Assessment of Cannabinoid Receptor Expression and Anti-Neoplastic Effects of Cannabidiol (CBD) in Canine Urothelial Carcinoma Cells

by

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ABSTRACT

ASSESSMENT OF CANNABINOID RECEPTOR EXPRESSION AND ANTI-NEOPLASTIC EFFECTS OF CANNABIDIOL (CBD) IN CANINE UROTHELIAL CARCINOMA CELLS

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Canine urothelial carcinoma (UC) is an invasive epithelial neoplasm and is the most common urogenital tumour in dogs. Despite the use of multimodal therapies, local and systemic control of this disease remain challenging. A particular difficulty with UC relates to the frequency of local treatment failure, which often results in urinary obstruction, however distant metastases also occur, highlighting the need for novel therapies to help improve outcomes.

Increasingly, cannabidiol (CBD) has been evaluated for anti-neoplastic effects *in vitro* and *in vivo*, yielding encouraging results. However, the mechanisms through which these effects occur remain incompletely understood. Although these effects have been studied in various human malignancies, including urogenital tumours, there is a lack of such information available for veterinary patients. The present work aimed to evaluate expression of various cannabinoid receptors in canine UC cell lines, examine the anti-neoplastic effects of CBD and investigate potential underlying mechanisms.

Three canine UC cell lines and one immortalized canine kidney epithelial cell line were evaluated. A major band was observed in all cell lines using an antibody directed against cannabinoid receptors CB1, CB2, PPARγ, GPR55 and TRPV1 via western blotting. Additionally, treatment with CBD resulted in dose dependent reduction of cell viability in all cell lines. However, apoptosis and autophagy were found not to be significantly increased compared to vehicle controls at 8 and 24 hours following administration of CBD, respectively. Lastly, treatment with cannabinoid receptor antagonists, to investigate whether the anti-neoplastic effects of CBD are mediated by these receptors, did not alter cell viability in comparison to treatment with CBD alone.

Based on this information, it can be concluded that while these cannabinoid receptors are expressed in canine UC *in vitro*, their biologic role and role in any potential anti-neoplastic effects remain unclear. Further investigation into possible underlying mechanisms of action of CBD is necessary prior to defining its role as a potential novel therapeutic agent for canine UC.

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DECLARATION OF THE WORK PERFORMED

All the work in this thesis was performed by me, Sarah Laliberte, apart from the following:

Jordon M. Inkol prepared CBD, assisted with maintenance of cell lines and assisted with western blot and flow cytometry assays.

Arata Matsuyama aided with data analysis and interpretation.

I, Sarah Laliberte, performed the remainder of experiments, data analysis, writing, graphing and table formatting in this thesis with editorial comments made by Drs. Anthony Mutsaers, Samuel Hocker and Darren Wood.

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LIST OF ABBREVIATIONS

Δ^9-THC	Δ^9-Tetrahydrocannabinol
2-AG	2-arachidonoylglycerol
AEA	Anandamide
AKT	Protein Kinase B
ALP	Alkaline phosphatase
AXA	K9TCC-Pu-AXA
BSA	Bovine serum albumin
CB1	Cannabinoid receptor type 1
CB2	Cannabinoid receptor type 2
CBD	Cannabidiol
C_{max}	Maximal plasma concentration
CNS	Central nervous system
COX	Cyclooxygenase
CR	Complete response
CREB	cAMP response element binding protein
DAG	Diacylglycerol
DMEM	Dulbecco's Modified Eagle Medium
ERK	Extracellular signal-regulated kinase
FAAH	Fatty acid amide hydrolase
FABP	Fatty acid binding protein
FBS	Fetal bovine serum

G-PCR	G protein-coupled receptor
GPR55	G protein-coupled receptor 55
IC50	Half-maximal inhibitory concentration
IHC	Immunohistochemistry
IM/IGRT	Intensity-modulated and image-guided radiation therapy
LPI	Lysophosphatidylinositol
MAGL	Monoacylglycerol lipase
MFI	Mean fluorescent intensity
mTOR	Mammalian Target of Rapamycin
MTT	3-(4,5-Dimethylthaizol-2-yl)-2,5-diphenyltetrazolium bromide
OEA	Oleoethanolamine
ORIG	K9TCC
PBS	Phosphate buffered saline
PCD	Programmed Cell Death
PI	Propidium Iodide
PLC	Phospholipase C
PNS	Peripheral nervous system
PPAR	Nuclear Peroxisome Proliferator-Activated Receptor
PR	Partial response
PVDF	Polyvinylidene difluoride
RhoA	Ras homologue gene family member A
ROCK	Rho-associated protein kinase
ROS	Reactive Oxygen Species

RXR	9-cis-retinoic acid receptor
SH	K9TCC-Pu-Sh
$t_{1/2}$	Half-life
TCC	Transitional Cell Carcinoma
TRP	Transient receptor potential
TRPV1	Transient ion channel Receptor Potential Vanilloid type 1
UC	Urothelial carcinoma
WHO	World Health Organization

1 General Literature review

1.1 Review of canine urothelial carcinoma

1.1.1 Tumour behaviour and staging

Urothelial Carcinoma (UC), previously referred to as transitional cell carcinoma (TCC), is the most commonly diagnosed urinary bladder neoplasm in dogs, comprising approximately 2% of all reported canine cancers.[1–4] This cancer affects thousands of dogs worldwide and over the last 30 years, its prevalence in university teaching hospitals across North America continues to increase.[2] While this increase may relate to several factors, including an expanding awareness of available treatment options among clients and referring veterinarians, or active case recruitment for research programs given the status of this disease as a relevant animal model for human UC, it represents a growing problem nonetheless.[2,5]

Canine UC is a highly, locally infiltrative disease with most tumours invading the bladder wall or neighboring organs at the time of diagnosis.[2,4,6] A World Health Organization (WHO) classification scheme exists for canine urinary bladder tumours based on the tumour (T), node (N) and metastasis (M), status of a patient [**Table 1-1**].[7]

In a previous report of approximately 100 dogs diagnosed with UC of the bladder, 78% were found to have invasion of the tumour into the bladder wall (clinical stage T2) at the time of diagnosis while 20% were found to have invasion of the neighboring organs (clinical stage T3).[6] Additionally, most canine UC (>95%) were found to

represent intermediate to high grade tumours following histopathologic evaluation of tumour samples in a group of 74 dogs.[6] Approximately 60% of these tumours were found to have an infiltrative growth pattern with papillary tumours being the most common histologic subtype.[6]

Table 1-1-1. WHO clinical stage (TNM) classification system for canine urinary bladder cancer[7]

T: Primary tumour	
Tis	Carcinoma in situ
T0	No evidence of primary tumour
T1	Superficial papillary tumour
T2	Tumour invading the bladder wall, with induration
T3	Tumour invading neighboring organs (prostate, uterus, vagina, pelvic canal)
N: Regional lymph node (internal and external iliac lymph node)	
N0	No regional lymph node involved
N1	Regional lymph node involved
N2	Regional lymph node and juxta regional lymph node involved
M: Distant metastases	
M1	No evidence of metastasis
M2	Distant metastasis present

Common challenges associated with the management of UC arise from the frequent trigonal location within the urinary bladder and urethral or prostatic involvement.[2,4] Knapp et al., found concurrent involvement of the urethra occurring in 56%, and the prostate was involved in 29% of male dogs, among a group of over 100 dogs diagnosed with UC.[6] Collectively, these locations pose a therapeutic challenge due to the potential for urinary outflow tract obstruction, which frequently occurs with

growth of the primary tumour. Clinical signs of UC commonly include dysuria, pollakiuria and hematuria, and can often be debilitating, particularly in the event of complete obstruction of the ureters or urethra, which may also progress to hydroureter, hydronephrosis or even renal failure.[1,2,4]

Distant metastases also occur in canine UC with rates of 20% reported at the time of diagnosis and of over 50% by the time of death, based on necropsy examination.[2,4] Common locations for metastases include regional or distant lymph nodes, lung and bone; however other reported locations include liver, kidney, adrenal glands, skin, spleen, gastrointestinal tract, heart and brain.[2] Despite this, a particular tragedy with this disease relates to the frequency of local treatment failure resulting in urinary obstruction and leading to euthanasia in most cases. In a group of 85 dogs with UC for which the cause of death was known, local disease more commonly lead to death than metastatic disease in 61% and 14% of dogs respectively.[6] As this often occurs in otherwise bright and healthy dogs, it can become difficult for pet owners and clinicians to come to terms with such treatment failure.

