancer cells. In Figure 6a above Mia-PaCa2 cells were tested against varying concentrations of THCV and HHC. In Figure 6b above Mia-PaCa2 cells were tested against varying concentrations of HHCV. Above in Figure 6c is the PANC-1 cells tested against varying concentrations of HHCV.

The compiled data of the compounds tested on various cell lines was compared to PARP inhibitors currently on the market or in clinical trials shown in figure 7, to provide more objective evidence towards the usage of HHC and THCV as possible pancreatic antineoplastic compounds.

Poly(ADP)-ribose polymerase (PARP) is a type of nuclear enzyme that helps repair DNA damage in cells [41]. PARP inhibitors work by preventing cancer cells from repairing, allowing apoptosis to occur [42]. These drugs are a type of targeted therapy used to help treat cancers. The use of these inhibitors as a control to compare THCV and HHC against the inhibitors show efficacy towards the ability for the rare cannabinoids, to be considered semi-efficient antineoplastics.



Cell line	Drug	IC50 (μM)
MIA PaCa-2	Olaparib	9.115
MIA PaCa-2	Veliparib	25.358
HPAF-II	Olaparib	21.468
HPAF-II	Veliparib	35.378

Figure 7. Effect of PARP inhibitors on pancreatic cancer cell lines

The various PARP inhibitors that were tested on the same cell lines that THCV, HHC, and HHCV were tested on, had generally a weaker response than the cannabinoids, which show a less efficacy towards the treatment of pancreatic cancer unless a higher dose is used which would entail other side effects that would be counterintuitive towards a treatment. PARP inhibitors are relatively new to the market but have been used in the treatment of ovarian, fallopian tube, and peritoneal cancer [43]. Indications that PARP Inhibitors can be used in the treatment of but not limited to lung, pancreatic, prostate, and kidney and bladder cancer are still being researched.

Conclusion

The introduction of THCV, HHC and HHCV as potential candidates [44] towards the treatment of pancreatic cancer through possible interaction of GPCR pathways that are found within PDAC cells could modulate and contribute to the pro-apoptotic and anti-proliferation properties that these cannabinoids produced in-vitro. The IC_{50} values of these compounds compared to PARP inhibitors which are known for treating various cancers, are much lower resulting in a more efficient compound for the specific treatment of pancreatic cancer. A semi-SAR study by hydrogenating THCV and producing HHCV, did provide slightly lower IC_{50} value on specific PDAC cell line. Although the IC_{50} values are lower compared to other active antineoplastic compounds on the market the treatment of Pancreatic cancer is still evolving and the need to produce antineoplastics is pertinent. Continued SAR and analogs studies are currently being conducted to increase bioavailability and increase IC_{50} values from micromolar to nanomolar concentrations.

Experimental Section

General Hydrogenation Conditions: A 20L flask equipped with a reflux condenser and an addition funnel was purged with argon for 10 minutes at 1 bar. Pd/C (0.1 molar %) was added to the reaction slowly using a powder funnel under argon. The flask was then purged with argon for 10 minutes at 1 bar. Ethanol (1L) was added slowly to avoid sparking the solvent. Cannabinoid (100 g) was dissolved in minimal amounts of ethanol. The solution was added to the flask under

argon and purged for 10 minutes at 1 bar. Afterwards, the atmosphere of argon was stopped, and an atmosphere of hydrogen (1 bar) was introduced. The reaction was then stirred at 25 °C for 3 hours or until complete by HPLC with a diode array detector. Upon completion, the reaction was purged with argon for 10 minutes at 1 bar. The reaction mixture was poured over 1–3-micron filter paper on a Buchner funnel and then concentrated *in-vacuo*. The crude oil was then dissolved in hexane and purified over silica (0 to 5% Ethyl Acetate). The fractions of interest were concentrated *in-vacuo* and then distilled to afford a yellow oil.

