

The expression of cannabinoid-related genes in multiple disease cell lines

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ABSTRACT

The endocannabinoid system comprises a series of ligands and receptors that have putative roles in regulating physiological and cognitive processes such as pre- and post-natal development, appetite, pain sensation, mood, and memory. Besides the endocannabinoids, these endogenous receptors are also capable of mediating the pharmacological effects of phytocannabinoids, which are derived from the cannabis plant. We sought to interrogate a panel of cell lines, representative of multiple human diseases, to characterize their cannabinoid-mediating gene expression. We found all lines expressed one or more gene product that rendered the cell potentially responsive to phytocannabinoids. Moreover, the expression profiles differed between normal and cancerous cells, as well as between cells derived from the brain, pancreas, and skin. Taken together, given the presence of one or more of these mediating gene products, our findings suggest a role for cell lines in studying the endocannabinoid system and potential therapeutic use of phytocannabinoids.

ABBREVIATIONS

5-HT_{1A}: 5-hydroxytryptamine receptor 1A; AEA: Anandamide (or arachidonoyl ethanolamide); ATCC: American Tissue Culture Collection; CB_{1R}: Cannabinoid type 1 receptor; CB_{2R}: Cannabinoid type 2 receptor; CB_{3R}: Cannabinoid type 3 receptor; CBD: Cannabidiol; CBG: Cannabigerol; COX: Cyclooxygenase; Ct: Cycle number threshold; FAAH: Fatty acid amide hydrolase; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; GBM: Glioblastoma; GPCR: G-protein-coupled receptor; GPR55: G-protein-coupled receptor 55; Id-1: Inhibitor of differentiation and DNA binding 1; JCRB: Japanese Collection of Research Biosources Cell Bank; NSAID: Non-steroidal anti-inflammatory drug; PPAR- γ : Peroxisome proliferator-activated receptor-gamma; PTGS2: Prostaglandin-endoperoxide synthase 2; RNA-seq: RNA sequencing; SCC: Squamous cell carcinoma; THC: Δ 9-tetrahydrocannabinol; TRP: Transient receptor potential;

INTRODUCTION

The cannabis plant (*Cannabis sativa* L.) contains a large number of pharmacologically active compounds called

phytocannabinoids. The most abundant of these compounds are Δ 9-tetrahydrocannabinol (THC) and cannabidiol (CBD), although the amounts and proportions of the various phytocannabinoids in each plant vary by strain and can be adjusted by breeding.

Many phytocannabinoids have been shown to be non-toxic and to possess anti-tumor activity in multiple cancer types [1]. Numerous mechanisms have been proposed to explain these anti-tumor effects, including the production of reactive oxygen species [2,3], promotion of apoptosis [4], a reduction in inflammation [5], activation of receptors that may inhibit drug transport [6], tumorigenesis [7] and potentiating the activity of multiple types of chemotherapy [8].

In spite of a wide spectrum of pharmacological activities, little is known about the molecular mechanism(s) of phytocannabinoid action. Several gene products, however, have been implicated in mediating the effects of phytocannabinoids. Activation of the endogenous cannabinoid type 1 receptor (CB_{1R}) and type 2 receptor (CB_{2R}) has been shown to lead to the inhibition of tumor progression [9]. CBD does not interact efficiently

Keywords

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Id-1
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with CB1R and CB2R, but cannabigerol (CBG), another non-psychoactive phytocannabinoid that interacts with specific targets involved in carcinogenesis, is a weak partial agonist of both CB1R and CB2R [10]. Phytocannabinoids also target transient receptor potential (TRP) channels [11], including TRPV1 [12] and TRPV2 [13], the cyclooxygenase (COX)-1 and COX-2 (also known as prostaglandin-endoperoxide synthase 2, or PTGS2) enzymes [14], the fatty acid amide hydrolase (FAAH) enzyme [11], the 5-hydroxytryptamine receptor 1A (5-HT_{1A}) and α 2-adrenergic receptor [10], the nuclear receptor peroxisome proliferator-activated receptor-gamma (PPAR- γ) [15], and the orphan G-protein-coupled receptor 55 (GPR55) [16].

Many of the effects produced by THC and its synthetic cousins depend on their ability to target cannabinoid receptors [17-19], which include the GPCRs CB1R [20] and CB2R [21].