1.1.2 Risk factors for canine urothelial carcinoma

Although the etiology of canine UC is likely multifactorial, several risk factors have been established. This includes exposure to older flea control products,[8,9] exposure to environmental lawn chemicals,[10,11] female sex, [2,4] and breed.[12] Scottish Terriers appear to be at highest risk with a reported odds-ratio of 21.12 for the development of UC when compared to mixed-breed dogs.[2] Other high-risk breeds

include American Eskimos, Shetland Sheepdogs, West Highland White Terriers, Keeshonds, Samoyeds, Beagles and Dalmatians with odds-ratios ranging from 2.43 to 6.58.[2] The risk of developing UC also appears to be higher in neutered dogs of both sexes though a reason for this has yet to be determined.[2,12] Although androgen and estrogen receptors are found in UC, their role in the development or progression of canine UC is currently unknown.[2]

1.1.3 Therapies for canine urothelial carcinoma

Therapeutic strategies for canine UC involve both local and systemic methods, including combinations of medical and radiation therapies as well as, occasionally, surgery or interventional urology. Tumours in trigonal or prostatic locations are rarely amenable to surgery; however, if this is attempted, careful techniques to avoid seeding cancerous cells into surrounding structures are crucial. Dogs undergoing cystotomy are at significantly higher risk of developing UC in the abdominal wall than dogs that do not.[13] Surgery also has a palliative role through the use of cystostomy catheters or tubes to relieve urethral obstruction and restore or maintain urine flow in obstructed or nearly obstructed dogs.[14,15] Minimally invasive interventional radiology approaches can also be applied for this purpose – and tend to be favoured over surgical techniques in recent years – with the use of urethral stent placement, which can be accomplished cystoscopically and/or with fluoroscopic guidance.[16,17] Of course, these procedures are not without inherent risk, and urinary incontinence is reported in 26%-39% of dogs following urethral stent placement.[16,17] Additionally, both stent migration and re-

obstruction can occur, and dogs are at higher risk of urinary tract infection following cystostomy tube and stent placement.[1,15,16] For these reasons, these surgical or minimally invasive approaches do not typically represent the standard of care for this tumour type.

The early use of radiation therapy in UC was characterized by a large treatment field and frequent side effects including chronic colitis, cystitis and urethral stricture, with only minimal survival advantages compared to medical therapy alone.[18,19] More recently, the use of intensity-modulated and image-guided radiation therapy (IM/IGRT) has become more widespread, leading to a reduction in early and late side effects but still only limited improvements in median survival times have been observed.[20,21] Nolan et al. describe a 60% response rate and median survival time of 654 days in a group of dogs treated with various combinations of definitive intent IM/IGRT, cyclooxygenase (COX) inhibitors, adjuvant chemotherapy and cytoreductive surgeries.[20] Overall, radiation therapy was well tolerated in that group of 21 dogs, with 19% developing late radiation-associated complications.[20] A more recent retrospective study investigating the combined use of COX inhibitors, mitoxantrone chemotherapy and IM/IGRT for dogs with genitourinary carcinoma, reported an overall survival time of 510 days.[22] In this group of 51 dogs, 31% experienced permanent urinary incontinence while other toxicoses were mild and self-limiting.[22]

Given the limitations of the aforementioned local therapies, without a doubt, the mainstay of treatment for canine UC has long been systemic medical therapy. The

medical approach generally consists of various combinations of maximum tolerated dose chemotherapy and COX inhibition, although metronomic chemotherapy protocols have also been investigated.[1,4,23–27] Although complete responses (CR) are rare, various agents induce a partial tumour response (PR) or produce stable disease and because resistance does not tend to be indiscriminate, some of the best outcomes are seen in dogs receiving sequential treatment protocols. Among the most commonly used chemotherapeutic agents are mitoxantrone and vinblastine which result in remission and stable disease in approximately 8-35% and 45-70% of dogs, repectively.[4,23–25,28] Additionally, platinum agents have demonstrated antitumour activity and strong remission rates when combined with COX inhibition.[29–31] Even occasional reports of CR with cisplatin and piroxicam therapy exist, however this combination also appears most likely to induce severe side effects including renal, gastrointestinal and bone marrow toxicities, limiting its clinical use.[30–32] An alternative platinum agent, carboplatin, is therefore more commonly chosen, with remission rates of up to 38% when used in combination with COX inhibition.[24,29]

COX inhibition as a monotherapy also represents a valid option, particularly if palliative treatment is sought. Single agent oral piroxicam has demonstrated CR in 2.6% of dogs, with 18.4% and 59.2% PR and stable disease respectively.[2] Selective COX-2 inhibitors, deracoxib and firocoxib have also been investigated for use as single agents, and while response rates are similar to those observed with piroxicam, CR has not been documented with the use of either of these drugs.[32,33]

Combinations of chemotherapy and radiation have led to highly variable response rates of 8-57%, however overall survival times for patients with UC still range from 105-654 days with definitive intent therapies.[20–27,33,34] Emerging treatment strategies including therapies targeting folate receptors, bladder cancer specific peptides, aberrant DNA methylation and immune checkpoint inhibitors are in various stages of development; however, despite this, effective new strategies are sorely needed in order to improve outcomes in dogs with UC.[1,35]

1.1.4 Canine urothelial carcinoma cell lines as a translational model

Pre-clinical models generally represent a critical first step prior to *in vivo* research investigating novel cancer therapies. Among these pre-clinical tumour models, monoculture cell lines are indispensable for *in vitro* assessment of potential new therapies. In addition to acting as a pre-clinical model for treatment of canine neoplasms, canine tumour cell lines can also provide a translational model prior to pursuit of human clinical trials. Canine UC has been established as a particularly valuable comparative model given the tendency for naturally-occurring disease to demonstrate invasive behaviour equivalent to muscle invasive UC in humans, a more commonly lethal manifestation of the disease.[36] In contrast, other models of bladder cancer, including experimentally induced tumours in rodents, tend to resemble lower grade, less invasive neoplasms.[36] For that reason, several cell lines have been established from invasive canine UC to serve as both a species specific and translational model for *in vitro* research into this disease.[36]

Three such cell lines were used in the present study, namely K9TCC (ORIG), K9TCC-PU-AxA (AXA) and K9TCC-PU-Sh (SH). These were among a group of 8 cell lines developed and reported in 2009 by a research group at Purdue University.[36] To establish these cell lines, tissue samples were collected from female dogs with UC, ranging in age from 8-12 years, at the time of either diagnostic procedures (cystoscopy or surgery), or euthanasia. None of these dogs had received chemotherapy prior to sample collection and all tumours were histologically diagnosed as grade 2 or 3 invasive UC.[36] After progression through 10-15 passages, all cell lines were characterized and their identity as canine UC confirmed using immunohistochemistry (IHC), which demonstrated moderate to strong positivity for vimentin, E-Cadherin and cytokeratin expression. Western blot for proteins of importance in human bladder cancer (pRb, E-Cadherin, COX-2 and p53) were also assessed, revealing comparatively lower expression of COX-2, pRb and p53 in SH.[36] Xenograft studies were subsequently performed, assessing for tumourigenicity following subcutaneous injection into athymic mice. Among the three cell lines listed above, few differences in behaviour were observed. Notably, SH demonstrated frequent multinucleate giant cells, while these were rare in ORIG and AXA. Additionally, this cell line demonstrated metastasis to a regional lymph node following engraftment, while the others did not.[36]

Ultimately, the development and existence of these cell lines have provided an indispensable tool, allowing continued advancements in cancer research through *in vitro* work such as that presented here.

1.2 Introduction to phytocannabinoids, the endocannabinoid system and cannabidiol

The role of the *Cannabis sativa* plant in medicine has a prolonged, and often controversial, history with reports of its use dating back over 5000 years.[37] The term cannabinoid refers to a group of over 90 terpenophenols derived from *C. sativa*, which are further classified based on their origins as either plant-derived cannabinoids (phytocannabinoids), laboratory synthesized cannabinoids or endogenously produced cannabinoids (endocannabinoids).[38,39] Regardless of their origin, all cannabinoids function through the complex endocannabinoid system, which has documented physiologic effects in a wide variety of body systems.[39,40]

Cannabinol, the main cannabinoid produced by *C. sativa*, forms from delta–9-tetrahydrocannabinol (Δ^9-THC) during storage and harvesting, and was the first plant-derived cannabinoid isolated in the late 19th century.[37,39] Several years later, in 1940, a second major component of the plant, cannabidiol (CBD), was also isolated.[37,39] Unlike Δ^9-THC, this second phytocannabinoid was not associated with changes in behaviour, mood, or consciousness, making it attractive for use as a therapeutic agent. The first evidence of this resulted from early work by Loewe in 1946, where Δ^9-THC was found to induce catalepsy in mice, while CBD did not, a sign that is now accepted to correlate with psychotropic activity.[37,39,41]

All cannabinoids exert their effects through the endocannabinoid system, which includes a variety of endogenous cannabinoid receptors. A thorough understanding of this system remains elusive, however it has been traditionally accepted to consist of three major components: two well-known G protein-coupled receptors (G-PCR), namely cannabinoid receptor type 1 (CB1) and type 2 (CB2); two primary endogenous lipid endocannabinoid ligands, N-arachidonoylethanolamine (anandamide or AEA) and 2-arachidonoylglycerol (2-AG); as well as their associated enzymatic machinery allowing for biosynthesis and degradation.[38,42–44]

