CANNABINOL INHIBITS PROLIFERATION AND INDUCES CELL CYCLE ARREST AND APOPTOSIS IN GLIOBLASTOMA, HEPATOCELLULAR CARCINOMA AND BREAST CANCER CELLS

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DEDICATION

I dedicate this thesis to my parents for supporting me with love.

ABSTRACT

Cannabis sativa is an agriculturally and medicinally plant with many pharmaceutical properties. Cancer is a deadly disease; it is estimated that it will cause over 80 thousand deaths in 2019 in Canada. Although numerous studies have demonstrated that cannabinoids have anti-tumorous properties in various cancers, the anti-malignant activities of cannabinol (CBN) on carcinogenesis and underlying mechanisms remain largely unknown. In this study, we provide evidence that CBN inhibits proliferation of A172, HB8065 and HCC1806 cells in a dose- and time-dependent manner. CBN regulates expression of cannabinoid receptors, CB2, GPR55 and GPR18 in different cell lines, while reducing levels of phosphorylated ERK1/2 in HCC1806 and phosphorylated AKT in A172 and HB8065 cells. We find that CBN induces apoptosis through downregulation of p21 and p27 and a G1 or S-phase cell cycle arrest through downregulation of cyclin E1, CDK1 and CDK2. These data support the medicinal potential of CBN in anti-cancer therapy.

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LIST OF ABBREVIATIONS

2-AG: 2-arachidonoylglycerol
AEA: Anandamide
AKT: serine/threonine-specific protein kinase B
CBs: Cannabinoids
CBD: cannabidiol
CBN: cannabinol
CBG: cannabigerol
THC: Tetrahydrocannabinol
CDK: cyclin-dependent kinase
ERK: extracellular signal-regulated kinases
MAPK: mitogen-activated protein kinases
RB: Retinoblastoma tumor suppressor protein
E2F: a family of transcription factors in eukaryotes
TRPA1: transient receptor potential ankyrin channel 1
TRPV2: transient receptor potential cation channel 2
PPARs: peroxisome proliferator-activated receptors
FAAH: Fatty acid amide hydrolase
MAGL: monoacyl-glycerol lipase
FADD: Fas-associated protein with death domain
IL-2: interleukin-2
IL-12: interleukin-12
IL-6: interleukin-6
IL-8: interleukin-8
IL-10: interleukin-10
TNF: tumor necrosis factor
PTEN: phosphatase and tensin homolog
PIP_3: Phosphatidylinositol (3,4,5)-trisphosphate
AC: adenylyl cyclase
ER: endoplasmic reticulum
PKA: protein kinase A
PI3K: phosphatidylinositol 3-kinase
mTOR: mammalian target of rapamycin
ROS: reactive oxygen species
p21/p27: cyclin-dependent kinase inhibitor proteins.
PPARs: peroxisome proliferator-activated receptors
FA2H: fatty acid 2-hydroxylase
PCNA: DNA polymerase processivity factor

1. INTRODUCTION

1.1 Cannabis plants, Cannabinoids, and Cannabinol

Cannabis plants have been applied as traditional medicine for thousands of years and the medical properties of cannabis have been studied for several decades[1]; medical cannabis has been legal in Canada since October 2018. Species of *Cannabis sativa* belong to the family of *Cannabaceae*. There are two groups of cannabis plants, marijuana and hemp, with different levels of psychoactive cannabinoid tetrahydrocannabinol (THC). Hemp plants are mainly cultivated for seeds and fiber and have THC lower than 0.3% (in Canada). To date, over 120 phytocannabinoids have been discovered in cannabis plants, including the two most abundant plant-derived phytocannabinoids - Δ^9-tetrahydrocannabinol (Δ^9-THC) and cannabidiol (CBD), which have been extensively studied for their potential in regulation of inflammation and anti-cancer properties.[2]

Based on origin, cannabinoids could be classified into three groups. The first group is composed of endocannabinoids, which are endogenous ligands and act as neurotransmitters activating cannabinoid receptors (CBRs) on the surface of cells. The main endocannabinoids are anandamide and 2-arachidonoyl glycerol (2-AG). The second group consists of the natural plant-derived cannabinoids which are isolated from the Cannabis plants. Over 100 bio-active compounds are identified as the phytocannabinoids that interact with the cannabinoid receptors throughout the human body, including the psychoactive compounds [mainly Δ^9-THC and to some extent Δ^8-THC and cannabigerol (CBG)] and non-psychoactive compounds [cannabinol (CBN), CBD and others]. The last

group is composed of designed drugs that have similar bio-active effects with endocannabinoids and natural cannabinoids in binding CBRs - synthetic cannabinoids, such as CP559040, HU-211 and WIN 55.212-2. In addition, combinations of natural cannabinoids sometimes show more potent effect than single cannabinoids. Sativex/Nabiximols, a 1:1 formulation of CBD and THC extracted and purified from cannabis plants, has been utilized to relieve pain in European and North American populations[3]. The cannabis botanical drug preparation (BDP) effectively contributes to antitumor responses (inhibition of proliferation and reduction of tumor size) in ER+/PR+, HER2+ and triple-negative breast cancer, compared to the signal pure THC cannabinoid[4].

Cannabinoids are produced and accumulated as cannabinoid acids in the fresh plants. However, the cannabinoid acids are unstable and they change into another form - cannabinoids including Δ^9-THC, CBD and CBG, upon prolonged storage, drying or exposure to heat[5]. In addition, Δ^9-THC is slowly degraded to CBN and this process can be expedited by exposure to heat and pressure.

Δ^9-THC, the most abundant psychoactive cannabinoid in cannabis, has been shown to be effective in many disorders in animals and humans. Δ^9-THC shares similar activities as anandamide, which is a potent agonist for both CB1 and CB2 receptors to regulate a range of molecular mechanisms. Some studies have indicated that Δ^9-THC inhibits proliferation and metastasis of tumorous cells and induces apoptosis and cell cycle arrests[6]. Additionally, Δ^9-THC may also interact with other cannabinoid-associated receptors, for example, the receptors of PPARs family[7]. Japanese researchers have found that Δ^9-THC upregulates the PPARα levels to stimulate FA2H (fatty acid 2-hydroxylase) expression in

human breast cancer MDA-MB-231 cells[8]; this activity may associate with the anti-cancer properties since FA2H is a tumor suppressor in breast cancer[9].

CBD, another well-researched cannabinoid, is produced from CBDA by degradation. Animal studies have shown that CBD lacks the psychoactive effects; therefore, CBD has the medicinal potential for numerous diseases, such as cancer or diseases with an inflammation component[10]. CBD is initially believed to interact with CB1 and CB2 receptors in a reasonably low concentration (1 uM), even though CBD has a low affinity for CB1 and CB2[11]. Recent studies have demonstrated that activities of CBD are associated with CB2 expression and CBD acts as an allosteric modulator to activate the CB2 signaling pathways[12]. Moreover, CBD has been shown to upregulate the phosphorylated ERK, consequently inhibiting the expression of *Id-1* gene to reduce cell proliferation and invasion[13]. Several lines of evidence also indicated that CBD up-regulates the expression of endocannabinoids, AEA and 2-AG[14,15]. The increase of 2-AG was observed in Caco-2 cells in response to CBD-treatment, which may contribute to CBD-mediated suppression of colon cancer[15].

CBN is metabolized from THC by breakdown of the THC molecules[16] (Figure 1), therefore some of the bio-active properties of CBN could be similar to CBD and THC. As a sub-product of THC, CBN is slightly psychoactive, with only 10% of the activity of Δ^9-THC[17]. Also, CBN has a low affinity to CB1 receptor but a high affinity to CB2 receptor[2,18]. It was pointed out that the activation of the G-protein-coupled receptor (GPCR) with binding to CBN is associated with cell signaling networks, such as PI3K/AKT and ERK signaling pathway, to mediate cell survival[19]. CBN and other cannabinoids, such as CBD,

have also been found to interact with transient receptor potential (TRP) channels which may act as ionotropic cannabinoid receptors[20,21]. CBN has been shown to potently activate TRPA1 while having a weak effect on TRPV2[22]. This ability may be associated with the anti-inflammatory and anti-cancer functions. Additionally, CBN, Δ^9-THC, and CBD could reverse the drug-resistant phenotype induced by multidrug resistance protein ABCG2 via inhibition of its ATPase activity in vitro[23]. On the other hand, CBN has anti-inflammatory properties. In a mouse study, CBN up-regulates the ERK-signaling protein kinases – protein kinase C to increase the secretion of IL-2 which is the essential cytokine to activate the T cell for immune response[24]. Other research has indicated that CBN, CBD and THC induced IL-6, IL-8, IL-12 and TNF release while increasing the release of the anti-inflammatory cytokine, IL-10 in human gingival epithelial cells; this immune suppression is associated with the CB2 – PI3K signaling[25]. Studies of the CBN activities however are still limited, so more research needs to be done.

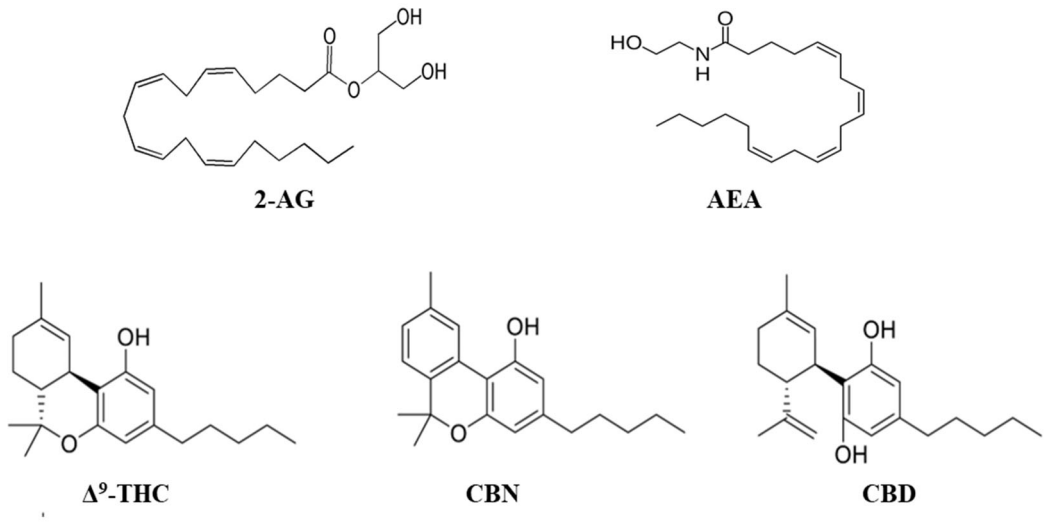

Figure 1. Structures of 2-AG, AEA, Δ^9-THC, CBN and CBD. 2-AG, 2-Arachidonoylglycerol; AEA, Anandamide; CBD, Cannabidiol; CBN, Cannabinol; Δ^9-THC, Δ^9-Tetrahydrocannabinol. Reprinted from *Progress in the Chemistry of Organic Natural Products,* by P. Morales, 2017, p. 19. Copyright © 2017 by Springer International Publishing. Reprinted with permission.

1.2 Endocannabinoid System

The endocannabinoid system is a lipid signaling network that consists of endogenous cannabinoids, cannabinoid receptors, and signaling molecules, such as the metabolic emzymes[26].

1.2.1 Endocannabinoids

Endocannabinoids are a group of lipophilic molecules produced and released from animal cells that regulate a range of physiological processes through binding to cannabinoid receptors[27]. AEA and 2-AG expressed in animals are two well-studied endocannabinoids. AEA is primarily released in high amounts in the brain and nervous tissues and has been shown to have a higher affinity to the CB1 receptor. 2-AG is expressed throughout the

whole body and can activate both CB1 and CB2 receptors. The amount and activity of these two well-researched endocannabinoids have been linked to many disorders, including inflammation, obesity and malignant diseases[26].

1.2.2 Cannabinoid receptors

Cannabinoid receptors located on cell membrane throughout the whole body are involved in regulation of many physiological processes. The two well-known cannabinoid receptors are CB1 and CB2, which can be activated by endogenous, plant-derived and synthetic cannabinoids[26]. The CB1 receptor is widely expressed in the central nervous system (CNS) and peripheral neurons where it has been shown to mediate release of neurotransmitters such as dopamine and glutamate[28]. In addition, the CB1 receptor is also expressed in other tissues, for instance intestine, lung and spleen[28–30]. The expression of CB1 receptor has been linked to cancer. Loss or inhibition of CB1 receptor enhanced growth of human colorectal cancers (CRC), while upregulation and activation of the CB1 receptor induced CRC cell death and apoptosis via the cAMP-dependent PKA pathway[31]. However, CB2 receptor exhibits abundant expression primarily in peripheral tissues, such as intestine, liver, and endothelium as well as the immune system, including bone marrow and immune cells. Recent studies indicated that CB2 receptor interacts with HER2 to form a HER2-CB2 complex in breast cancer, whereas inhibitors, such as Δ^9-THC, disrupt this complex and bind to CB2 receptor to promote anticancer activies[32]. CB1 and CB2 receptors belong to the G protein-coupled receptor (GPCR) superfamily. Plant-originating cannabinoid Δ^9-THC is a potent agonist that binds to both CB1 and CB2 receptors and the response to Δ^9-THC is influenced by expression of CB receptors and endocannabinoids. In contrast, CBD behaves as an antagonist of CB1 and CB2 receptors and is also involved in

suppression of immune cell migration through interaction with CB2 receptor[11]. The activation signal mediated by CB1 and CB2 receptors is transduced via $G_{\alpha\ i/o}$ signaling pathway, known as MAPK/ERK and AKT signaling pathways[33].