CB1Rs are located primarily in central and peripheral neurons; CB2Rs, predominantly in immune cells. CB1Rs are also expressed by some non-neuronal cells, including immune cells, and CB2Rs by some neurons both within and outside the brain, reviewed in [22].

Although CBD has very low affinity for both CB1R and CB2R [23], there is evidence that CBD can interact with these receptors at reasonably low concentrations [22], yielding such varied effects as GPR55 antagonism [24,25], activation of TRPV1 [26] and decreased cancer cell proliferation and increased neuroprotection [27].

FAAH is an intracellular enzyme that catalyzes the hydrolysis of the endogenous cannabinoid ligand anandamide (arachidonylethanolamide, AEA) [28,29]. CBD has been reported to inhibit both FAAH-mediated degradation of AEA [30] and AEA transporter activity [31]. These findings suggest that some of the pharmacological actions of CBD might be due to inhibition of AEA degradation.

GPR55 is an orphan G protein-coupled receptor (GPCR), meaning it has no known ligand. The *GPR55* gene is expressed in several brain areas including the hippocampus, hypothalamus, frontal cortex, and cerebellum [16]. The function of GPR55 in the periphery is varied: vasodilatation, cellular proliferation and migration, bone dynamics, energy balance, gastrointestinal processes, and inflammation and pain, reviewed in [16]. In the central nervous system, studies suggest GPR55 could modulate procedural memory, motor coordination, anxiety, and hippocampal release of glutamate, reviewed in [26].

GPR55 can be activated by endocannabinoids, though the extent to which the receptor can be fully activated is still unknown [25]. Despite poor sequence homology, many studies have reported significant functional interaction between GPR55 and CB1R/CB2R [32-34]. As such, GPR55 is being considered as the cannabinoid receptor type 3 (CB3R) by

the International Union of Basic and Clinical Pharmacology Committee on Receptor Nomenclature and Drug Classification [35, 32]. Given its role in motor coordination, modulation of GPR55 activity has been proposed as a therapeutic target for epilepsy. CBD was recently approved by the US FDA as a treatment for certain childhood forms of epilepsy (Epidiolex®, GW Pharmaceuticals), leading to the hypothesis that one mechanism of action of CBD is GPR55 inhibition [25].

CBD has been shown to reduce the expression of the inhibitor of differentiation and DNA binding 1 (Id-1). Id family members negatively regulate the activity of basic helix-loop-helix proteins, which are critical components of developmental processes such as sex determination and neurogenesis [36]. Constitutive expression of Id proteins can inhibit the differentiation of various tissues [37,38]. Perhaps most relevant, the Id proteins have been shown to be involved in the pathogenesis of human cancers [39,40].

The level of expression of the *ID1* gene has been shown to be strongly correlated to the invasive and metastatic behavior of breast cancer [41,42] and glioblastoma (GBM) [36] cells. Higher levels of *ID1* have been documented for a variety of aggressive tumor types including melanoma and cancers of the head and neck (squamous cell carcinoma [SCC]), esophagus (SCC), oral cavity (SCC), liver, colon, pancreas, thyroid, ovary, cervix, endometrium, and prostate (reviewed in (Fong, Debs and Desprez, 2004 [17])), especially when compared to normal cells of the same tissue origin [39,40]).

The *PTGS2* gene encodes the COX-2 enzyme, which is one of two cyclooxygenases in humans. It is involved in the conversion of arachidonic acid to prostaglandin H₂, an important precursor of prostacyclin, thromboxane, and other prostaglandins, which are all expressed in inflammation. PTGS2 is not expressed in most cells under normal conditions, but elevated levels are found during inflammation. Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit prostaglandin production by both PTGS1 (COX-1) and PTGS2.

The expression of *PTGS2* is upregulated in many cancers. Inhibiting *PTGS2* expression may therefore be beneficial in preventing and treating certain types of cancer [43].

COX-2 plays a significant role in intraneuronal CB1R signal transduction and modulation of *PTGS2* and *CNRI* gene expression [44]. CBD and THC have been found to increase expression and activity of PTGS2, with *PTGS2* being one of the 25 most up-regulated genes upon treatment with either cannabinoid in T98G (but not U-87 MG) cells [45].

TRP channels are pore-forming transmembrane proteins that allow ions and xenobiotics to permeate biological membranes [46]. It has been suggested that TRP channel-expressing cancer cells may allow entry of charged molecules [47,48,49,50] and certain anti-proliferative and cytotoxic molecules [47] by permeation.