Endocannabinoids are synthesized in animal tissues on demand via cleavage of the cell membrane phospholipid, arachidonic acid.[45] For AEA, this also involves N-acylation of phosphatidylethanolamine to generate N-acyl-phosphatidylethanolamine, followed by release of N-acylethanolamine by a phospholipase D type phosphodiesterase.[45,46] For 2-AG, the more abundant endocannabinoid, two diacylglycerol (DAG) lipases (DAGLα and DAGLβ), catalyze the hydrolysis of DAG to 2-AG.[45,47] As lipid-derived transmitters, endocannabinoids are lipophilic and therefore do not undergo storage within cellular vesicles. Instead they are immediately released from the cell following production, where they act on local cannabinoid receptors.[45] Subsequent degradation of endocannabinoids occurs via enzymatic hydrolysis performed primarily by fatty acid amide hydrolase (FAAH), also referred to as oleamide, for AEA and monoacylglycerol lipase (MAGL) for 2-AG.[45,48]

The above description however represents a gross simplification of the endocannabinoid system as a number of additional exogenous cannabinoids and molecular targets have since been identified and characterized.[44] While CB1 and CB2 are the most prevalent and are considered to be canonical receptors of the endocannabinoid system, several others have also been implicated in cannabinoid signaling, including the transient ion channel receptor potential vanilloid type 1 (TRPV1)[43,49–55]; G protein-coupled receptor 55 (GPR55)[43,50,51,56,57]; and nuclear peroxisome proliferator-activated receptor gamma (PPARγ) [**Figure 1**].[43,56–59] Several additional ligands in the extended endocannabinoid family have also been established including oleoylethanolamine (OEA) and palmitoylethanolamide (PEA). While not considered "true" endocannabinoids, due to their low affinity for canonical cannabinoid receptors CB1 and CB2, these fatty-acid derivatives are ligands at other receptors implicated in cannabinoid signaling including PPARs and vanilloid receptors.[44,60,61] Ultimately, these ligands and receptors all function in a series of complex signaling pathways with roles in homeostasis, development, cognition, learning, memory, anxiety, pain sensation, appetite, as well as neurologic, inflammatory and immune system function.[38–40,49,62]

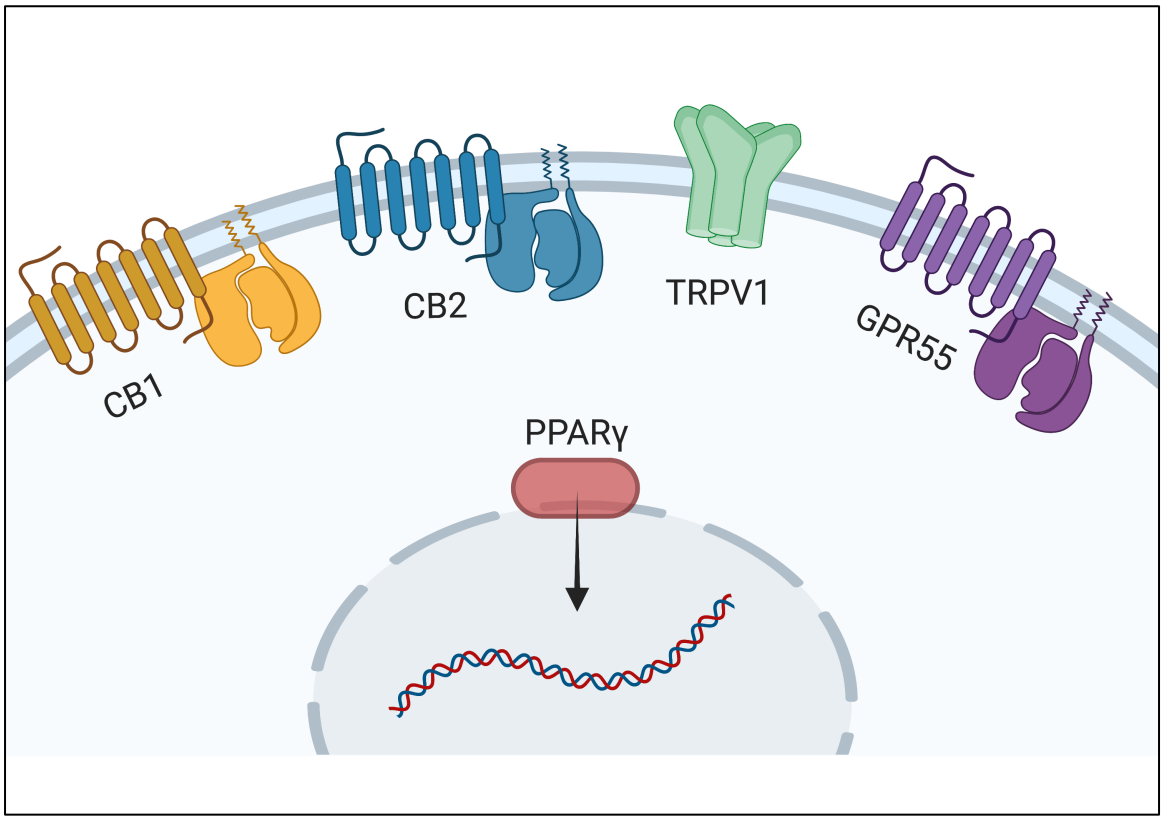


Figure 1. Major cannabinoid receptors and their reported cellular locations.
Cannabinoid receptor 1 (CB1), 2 (CB2) and G protein-coupled receptor 55 (GPR55) are membrane-bound G-PCRs. Transient Receptor Potential Vanilloid 1 (TRPV1) is a membrane-bound cation channel and Peroxisome Proliferator-Activated Receptor-gamma (PPARγ) is a nuclear receptor and regulator of transcription. Created with BioRender.com.

1.3 Cannabinoid receptors

1.3.1 Canonical cannabinoid receptors

The two canonical cannabinoid receptors, CB1 and CB2 were identified and cloned in the early 1990s.[40] Both are 7-transmembrane-domain G-PCRs located within the plasma membrane with a high affinity for their endogenous cannabinoid ligands AEA and 2-AG.[38,44,45]

The first evidence of their existence emerged in the mid-1980s through early radiolabeling techniques. Unlabeled cannabinoids were found to displace labeled synthetic cannabinoids from their binding sites in rat brain cell membranes.[63] This, combined with findings that psychotropic cannabinoids could inhibit adenylate cyclase through interactions with G proteins, which correlated with cannabimimetic responses *in vivo* in mice, cemented the existence of specific cannabinoid receptors functioning through G-PCR-mediated mechanisms.[37] Currently, it is generally accepted that these two major cannabinoid receptors inhibit adenylate cyclase and activate mitogen-activated protein kinase and a variety of downstream transcription factors.[37]

1.3.1.1 Cannabinoid Receptor Type 1

CB1 receptors are primarily found at sites of nerve terminals within the central nervous system (CNS) with lower concentrations in the peripheral nervous system (PNS).[37,38,49] These receptors are credited for the psychotropic effects of cannabinoids such as Δ^9-THC, and their activation leads to release of multiple neurotransmitters including acetylcholine, dopamine, GABA, serotonin, histamine and glutamate.[49] They

have roles in cognitive function, emotion, locomotor activity, hunger and neuro-protection as well as cardiovascular, gastrointestinal and respiratory effects.[49] Additionally, CB1 receptors have been implicated in pain pathways both within the CNS and at peripheral terminals of primary afferent neurons.[64]

1.3.1.2 Cannabinoid Receptor Type 2

CB2 receptors are mainly present outside of the CNS and, unlike CB1, are not generally considered to mediate psychotropic effects.[49,64] These receptors are primarily expressed on cells of the immune system, particularly B-lymphocytes and natural killer cells, but have also been found within other immune system tissues including mast cells, macrophages, spleen, lymph nodes and tonsils.[50,64] The activation of CB2 receptors has been shown to modulate immune cell migration, inhibit pro-inflammatory cytokine production and promote anti-inflammatory cytokine release, explaining their role in chronic pain relief and in management of inflammation.[37,42,49,65,66]

1.3.1.3 Activity of cannabinoids on CB1 and CB2

Among the endocannabinoids, AEA acts as a partial agonist on both classical cannabinoid receptors, binding with slightly greater affinity on CB1 than CB2 and inducing much greater biologic response via CB1 than CB2.[40] Alternatively, 2-AG has a similar affinity at both receptors with higher activity at CB2 than AEA, demonstrating 3-fold higher potency at this receptor.[40,67]

Among the phytocannabinoids, Δ^9-THC appears to demonstrate similar activity to AEA, binding with equal affinity to CB1 and CB2, and producing lower activity at the CB2 receptor.[40,64] Comparatively, CBD demonstrates low CB1 and CB2 receptor affinity and acts as a non-competitive negative allosteric antagonist of CB1, and an inverse agonist of CB2.[68–70]