Recently, more endocannabinoid-related receptors, known as orphan G protein-coupled receptors, have been identified, including GPR18, GPR19 and GPR55[10,34]. GPR55, also known as the third cannabinoid receptor (CB3), was detected in both brain and peripheral tissues and was shown to stimulate the $G_{\alpha 12/13}$ signaling pathway, known as JNK and p38 MAPKs signaling pathways via anandamide and 2-AG binding[35]. Moreover, the upregulated GPR55 triggered metastasis in triple-negative breast cancer, this finding supports GPR55 being an underlying biomarker for specific cancer[36]. N-Arachidonyl glycine receptor (NaGly receptor), also known as GPR18, shares a similar role with the CB2 receptor in the peripheral tissue, immune cells, and CNS. Anandamide and Δ^9-THC are potent agonists of GPR18[37]. GPR19 was recently discovered as a novel cannabinoid receptor, while its function is unclear. Cannabinoid receptors play an important role in understanding of cellular physiological processes of cannabinoids and analysis of their potential medicinal properties[38].

1.2.3 Endocannabinoid signaling network

The endocannabinoid system is involved in various physiological and pathological processes, including multiple diseases, inflammation, and pain[27]. Endocannabinoids are rapidly released, transported, uptaken and degraded in cells to tightly control cell activities such as proliferation, cell cycle progression, and apoptosis (Figure 2). Activities of endocannabinoids are primarily determined by metabolic specific enzymes – fatty acid amide hydrolase (FAAH) and monoacyl-glycerol lipase (MAGL); FAAH is recognized to

degrade AEA and in part 2-AG, while the MAGL metabolized 2-AG after endocannabinoid uptake in animal cells[39]. Furthermore, high expression of FAAH and MAGL has been found in various cancers, including prostate, colorectal, and breast cancers[40]. Moreover, research has indicated that THC and CBD bind to fatty acid-binding proteins (FABPs) to reduce AEA transport to FAAH[41]. Interestingly, downregulation of FAAH has been demonstrated to promote anticancer activities of AEA by inhibiting the ERK, AKT and NF-κB pathways in lung cancer[42].

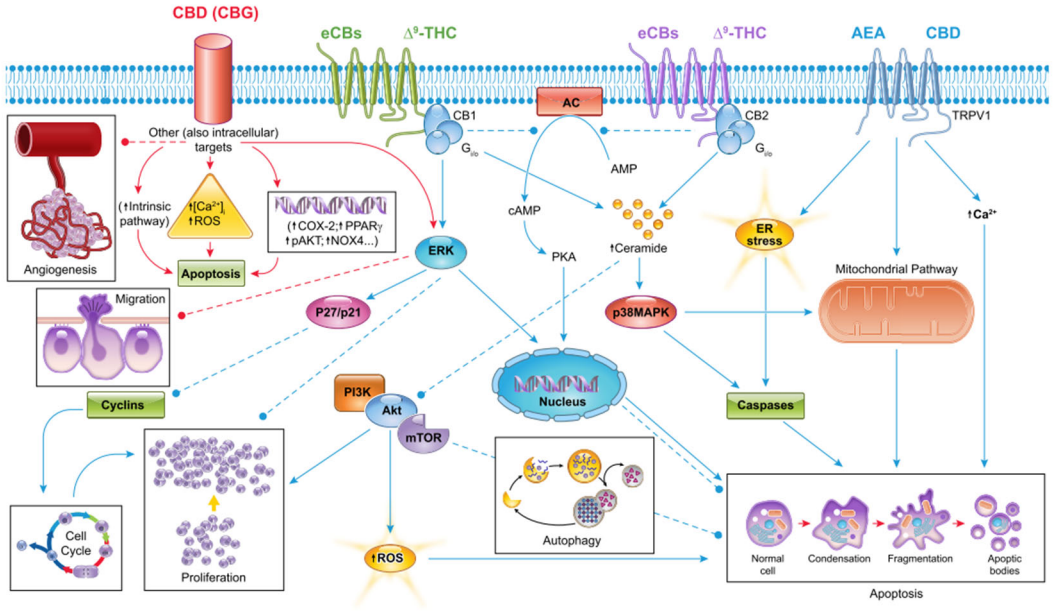

Figure 2. The endocannabinoid system and main downstream intracellular signaling pathways in cancer cells. AC, adenylyl cyclase; CBD, cannabidiol; CBG, cannabigerol; eCBs, endocannabinoids; ER, endoplasmic reticulum; Δ^9-THC, Δ^9-Tetrahydrocannabinol; PKA, protein kinase A; AKT, protein kinase B; PI3K, phosphatidylinositol 3-kinase; ERK, extracellular regulated kinase; MAPK, mitogen-activated protein kinase, mTOR, mammalian target of rapamycin; ROS, reactive oxygen species; p21/p27, cyclin-dependent

kinase inhibitor proteins. Blue arrows show pathways associated with cannabinoid/vanilloid receptor signaling mechanisms. Red arrows indicate the non-cannabinoid receptor pathways. Continuous lines mean stimulation and dotted lines mean inhibition. Adapted from "From Phytocannabinoids to Cannabinoid Receptors and Endocannabinoids: Pleiotropic Physiological and Pathological Roles Through Complex Pharmacology," by A. Ligresti, 2016, Physiological Reviews, 96, 4, p. 1599. Copyright © 2016 the American Physiological. Reprinted with permission.

1.3 Cancer and cannabinoids

Many Canadians are diagnosed with cancer and die from cancer every year. According to cancer statistics from the Canadian Cancer Society in 2019, cancer is the leading cause of death with approximately 30% of all deaths. Notably, breast cancer, the main cancer type in women, represents predictably 25% of all cancer types in women[43]. Numerous cancer studies have indicated that cancer development is complicated, can be caused by single-gene or multiple-gene mutations and epigenetic changes, by tumor microenvironment or as a result of metastases[44]. Cancer is a type of disease caused by out-of-control cell proliferation. Cancer progression is strongly associated with cell signaling pathways and the cell cycle. Studies have demonstrated that CB receptors were aberrantly expressed in several malignancies, including breast[45], leukemia[46], prostate[47], brain[48], and lung cancers[49]. Anti-tumorous properties of cannabinoids are thought to be associated with changes in the expression of CB receptors[27].

1.3.1 CB receptor signaling networks in cancer

Medicinal properties of cannabinoids are associated with numerous cellular

molecules and their signaling networks. Many reports have indicated that cannabinoids have remarkable anti-tumorous activities decreasing cell proliferation and cancer progression via stimulation of the CB receptors and subsequent signaling pathways[50]. Two cell signaling pathways, MAPK/ERK and PI3K/AKT signaling pathways have been shown to be negatively regulated by different cannabinoids via activation of CB receptors in cancer[51,52].

PI3K-AKT-mTOR signaling pathway has been demonstrated to regulate various cell processes, such as cell growth, apoptosis and autophagy and it is one of the most frequently dysregulated pathways in various human malignancies[53]. The autophagic and apoptotic induction of endoplasmic reticulum (ER) stress is tightly associated with down-regulation of AKT signaling[54]. Moreover, there are numerous inhibitors of the AKT pathway, including cannabinoids. CB2 receptor agonists, Δ^9-THC and JWH-133, reduce the growth of ErbB2-overexpressed breast cancer cells via downregulation of the AKT signaling pathway[45]. In hepatocellular carcinoma and glioblastoma cells, Δ^9-THC and JWH-133 also reduce the phosphorylated AKT levels to downregulate the autophagy-inhibitor, mTORC1 and stimulate ER stress to subsequently promote anti-tumorous action in cells[55,56]. CBD has been reported to promote ER stress and further induce autophagy and apoptosis via inhibiting AKT/mTOR signaling pathway and downregulating cyclin D1 which is an essential regulator in cell-cycle G1 phase[57].

The MAPK/ERK signaling pathway is another essential cell survival pathway and is usually a target for inhibiting proliferation of multiple cancer cell types[58]. Several studies have indicated that cannabinoids are promising drugs to inhibit the ERK signaling network.

Evidence exists that cannabinoids trigger both MAPK/ERK and PI3K/AKT signaling pathways to inhibit the cancer cells. CBD led to a dose-dependent down-regulation of ERK and AKT signalling in U87-MG and T98G glioma cells to suppress cancer cell growth, invasion, and angiogenesis[59]. Furthermore, in breast cancer, CBD inhibits activation of EGF-mediated ERK and AKT signaling and attenuates cell survival[60]. Moreover, Δ^9-THC has been shown to reduce activation of MAPK/ERK and PI3K/AKT signalling, resulting in the BAD-mediated apoptosis in colorectal cancer and Jurkat leukemia cells[52,61].

In summary, inhibition of AKT and/or ERK signaling pathways might be a potential pharmacological activity for cannabinoids.

1.3.2 Cell cycle and cannabinoids

Proliferation of cells in different tissues throughout animal bodies is individually regulated by cell cycle process. There are four phases in the cell cycle: G1 (Gap1), S (DNA synthesis), G2 (Gap2) and M (Mitosis) phases. Cells prepare DNA synthesis-associated proteins during the G1 phase. In the following S phase, chromosomal DNA is replicated. The production of other essential proteins occurs in G2 phase before entering mitosis. The distribution of chromosomes between two daughter cells occurs in M phase. When cells finish cell cycle, they enter the non-dividing condition called G0 phase[62,63].

The progression of cell cycle in cells is regulated by three protein families: cyclins, cyclin-dependent kinases (CDKs) and cyclin-dependent kinase inhibitors. Cyclin and CDK proteins are essentially required for cell cycle. CDKs interact with cyclins to form a CDK-cyclin complex, which is involved in every step of the cell cycle (Figure 2)[64]. Cyclins are a large family of proteins including cyclin A, cyclin B, cyclin D and cyclin E, which

participate in different steps of the cell cycle while specific cyclins are abundantly expressed in different time[65]. CDKs, the catalytic partners of cyclins, are crucial controllers of the cell cycle, and regulate cyclins and CDK inhibitors from one phase to the next phase[66]. The complex of CDK4, CDK6 and cyclin D (cyclin D1/2/3) is involved in DNA synthesis to drive the G1 phase into the S phase by phosphorylation of various critical proteins, RB protein in particular, which is a modulator of a pivotal transcription factor E2F[67]. The transition from G1 to S phase requires CDK2 - Cyclin E complex, whereas CDK2 - cyclin A complex triggers the beginning of DNA replication during S phase[68,69]. CDK2 - cyclin A complex as a key regulator promotes expression of CDK1, a crucial molecule for G2/M phase[70]. The G2/M phase is elicited by CDK1 – cyclin A and CDK1 – cyclin B complexes. CDK1 – cyclin A complex is detected in the middle-late S phase and is required for transition to the G2 phase[71]. CDK1 – cyclin B complex is known to act on late G2 phase and trigger mitosis in most animal cells via activation of various substrates[72]. Therefore, developing inhibitors of cyclins and CDKs may provide opportunities for cancer therapeutic intervention, because cyclins and CDKs are essential molecules for the survival of various cancer cells[73].

Downregulation of CDK activity by activating cyclin-dependent kinase inhibitors (CDK inhibitors) has been shown as a promising approach for the treatment of advanced cancers[74]. CDK inhibitors are a group of proteins including members of the INK4 family (e.g. p15, p16, p18, and p19) as well as members of the Kip family (e.g. p21, p27, and p57), which inhibit the activities of CDK to reduce cell cycle progression[75]. Several studies have demonstrated that p21 functions as a suppressor of the cell cycle to induce cell cycle arrest by inhibiting activities of CDK1 and CDK2[76]. Upregulation of p21 is triggered by a tumor

suppressor p53 which displays multiple anti-tumorous properties, including eliciting cell cycle arrest and apoptosis[75]. p27 is a tumor suppressor that regulates the cell-cycle kinases in G0 phase and S phase. p27 is thought to promote assembly of CDK4/6 – cyclin D1 complex in G0 phase whereas inhibit the assembly of CDK2 - cyclin E in G1 phase[77]. During the G1 – S transition and S phase, p27 reduces the suppressive effect on CDK2 – cyclin E complex and CDK2 – cyclin A complexes allow to activate initiation of DNA replication[77].