CBD has been found to be a full, though weak, agonist of human TRPV1 [26]. The TRPV2 channel is triggered by agonists such as THC and CBD [51, 52].

The expression of some of these genes has been assessed in some cell lines and tissue types [53,54,55] however, they have not been characterized in some of the representative diseases presented here. Given the importance of each of these genes to normal and pathological processes, we sought to assess the level of each across a panel of human cell lines.

METHODS

Cell lines

The GBM lines T98G and U-87 MG ; pancreatic cancer lines MIA PaCa-2 and PANC-1; actinic keratosis line HT 297.T; melanoma lines A-375 and A101D; and normal skin fibroblasts BJ and Detroit 551 were all obtained from the American Tissue Culture Collection (ATCC) and grown according to the manufacturer's recommendations. All assays were performed using cells that had been passaged fewer than 20 times upon arrival.

The SCC (skin) line HSC-5 (Hozumi *et al.*, 1990 [22]) was obtained from the Japanese Collection of Research Biosources Cell Bank (JCRB) and Sekisui XenoTech.

RNA extraction

RNA from cell samples was generated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions.

Generation of complementary DNA (cDNA)

RNA was converted to cDNA using the RT² First Strand Kit (Qiagen) according to the manufacturer's instructions.

Quantitative PCR (qPCR)

The expression of *ACTB* (β -actin), *CNR1* (CB1R), *CNR2* (CB2R), *FAAH*, *GAPDH*, *GPR55*, *ID1*, *PTGS2*, *TRPV1*, and

TRPV2 was measured by qPCR using validated human primer sets (RT² qPCR Primer Assays, Qiagen) and the RT² SYBR Green qPCR Mastermix (Qiagen). Values for gene expression were determined using the Δ Ct method, where the cycle number threshold (Ct) of either glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or β -actin (as housekeeping genes) is subtracted from the Ct value of each gene. Expression levels for the housekeeping gene transcripts were normalized to one million copies. The results presented throughout the paper represent the means of at least three independent experiments.

Statistical analysis

The differences in the mean values for expression of each gene for each cell line were evaluated for statistical significance. P values were calculated by independent sample t-test with equal variance, confidence interval = 95%.

RESULTS

Glioblastoma

We compared the level of expression of the set of cannabinoid-related genes across two different GBM lines, T98G and U-87 MG. T98G cells had the highest expression of GPR55, followed by ID1 (Figure 1A). U-87 MG cells exhibited the highest expression of *ID1* among all the genes analyzed (Figure 1B), as well as having fairly robust expression of *TRPV1*. U-87 MG cells also exhibited robust expression of *PTGS2* and *TRPV2*.

Pancreatic cancer

The pancreatic cancer line MIA PaCa-2 had observable and robust expression of all cannabinoid-related genes except *TRPV2* (Figure 2A). PANC-1 cells, on the other hand, were fairly low expressers of all genes except *FAAH*, *ID1*, and *TRPV1* (Figure 2B).