1.3.2 PPAR receptors

PPARγ functions as a ligand binding transcription factor and is a member of the peroxisome proliferator-activated receptor family, which exists within the nuclear receptor superfamily.[71] Following activation by specific ligands, PPARs form heterodimers with the 9-cis-retinoic acid receptor (RXR) and bind to DNA promoter regions called PPAR response elements to induce transcription of PPAR target genes involved in cellular differentiation, proliferation, lipid metabolism, inflammation and apoptosis.[61,71–73] Thanks to their large ligand-binding domains, PPARs can be activated by ligands of varying chemical structures including fatty acids, fatty acid derivatives and several plant extracts.[71] Additionally, although they can both activate (transactivation) or inhibit (transrepression) gene expression, regulation of gene transcription via transactivation is most common.[74]

Several PPAR isoforms exist including PPARα, PPARβ and PPARγ, all of which are found in the brain and PNS, however compared to other PPARs, PPARγ is found in reduced quantities in the brain.[71,73] Beyond this, three PPARγ variants exist, PPARγ1,

which is ubiquitously expressed, PPARγ2, found in adipose tissue and PPARγ3, found on macrophages.[73,75]

PPARγ is well known for its roles in lipid metabolism, differentiation of adipocytes and insulin sensitivity.[73,75] In fact, thiazolidinediones, a class of drugs commonly used in the treatment of type II diabetes, are established agonists.[73,75] As with other PPARs, PPARγ also has anti-inflammatory actions in various tissues.[71] This effect occurs via protein-protein interactions of PPARγ with other transcription factors or coregulatory complexes bound to the promoter regions of target genes, resulting in transrepression.[76] Known transcription factor targets of PPARγ inhibition include those involved in several inflammatory pathways such as nuclear factor-kB (NF-κB), AP-1 and STAT.[76] PPARγ also reduces secretion of pro-inflammatory cytokines by monocytes, including TNF-α, IL-1, and IL-6.[77]

PPAR has also been implicated in cannabinoid signaling. This connection was first established in a study examining lipoxygenase metabolism of the endocannabinoid 2-AG, where a metabolite in this process was found to bind with PPARγ, inducing transcriptional activity.[78] In subsequent years, a synthetic structural analog of Δ^9-THC, ajulemic acid, and AEA were also both found to increase transcriptional activity of PPARγ.[79,80] Further studies using PPARγ antagonists have shed additional light on PPARγ-mediated cellular and tissue responses. Such antagonists can inhibit the effects of Δ^9-THC on vasorelaxation in isolated segments of rat arteries, induce neuroprotection

in neuroblastoma cells exposed to various toxins and cytotoxicity in hepatocellular carcinoma cells.[81–83]

Before long, CBD was also shown to bind and activate PPARγ in rat aortae through functional vascular studies and myography. This effect could be inhibited by PPARγ antagonists, but not by selective antagonists of canonical cannabinoid receptors CB1 and CB2.[84] PPARγ antagonists were subsequently confirmed to block anti-inflammatory and neuroprotective effects of CBD following administration of β-amyloid, and to counteract the pro-apoptotic effects exerted by CBD in human lung cancer cells.[59,85] Building on these findings, a wide range of publications have established CBD as a PPARγ ligand with roles in the cardiovascular system, immune system, inflammation, antineoplastic activity and neuroprotective effects, among others.[58,59,71,86–88]

Despite evidence that PPAR has a major role in cannabinoid and CBD signaling, the mechanism of this interaction remains elusive to date. Various pathways have been proposed, including direct binding of cannabinoids to PPARs within the nucleus, conversion of cannabinoids into PPAR-active metabolites prior to binding the PPAR target, activation of cell surface cannabinoids to bring about intracellular signaling cascades leading to indirect activation of PPARs within the nucleus, or a combination of all three [**Figure 2**].[73] Recently, evidence has emerged supporting the active transportation of cannabinoids, including CBD, via fatty acid binding proteins (FAPBs) to PPARs within the nucleus.[89] FABPs are intracellular, lipid-binding proteins that

transport and direct fatty acids throughout the cell and nucleus. To date, it has been shown that the endocannabinoid AEA is transported by FABPs 5 and 7 from the plasma membrane to fatty acid amide hydrolase (FAAH) for hydrolysis and degradation and that activation of PPARα by OEA, another endocannabinoid-like compound, is reduced via FABP inhibition.[90,91] CBD and Δ^9-THC are also transported intracellularly by FABPs, which may represent the mechanism by which they are moved to the nucleus for PPAR activation.[89] From this information, it has been postulated that FABP transportation can direct the movement of cannabinoids within the cell, either to enzymes including FAAH for degradation, or to PPAR in the nucleus for activation of transcription [**Figure 2**].[61,71,73]

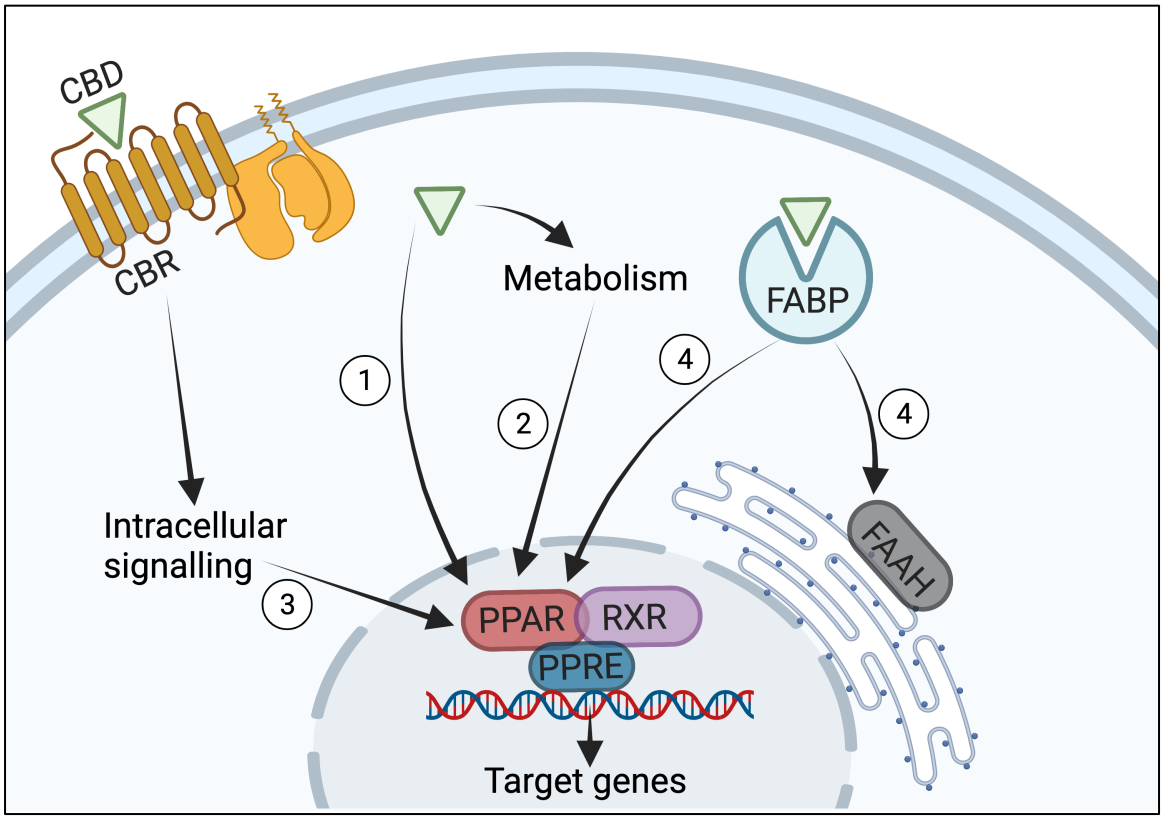


Figure 2. Four potential mechanisms of cannabinoid and PPAR signaling.

(1) Direct binding of cannabinoids, such as CBD, with PPARs within the nucleus leading to induction of transcription of target genes. (2) Conversion of cannabinoids into metabolites for subsequent transfer to the nucleus and activations of PPARs. (3) Binding of cannabinoid ligands to cell surface cannabinoid receptors leading to induction of an intracellular signaling cascade and indirect activation of PPAR activity. (4) Binding of intracellular cannabinoids to FABPs resulting in either transport to FAAH for hydrolysis and degradation, or intranuclear transport to PPAR for activation of transcription. Created with BioRender.com.