There is an increasing body of evidence that cannabinoids promote cell cycle arrest and inhibit proliferation of various cancer types. WIN 55.212-2 is a synthetic cannabinoid that binds to the CB2 receptor. WIN 55.212-2 inhibited cell proliferation and triggered cell cycle arrest at G0/G1 phase through up-regulation of p27 and down-regulation of CDK4[78]. Anandamide has a high affinity to CB1 receptor and is able to induce cell cycle arrest in S phase by downregulating the activities of CDK2 and Cdc25A, but up-regulating p21 and p27 levels in human breast cancer MDA-MB-231 cells[79]. THC contributed to cell cycle arrest in G2/M phase via enhancing p21 expression in human breast cancer cells[80]. The cells were arrested in G0/G1 phase after treatment with CBD which upregulated p53 protein level but downregulated p21 protein level, and subsequently reduced expression of CDK2 and cyclin E in human gastric cancer SGC-7901 cells[81].

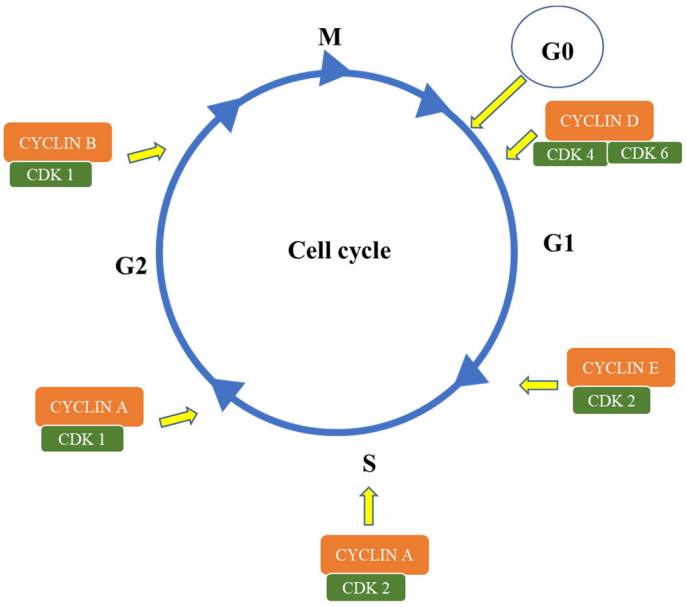

Figure 3. Cyclins and CDKs in general cell cycle progression of human cells. G0, gap 0 phase; G1, gap 1 phase; S, synthesis phase; G2, gap 2 phase. CDK, cyclin-dependent kinase.

1.3.3 Apoptosis and cannabinoids

Apoptosis, known as the process of programmed cell death, is an essential cell activity that occurs in various animal cells, including aging, damaged, mutated, and diseased cells[82]. Apoptosis is regulated by two major pathways: intrinsic and extrinsic. The intrinsic pathway, known as the mitochondrial pathway is primarily controlled by BCL-2 protein family, while the extrinsic pathway functions through Fas receptors on cell membranes activating the apoptotic downstream molecules via FADD and TRADD[83]. Cell survival-associated PI3K/AKT and MAPK/ERK signaling pathways block apoptosis by inhibition of pro-apoptotic molecules[84,85]; however, several lines of evidence also indicated that MAPK/ERK pathway might promote pro-apoptosis by activating the intrinsic and

extrinsic pathways[86]. Currently, anti-cancer studies explore multiple approaches to trigger cancer cell death, one of the most important strategies is to develop anti-cancer drugs that activate the apoptotic signaling pathways[87].

Over the last two decades, cannabinoids have been demonstrated to exhibit the control of cancer cell proliferation, autophagy and apoptosis in many in vitro and in vivo experiments[88]. Many studies have shown that cannabinoids stimulate the process of apoptosis in various cancer cell lines, such as glioma[51], breast[89], liver[55], prostate[90] cancers. In prostate cancer LNCaP and PC3 cells, synthetic cannabinoid WIN-55,212-2 triggers cell cycle arrest in G0/G1 phase and subsequently induces apoptosis via inhibition of PIK3/AKT pathway[90]. Other studies indicated that CBD significantly inhibits cell proliferation and induces the pro-apoptosis through activation of intrinsic apoptotic pathways in prostate cancer cells; moreover, the complex of CBD-BDS (Cannabis extract from GW Pharmaceuticals Ltd.) reduced LNCaP xenograft growth in mice[91]. CBD inhibits cell proliferation and induces apoptosis through up-regulation of reactive oxygen species (ROS) in glioma stem cells[92]. In addition, once activated by specific cannabinoids including WIN-55212, JWH133 and THC, cannabinoid receptors may interact with the Fas receptors, such as the TNF receptor, subsequently inducing tumor cell apoptosis[93,94].

In summary, cannabinoids have shown pro-apoptotic activities in numerous in vitro and in vivo cancer studies and thus may represent potential agents for cancer therapies.

1.3.4 The main challenges currently in cancer treatment

To date, tumor treatment primarily includes surgical removal, radiation exposure and chemotherapy or a combination of those three methods by cancer type, tumor size and

location (NIH, 2019). Chemical treatment is a novel approach to prevent cancer development in clinical therapy; however, chemotherapy can lead to drug resistance[95], eventually leading to tumor re-development and metastasis. The strategy to overcome drug resistance is delivering higher doses of chemotherapy or a combination of different agents, such as inhibitor of drug-resistance molecule ABCG2 family[96]. This, however, may result in selection of cancer cells with higher drug tolerance[97]. Therefore, development of new drugs is a necessity for anti-cancer chemotherapy.

In the last decade, studies showed many anti-cancer properties of medical cannabis and cannabinoids both in vitro and in vivo. Some products of medical cannabis have been applied in clinical trials, such as Sativex and Nabilone[50]. Interestingly, THC, CBD and CBN have been demonstrated to reverse the drug-resistant phenotype induced by multidrug-resistant protein ABCG2 via inhibition of its ATPase activity in vitro[23]. With considerable evidences of anti-cancer activities and lack of psychoactive properties, CBN may be a potential therapeutic strategy for cancer patients.

In summary, some previous studies have demonstrated promising therapeutic properties of cannabinoids against the progression of various types of malignancies, including prostate, breast, liver, lung, and brain cancers. CBN is a natural cannabinoid with a structure similar to THC and CBD, and primary affinity to CB2 receptor. It has shown the anti-proliferative properties in prostate cancer cells[91]. However, the role of CBN in blocking carcinogenesis and the underlying mechanisms remain largely unknown. In this study, we investigated the anti-cancer effects of CBN on glioblastoma, hepatocellular carcinoma, and breast cancer cells.

2. Hypothesis

In this study, I hypothesized that CBN inhibits proliferation of cancer cells via suppressing proliferative signaling pathway(s) and induces cell cycle arrest and apoptosis.

3. Materials and methods

3.1 Main reagents

Cannabinoids: Δ^9-THC (Cat#T4764), CBD (Cat#C-045) and CBN (Cat#C-046) were purchased from Sigma. 1.0 mg/ml stock solutions were prepared by dissolving cannabinoids in methanol and stored at -20 ℃.

3.2 Cell culture and maintenance

Human lung normal fibroblast WI-38 (CCL-75), Human embryonic kidney epithelial cells HEK293 (CRL-1573) and HepG2 human hepatoblastoma cells (HB-8065) purchased from ATCC (Rockville, MD, USA) were cultured in Eagle's Minimum Essential Medium (Cat# 320-026-CL, WISENT INC., Quebec, Canada) supplemented with a final concentration of 10% heat-inactivated Premium Grade Fetal Bovine Serum (Cat# 97068-085, VWR International LLC, Radnor, USA), and 1% Penicillin and Streptomycin solution (10,000 IU Penicillin and 10000 ug/ml Streptomycin, Cat#450-201-EL, WISENT INC., Quebec, Canada). Human foreskin normal BJ-5ta cell line (CRL-4001) was purchased from ATCC (Rockville, MD, USA) and cultured in 4:1 mixture of Dulbecco's Modified Eagle's Medium (Cat# 319-005-CL, WISENT INC., Quebec, Canada) and Medium 199 containing 4 mM L-glutamine, 4.5 g/L glucose and 1.5 g/L sodium bicarbonate, 0.01 mg/ml Hygromycin B, 10% heat-inactivated Premium Grade Fetal Bovine Serum (Cat# 97068-085, VWR International LLC, Radnor, USA), and 1% Penicillin and Streptomycin solution. Human glioblastoma A172 cells (CRL-1620, mutant genes: CDKN2A and PTEN) were purchased from ATCC (Rockville, MD, USA) and cultured in Dulbecco's Modified Eagle's Medium (Cat# 319-005-CL, WISENT INC., Quebec, Canada) supplemented with a final

concentration of 10% heat-inactivated Premium Grade Fetal Bovine Serum (Cat# 97068-085, VWR International LLC, Radnor, USA), and 1% Penicillin and Streptomycin solution. Human mammary gland squamous cell carcinoma HCC1806 cells (CRL-2335, mutant genes: CDKN2A, KDM6A, STK11 and TP53) were purchased from ATCC (Rockville, MD, USA) are cultured in RPMI-1640 medium (Cat# 350-000-CL, WISENT INC., Quebec, Canada) supplemented with a final concentration of 10% heat-inactivated Premium Grade Fetal Bovine Serum (Cat# 97068-085, VWR International LLC, Radnor, USA), and 1% Penicillin and Streptomycin solution. All cell lines were grown in a BSL laboratory with limited personal access according to APPENDIX A. All cells were incubated at 37 ℃ in a humidified atmosphere of 5% CO_2. The culture medium was replaced with fresh complete medium every 2 or 3 days until cell confluency reached 90% for further experiments.

3.3 Cell Viability Test

Once grown to 90% confluency in a 10 cm culture dish, cells were detached using 1 to 1.5 ml TRYPSIN/EDTA (0.25% Trypsin and 2.21 mM EDTA-4Na, Cat#325-043-EL, WISENT INC., Quebec, Canada). Resuspended cells were seeded in 96-well plates at a density of 3×10^3 cells/well in 150 µl cultural medium. Cells were cultured at 37 ℃ in a humidified atmosphere of 95% fresh air and 5% CO_2. At 24 h after incubation, the culture media was removed, and cells were treated with the newly prepared complete media with a range of concentrations of THC, CBD, and CBN (0 µM to 20 µM). A broad range of concentrations was examined to figure out the appropriate effective/cytotoxic concentration for each cannabinoid. Treatment media were changed every 24 or 48 h of exposure depending on cell confluency. The concentration of methanol in culture medium was

normalized in all treatments.

Cell viability test was performed using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue) colorimetric metabolic activity assay with the Cell proliferation kit I (#11465007001, Roche, Ontario, Canada) according to the manufacturer's instructions (APPENDIX B). Cell proliferation rate was determined at specific time points (0, 1, 2, 3, 4 and 5 days). Briefly, the 96-well plate was removed from incubator, 15 µl of MTT labeling reagent was added to each well, the cells were then placed back in the incubator. At 4 h after incubation, 150 µl of MTT solution was added to each well and the plate continued to incubate in the CO_2 incubator overnight. Absorbance was measured using a microplate reader at 595 nm wavelength. The cell viability was calculated by comparing to the control treatment. All experiments were repeated three times, each test was done in triplicate.

3.4 Cell cycle and apoptosis analyses

Once grown to 90% confluency in a 10 cm culture dish, cells were detached using TRYPSIN/EDTA (0.25% Trypsin and 2.21 mM EDTA-4Na, Cat#325-043-EL, WISENT INC., Quebec, Canada). The cells were replated in 6-well culture plates at a density of 6×10^4 cells/well. At 24 h after incubation, cells were exposed to 10 µM and 15 µM CBN or to methanol as control. Culture medium was replaced every day with fresh treatment-medium until the recommended time point (96 h). The equivalent concentration of methanol in the culture medium was normalized in all treatments. At the endpoint of treatment, the cells were detached by using TRYPSIN/EDTA and harvested by centrifugation at 1000 rpm for 5 min. The cell pellet was washed twice with 6 ml ice-cold PBS followed by centrifugation

at 1000 rpm for 5 min each.

For cell cycle arrest analysis, the cell pellet was resuspended in 1ml ice-cold PBS and then resuspended cells were fixed in 9 ml ice-cold absolute ethanol overnight at -20 ℃. The fixed cells could be stored at -20 ℃ for up to 2 months. The cells were centrifuged at 1000 rpm for 5 min to remove the ethanol. After being washed twice with 6 ml ice-cold PBS-Tween-20 buffer, the cells were centrifuged at 1000 rpm for 5 min. The pellet was resuspended and incubated with a 0.5 ml propidium iodide (PI) working solution (100 ug/ml PI and 100 ug/ml RNase A in ice-cold PBS-Tween-20 buffer) and incubated for 30 min at room temperature. 0.5 ml mixture was transferred to a flow cytometer test tube. The cell cycle stage was analyzed using a FACS flow cytometer, BD FACSAria Fusion (BD Biosciences, California, United States). Data are analysed from three independent measurements, and at least 10,000 cells were counted for each measurement.