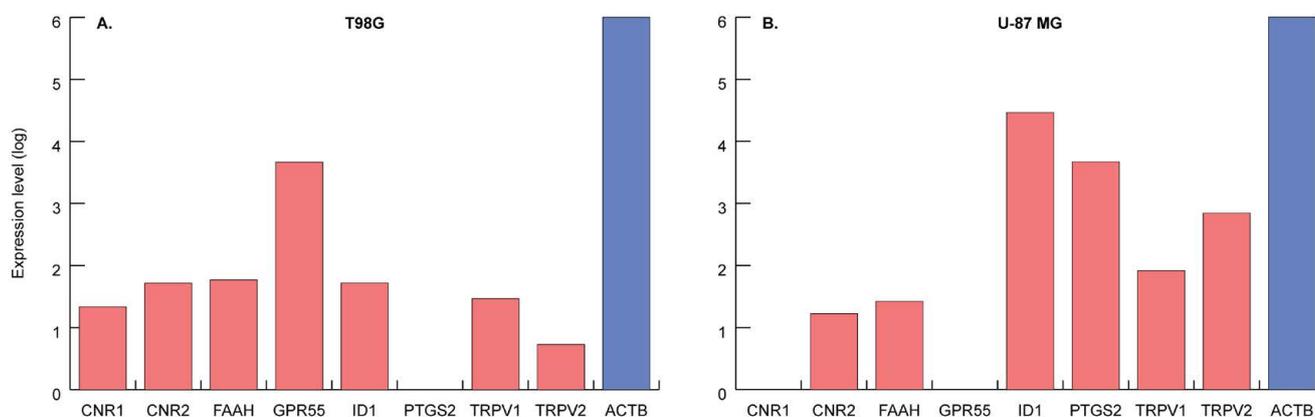


Figure 1. Expression of cannabinoid-related genes in glioblastoma cells. Expression levels in (A) T98G cells and (B) U-87 MG cells were normalized to one million copies of β -actin. The standard error of the mean (SEM) for all histograms was \pm 10%.

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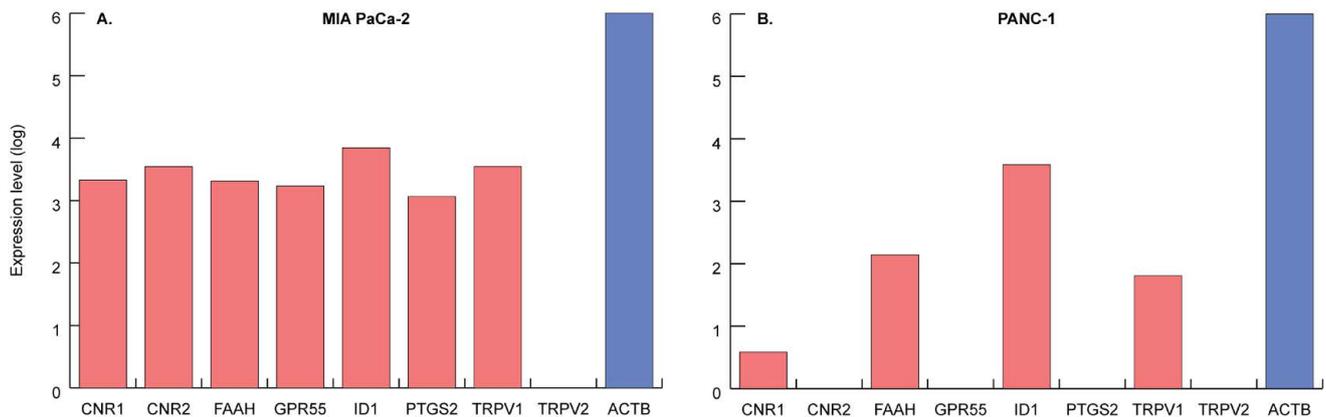


Figure 2. Expression of cannabinoid-related genes in pancreatic cancer cells. Expression levels in (A) MIA PaCa-2 cells and (B) PANC-1 cells were normalized to one million copies of β -actin. The SEM for all histograms was $\pm 15\%$.