1.3.3 GPR55 receptors

More recently, GPR55 has been implicated as a novel class of receptor involved in endocannabinoid signaling.[45,92] This G-PCR was first identified and cloned in 1999 and at that time was considered an orphan G-PCR, implying that a known ligand had yet to be determined.[93] This receptor was found to be highly expressed in the human brain and was mapped to chromosome 2q37 using fluorescence in situ hybridization.[93]

Shortly following its discovery, two industrial patents suggested a role for GPR55 as a cannabinoid receptor and the first endogenous ligand, lysophosphatidylinositol (LPI), was established.[94,95] That same year, another group corroborated its identity as a cannabinoid receptor confirming binding and activation by the synthetic cannabinoid CP55940 in HEK293 cells.[92] The effects of CBD were also of note in this study, showing no agonistic effect on GPR55 and acting to antagonize the effect of CP55940.[92]

In the ensuing years, investigation into the activity of other cannabinoids on GPR55 have produced conflicting results. AEA has been variably found to act as an agonist or partial agonist of GPR55, with enhancing effects at low concentrations and inhibitory ones at high concentrations, while other groups failed to identify any agonistic effects.[92,96–98] Other synthetic cannabinoids also activate GPR55, though their effects are varied and at times contradictory, with the CB2 agonist HU210 and the CB1 antagonist AM251 both exhibiting agonist activity for GPR55.[99,100] CBD specifically, acts as a GPR55 antagonist while the synthetic CBD analogue O-1602 instead appears to activate this receptor.[92,101–103]

Interestingly, despite the fact that classical cannabinoid receptors CB1 and CB2 are also G-PCRs, only limited homology of GPR55 with these receptors (13.5 and 14.4% respectively) exists.[94] Studies examining the amino acid sequence of GPR55 have also revealed that it lacks the classical "cannabinoid binding pocket" found on both of these original receptors, suggesting that major differences exist in ligand binding sites between these G-PCRs.[104]

Also, despite their shared G-PCR identities, the downstream signal transduction pathways of GPR55 appear to differ considerably from those of CB1 and CB2. Following ligand binding, GPR55 appears to activate the ras homologue gene family member A (RhoA), cdc4 and rac1, leading to induction of the phospholipase C (PLC) pathway, which promotes release of Ca^{2+} from inositol triphosphate receptor-gated endoplasmic reticulum stores, increasing concentrations of intracellular Ca^{2+}. This release in turn leads to phosphorylation and activation of extracellular signal-regulated kinase (ERK) and downstream target gene expression.[94,98,99,105,106] However, this pathway does not provide the full picture of GPR55 signaling, as other studies have found that GPR55 does not consistently activate ERK phosphorylation above basal levels.[102] Still others suggest that GPR55 activation leads to activation of a slightly different signaling pathway, also implicating RhoA, followed by activation of Rho-associated protein kinase (ROCK), PLC and intracellular calcium release, finally culminating in activation and nuclear translocation of the nuclear factor of activated T cells (NFAT) transcription factor.[97,103] Additional transcription factors have also been

implicated in the downstream signaling pathway of GPR55, including NF-kB and cAMP response element binding protein (CREB).[100]

To further complicate matters, GPR55 signaling also appears to be modulated by heteromerization and co-localization with canonical cannabinoid G-PCRs. Variable co-expression of CB1/CB2 and GPR55 exist in different cell types. For instance, GPR55 and CB1 are both highly expressed in several regions of the brain and CNS while CB2 and GPR55 are both found on neutrophils and various cancer cells.[107–109] Co-expression of GPR55 with CB1 appears to result in a physical interaction between the two receptors in the absence of ligand signaling. Additionally, un-stimulated CB1 has been shown to inhibit GPR55-mediated transcription factor activation of both NFAT and ERK while paradoxically, in the same study, CB1 activity was amplified by the presence of GPR55.[107]

In neutrophils, where CB2 and GPR55 co-localization occurs, GPR55 activation appears to inhibit CB2-mediated ROS production, while promoting CB2-mediated cell polarization and chemotaxis.[109] Finally in certain cancer cells, co-expression of CB2 and GPR55 results in heteromerization, cross-talk between receptors and ligand-concentration dependent signaling. At low Δ^9-THC concentrations, CB2 agonism occurs while at high concentrations, GPR55 antagonism occurs and results in uni-directional, cross-antagonism of CB2 through the heteromer.[108]

Overexpression of GPR55 occurs in a variety of cancer cells and is generally linked with cell growth and proliferation.[105,110,111] Considering its role as a major ligand of GPR55, it is also interesting to note that LPI and other major lysophospholipids, are also elevated in certain human cancers.[112,113] Working with GPR55, this ligand plays a role in cell migration, orientation and polarization of breast cancer cells, key characteristics of their metastatic behaviour.[111,112] Additionally, GPR55 overexpression has been correlated with higher grade tumours and reduced survival times in human glioblastoma and breast cancer.[111] Evidence that GPR55 plays a role in promoting tumour proliferation is also supported by studies in which tumour growth was slowed by knockdown of GPR55 in nude mice xenografted with T98G glioma cells.[111] Tumours with lower levels of GPR55 also showed reduced Ki-67 positivity and decreased levels of phosphorylated ERK.[111]

1.3.4 TRPV1 receptors

TRPV1, also known as the capsaicin receptor, is a cell membrane receptor in the transient receptor potential (TRP) channel family.[55] TRP channels are a superfamily of ion channels found in the plasma membrane that modulate ion entry into the cell and mediate signal transduction in response to various physical and chemical stimuli.[55] TRPV1 was the first member of the TRPV family to be identified and, as for other members of the TRP superfamily, acts as a nonselective cation channel for divalent cations, including Mg^{2+}, Ca^{2+} and Na^{2+}. Following ligand binding and activation, calcium preferentially moves through the pore into the cell and stimulates a series of calcium-

dependent processes, resulting in the integration of noxious stimuli along small diameter afferent nerve fibers.[55,114,115]

Although it is primarily activated by noxious stimuli, including heat and acids, several natural compounds from vanilloids, capsaicin (found in chili peppers) and allicin (found in garlic) can also activate specific TRP channels.[55] In addition, modulation by cannabinoid receptors has also been established. TRPV1 is an important site of activity for endogenous cannabinoids and AEA was the first endocannabinoid agonist to be identified for this receptor.[55,116] Numerous *in vitro* studies have since firmly established TRPV1's role in the endocannabinoid system with assays involving human and rat cell lines and *ex vivo* tissues from various mammalian species.[116,117]

CBD has also been found to have an antihyperalgesic effect *in vivo* in rats, which could be reversed using a TRPV1 antagonist, capsazepine, suggesting action of CBD at this cannabinoid receptor as well.[118] Interestingly, in the same report, neither antagonists for CB1 or CB2 modified the antihyperalgesic effects of CBD, suggesting a limited role for these receptors in that process.[118] Ultimately, CBD is reported to be potent at the TRPV1 receptor with comparatively less affinity for the canonical CB1 and CB2 receptors.[44,114,118,119] Further, recent computational studies have demonstrated higher calculated binding energy for the binding of CBD to TRPV2 compared to TRPV1, which suggest higher affinity for the former.[120] In comparison, Δ^9-THC, preferentially modulates TRPV2 activity with moderate activity on TRPV3 and TRPV4 but does not modulate TRPV1 activity.[119]

1.4 Cannabinoids in cancer treatment

A variety of research has been undertaken to better understand and exploit the endocannabinoid system with the goals of reducing symptoms or delaying progression of numerous disease processes. This includes schizophrenia, pain, multiple sclerosis, intestinal and cardiovascular diseases, among others.[37,39] Additionally, in the realm of cancer therapy, the presence of several cannabinoid receptors in assorted human malignancies, including UC, has generated interest in their potential role as an antineoplastic agent.[58,121–125]

Cannabinoids were first introduced to the field of cancer treatment for their palliative properties, with demonstrated roles in the reduction of nausea and anorexia, and in the management of pain.[37,50,56] In the 1980s, dronabinol, a Δ^9-THC product and its synthetic analogue, nabilone, were both licensed for suppression of nausea in patients receiving chemotherapy.[37] Subsequently, a product containing both Δ^9-THC and CBD, Sativex, was developed and licensed in Canada for the treatment of neuropathic pain in individuals diagnosed with multiple sclerosis and was later also explored for the treatment of pain in individuals undergoing cancer therapy.[37]

With progression of basic scientific and clinical research since the end of the 20th century, the potential applications for cannabinoids as therapeutic agents have greatly expanded. The endocannabinoid system has now also been studied for its anti-neoplastic effects and multiple cannabinoids, including the well-characterized Δ^9-THC and CBD, have demonstrated these effects both *in vitro* and *in vivo.*[58,121–125] Due to its

psychoactive effects there are limited therapeutic applications of Δ^9-THC; however, this limitation has opened the door for CBD to become a focus in the investigation of cannabinoids for cancer treatment.[38] As previously noted, CBD is thought to act as a negative allosteric inhibitor of CB1, potentially contributing to this reduced psychoactive profile and thus broadening its potential use as a therapeutic agent.[56]

1.4.1 Anti-neoplastic activity and mechanisms of action of Cannabidiol

Cannabinoids have been shown to exert their anti-cancer effects in some human cell lines and *in vivo* models through CB1 and CB2 receptors,[126,127] however in many cases, they also function in a CB1 and CB2 independent manner.[128] CBD in particular has a relatively low affinity for these receptors with reported antagonistic effects on CB1 and inverse agonistic effects on CB2.[38,68,69] Various studies of the anti-neoplastic effects of CBD have also implicated several mechanisms like apoptosis,[125,129] autophagy,[125,130] induction of reactive oxygen species (ROS)[131] or inhibition of the AKT/mTOR cellular proliferation pathway.[125,128] CBD has even been found to act synergistically with, and aid in the perfusion of, other chemotherapeutic agents.[50,132–134] Two specific mechanisms of activity frequently implicated in the anti-cancer effects of CBD, apoptosis and autophagy, are classified as programmed cell death (PCD). PCD is a process of cellular suicide, which is critical to normal tissue development and homeostasis. Apoptosis generally involves the activation of caspases, a family of proteins which initiate orderly cellular disassembly, involving phosphotidyl serine inversion on the plasma membrane, DNA fragmentation and cell death.[135] Autophagy is

a more recently described mechanism of PCD. This mechanism involves production of de novo-synthesized, double membrane enclosed vesicles, which engulf and degrade cellular components.[136]