Cell apoptosis was analyzed using FITC Annexin V Apoptosis Detection Kit (Cat#556547, DB Pharmingen, Mississauga, Canada) according to the manufacturer's instructions. Briefly, at 96 h after treatment, floating cells in the medium were collected. The adherent cells were detached using TRYPSIN/EDTA and harvested by centrifugation at 1000 rpm for 5 min. After two washes with cold PBS, the cell pellet was resuspended in 1 × Binding buffer at a concentration of 1×10^6 cells/ml. The suspension was transferred to a 5 ml test tube when 5 ul of FITC Annexin V and 5 ul propidium iodide (PI) were added. The cells were gently vortexed and incubated for 15 min at room temperature in the dark. The reaction was stopped by adding 400 ul of 1 × Binding Buffer. Untreated cells were also used as a control for double staining (single staining for PI stain and FITC Annexin V stain).

The cells were analyzed immediately (within an hour) after staining using a FACScan flow cytometer BD FACSAria Fusion (BD Biosciences, California, United States). Data are analysed from three independent measurements, and at least 10,000 cells were counted for each measurement.

3.5 Exposure of cancer cells to CBN

Once grown to 90% confluency in 10 cm culture dish, cells were detached using TRYPSIN/EDTA (0.25% Trypsin and 2.21 mM EDTA-4Na, Cat#325-043-EL, WISENT INC., Quebec, Canada) and re-plated in 6-well plates. Medium was replaced every 2 or 3 days until cell confluency reached to 80%. Cells were then exposed to either 10 μM and 15 μM CBN or methanol as control, and the medium was replaced with fresh treatment-medium every day until the recommended time point (96 h). The concentration of methanol in the culture medium was normalized in all treatments. In the period of 96-hour treatment, cells may grow over 90% confluency. In this case, cells would be detached, resuspended and kept growing for 96 h. At the endpoint, cells were harvested using TRYPSIN/EDTA for further experiments.

3.6 Western blot analysis

The detailed Western blotting protocols are in APPENDIX C. Briefly, after being grown to 90% confluency in 10 cm culture plate, the cells were washed twice with 10 ml cold PBS. Whole cellular protein lysate was prepared in 100-150 μl RIPA lysis buffer (6-well plate, 60 μl of RIPA buffer would be used) with 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% deoxycholate, 1 mM sodium orthovanadate, and 1 mM PMSF. After sonication, the protein solution was centrifuged at

12,000 ×g for 10 min. The supernatant was transferred to a new microtube and stored. A small aliquot (2 ul) of lysates were diluted with sterile ultra-pure water in a 1:40 ratio and reserved for protein determination using protein assay reagents from Bio-Rad. 25 µl of diluted protein solution was added to 1.25 ml Bio-Rad assay reagent followed by incubation at room temperature for 10 min. Protein concentration was determined immediately using a NanoDrop 2000/2000c Spectrophotometer (ThermoFisher Scientific Company, Wilmington, DE). An equal amount of protein sample (60-100 ug) was prepared with 4 × loading buffer (0.0625 M Tris, 2% SDS, 10% glycerol, 0.01% bromophenol blue and 1% 2-mercaptoethanol) and RIPA lysis buffer and heated at 95℃ for 10 min. The protein sample and PageRuler Plus Prestained Protein Ladder (Cat#26620, Thermo Scientific, Massachusetts, USA) were loaded and electrophoretically separated on a 10% SDS-PAGE in slab gels of 10% polyacrylamide at 100V, and electrically transferred to polyvinylidene difluoride membrane (GE Healthcare Biosciences). After transfer, the membrane was blocked with 5% (w/v) non-fat dry milk for 1h.

Following the protocol, membranes were incubated with primary antibodies at 4 ℃ overnight. Cannabinoid receptor 1 expression levels were detected using 1:100 dilution of mouse monoclonal CB1 Antibody (2F9) (Cat#sc-293419, Santa Cruz Biotechnology, Inc., Texas, United States). Cannabinoid receptor 2 expression levels were detected using 1:200 dilution of mouse monoclonal CB2 Antibody (3C7) (Cat#sc-293188, Santa Cruz Biotechnology, Inc., Texas, United States). GPR55 expression levels were detected using 1:500 dilution of rabbit monoclonal GPR55 antibody (Cat# LS-C747960, Lifespan Biosciences, Washington, United States). GPR18 expression levels were detected using 1:500 dilution of rabbit monoclonal GPR18 antibody (Cat# LS-C352266, Lifespan

Biosciences, Washington, United States). GPR19 expression levels were detected using 1:500 dilution of rabbit monoclonal GPR19 antibody (Cat# LS-C352257, Lifespan Biosciences, Washington, United States). p-ERK1/2 levels were detected using a 1:1,000 dilution of rabbit monoclonal phospho-p44/p42 MAPK (ERK 1/2) antibody (Cat# CS-9101, Cell Signaling Technologies, Massachusetts, United States). ERK1/2 expression levels were detected using 1:1,000 dilution of rabbit monoclonal p44/p42 MAPK (ERK1/2) antibody (Cat#CS-4595, Cell Signaling Technologies, Massachusetts, United States). p-AKT1/2/3 levels were detected using 1:200 dilution of rabbit monoclonal p-AKT1/2/3 Thr308 antibody (Cat#sc-16646, Santa Cruz Biotechnology, Inc., Texas, United States). AKT1 expression levels were detected using 1:1,000 dilution of rabbit monoclonal AKT1 antibody (Cat#ab32505, Abcam Inc, Cambridge, United Kingdom). P21 expression levels were detected using 1:500 dilution of rabbit monoclonal p21 waf1.Cip1 (12D1) antibody (Cat#cs-2947, Cell Signaling Technologies, Massachusetts, United States). p27 expression levels were detected using 1: 500 dilution of rabbit monoclonal p27 antibody (Cat#cs-2552, Cell Signaling Technologies, Massachusetts, United States). CDK1 expression levels were detected using 1: 500 dilution of rabbit monoclonal CDK1 antibody (Cat#ab-32384, Abcam Inc, Cambridge, United Kingdom). CDK2 expression levels were detected using 1: 500 dilution of rabbit monoclonal CDK2 antibody (Cat#cs-2546, Cell Signaling Technologies, Massachusetts, United States), CDK4 expression levels were detected using 1:500 dilution of mouse monoclonal CDK4 antibody (Cat#cs-2906, Cell Signaling Technologies, Massachusetts, United States), Cyclin A2 expression levels were detected using 1:500 dilution of mouse monoclonal Cyclin A2 antibody (Cat#cs-4656, Cell Signaling Technologies, Massachusetts, United States), Cyclin B1 expression levels were detected

using 1:500 dilution of mouse monoclonal Cyclin B1 antibody (Cat#cs-4135, Cell Signaling Technologies, Massachusetts, United States), Cyclin E1 expression levels were detected using 1:500 dilution of mouse monoclonal Cyclin E1 antibody (Cat#cs-4129, Cell Signaling Technologies, Massachusetts, United States).

GAPDH and β-actin protein levels were detected as loading controls, using 1:1,000 dilution of mouse monoclonal GAPDH antibody (Cat#sc-47724, Santa Cruz Biotechnology, Inc., Texas, United States) and 1:1,000 dilution of rabbit monoclonal Actin antibody (Cat#ab-179467, Abcam Inc, Cambridge, United Kingdom), respectively. After overnight incubation, the membrane was washed three times with 0.1% Tween-20 in PBS (PBS-T). The blot was then incubated with 1:10,000 dilution of either Bovine anti-mouse secondary antibody (Cat#sc-2371, Santa Cruz Biotechnology, Inc., Texas, United States) or Donkey anti-Rabbit secondary antibodies (Cat#sc-2313, Santa Cruz Biotechnology, Inc., Texas, United States) for 1 h at room temperature. After three washes with PBS-T, the membrane was exposed to ECL Prime Western Blotting System (Cat#GERPN2232, GE Healthcare, Chicago, USA) and visualized using the FluorChem HD2 Imaging System (Cell Biosciences, California, United States). Date of protein expression are analysed from three measurements of western blot results by ImageJ programme.

3.7 Statistical Analysis

Results are examined as mean ± SD of data from ≥3 independent experiments for each treatment group. Student's two-tailed t-test was used to compare treatment groups to the vehicle control group by Microsoft® Office Excel 365 Proplus. Differences between groups were considered significant at the level of $p < 0.05$ (*) and $p < 0.01$ (**).

4. RESULTS

4.1 CBN inhibits proliferation of cancer cells via AKT and ERK1/2 pathways

To establish a cell model system that allows us to determine the effect of CBN on cancer cell biology, we measured protein levels of cannabinoid-binding receptors CB1 and CB2 in normal cell lines – WI38, BJ-5ta and HEK-293, and cancer cell lines HCC1806, A-172, HB-8065 and TIB-152. Western blot analysis showed that CB1 and CB2 were expressed in both non-tumorous and tumorous cell lines examined (Fig. 1A). A previous report has indicated that CBN has a high affinity to CB2 receptor and low to CB1 receptor[18], suggesting that the cancer cell lines (A172, HCC1806, and HB8065) with high expression levels of CB2 would provide a good model system to determine the effect of CBN on cell proliferation. MTT assay indicated that CBN suppressed proliferation of all three examined cancer cell lines in a time- and dose-dependent manner (Fig. 1B). Interestingly, the same dose of CBN had no effect on normal fibroblast (WI-38) proliferation (Fig. 1B). To see the contributing role of cannabinoid receptors and downstream signaling pathways in CBN-mediated inhibition of proliferation, we determined the receptor protein levels and measured the status of ERK1/2 and AKT pathways. Western blot analysis showed that both 10 μM and 15 μM CBN enhanced CB2 expression in A-172 and HB8065 cells, suppressed GPR18 expression in A-172 cells and reduced GPR55 expression in HB8065 cells (Fig. 1C). In HCC1806 cells, 15 μM CBN inhibited expression of GPR55 and CB2 but highly increased the GPR18 protein level. 10 μM CBN, however, had not much effect (Fig. 1C). Furthermore, the phosphorylated ERK1/2 was upregulated in A-172 and HB8065 cells in response to CBN in a dose-dependent manner, while it was downregulated in HCC18065

cells (Fig. 1D). Moreover, phosphorylated AKT was downregulated in A-172 and HB8065 cells in response to CBN, whereas it was upregulated in HCC1806 cells (Fig. 1D). Taken together, these results may suggest that CBN inhibits proliferation of malignant cells through interaction with their receptors and suppression of ERK1/2 and AKT pathways.