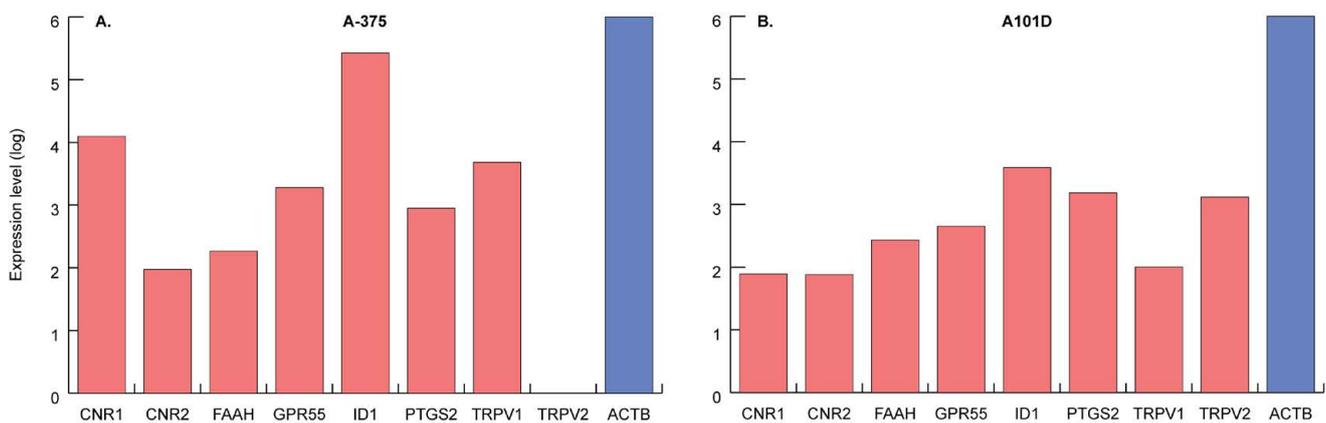


Figure 3. Expression of cannabinoid-related genes in melanoma cells. Expression levels in (A) A-375 cells and (B) A101D cells were normalized to one million copies of β -actin. The SEM for all histograms was $\pm 10\%$.

Skin

The malignant melanoma line A-375 had observable expression of all cannabinoid-related genes except *TRPV2* (Figure 3A). The genes with the highest levels of expression were *CNR1*, *GPR55*, *ID1*, and *TRPV1*. Another melanoma line, A101D, had observable expression of all genes (Figure 3B). It, too, had *ID1* as the highest-expressing gene.

The SCC line HSC-5 (Figure 4) and actinic keratosis line HT 297.T (Figure 5) both had observable expression of *FAAH*, *ID1*, *PTGS2*, and *TRPV1*; HSC-5 cells also expressed *CNR1*. Similar to other lines, *ID1* expression was the highest of all genes tested.

We also examined gene expression in normal skin fibroblasts. The two lines studied, BJ and Detroit 551, exhibited different patterns of expression. BJ cells had high levels of *PTGS2* and *TRPV2* expression (Figure 6A), while Detroit 551 cells had

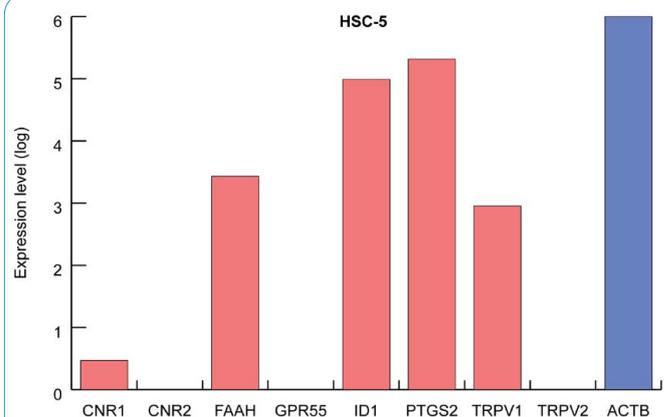


Figure 4. Expression of cannabinoid-related genes in HSC-5 cells, a squamous cell carcinoma line derived from skin. Expression levels were normalized to one million copies of β -actin. The SEM for all histograms was $\pm 10\%$.

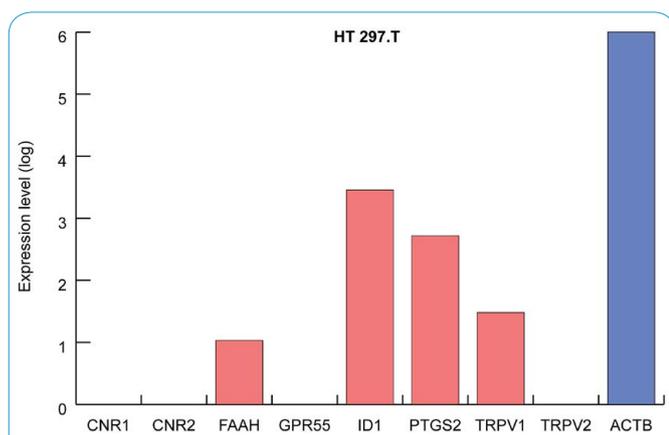


Figure 5. Expression of cannabinoid-related genes in HT 297.T cells, an actinic keratosis line. Expression levels were normalized to one million copies of β -actin. The SEM for all histograms was $\pm 10\%$.

high levels of *ID1* and *PTGS2* (**Figure 6B**). Interestingly, BJ cells had completely undetectable levels of *ID1*.

A summary of the expression levels of the cannabinoid-related genes can be found in **Table 1**. A heatmap visualization of the same data can be found in **Figure 7**. When visualized in the aggregate, all cell types expressed cannabinoid-related genes, though to varying degrees. The highest total gene-expressing cell line was MIA PaCa-2, while the highest gene-expressing cell type was melanoma.