A range of literature exists to support the antineoplastic effects of CBD in human neoplasia. Early *in vitro* research into its effects on human glioma cells demonstrated an ability to inhibit cell migration[128] and trigger oxidative stress and caspase activation, leading to apoptosis.[137] CBD-associated apoptosis was also demonstrated in human leukemia cell lines through the activation of caspase pathways.[131] In human breast cancer cell lines, it has been shown to inhibit cell growth, down-regulate ID-1,[138] a regulator of metastasis, and induce cross-talk between apoptosis and autophagy.[58,125,139] Even pancreatic carcinoma has been the subject of investigation for the anti-proliferative and pro-apoptotic effects of CBD, which appear to be primarily mediated through CB1, CB2 and GPR55 pathways.[124] Interestingly, despite this variety of anti-neoplastic mechanisms, CBD has been shown to preferentially kill cancerous cells compared to non-cancerous ones when used *in vitro* at concentrations previously shown to induce cell death.[125,139]

1.4.2 Cannabidiol and human urogenital neoplasms

As it pertains to human urogenital malignancies, a recent systematic literature review investigating the use of cannabinoids in human urological cancers identified 23 studies performed between 1946 and 2016.[140] The majority of these focused on the role of cannabinoids in the treatment of prostate cancer and CBD appears to inhibit prostatic

carcinoma cell growth *in vitro* and *in vivo* through several mechanisms including apoptosis and production of ROS.[129] Interestingly, high CB1 immunoreactivity at diagnosis has been associated with poorer outcomes, and increased CB1 and CB2 expression have been found in samples from tumours with a higher Gleason score, suggesting a more advanced disease stage.[141,142]

Fewer studies have examined the effects of cannabinoids on human bladder cancers. One report, exploring the roles of CB1, CB2 and endogenous cannabinoids AEA and 2-AG in human bladder UC, demonstrated a role for CB1 in proliferation and for CB2 in triggering the pro-inflammatory state.[143] Another recent study also demonstrated *in vitro* antineoplastic effects, including apoptosis, down-regulation of AKT and decreased cell mobility, of human bladder UC cells through CB2 mediated pathways.[121] Furthermore, CB2 was upregulated in neoplastic tissues while expression of CB1 was similar between healthy and neoplastic ones, when assessed using IHC. Quantitative PCR analysis also revealed upregulation of both receptors, though CB2 levels were higher, when primary muscle invasive UC was compared with paired normal tissues.[121]

Moving beyond the canonical cannabinoid receptors, TRPV receptor expression in human bladder UC has also been correlated with high-grade disease.[144] In addition, TRPV2 receptors have been implicated in CBD-induced apoptosis secondary to calcium influx.[144] Administration of CBD also resulted in a dose dependent reduction in cell

viability of a poorly differentiated human UC cell line, while those of a well-differentiated UC cell line were relatively unaffected.[144]

Given the predominantly *in vitro* nature of much of this research, and a lack of clinical trials performed to date, the role of CBD in the management of urological malignancies is evidently an area of active research with significant knowledge gaps awaiting further exploration.

1.5 Cannabinoids in veterinary medicine

1.5.1 Cannabinoid receptor expression

Despite the wealth of basic scientific literature examining the anti-neoplastic effects of CBD in human neoplasms, there is a lack of such information available for veterinary patients. Even evidence to support the presence of cannabinoid receptors in canine malignancies, a required early step in such preclinical investigation, is currently lacking.

In normal canine tissues, as in human tissues, the presence of CB1 has been well established within *ex vivo* canine brain and peripheral nerve tissues.[145–147] CB1 expression has also been identified through IHC in canine skin and hair follicles, salivary tissues as well as sensory structures (inner ear, olfactory epithelium and eye) and thyroid in developing canine embryos.[147–150]

Building on these identification studies, the cellular distribution of canonical and "newer" cannabinoid receptors within the canine cervical dorsal root ganglia have also been explored, using IHC to evaluate *ex vivo* samples for the presence of CB1, CB2, GPR55, PPARα, and TRPV1.[145] In this study, weak to moderate CB1 and TRPV1 immunoreactivity was present in 50% of neuronal cells, 100% demonstrated CB2 immunoreactivity and 40% also expressed GPR55. PPARα immunoreactivity was found in satellite glial cells and endothelial cells of local blood vessels. Although 5/8 canine patients whose tissues were obtained for use in this study died of neoplastic disease, these tumour tissues were not evaluated for cannabinoid receptor expression.[145]

The gastro-intestinal tract of both normal dogs and those suffering from chronic colonic dysmotility has also been evaluated for localization of various cannabinoid receptors.[57,145,151] In *ex vivo* tissues extracted from normal canine gastrointestinal tract of 3 dogs dying of various causes, CB1, CB2, PPARα and GPR55 were widely distributed within the canine pylorus, descending duodenum, ileum, and distal colon using IHC.[57] The specificity of anti-cannabinoid receptor antibodies for each of these receptors in canine small intestinal samples has also been assessed via western blot.[57,145] CB1 and CB2 expression has also been confirmed in epithelial cells of samples of canine colonic mucosa collected via endoscopic biopsy.[151] However once again, no literature investigating the presence of either canonical or putative cannabinoid receptors in canine neoplastic tissues currently exists.

1.5.2 Cannabidiol in veterinary medicine

To date, administration of CBD in dogs has generally been found to be safe, with few observed side effects.[49,152–158] Previous literature has explored its use in the settings of chronic pain and epilepsy, and pharmacokinetic and safety studies examining tolerability at various doses exist as well.[152–158] Overall, these veterinary studies have found CBD to be well tolerated with side effects generally limited to mild to moderate, progressive serum alkaline phosphatase elevation associated with chronic use.[152,154] The use of cannabinoids as a palliative, appetite-stimulating tool for veterinary patients with oncological disease has been reported anecdotally, and the owner-directed use of CBD as a health or nutritional supplement is also increasingly common in dogs with cancer.[159–162] However literature describing the use of CBD or other cannabinoids in canines for its antineoplastic effects is very limited with reports restricted entirely to *in vitro* use.[133,134,163]

1.5.2.1 Pharmacokinetics and safety of cannabidiol in dogs

The first assessment of pharmacokinetic activity of CBD in canines was reported in 1988 with the goal of justifying its use as an antiepileptic agent.[155] In this study, both intravascular (IV) and oral administration of CBD were assessed, using high performance liquid chromatography to measure plasma levels of CBD. Rapid distribution with prolonged elimination and a half-life ($t_{1/2}$) of 7-9 hours occurred following IV administration of two doses (45 and 90mg). Following oral administration of a gelatin capsule containing 180mg CBD, only minimal absorption occurred with

undetectable plasma levels in 3/6 dogs studied. This low oral bioavailability was suspected to be due to a high first pass effect and incomplete absorption of the drug related to its highly lipophilic nature.[155]

More recently, a number of pharmacokinetic and safety studies of CBD using liquid chromatography-mass spectrometry (LC-MS) to measure plasma levels of CBD have been undertaken investigating additional formulations, dosages and delivery methods.[152,153,156,157] In a study of 30 healthy research dogs receiving 1 of 3 oral or transdermal formulations at 2 dosages for each (approximately 10mg/kg and 20mg/kg body weight per day), an oral CBD-infused oil was found to achieve the highest plasma levels and provide the most favorable pharmacokinetic profile.[153] At the highest dose, this formulation achieved a median maximal plasma concentration (C_{max}) of 903.68 ng/mL +/- 262.15 ng/mL (approximately 2.87 uM) and had a bioavailability of 100% and $t_{1/2}$ of 127.5 +/- 32.2 min. This formulation also produced the smallest inter-individual variability in plasma CBD levels. In this study, CBD was administered with food, which has also previously been reported to be advantageous.[158]

Another pharmacokinetic study compared lower oral doses of an oil-based CBD formulation with placebo in dogs with osteoarthritis and found a similar elimination $t_{1/2}$ (4.2 hrs) and median plasma CBD C_{max} of 102.3 ng/mL (180 nM) and 590.8 ng/mL (1.2 uM) for 2mg/kg and 8mg/kg doses respectively.[152] This study also reported adverse events and found no obvious psychoactive effects at any time point over the 4 weeks of treatment.[152] Serial evaluation of complete blood count and biochemical profile

demonstrated a significant increase in alkaline phosphatase (ALP) from baseline after 4 weeks of treatment in 9/16 treated dogs, with no other clinically relevant differences in serum chemistry or hemogram values between placebo and CBD oil groups.[152]

Another randomized, placebo-controlled, blinded, parallel study evaluating the safety of escalating cannabinoid doses in healthy research dogs also found a CBD-oil formulation to be well tolerated up to the highest administered dose of 62 mg/kg.[156] Although adverse events were reported in some dogs receiving CBD oil, they primarily consisted of mild gastrointestinal signs (nausea, diarrhea, emesis) and did not differ in frequency from those in the placebo group. This study also found mild elevation of ALP from baseline in dogs receiving CBD with a single dog in this group demonstrating a 2.9-fold elevation of ALP 24 hours after receiving the final dose and a downward trend in this value seen at day 7.[156] CBD plasma levels were measured following the 9th of 10 doses and also achieved the low micromolar dose range with mean peak C_{max} of 347.0 ng/mL (1.1uM) 24 hours after administration of the drug.[156]

Transient ataxia has also been reported in another study using a similar oral, oil-based formulation of CBD which achieved similar plasma concentrations in a group of dogs with intractable idiopathic epilepsy.[154] Significant increase in serum ALP over the 12 week study period was also reported in that group of dogs.[154] Although dogs in this study were also receiving additional anti-epileptic drugs, for which side effects also include ataxia and elevation of hepatic parameters, these effects were not seen in the placebo group.