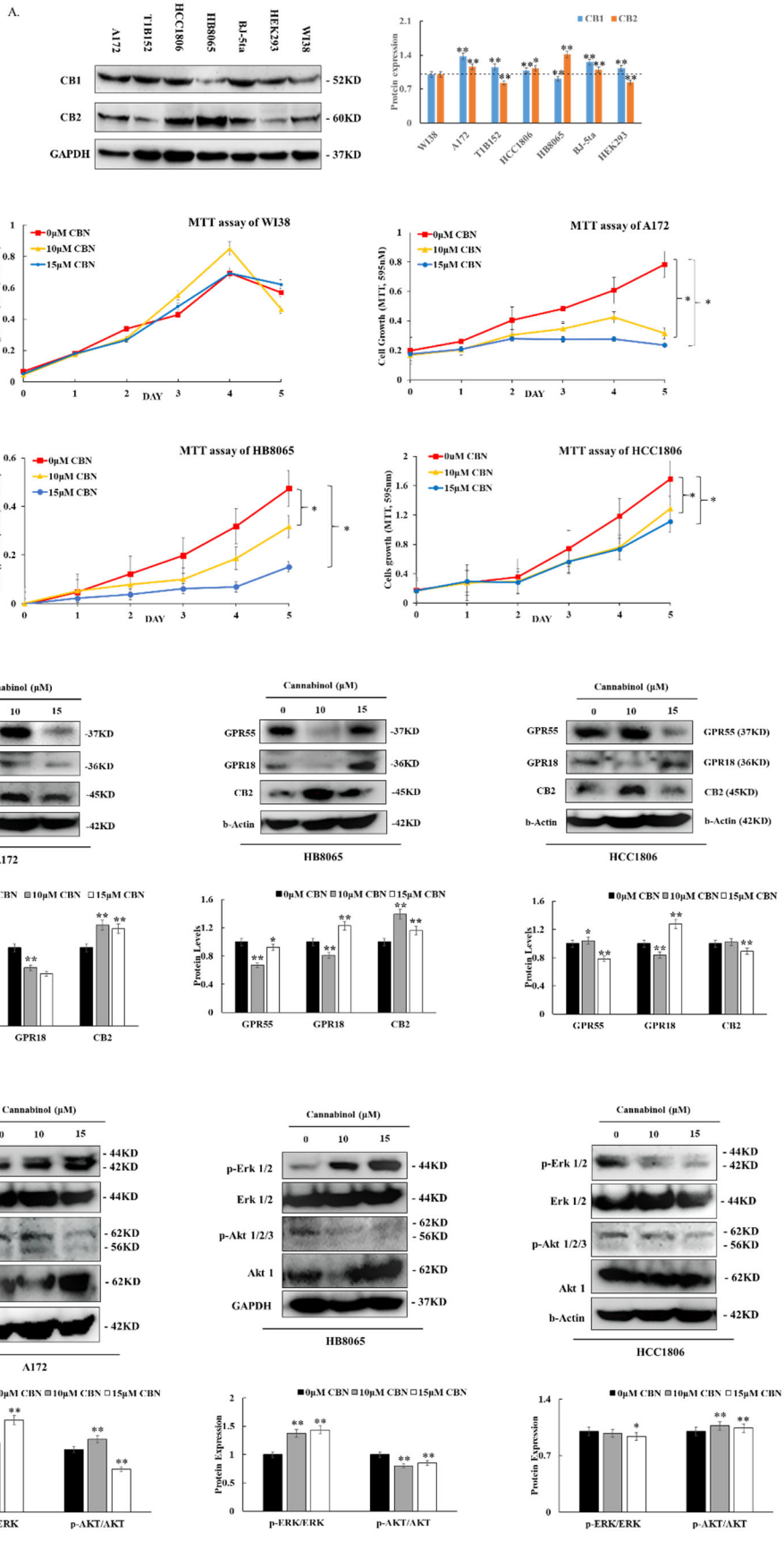
A.
A172
T1B152
HCC1806
HB8065
BJ-5ta
HEK293
WI38
CB1
CB2
GAPDH
- 52KD
- 60KD
- 37KD
Protein expression
CB1 CB2
B.
MTT assay of WI38
MTT assay of A172
MTT assay of HB8065
MTT assay of HCC1806
0μM CBN
10μM CBN
15μM CBN
Cell growth (MTT, 595nM)
Cell Growth (MTT, 595nM)
Cells growth (MTT, 595nm)
DAY
C.
Cannabinol (μM)
0 10 15
GPR55
GPR18
CB2
b-Actin
-37KD
-36KD
-45KD
-42KD
GPR55 (37KD)
GPR18 (36KD)
CB2 (45KD)
b-Actin (42KD)
A172
HB8065
HCC1806
Protein Levels
0μM CBN 10μM CBN 15μM CBN
D.
p-Erk 1/2
Erk 1/2
p-Akt 1/2/3
Akt 1
GAPDH
- 44KD
- 42KD
- 62KD
- 56KD
- 37KD
Protein Expression
p-ERK/ERK
p-AKT/AKT

Figure 4. Suppressive effect of CBN on cancer cell proliferation via AKT and ERK1/2 pathways. (A) Whole cellular lysates prepared from the indicated cell lines were subjected to western blot analysis using antibodies against CB1 and CB2. GAPDH was used as a loading control. Relative densitometry was presented as a ratio of target protein to b-actin or GAPDH. (B) A172, HB8065, HCC1806, and WI38 cells were treated with either 10 μM and 15 μM CBN; MTT assay was performed as described in the "Method". (C) At 96 h after treatment, whole cellular lysates were prepared and subjected to western blot analysis using antibodies to CB2, GPR18 and GPR55; actin was used as a loading control. Relative densitometry was presented as a ratio of target protein to b-actin. (D) At 96 h after treatment, whole cellular lysates were prepared and subjected to western blot analysis using antibodies to p-AKT1/2/3, AKT1, p-ERK1/2, ERK1/2; actin and GAPDH served as loading controls. Relative densitometry was presented as a ratio of target protein to b-actin (or GAPDH). Data are expressed as the mean ± SD (N=3), * $P<0.05$ and ** $P<0.01$ with comparison to the control group.

4.2 CBN modulates cell cycle progression of cancer cells

We next looked at the effect of CBN on cell cycle of three selected cancer cell lines A172, HB8065 and HCC1806 using flow cytometry. Cell cycle analysis indicated that 15 μM CBN induced S phase cell cycle arrest in A-172 cells and G1 arrest in HCC1806 cells, while 10 μM CBN had no effect (Fig. 5A, upper and lower panels). Interestingly, 15 μM CBN shortened S phase in HB8065 cells (Fig. 5A, middle panel). To understand the underlying mechanism, we determined protein levels of relevant cell cycle regulators. Western blot analysis showed that p21, p27, CDK1 and CDK2 were downregulated, while cyclin B2 was upregulated in A-172 cells exposed to CBN (Fig. 5B, left panel). P21 and cyclin A2 were downregulated in HB8065 cells in response to CBN (Fig. 5B, middle panel). Western blot analysis also indicated the downregulation of CDK2 and cyclin E1 and upregulation of CDK1 and CDK4 in HCC1806 cells exposed to CBN (Fig. 5B, right panel). These results suggest that CBN may induce cell cycle arrest via downregulating cyclins and/or CDKs, eventually contributing to the CBN-mediated proliferative inhibition of malignant cells.

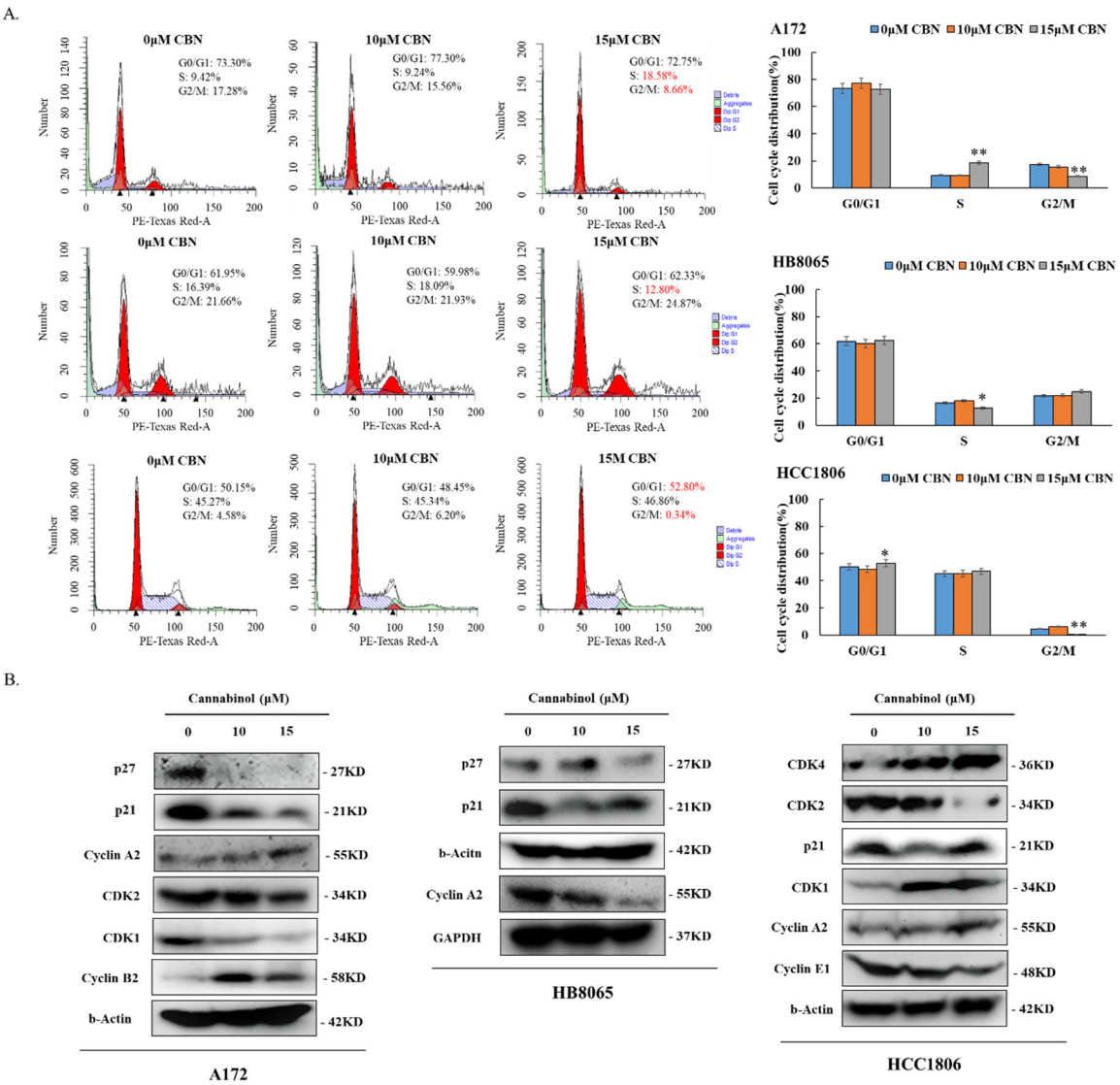

Figure 5. CBN induced cell cycle arrest in cancer cells. A-172, HB8065 and HCC1806 cells were exposed to the indicated concentrations of CBN. (A) At 96 h after exposure, the cells were harvested for cell cycle analysis using a flow cytometer as described in the "Methods". (B) At 96 h after treatment, whole cellular lysates were prepared and subjected to western blot analysis using antibodies to p21, p27, CDK1, CDK2, CDK4, Cyclin A2, Cyclin B2, Cyclin E1; b-Actin and GAPDH served as loading controls. Data are expressed as the mean ± SD (N=3), * $P<0.05$ and ** $P<0.01$ with comparison to the control group.

4.3 CBN induces apoptosis of cancer cells

To see the effect of CBN on cancer cell apoptosis, we treated cells with either 10 μM or 15 μM CBN for 96 hours and analyzed apoptosis with a flow cytometer. Apoptosis analysis indicated that CBN significantly induced apoptosis of A172, HB8065 and HCC1806 cells ($p<0.05$) compared to the control groups (Figure 6A-C). In A172 cells, CBN induced dose-dependent apoptosis (Fig. 6A). In HB8065 cells, 15 μM CBN caused a significant induction in apoptosis (Fig. 6B), 10 μM CBN had no effect. Interestingly, in HCC1806 cells, 10 μM CBN induced apoptosis, while 15 μM CBN attenuated apoptosis (Fig. 6C). Our results suggest that CBN induces cancer cell apoptosis that may also contribute to the CBN-mediated proliferative inhibition of malignant cells.

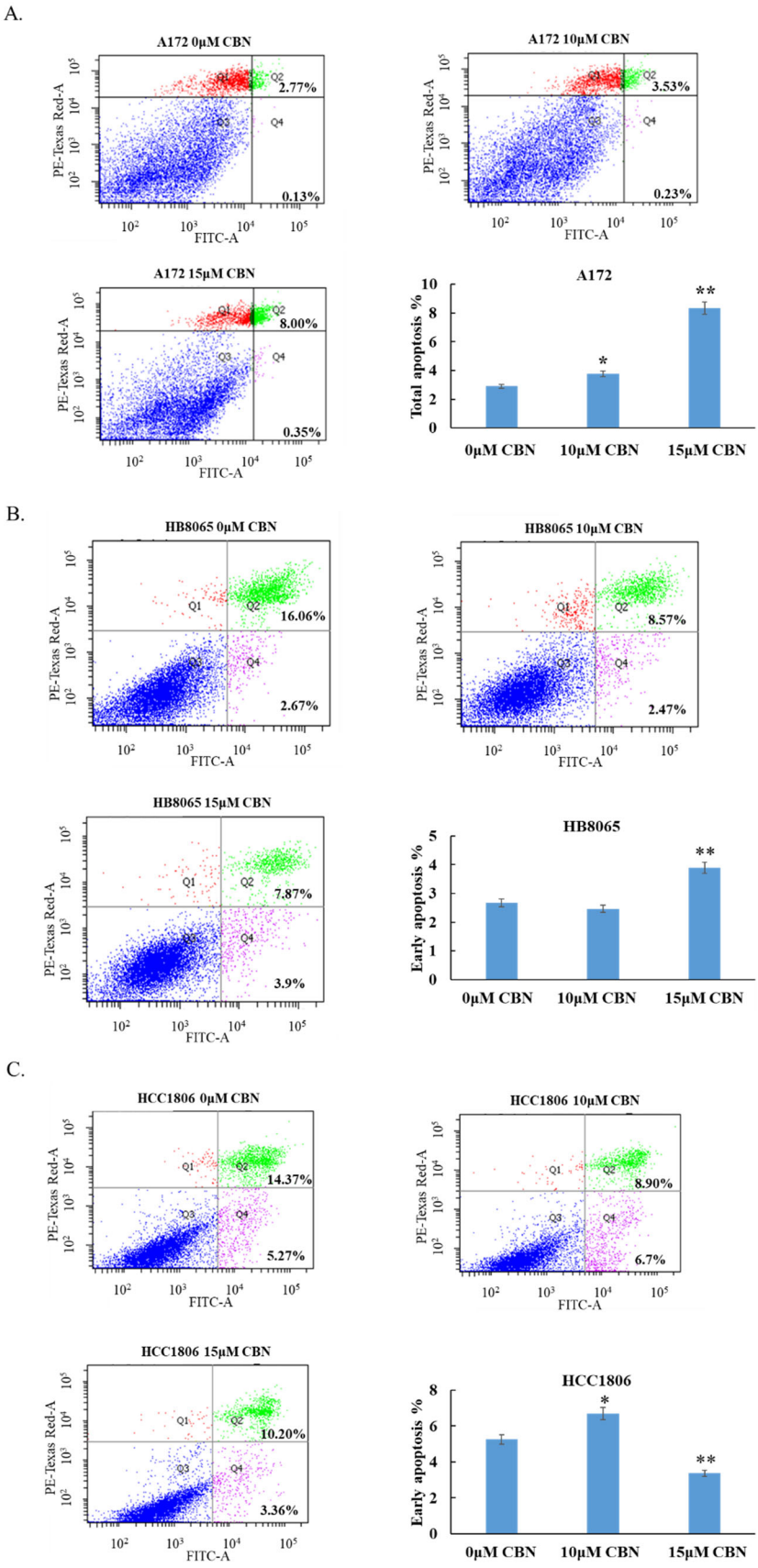
A.
A172 0μM CBN
2.77%
0.13%
A172 10μM CBN
3.53%
0.23%
A172 15μM CBN
8.00%
0.35%
A172
Total apoptosis %
0μM CBN
10μM CBN
15μM CBN
B.
HB8065 0μM CBN
16.06%
2.67%
HB8065 10μM CBN
8.57%
2.47%
HB8065 15μM CBN
7.87%
3.9%
HB8065
Early apoptosis %
C.
HCC1806 0μM CBN
14.37%
5.27%
HCC1806 10μM CBN
8.90%
6.7%
HCC1806 15μM CBN
10.20%
3.36%
HCC1806
PE-Texas Red-A
FITC-A