ID1 expression

Because of the special role *Id-1* appears to play in tumorigenesis, and the fact that CBD has been shown to down-regulate *ID1* gene expression and associated glioma cell invasiveness and self-renewal [36], we paid particular attention to its expression levels in the cell lines assessed in this study. We found that expression of *ID1* was consistently high in cancerous cell lines,

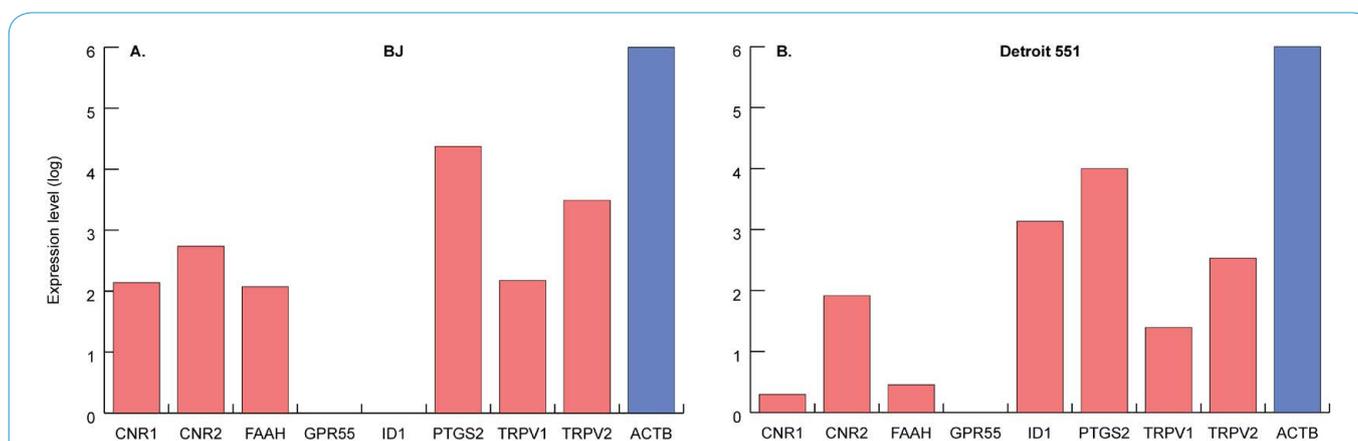


Figure 6. Expression of cannabinoid-related genes in normal skin fibroblasts. Expression levels in (A) BJ cells and (B) Detroit 551 cells were normalized to one million copies of β -actin. The SEM for all histograms was $\pm 15\%$.

Table 1: Expression of cannabinoid-related genes across multiple cell lines.

Cell line	<i>CNR1</i>	<i>CNR2</i>	<i>FAAH</i>	<i>GPR55</i>	<i>ID1</i>	<i>PTGS2</i>	<i>TRPV1</i>	<i>TRPV2</i>
<i>Glioblastoma</i>								
T98G	+	+	+	++	+	ND	+	+
U-87 MG	ND	+	+	ND	++	++	+	+
<i>Pancreatic cancer</i>								
MIA PaCa-2	++	++	++	++	++	++	++	ND
PANC-1	+	ND	+	ND	++	ND	+	ND
<i>Skin – Squamous cell carcinoma</i>								
HSC-5	+	ND	++	ND	++	+++	+	ND
<i>Skin – Actinic keratosis</i>								
HT 297.T	ND	ND	+	ND	++	+	+	ND
<i>Skin – Melanoma</i>								
A-375	++	+	+	++	+++	+	++	ND
A101D	+	+	+	+	++	++	+	++
<i>Skin – Normal</i>								
BJ	+	+	+	+	ND	++	+	++
Detroit 551	+	+	+	ND	++	++	+	+

Expression levels: +++, < 1 log difference of β -actin expression; ++, > 1 and < 3 logs difference of β -actin expression; +, > 3 logs difference of β -actin expression; ND, not detected; The differences between each category of expression level (+++, ++, +, and ND) are all statistically significant ($P < 0.05$).