1.5.3 The use of Cannabidiol in canine oncology

As previously noted, veterinary literature describing the use of CBD in canine neoplasia is very limited and to date, restricted to cell culture models. Two reports published in 2021 explore the use of CBD in combination with common chemotherapeutic drugs in cell lines of various common canine neoplasms.[133,134] Henry et al. examined the effects of CBD on several major proliferative signaling pathways including p38, JNK, AKT and mTOR.[134] This report found that CBD reduced canine cancer cell proliferation, assessed via 3-(4,5-Dimethylthaizol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays, across canine cell lines from various neoplasms including osteosarcoma, mammary carcinoma and lymphoma. In this study, drug concentrations in the range of 2.5-10 ug/mL were used (7.9 – 31 uM), which are well above reported plasma levels that have been achieved *in vivo*, ranging from approximately 1.1-2.87 uM.[152,153,156] In this report, combined treatment with vincristine and CBD resulted in a synergistic or additive reduction in cell proliferation across all cell lines while combination with doxorubicin showed more variable results, with synergy or additive effects at higher CBD doses and antagonistic effects at lower CBD doses. CBD was also found to rapidly induce apoptosis within 4-8 hours of treatment, which was followed by autophagy across all cell lines. Using western blot to assess for changes in major signaling molecules, there was no alteration in the PI3K-AKT-mTOR signaling at the time points of induction of apoptosis or autophagy. Instead, repeatable increases in both ERK and JNK phosphorylation were seen in all cell lines in the 8 hours following treatment, suggesting activation of the MAP kinase pathway. These changes

corresponded with changes in autophagic markers LC3II and p62, and a marker of apoptosis, caspase 3, suggesting a role for ERK and JNK signaling in CBD-induced autophagy and apoptosis.[134]

The second report of the use of CBD in canine neoplasia specifically explored its use in 3 canine UC cell lines.[133] In this report, produced by the same research group as the present study, treatment with CBD resulted in a dose dependent reduction in cell viability, as assessed by crystal violet assays, with IC_{50}s in the 5-6 uM range. As with the previous report, this mildly exceeds doses which have been achieved in canine plasma.[134,152,153,156] This study also assessed combination therapy of CBD with several chemotherapeutic drugs including vinblastine, mitoxantrone and carboplatin. A synergistic relationship was found between CBD and the first two agents while an antagonistic relationship of CBD with carboplatin was found.[133] Based on the major role of COX inhibitors in the treatment of canine UC, combinations of piroxicam with both CBD and the above chemotherapeutic agents were also assessed, with no interaction or effect found when piroxicam was combined with CBD.[133]

It is also worth noting that, previously, another study investigated the treatment of a canine osteosarcoma cell line with cannabinoids, though that project focused exclusively on the use of a synthetic cannabinoid WIN-55,212-2, rather than CBD. This study found a 35% reduction in expression of VEGF-A, a key stimulator/regulator of angiogenesis, following administration of WIN-55,212-2 *in vitro*. However this report did

not assess for the presence of cannabinoid receptors in the cells, investigate major signaling pathways or further explore potential anti-neoplastic mechanisms.[163]

1.6 Study Rationale, Hypotheses and Objectives

When considering the documented anti-cancer effects in human neoplasia, emerging evidence of anti-neoplastic effects in canine UC cells and other neoplasms, and minimal reported side effects in dogs, CBD represents an interesting candidate for a possible novel therapy for canine UC.

While CBD may prove to be a viable anti-cancer therapeutic, prior to its use in veterinary oncology patients, it must be carefully evaluated to better understand both its mechanisms of action and effectiveness. This project represents a unique opportunity to fill a knowledge gap and gain insight into the expression of cannabinoid receptors in canine UC, while simultaneously exploring the mechanisms by which CBD might exert anti-neoplastic effects. This project is the first, to the author's knowledge, to evaluate the expression of cannabinoid receptors in canine cancer cells and provides further elucidation of the potential anti-neoplastic mechanisms of CBD on canine UC cell lines *in vitro.* This investigation establishes preliminary information to pave the way for new tools in the treatment of dogs with UC, a disease in great need of novel therapeutic agents.

The main objectives of this study were to:

1. Evaluate expression of known cannabinoid receptors CB1, CB2, PPARγ, GPR55, and TRPV1 in canine UC cell lines.
2. Establish half-maximal inhibitory concentration (IC_{50}) values for CBD in canine UC cell lines.
3. Assess for CBD-induced reductions in cell viability in non-cancerous, immortalized canine renal epithelial cells.
4. Investigate whether treatment of canine UC and renal epithelial cell lines with CBD induces apoptosis and autophagy.
5. Assess whether CBD-induced reductions in cell viability occurring in UC cell lines and a canine renal epithelial cell line occur through CB1, CB2, PPARγ, GPR55 and TRPV1 mediated mechanisms.

Hypotheses:

1. Cannabinoid receptors CB1, CB2, PPARγ, GPR55 and TRPV1 are expressed in canine urothelial carcinoma cell lines.
2. Treatment with CBD will result in decreased cell viability in canine UC in a dose dependent manner.
3. Treatment with CBD will not result in decreased cell viability in non-cancerous canine renal epithelial cell lines.
4. Treatment with CBD will induce cell death by apoptosis and autophagy.

5. Treatment with CBD will result in decreased cell viability of UC cell lines through CB1, CB2, PPARγ, GPR55 and TPRV1 receptor mediated mechanisms

2 Materials and Methods

2.1 Cell lines and culture conditions

Three canine UC cell lines were used for all experiments: ORIG, AXA and SH. Additionally, a non-cancerous, immortalized canine renal epithelial cell line, MDCK, was used as a negative control, representing normal tissue. All canine urothelial carcinoma cell lines were generously provided by Dr. Deborah Knapp (Purdue University, West Lafayette, IN, USA). MDCK was obtained from American Type Culture Collection and was harvested from a normal female adult cocker spaniel kidney in 1958 and since immortalized. A human breast carcinoma cell line, MDA-MB-231 (MB-231) was also used as a positive control in immunoblotting experiments as it has been previously demonstrated to express all cannabinoid receptors assessed in this study.[112,164–167] All cell lines were tested negative for mycoplasma spp. prior to experimentation.

Cells were grown in monolayer culture in Dulbecco's Modified Eagle medium (DMEM) (Wisent Bioproducts, Quebec, Canada), supplemented with 10% Fetal bovine serum (FBS) (Wisent Bioproducts, Quebec, Canada), 100 U/mL penicillin-streptomycin (Thermo Fisher Scientific, Massachusetts, USA) and 0.05mg/mL plasmocin prophylactic (InvivoGen, San Diego, USA). Cells were incubated in standard conditions at 37°C and 5% CO_2 in a humidified incubator.

2.2 Chemical Reagents

CBD was obtained as a resin from MediPharm Labs and third-party analysis was performed (Eurofins Experchem Laboratories, Inc., Ontario, Canada), revealing a

product with approximately 75.8% CBD, 0.8% CBDA and 3.36% THC. The product was resuspended at a stock concentration of 50mM in a vehicle solution containing 5% ethanol, 5% Tween, and 90% Phosphate buffered saline (PBS) (HyClone, Utah, USA). Aliquots were then prepared and stored at -20°C for up to 4 months prior to use. Doxorubicin (Accord Healthcare, Quebec, Canada) was obtained from the Ontario Veterinary College Health Sciences Center pharmacy and maintained at a stock concentration of 3.68mM. All cannabinoid receptor antagonists (AM 251, SCH 336, T0070907, capsazepine, ML 193) [**Table 2-1**]. were obtained from Tocris Biosciences (Bristol, UK) and were resuspended in DMSO and maintained at stock concentration of 10mM (AM 251) or 20mM (SCH 336, T0070907, capsazepine, ML 193).

Table 2-1 Cannabinoid receptors and their antagonists.