Figure 6. CBN induced apoptosis in cancer cells. (A-C) A172, HB8065 and HCC1086 cells were treated with the indicated concentration of CBN; at 96 h after treatment, cells were harvested for apoptosis analysis using flow cytometer as described in the "Methods". Data are expressed as the mean ± SD (N=3), * $P<0.05$ and ** $P<0.01$ with comparison to the control group.

5. DISCUSSION

The anticancer activities of cannabinoids, especially Δ^9-THC and CBD, have been studied in many cancers in the last decade. Δ^9-THC is known to have unwanted psychoactive side effects likely due to the activation of the CB1 receptor which is mainly located in the nervous system[17]. In recent years, one of the most-well studied non-psychoactive cannabinoids, CBD, has been demonstrated to induce apoptosis and to reduce cancer cell growth and tumor size[10,17]. CBN is another cannabinoid that has weak to no psychoactive effect. Previous reports have shown that it has a high affinity to the CB2 receptor and a weak psychoactive effect in animals[2]. CBN appears to have various medicinal properties such as analgesic[98], antibacterial[99], anti-inflammatory[24] and anti-tumorous[100] activities. Munson (1975) showed that CBN effectively decreased tumor size in lung cancer[100]. The effect of CBN on cancer is still poorly understood. In this study, we have demonstrated an anti-cancer feature of CBN in glioblastoma, hepatocellular carcinoma and breast cancer cells through suppressing cell proliferation and inducing apoptosis and cell-cycle arrest. Notably, Δ^9-THC and CBD displayed a dose- and time-dependent anti-proliferation role in normal WI-38 cells (Supplement 1), whereas CBN had no effect (Fig. 4B). Additionally, we confirmed that CBN inhibited A172, HB8065 and HCC1806 cell proliferation. HCC1806 cells appeared to be least sensitive to both 10 uM and 15 uM CBN treatment. For instance, exposure to 15 uM CBN for 96 h inhibited cell viability 70%-80% in A172 and HB8065 cells, whereas the same concentration and duration of CBN treatment reduced cell viability in HCC1806 cells by about 50% (Figure 4B). According to the literature, p53 protein plays a crucial role in modulating the expression of numerous genes which are engaged in various cell processes, including DNA

damage repair and induction of cell cycle arrest and apoptosis[101]. TP53 gene is one of the most commonly deleted or mutated genes across different human cancer types[102]. The absence of P53 gene promotes tumor cell survival and cancer development[102]. Cancer cell lines with mutant TP53 gene ($p53^-$) were also reported to be undruggable or have stronger resistance to chemotherapeutic drugs[103]. Given that HCC1086 cell line is a TP53 gene deficient – triple negative breast cancer cell line[104], it may be a reason why HCC1806 cells are more resistant to CBN than other two cell lines. This finding indicated that CBN has the anti-tumor effect on the TP53-deficient tumor and triple negative breast tumor which is the most aggressive breast cancer type[105].

We found that CBN might suppresses cancer cell proliferation via inhibition of the ERK and AKT pathway. Cell signaling pathways play a critical role in regulation of cell proliferation. Upregulation of ERK and AKT signaling pathways is often believed to promote tumor growth and is associated with drug resistance in animal studies[58,106,107]. Therefore, inhibiting ERK and AKT pathways is an important strategy for the development of anticancer chemotherapeutic drugs. There are several known inhibitors of ERK and AKT pathways used in clinical therapies. Several BRAF and MEK (two molecules from ERK pathway) inhibitors, such as encorafenib, dabrafenib and cobimetinib, have been approved by the FDA[108]. An AKT inhibitor, MK-2206, has progressed to the phase II trial treatment in breast cancer patients[109]. Concerning cannabinoids, Δ^9-THC and CBD have been shown to be inhibitors of MAPK/ERK and AKT pathways. Δ9-THC and CBD attenuate proliferation and induce apoptosis and autophagy in breast cancer, hepatocellular carcinoma and glioblastoma cells via downregulating the AKT signaling pathway[45,55–57]. Δ^9-THC also induces cell apoptosis in colorectal cancer and Jurkat leukemia cells by

inhibition of MAPK/ERK and PI3K/AKT signaling pathways[52,61].

Mutations or deletions of various genes in various cancer cells are associated with irregular activation of MAPK/ERK or PI3K/AKT signaling pathways and subsequently related to promotion of cell proliferation[110]. One of the tumor suppressor protein – phosphatase and tensin homolog (PTEN) plays a critical role on inhibition of cell proliferation, particularly, on suppression of PI3K-AKT signaling pathway via dephosphorylation of the AKT endogenous activator - PIP_3[111]. Cells deficient for PTEN exhibit a loss of the negative regulation of the PI3K-AKT pathways consequently contributing to cancer development in various organs[112]. PTEN gene also is one of the most commonly deleted or mutated genes in various human cancer cell line[113], including A172[114] and HB8065[115] cell lines, with abnormally high PI3K-AKT activation promoting cell proliferation and suppressing cell death[116]. Our research showed that the AKT phosphorylation was significantly decreased with CBN treatment in both PTEN-deficient cell lines, A172 and HB8065. This finding suggests that CBN may serve as a promising AKT inhibitor in specific PTEN-deficient cancer types. On the other hand, HCC1806 cell line has been classified to be resistant to AKT inhibitor and more sensitive to MEK inhibitor[117]. Our results showed that the expression of phosphorylated ERK was notably inhibited with CBN treatment in a dose-dependent manner in HCC1806 cells. The serine/tyrosine/threonine kinase MEK is one of the upstream signaling molecules to act on ERK activation and the MEK inhibitors are usually used to suppress the ERK activation and to further inhibit the ERK signaling pathway[108]. Therefore, our finding indicates that CBN may have anti-tumor activity via downregulation of MAPK/ERK signaling pathway even though the tumors have AKT-inhibitor-resistant properties.

The cell cycle is a process to produce new cells from the mother cell, which is mainly regulated by two groups of proteins, CDKs and cyclins[64]. Dysregulation of the cell cycle impairs cell division[118]. Current anticancer strategies aim to develop chemotherapeutic drugs that induce cell cycle arrest. Cannabinoids have been demonstrated to induce cell cycle arrest in tumors. Δ^9-THC increases p21 level to trigger cell cycle arrest in G2/M phase in breast cancer[80]. In addition, CBD enhances expression of p53 and reduces levels of CDK2 and cyclin E to contribute to cell cycle arrest in G0/G1 phase in gastric cancer SGC-7901 cells[81]. In this study, we reveal for the first time that CBN may induce cancer cell cycle arrest via downregulation of expression of cyclins and/or CDKs. Cell cycle arrest occurred in different phases under CBN treatment. CBN caused S phase cell cycle arrest in A172 glioblastoma cell line and was correlated with downregulation of CDK1 and CDK2 (Fig. 5A and B), while it induced G1 arrest in HCC1806 breast cancer cells line correlated with downregulation of CDK2 and cyclin E1. S phase is a period of DNA synthesis. When cells enter the S phase, CDK2 combines with cyclin A to form the CDK2-Cyclin A complex. CDK1 is rapidly activated at S/G2 transition[119]. Therefore, downregulation of CDK1 and/or CDK2 will lead to S-phase cell cycle arrest, supporting our findings. Two complexes, CDK4/6-Cyclin D and CDK2-Cyclin E, play critical roles in regulating G0/G1 phase activities. Downregulation of CDK2 and cyclin E1 has been shown to induce G1 arrest[120], which could explain CBN-triggered G1 arrest in HCC1806 breast cancer cells.

Apoptosis is the process of programmed cell death in aging, diseases and infection; apoptosis is regulated by two pathways – intrinsic and extrinsic apoptotic pathways[82]. Cancer is caused by loss of balance between cell growth and cell death[121]. The death of

cancer cells can be induced by the up-regulation of the levels of pro-apoptotic proteins[121]. Cannabinoids have been found to enhance apoptosis both in vitro and in vivo. For example, CBD contributes to pro-apoptosis via the activation of the intrinsic apoptotic pathway in prostate cancer cells[91] and induces apoptosis via reactive oxygen species in glioma stem cells[92]. In this study, we demonstrated that CBN may induced apoptosis of glioblastoma, hepatocellular carcinoma and breast cancer cells. Downregulation of p21 may contribute to CBN-induced apoptosis in 3 tested cancer cell lines (Fig. 5B and 6) because p21 has been shown to function as an apoptotic inhibitor[122]. Interestingly, we also noted that low concentration of CBN (10 μM) induced apoptosis (Fig. 6C), whereas high concentration of CBN (15 μM) attenuated apoptosis in HCC1806 cells, and the phenotypic changes correlated with p21 levels (Fig. 5B). The reason for low apoptotic rates may be an extended period of CBN treatment (96 hours). It is possible that the reduction of time of treatment to 48 or 72 h may result in an increase in the rate of apoptosis. In addition, AKT activation and high level of p21 in response to 15 uM CBN treatment may play an important role in the decrease of apoptosis in HCC1806 cells (Figure 4D and Figure 5B). AKT is a potential inhibitor of pro-apoptotic molecules such as caspase-9 and contributes to prevention of the chemotherapy-mediated apoptosis[123,124]. This abnormal activation, AKT activation, may associate with the loss of TP53 gene in HCC1806 cell line[104]. The tumor suppressor P53 is known as the transcriptional factor to control the transcription of PTEN which is a novel endogenous suppressor of PI3K/AKT pathway[112]. Moreover, AKT activation by CBN may be due to prevention of cell death response in HCC1806 cells due to the anti-apoptotic activity of AKT. In short, the level of AKT activation is not likely a marker for selection of the underlying anti-cancer drugs for breast cancer cells. Moreover, Given that p21 is a

tumor suppressor and cell-cycle kinase inhibitor that promotes cell cycle arrest in p53-dependent and p53-independent pathways[76] and has the anti-apoptosis activity in tumor development[125], the activation of p21 level may have two potential outcomes in response to 15 uM CBN in HCC1806 cells. On one hand, p21 is a crucial regulator that induces G1 and G2 cell cycle arrest in p53-deficient cells through binding and inhibiting the essential DNA replication factor - DNA polymerase processivity factor PCNA[126]. Our result indicated that 15uM CBN induced the G1 cell cycle arrest in the p53-deficient HCC1806 cells (Figure 5). It has to be shown whether there are change in PCNA expression in our experiments and whether the activation of p21 is associated with the induction of G1 cell cycle arrest in HCC1806 cells. This finding may provide another evidence that CBN may be an effective weapon against the p53-deficient tumor. On the other hand, p21 is a important marker of anti-apoptosis[127] and also acts as an tumor-promoting factor in the p53-deficient cells[128]. P21 activation by CBN may be owing to inhibition of cell death response in HCC1806 cells due to the anti-apoptotic function of p21.

In conclusion, CBN suppresses proliferation of glioblastoma, hepatocellular carcinoma and breast cancer cells via induction of cell cycle arrest and apoptosis, highlighting the medicinal potential of CBN in anti-cancer therapy.