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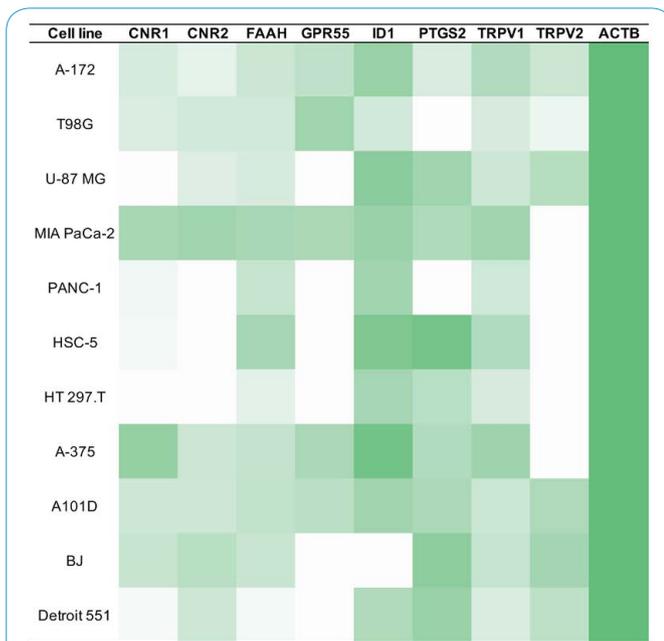


Figure 7. Heatmap depicting the expression of cannabinoid-related genes across multiple cell lines. The colors indicate levels of gene expression and range from white (nadir of expression) to dark green (zenith of expression).

with the exception of T98G cells (**Figure 8**). Two normal skin fibroblast lines, BJ and Detroit 551, showed different levels of *ID1* expression, with BJ having undetectable *ID1* levels.

DISCUSSION

Phytocannabinoids, including THC and CBD, have been proposed as therapeutic agents for a wide variety of diseases. It has been demonstrated that the endogenous expression of enzymes, receptors, signaling molecules, and other mediators that comprise the endocannabinoid system, many of which were analyzed in this study, render the human body capable of functional responses to this class of compounds. To our knowledge, this study is the first systematic assessment of cannabinoid-related genes across multiple cell lines that span human diseases.

Virtually all of the cell lines we tested exhibited robust expression of *ID1*, a gene that has been implicated in the pathogenesis of human cancers [39,40], particularly glioblastoma [57]. As CBD has been shown to reduce the expression of *ID1*, thereby reducing either the likelihood of cellular transformation or the aggressiveness of existing tumors [58], these results suggest therapeutic targeting of these diseases with CBD may improve outcomes.

The expression of *PTGS2* was second only to *ID1* across the cell lines tested. Given its role in mediating inflammation, it was not surprising to observe fairly high levels in transformed

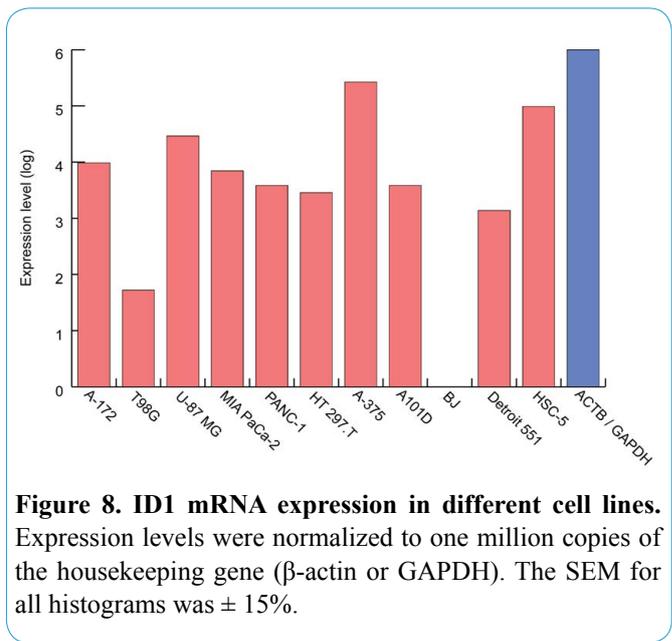


Figure 8. ID1 mRNA expression in different cell lines. Expression levels were normalized to one million copies of the housekeeping gene (β -actin or GAPDH). The SEM for all histograms was $\pm 15\%$.

cell lines (even if some are characterized as “normal”). This observation is consistent with the finding that *PTGS2* expression is upregulated in many cancers. What is counter-intuitive, however, is the observation that CBD was found to up-regulate expression of *PTGS2*, as well as increase the activity of the COX-2 enzyme [45]. Increasing the activity of a pro-inflammatory mediator such as COX-2 contradicts the narrative that CBD and other cannabinoids are potent anti-inflammatory drugs, exerting their effects through induction of apoptosis, inhibition of cell proliferation, suppression of cytokine production, and induction of regulatory T cells [5]. Further investigation into the effect phytocannabinoid treatment of these cell lines has on *PTGS2* expression is warranted.