HHC ^{1}H NMR (500 MHz, CD3CN) δ 6.72 (s, 1H), 6.13 (d, J = 1.7 Hz, 1H), 6.08 (d, J = 1.7 Hz, 1H), 3.88 (pd, J = 6.1, 4.3 Hz, 0H), 3.11–3.03 (m, 1H), 2.45 – 2.34 (m, 3H), 2.18 (s, 1H), 1.87-1.79 (m, 2H), 1.63–1.48 (m, 2H), 1.42–1.23 (m, 5H), 1.31 (s, 3H), 1.19–1.03 (m, 3H), 1.00 (s, 3H), 0.95–0.85 (m, 5H),0.64 (dt, J = 12.8, 11.4 Hz, 1H).

HHC ^{13}C NMR (101 MHz, CD3CN) δ 157.07, 155.91, 143.39, 118.36, 111.32, 109.96, 108.38, 77.53, 64.22,50.26, 39.81, 36.40, 36.06, 33.69, 32.37, 31.70, 28.83, 28.14, 25.68, 23.31, 23.04, 19.37, 14.43, 2.01, 1.80,1.60, 1.39, 1.19, 0.98, 0.77.

HHCV ^{1}H NMR (500 MHz, C6D6) δ 6.36 (1H), 5.87 (1H), 4.95 (1H), 3.09 (1H), 2.53 (1H), 2.33 (2H), 1.69 (1H), 1.48 (4H), 1.29 (4H), 1.09 (1H), 0.98 (3H), 0.95 (1H), 0.90 (2H), 0.84 (3H), 0.74 (1H).

HHCV ^{13}C NMR (101 MHz, CD3CN) δ 155.84, 155.57, 142.63, 111.42, 110.71, 108.73, 77.67, 50.74, 49.92, 39.71, 38.30, 36.25, 33.18, 30.27, 28.69, 28.14, 24.75, 19.49, 14.49.

Synthesis of THCV: In a 20L reactor, under argon, was added CBDV (3 kg, 10.5 mol) and DCM (10L) set to stir for 1 hour. To the solution was added dropwise Triisobutylaluminum 1M solution in hexanes (2 L, 2 mol) over a period of 1 hour. The reaction was stirred at room temperature overnight. HPLC with a diode array detector showed no starting material, only desired product 10:1 D9-THCV: D8-THCV. The reaction was quenched with water and extracted with DCM. The combined organic was washed with brine, dried (Na_2SO_4) and concentrated in vacuo to give a dark red oil. The oil was purified via wiped film distillation. The NMR of the final product matches literature data [45].

THCV ^{1}H NMR (500 MHz, CD3CN) 6.19 (1H), 6.14 (1H) 5.44 (1H), 3.26 (1H), 2.66 (1H), 2.43 (2H), 2.16 (1H), 1.85 (1H), 1.73 (1H), 1.68 (3H), 1.61 (2H), 1.34 (3H), 1.05 (3H), 0.93 (3H)

THCV ^{13}C NMR (101 MHz, CD3CN) δ 155.2, 153.9, 141.4, 133.5, 118.7, 109.7, 108.3, 106.6, 75.4, 44.3, 36.5, 35.0, 30.8, 26.9, 26.2, 23.3, 22.0, 17.0, 12.5

List of Abbreviation

HPLC: High performance liquid chromatography
NMR: Nuclear magnetic resonance
MeCN: Acetonitrile
DCM: Dichloromethane
ATM: Atmosphere
Pd/C: Palladium on carbon
HRMS: High Resolution-Mass Spectrometry
GCMS: Gas Chromatography-Mass Spectrometry

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Supplementary Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

Conceptualization: ASA, PGJ, KPR, WC. Methodology: ASA, PGJ, KPR, WC. Investigation: YM, HYK, OA, GAR, ACC, TTT. Data Curation: YM, HYK, OA, GAR. Formal Analysis: GAR, PGJ, WC. Writing – Original Draft: GAR, TTT, PGJ, WC. Writing – Review and Editing: GAR, ASA, HYK, PGJ, WC. Supervision: ASA, PGJ, KPR, WC. Project Administration: ASA, PGJ, KPR, WC. All authors read and approved the final manuscript.

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Competing interests

The authors declare no competing interests

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