6. Limitations and Future Studies

This study has attempted to investigate the anti-tumor role of CBN in various cancers. First, we have demonstrated that CBN regulates the expression of cannabinoid receptors, CB2, GPR55 and GPR18. Activation of cannabinoid receptors leads to changes in the downstream signaling pathways and cell processes including cell cycle and apoptosis. Binding to cannabinoid receptors may be critical for inducing anti-proliferative effects in cancers. For example, the anticancer effects of a synthetic cannabinoid – WIN55,212-2 is reversed in the prostate cancer with pre-treatment with AM630 which is a specific CB2 antagonist[78]. One limitation of this study is that it did not answer which receptor CBN could bind in our model system, although an increase in the expression of CB2 receptor was the most prominent. More experiments need to further understand what receptors are important for CBN to have anti-cancer effect. Future studies may include, for instance, knock-out of cannabinoid receptors via CRISPR gene editing or knock-down with inhibitor application; thesis studies, may assist to determine whether the anti-caner properties of CBN are associated with binding to the cannabinoid receptors.

Second, our study has indicated that CBN inhibits cell proliferation in three types of cancers and this activity may be associated with the suppression of cell signaling pathways, MAPK/ERK and PI3K/AKT pathways. It is necessary to confirm the regulation of signaling pathways conducted by CBN with more experiments. For instance, to determine whether CBN has the downregulation effect on cell signaling pathways, we could use the same concentrations and durations of CBN treatment while pre-treating cells with AKT or ERK activators. Applying the inhibitors of ERK and AKT to block

downstream signaling can be another approach to further study whether two pathways respond to cannabinoids independently.

Moreover, we have demonstrated that CBN induced apoptosis and cell cycle arrest via regulation of multiple cell regulators, such as cell cycle regulators, CDK2 and cyclin A and apoptotic regulators, p21 and p27. Those cell regulators are closely associate with some transcription factors. For example, our result showed that CBN reduce the expression of CDK2 to induce G1 phase arrest in HCC1806 cells and S phase arrest in A172 cells (Figure 5). CDK2 is a crucial regulator of G1 phase and S phase and the complex cyclin E-CDK2 phosphorylates tumor suppressor factor Rb and its target – transcription factor E2F to control the cell cycle progression[129]. To better understand genes contributing to the suppressive role of CBN in carcinogenesis, it is essential to look at key transcription factor(s) and target genes via mRNA sequencing and analysis of relevant protein levels.

Last but not least, all our findings were based on the cell model system. Future research direction could include tumor xenograft animal model to further confirm tumor-inhibitory role of CBN in vivo.

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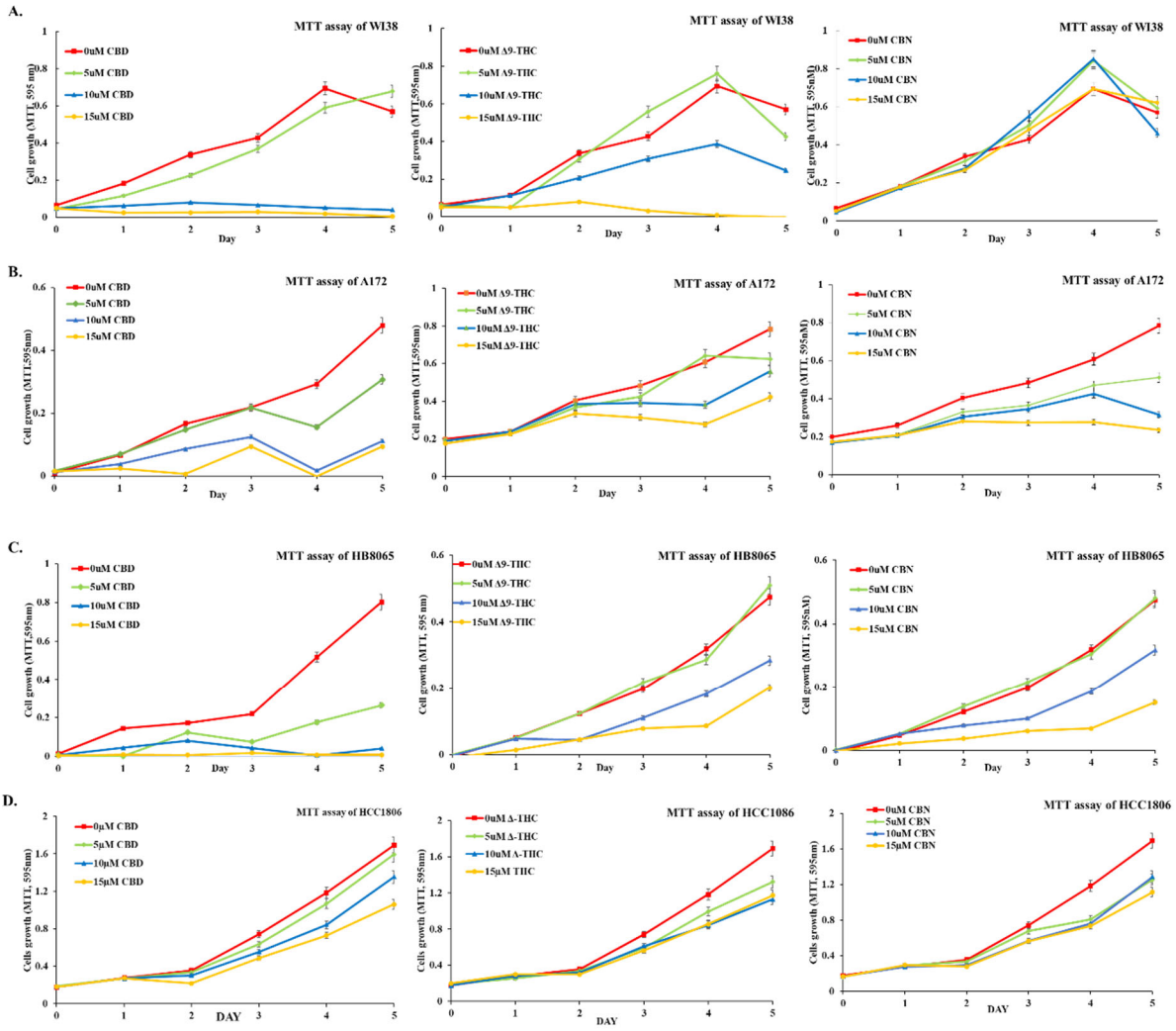
Supplementary information:

The results of cell viability in normal, glioblastoma, hepatocellular carcinoma and breast cancer cells.



APPENDIX A: Cell Culture and Maintenance Protocol

Thawing frozen cells, passaging and freezing cells:

1. Turn on the water bath and keep on 37 ℃, warm the medium before the experiment. Approximate time = 15-30 min.
2. Spray all the cell culture stuff, for example, pipettes, autoclaved-tips, stile-cell culture dishes, with 70% EtOH before putting in the BSL-2 hood, exposure ultraviolet light around 15-20 min.

   Thawing Frozen Cells (protocol from ThermoFisher Scientific website: https://www.thermofisher.com/ca/en/home/references/gibco-cell-culture-basics/cell-culture-protocols/thawing-cells.html)
3. Remove the cryovial with the frozen cells from -80 ℃ freezer or liquid nitrogen, spray the cryovial with 70% EtOH and immediately warm it up into a 37 ℃ water bath until there is a little place of ice left in the vial.
4. Spray the hand gloves and the outside of the vial with 70% EtOH.
5. Transfer the cells into the 15 ml centrifuge tube containing around 6 ml pre-warm growth medium.
6. Centrifuge the cell suspension at 1000 rpm for 5 min.
7. Gently resuspend the cell pellet with 1 ml pre-warm medium and transfer them into the cell culture dish with 9 ml pre-warm medium. Cells grow into a CO_2 incubator.

   Cell Passaging and Freezing:
8. Spray the hand gloves and incubator door with 70% EtOH before opening the CO_2 incubator.
9. Observe cells under the cleaned microscope (cleaned with Kimwipe and 70% EtOH). Check cells with 10 x or 20 x objective: 1) confluency or cell number; 2) contaminates; 3) cell shapes; 4) adhering cell status.
10. If the cells not confluent, change the fresh medium every 2 or 3 days by adding 10ml of medium to 10 cm cell culture dishes.
11. If cell number reach 80%-90% confluent, the cells were readied for passaging (splitting) or Freezing.
12. For splitting:
    a. Remove the old medium using the vacuum apparatus and a 2 ml sterilized Pasteur pipette.
    b. Add 1.5 ml TRYPSIN/EDTA to resuspend the adherent cells and incubate at room temperature or 37 ℃ for 1-10 min (The incubated time depends on how the adherent condition on different cell lines).
    c. observe cells under the microscope between incubation, if a large part of cells is floating, return to the BSL-2 hood and stopped the trypsin by 6 ml complete medium.
    d. Transfer the trypsinized cells to a sterile 15 ml centrifuge tube and centrifuge at 1000 rpm for 5 min.
13. For passaging,
    a. Return the centrifuge tube to the BSL-2 hood and use the vacuum apparatus and a 2 ml sterilized Pasteur pipette to remove supernatant.

b. Pipette 1 ml of complete medium to suspend the cell pellet by gentle vortex using pipette. Pipette 200 or 333 ul into each 10 cm culture dish. Gently mix by a handshake, be careful not to splash medium.

14. For freezing,
    a. Make freezing medium before resuspended the cell pellet. Recipe: 90% Complete medium, 10% DMSO or 20% FBS, 70% Complete medium and 10% DMSO.
    b. Following step 12, Return the centrifuge tube to the BSL-2 hood and use the vacuum apparatus and a 2 ml sterilized Pasteur pipette to remove supernatant.
    c. Pipette 1 ml freezing medium into the tube and resuspend the cell pellet by gently vortex by pipette. Add more 2 ml freezing medium to dilute the cell mixture. Pipette 1 ml resuspended cells into 2 ml cryotube. Freeze slowly by first placing at -20 ℃ and second placing at -8 0℃ for several days (maximum up to one year) and then stored in liquid nitrogen.

APPENDIX B: Details of Cell Proliferation Kit (MTT assay)

Cell Proliferation Kit I (MTT)

Colorimetric assay (MTT based) for the non-radioactive quantification of cell proliferation and viability

Cat. No. 11 465 007 001

1 Kit (for 2,500 tests)

Version 19
Content version: March 2016

Store at –15 to –25°C

1. Kit contents

Bottle	Label	Contents
1	MTT labeling reagent	• 5 vials containing 5 ml MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromid) labeling reagent (1 ×), • 5 mg/ml in phosphate buffered saline (PBS), • non-sterile • ready to use.
2	Solubilization solution (1 ×, ready-to-use)	• 3 bottles with 90 ml • 10% SDS in 0.01 M HCl

2. Introduction

2.1 Product overview

Assay principle

The assay is based on the cleavage of the yellow tetrazolium salt MTT to purple formazan crystals by metabolic active cells (Fig. 1) (6, 7, 35).

This cellular reduction involves the pyridine nucleotide cofactors NADH and NADPH (36). The formazan crystals formed are solubilized and the resulting colored solution is quantified using a scanning multiwell spectrophotometer (ELISA reader).

This ensures a high degree of accuracy, enables online computer processing of the data (data collection, calculation and report generation) and, thereby, allows the rapid and convenient handling of a high number of samples.

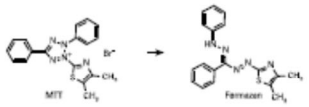


Fig.1: Metabolization of MTT to a formazan salt by viable cells.

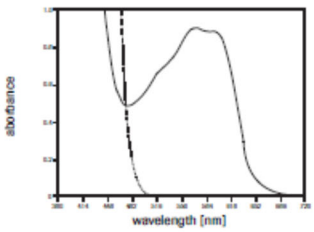


Fig. 2: Comparison of UV-spectra of MTT labeling reagent (dotted line) and the formazan salt after solubilization with solubilization solution.

Basic steps

Stage	Description
1	Cells, grown in a 96 well tissue culture plate, are incubated with the yellow MTT solution for approx. 4 h.
2	After this incubation period, purple formazan salt crystals are formed. These salt crystals are insoluble in aqueous solution, but may be solubilized by adding the solubilization solution and incubating the plates overnight in humidified atmosphere (*e.g.*, +37°C, 5 - 6.5% CO_2).
3	The solubilized formazan product is spectrophotometrically quantified using an ELISA reader. An increase in number of living cells results in an increase in the total metabolic activity in the sample. This increase directly correlates to the amount of purple formazan crystals formed, as monitored by the absorbance (see Fig. 3).

Background information

The determination of cellular proliferation, viability and activation are key areas in a wide variety of cell biological approaches. The need for sensitive, quantitative, reliable and automated methods led to the development of standard assays. Such an example is based on the capability of the cells to incorporate a radioactively labeled substance ([^{3}H]- thymidine), or to release a radioisotope such as [^{51}Cr] after cell lysis. Alternatively, the incorporation of 5-bromo-2'-deoxyuridine (BrdU)* in place of thymidine is monitored as a parameter for DNA synthesis and cellular proliferation in immuno-histo-and cytochemistry, in a cell ELISA and FACS analysis. (kits and reagents for these applications are available from Roche Diagnostics). Cell proliferation and viability assays are of particular importance for routine applications. Tetrazolium salts (*e.g.*, MTT, XTT, WST-1) are especially useful for assaying the quantification of viable cells, because they are cleaved to form a formazan dye (Fig. 1; for UV absorbance spectrum, see Fig. 2) only by metabolic active cells.