CBD has very low affinity for both CB1R and CB2R [23]. In addition, CBD has been reported to inhibit both FAAH-mediated degradation of the endogenous cannabinoid ligand, anandamide [30], and the RBL-2H3 cell anandamide transporter activity [31]. These findings raise the possibility that some of CBD’s pharmacological actions might be due to inhibiting the degradation of anandamide. Anandamide has been identified as an endogenous CB1R agonist and as a ligand for CB2R (despite low affinity).

Another important site at which anandamide acts is TRPV1 [59], which acts as a molecular integrator of noxious stimuli and is important for thermal nociception and inflammatory hyperalgesia [60,61]. CBD has been shown to be a full, although weak, agonist of human TRPV1 [26]. Thus, CBD could activate either cannabinoid receptors indirectly through anandamide or vanilloid receptors directly or indirectly via anandamide.

A comprehensive survey of gene expression in human tissues was conducted by RNA sequencing (RNA-seq) [55]). Among

27 different human tissues assessed by RNA-seq, *CB1R* expression was highest in the brain; skin was fairly low, pancreas was virtually undetectable. *CB2R* expression, highest in the lymph nodes, was virtually undetectable in the brain, pancreas, and skin. *FAAH* expression, highest in the prostate, was approximately equivalent in the brain and skin and twice that in the pancreas. *GPR55* expression was highest in the testis; expression in the brain and skin was approximately the same, with virtually undetectable levels in the pancreas. *IDI1* expression was highest in the colon and lung, with intermediate expression in the brain and skin, and virtually undetectable levels in the pancreas. *PTGS2* expression, highest in the bone marrow, was low in the brain and virtually undetectable in the pancreas and skin. *TRPV1* expression was highest in the small intestine; expression in the skin was approximately twice that in the brain and fourfold that in the pancreas. Finally, *TRPV2* expression was highest in the lung; expression in the brain and skin was approximately the same, and fivefold that in the pancreas.

In cancer, the situation is somewhat different. *CB1R* and *CB2R* are both overexpressed in several tumors, including pancreatic adenocarcinoma [54], suggesting that phytocannabinoid-based therapy may activate cell death pathways predominantly in tumor cells. In cell lines, our results are somewhat different than those reported previously [53]. In the GBM line T98G, the authors found low expression of *GPR55*, *TRPV1*, and *TRPV2*, and undetectable levels of *CB1R* and *CB2R*; we report T98G cells as expressing intermediate levels of *GPR55*, and low levels of the other three genes. For another GBM line, U-87 MG, found low levels of all genes except *CB2R*, which was undetectable; similarly, we report intermediate to low levels of each gene. In the malignant melanoma line A-375, [53] reported high levels of *CB1R* and *TRPV2*; in our study, we confirm fairly high levels of *CB1R* but did not detect *TRPV2* expression. These discrepancies, while minor, may be the result of several experimental factors, including media conditions, passage number of the cell lines, primer and enzyme efficiency during amplification, etc.

In conclusion, the cell types assessed in this study – diseased and normal cell types, cancer and non-cancerous cell types – all exhibited expression of one or more gene products that have been shown to mediate the pharmacological effects of cannabinoids. Moreover, the diversity of these cell types is consistent with the notion that our bodies, whether healthy or sick, are innately capable of responding to phytocannabinoids. Although this paper reports levels of RNA transcripts, we cannot conclude that the expression patterns are similar for the corresponding proteins; further investigation is therefore warranted. Our findings strongly suggest, however, there is a potential therapeutic role for phytocannabinoids in the context of disease.

DECLARATIONS

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

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

Competing interests

PAA is a shareholder in Diverse Biotech, Inc.

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Authors' contributions

PAA conceived and designed the work, interpreted the data, and drafted the manuscript.

XW acquired and analyzed the data.

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