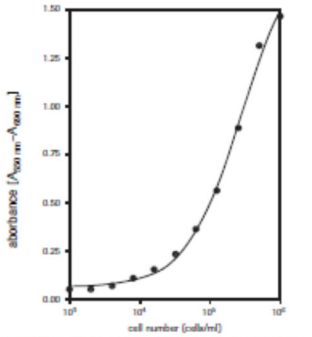


Fig. 3: Effect of different numbers of cells on color formation (example given, using Ag8 cells).

Application

The non-radioactive, colorimetric assay system using MTT was first described by Mosmann, T. *et al.* (1) and improved in subsequent years by several other investigators (2–6).

The assay is designed for the spectrophotometric quantification of cell growth and viability (1, 3, 5–7) without the use of radioactive isotopes.

- It is used for the measurement of cell proliferation in response to growth factors, cytokines and nutrients (1–3, 6, 8–12) (see fig. 4).
- The MTT assay is also useful for the measurement of cytotoxicity. Examples are the quantification of tumor necrosis factor-a or -b effects (13, 14). (see fig. 5) or macrophage induced cell death (15, 16) and the assessment of cytotoxic (17–34) or growth inhibiting agents such as inhibitory antibodies (see fig. 6).
- For the replacement of the radioactive [^{51}Cr]-release cytotoxicity assay, protocols using MTT have been developed. The MTT assay is as sensitive as the radioactive method, but shows a significantly lower background especially after long term incubation (34).
- The MTT assay can also be used to study cell activation (4).

Storage and stability

Stable at -15 to -25°C until the expiration date printed on the label.

Note: Protect from light. Repeated thaw-freeze cycles do not affect product stability. Precipitates may form during shipment or storage, in which case the container should be warmed to +37°C and thoroughly mixed.

After thawing, the MTT labeling reagents may be stored protected from light at +2 to +8°C for up to 4 weeks, in which case a sterile filtration of the reagent is recommended.

Advantages

Compared to radioactive isotope techniques, the Cell Proliferation Kit I (MTT) shows the following benefits.

Benefit	Feature
Safe	No radioactive isotopes are used.
Accurate	The absorbance revealed, strongly correlates to the cell number, (see fig. 3).
Sensitive	Low cell numbers are detected (see fig. 3).
Fast	The use of multiwell-ELISA readers allows for processing a large number of samples.
Easy	No washing steps and no additional reagents are required.

3. Protocols and required material

3.1 Assay procedure

Overview

Please refer to the following table.

Step	Description	Volume/ well	Time/ Temp
	Perform tissue culture using 96 well microplates (tissue culture grade, flat-bottom)	100 µl	24–96 h +37°C.
1	Add MTT labeling reagent and incubate in a humidified atmosphere	10 µl	4 h +37°C.
2	Add solubilization solution and incubate in a humidified atmosphere	100 µl	overnight +37°C
3	Evaluate microplate with the use of an ELISA reader at 550–600 nm with a reference wavelength of >650 nm.		

Handling instruction for larger volumes

If for the initial incubation of the cells a larger volume of culture medium is required, increase the amount of MTT labeling reagent correspondingly (*e.g.*, 20 µl MTT labeling reagent, when cells are cultured in 200 µl culture medium).

Protocol

Please refer to the following table.

Note: If for the initial incubation of the cells a larger volume of culture medium is required, increase then amount of MTT labeling reagent correspondingly (*e.g.*, 20 µl MTT labeling reagent, when cells are cultured in 200 µl culture medium).

Step	Action
1	Cells are grown in microplates (tissue culture grade, 96 wells, flat bottom) in a final volume of 100 µl culture medium per well, according to the media needs of the cells, in a humidified atmosphere (*e.g.*, +37°C, 5 - 6.5% CO_2). The incubation period of the cell cultures depends on the particular experimental approach and on the cell line used for the assay. For most experimental setups, the incubation of cells for 24 to 96 h is appropriate.
2	After the incubation period, add 10 µl of the MTT labeling reagent (final concentration 0.5 mg/ml) to each well.
3	Incubate the microplate for 4 h in a humidified atmosphere (*e.g.*, +37°C, 5 - 6.5% CO_2).
4	Add 100 µl of the Solubilization solution into each well.
5	Allow the plate to stand overnight in the incubator in a humidified atmosphere (*e.g.*, +37°C, 5 - 6.5% CO_2).
6	Check for complete solubilization of the purple formazan crystals and measure the spectrophotometrical absorbance of the samples using a microplate (ELISA) reader. The wavelength to measure absorbance of the formazan product is between 550 and 600 nm according to the filters available for the ELISA reader, used. The reference wavelength should be more than 650 nm.

3.2 Examples

3.2.1. Cell growth assay procedure

Additional reagents required

- Culture medium, *e.g.*, DMEM containing 10% heat inactivated FCS (fetal calf serum), 2 mM glutamine, 0.55 mM L-arginine, 0.24 mM L-asparagine-monohydrate, 50 µM 2-mercaptoethanol, HT-media supplement (1×), containing 0.1 mM hypoxanthine and 16 µM thymidine. If an antibiotic is to be used, additionally supplement media with penicillin/streptomycin or gentamicin *
- Interleukin-6, human (hIL-6) (200,000 U/ml, 2 µg/ml) sterile*.

Protocol

For the determination of human interleukin-6 (hIL-6) activity on 7TD1 cells (mouse-mouse hybridoma) (see fig. 4).

Step	Action
1	Seed 7TD1 cells at a concentration of 2 × 10^5 cells/well in 100 µl culture medium containing various amounts of IL-6 [final concentration *e.g.*, 0.1–10 U/ml (0.001–0.1 ng/ml)] into microplates (tissue culture grade, 96 wells, flat bottom).
2	Incubate cell cultures for 4 days at +37°C and 5 - 6.5% CO_2.
3	After the incubation period, add 10 µl of the MTT labeling reagent (final concentration 0.5 mg/ml) to each well.
4	Incubate the microplate for 4 h in a humidified atmosphere (*e.g.*, +37°C, 5 - 6.5% CO_2).
5	Add 100 µl of the Solubilization solution into each well.
6	Allow the plate to stand overnight in the incubator in a humidified atmosphere (*e.g.*, +37°C, 5 - 6.5% CO_2).
7	Check for complete solubilization of the purple formazan crystals and measure the spectrophotometrical absorbance of the samples using a microplate (ELISA) reader. The wavelength to measure absorbance of the formazan product is between 550 and 600 nm according to the filters available for the ELISA reader, used. The reference wavelength should be more than 650 nm.

APPENDIX C: Details of Western Blot Analysis

The western blotting analysis is to separate the protein molecules; there are four main steps: electrophoretic separation of proteins, transferring proteins to membrane, binding primary and secondary antibodies and detection the results. (details of western blot protocol according to Bio-Rad website: http://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6376.pdf and ThermoFisher Scientific website: https://www.thermofisher.com/ca/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/overview-western-blotting.html#introduction)

1. Electrophoretic separation of proteins

Casting the Gel:

1) Assemble glass plates in the gel casting apparatus.
2) Mix the reagents for the resolving gel:

	10% (10 ml)	12% (10 ml)
DD H_2O (ml)	4.0	3.3
1.5M Tris-HCl (PH8.8) (ml)	2.5	2.5
30% Acrylamide (ml)	3.3	4.0
10% SDS (ml)	0.1	0.1
10% APS (ml)	0.1	0.1
TEMED (ml)	0.004	0.004

3) Add the resolving gel mixture into the space between the glass plates to around 1.5 cm below the top of the shorter plate.
4) Equally pace 70% ethanol onto the top of the resolving gel to prevent meniscus formation in the gel.
5) Stand the gel 20 – 30 min at room temperature.
6) Drain the 70% ethanol and rinse the top of the gel with dd H_2O, and wick dd H_2O away with Kimwipe.
7) Mix component for stacking gel.

	2ml (1 gel)	4ml (2 gel)
dd H2O (ml)	1.4	2.7
1.0M Tris-HCl (ph6.8) (ml)	0.33	0.67
30% Acrylamide (ml)	0.25	0.5
10% SDS (ml)	0.02	0.04

10% APS (ml)	0.02	0.04
TEMED (ml)	0.002	0.004

8) Add stacking gel onto the top of resolving gel until filling full, insert comb to the top of the spacers. (10-well comb can load up to 60 ul of sample and 15-well comb can load up to 40 ul of the sample)
9) Stand gel for around 30 mins to an hour at room temperature, run the gel for experiment or store the gel at 4 ℃ up to 1 week.

Sample preparation:

1) Harvest cell and wash twice by 10 ml cold-PBS.
2) Resuspend cells in 100 ul RIPA buffer
3) Sonication: set up the sonicator probe at a frequency of 20 kHz, place the probe gently under the sample (do not touch the side of the microcentrifuge tube). The probe vibrates the sample for 5 X 10 seconds. Keep samples on ice.
4) Centrifuge at 1,2000 xg for 5 min, transfer the supernatant in the other 1.5 ml microcentrifuge tube.
5) Determine the protein concentration for all samples: diluted the protein sample in dd H_2O to 1:40, mix 25 ul diluted protein with 1.25 ml Bradford reagent in a 10 x 4 x 25 mm cuvette; incubate the mixture at room temperature for 6 minutes; measure the protein concentration using the Nano-drop 2000/2000c spectrophotometers.
6) An equal amount of protein (60-100 ug) was normalized with 4 x loading buffer and RIPA lysis buffer.
7) Incubate at 95 ℃ for 10 min.

Running the Gel:

1) Remove the comb and assemble cast gel into the Bio-Rad vertical apparatus.
2) Add fresh Gel Running buffer to the apparatus. Load equal amount of protein into each well along with a marker, PageRuler Plus Prestained Protein Ladder.
3) Run the part of stacking gel for 30 min at 60 V.
4) Increase the voltage to 100 V to finish the gel-running at 1-1.5 h.

2. Transferring the protein from the gel to the membrane
   1) Activate the polyvinylidene difluoride membrane in methanol.
   2) Place the gel, methanol and filter paper in 1 X transfer buffer for 5-10 min.
   3) Assemble the transfer sandwich and move the air bubbles out of the sandwich.
   4) Assemble the cassette into the Bio-Rad transfer tank and place an icebox in the tank as well as surround the tank with ice to keep it in low temperature.
   5) Transfer for 1.5-2 h at 100 V.

3. Antibody incubation
   1) The membrane is blocked in 1% non-fat milk in PBS-T at room temperature for 1

h.
   2) Incubate in specific primary antibodies against the target protein at 4 ℃, overnight.
   3) Rinse the membrane with washing buffer PBS-T 3 times, 15 min.
   4) Incubate with conjugated secondary antibodies for an hour at room temperature.
   5) Secondly rinse the membrane with washing buffer PBS-T 3 times, 15 min.
4. Imaging analysis
   1) Apply the reagent from ECL Prime Western Blotting System to the membrane and incubate for 10 min.
   2) Capture the chemiluminescent signals using the FluorChem HD2 Imaging System.
   3) Use Image J software to count the band.
5. Stripping and re-blotting
   1) Add 10 ml stripping buffer onto the membrane, wash twice and 10min each time.
   2) Wash twice, 10 min with PBS, and follow with another twice wash with PBS-T.
   3) Block the membrane in 1% non-fat milk in PBS-T at room temperature for 1 h.
   4) Incubate in specific primary antibodies against the target protein at 4 ℃, overnight.
   5) Rinse the membrane with washing buffer PBS-T 3 times, 15 min.
   6) Incubate with conjugated secondary antibodies for 1 h at room temperature.
   7) Secondly rinse the membrane with washing buffer PBS-T 3 times, 15 min.

Essential buffers:

Radioimmunoprecipitation assay buffer (RIPA buffer)

50 mM Tris-HCl, PH 8.0; 150 mM NaCl

4X Loading buffer

40% glycerol; 240 nM Tris-HCl (PH6.8); 0.04% bromophenol blue; 5% beta-mercaptoethanol; 8% SDS.

Running buffer:

25 mM Tri; 190 mM glycine; 0.1% SDS

Transfer buffer:

25 mM Tris; 190 mM glycine; 20% methanol

Washing buffer: (PBST: PBS+0.1% Tween 20)

1 X PBS: Start with 800 ml of dd H_2O, add 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, adjust the PH to 7.4 with 1 M HCl, add dd H_2O to the total volume of 1 L.

Blocking buffer

1% Non-fat dry milk in PBST

Stripping buffer (200 ml)

Glycine 3 g; 20% SDS 2 ml; Tween-20 2 ml; adjust PH to 2.2; add water up to 200 ml