Antagonist	Cannabinoid Receptor Target	Product	Source[a]
AM 251	CB1	1117	Tocris
SCH 336	CB2	5815	Tocris
T0070907	PPARγ	2301	Tocris
ML 193	GPR55	4860	Tocris
Capsazepine	TRPV1	0464	Tocris

[a]*Tocris Biosciences, Bristol, UK.*

2.3 Protein extraction

Cell lines were grown in conditions described above until 95% confluence was observed. Cell lysis for protein harvest was performed using a complete lysis buffer (Cell Signaling Technology, Massachusetts, USA) made up of the following components: 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA,

1% Triton-X 100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 µg/mL leupeptin, 1 mM PMSF, 2 µg/mL aprotinin, and 1% phosphatase inhibitor cocktail II. Ten cm cell culture plates were placed on ice, media was removed, and cells were washed with PBS prior to the addition of the above lysis buffer. After five minutes, cells were scraped and protein harvested into a 1.5mL microcentrifuge tube, which was also kept on ice for twenty minutes. Tubes containing cell lysate were centrifuged at 15,000 rpm for 20 minutes at 4°C. The supernatant was aliquoted into a 0.5mL tube and stored at -80°C until protein quantification or further use. Prior to Western blot assays, protein concentrations were evaluated via Bradford assay using a bovine serum albumin (BSA) standard curve.

2.4 Immunoblotting

CB1, CB2, PPARγ, GPR55 and TRPV1 receptor protein expression was evaluated in all canine UC (ORIG, AXA, SH) and non-cancerous immortalized cell lines (MDCK) using Western blot analysis. As previously noted, MB-231, a human breast carcinoma cell line, was also used as a positive control for immunoblotting purposes. All primary antibodies used in the present study are listed in **Table 2-2**.

Table 2-2 Primary antibodies used.

Primary Antibody	Host	Catalog No.	Source[a]
CB1	Rabbit	Orb10430	Biorbyt
CB2	Rabbit	Ab 3561	Abcam
PPARγ	Rabbit	PA3 – 821A	Invitrogen
GPR55	Rabbit	NLS 6817	Novus
TRPV1	Rabbit	NBP1 – 97417	Novus

β-actin	Rabbit	4970S	Cell Signaling

[a]*Suppliers of primary antibodies: Biorbyt Ltd., Cambridge, UK; Abcam Cambridge, UK; Novus Biologicals, Colorado, USA; Invitrogen, Massachusetts, USA; Cell signaling* Technology, Massachusetts, USA.

Protein lysates were resolved by 10% reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis using 30-50 µg of protein [**Table 2-3**]. A Polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, California) was subsequently activated in methanol and protein lysates were electrotransferred to activated PVDF membrane at 100V for 2 hours. Following transfer, membranes were incubated for 1 hour at room temperature with gentle rocking in a blocking solution of either 5% skim milk or 5% BSA (Wisent Bioproducts, Quebec, Canada) in Tris Buffered Saline + 0.01% Tween (pH = 7.6, TBST) [**Table 2-3**].

Membranes were then incubated overnight at 4°C on a rocking platform with primary polyclonal rabbit antibody diluted to either 1:500 or 1:1000 in 5% skim milk or 5% BSA [**Table 2-3**]. The rabbit primary monoclonal antibody β-actin (Cell Signaling Technology, Massachusetts, USA) diluted 1:10,000 in BSA, was used as a loading control.

Table 2-3 Western blotting conditions and antibody dilutions used in this study.

Primary Antibody	Gel %	Protein quantity	Blocking solution	Dilution	Primary antibody diluent	Secondary antibody diluent
CB1	10%	30 µg	5% milk	1:500	5% milk	5% milk
CB2	10%	50 µg	5% BSA	1:1000	5% BSA	2.5% milk, 2.5% BSA
PPARγ	10%	30 µg	5% milk	1:1000	5% milk	5% milk
GPR55	10%	40 µg	5% BSA	1:1000	5% BSA	5% milk
TRPV1	10%	30 µg	5% milk	1:1000	5% milk	5% milk

The following day, membranes were washed three times with TBST (15 minutes/wash) prior to incubation for 1 hour at room temperature with secondary anti-rabbit antibodies conjugated to horseradish peroxidase (Cell Signaling Technology, Danvers, Massachusetts) diluted 1:10 000 in skim milk or BSA [**Table 2-3**]. Membranes were subsequently washed again three times with TBST and chemiluminescence imaging was performed by a ChemiDoc MP imaging system (Bio-Rad, Hercules, California)

2.5 Cell viability assays

The crystal violet colorimetric assay was used to assess cell viability. These assays were used in the generation of half-maximal inhibitory concentration (IC50) curves for CBD, determination of cannabinoid receptor antagonist dosing and evaluation of mechanisms of action of CBD through the examination of its combination with known cannabinoid receptor antagonists and the ensuing effects on cell viability.

2.5.1 Half maximal inhibition concentration curves for CBD

For IC50 curves, cells lines ORIG, AXA, SH and MDCK were used. Adherent cells in culture were harvested using 0.25% trypsin/1mM EDTA (Wisent Bioproducts, Quebec, Canada). Cells were counted with a hemocytometer and seeded at 1.2×10^4 cells/well in 96-well plates with DMEM media containing 10% FBS. After 24 hours, media was removed and replaced with DMEM media free of FBS. Each cell line was

treated with increasing doses of CBD (0.01–100 μM) and colourimetric assay using crystal violet was performed after a 24-hour incubation period.

For colourimetric assay, all media was first aspirated and removed from the wells and replaced by 0.5% crystal violet (Thermo Fisher Scientific, Massachusetts, USA) diluted in 20% methanol (Thermo Fisher Scientific, Massachusetts, USA). The stain was left in wells for 20 minutes while plates underwent gentle rocking. Stain was then rinsed with deionized water and plates were left to dry overnight. After the drying step, 10% acetic acid (Thermo Fisher Scientific, Waltham, Massachusetts) was added to all wells and plates were placed on a rocker for 15 minutes. Absorbance was read at 590nM using a Synergy 2 microplate reader (BioTek, Vermont, USA). Results of wells treated with CBD were normalized to a vehicle control of FBS-free culture medium with 5% ethanol, 5% Tween, and 90% PBS (suspension vehicle for CBD). Three technical replicates, each consisting of three identical biological replicates, were conducted for each cell line.

2.5.2 Dose escalation experiments for cannabinoid receptor antagonists

Cell harvesting and counting for all cell lines were performed as above and cells were seeded at the same density in 96-well plates. After 24 hours, media containing FBS was replaced with media free of FBS. Each cell line was treated with increasing doses of each of five cannabinoid receptor antagonists for 24 hours: AM 251, SCH 336, T0070907, ML 193 and capsazepine (0.01, 0.1, 1, 5 and 10 μM). Staining with crystal violet and microplate readings were performed as above, and results were normalized

to DMSO vehicle control. From this data, a dose for use in subsequent experiments was selected as the highest dose producing minimal to no reduction of cell viability among all cell lines. Three technical replicates, each consisting of two biological replicates, were conducted with each cell line for each drug.

2.5.3 Combination experiments: CBD & cannabinoid receptor antagonists

Cells from all lines were seeded in 96-well plates as above. After 24 hours, media was removed, and cells were incubated with a single cannabinoid receptor antagonist (1 μM) in FBS-free media for 1 hour. Following incubation, media was again replaced, and cells were treated with CBD (5 or 10μM) in FBS-free media for 24 hours. Vehicle controls of both DMSO and CBD vehicle (5% ethanol, 5% Tween, and 90% PBS) were also evaluated. Staining with crystal violet and absorbance readings were performed as above. Three technical replicates, each consisting of three biological replicates, were carried out for each cell line and for each cannabinoid receptor antagonist [**Table 2-1**], both alone and in combination with CBD.

2.6 Flow cytometry

2.6.1 Annexin V/Propidium iodide apoptosis assays

Flow cytometry was used to quantify apoptotic and dead cells using fluorescein isothiocyanate (FITC)-conjugated annexin V and propidium iodide (PI) (eBioscience, San Diego, California). Adherent cells in culture, from MDCK and all UC cell lines, were harvested using 0.25% trypsin/1mM EDTA and counted manually with a hemocytometer. Cells were seeded into 6-well plates at 5 x 10^5 cells per well and

incubated for 24 hours. Cells were treated with CBD (5 μM), CBD vehicle (5% ethanol, 5% Tween, and 90% PBS) alone as a negative control or Doxorubicin (5 μM) as a positive control for 8 hours in FBS-free media. This time point was selected based on previous work performed in our research group in canine UC cell lines and published literature investigating the role of CBD in the induction of apoptosis in canine neoplastic cell lines of various other tumour types.[133,134]

After 8 hours, cells were rinsed with PBS, lifted using 0.25% trypsin/1mM EDTA, spun (400G for 10min), resuspended in manufacturer-provided assay buffer and stained with Annexin V-FITC and PI (eBioscience, San Diego, California) to detect apoptosis by flow cytometry. Evaluation of cell fractions was performed on a BD Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, New Jersey) at 5 x 10^4 events per group. Laser intensities were based upon control readings and spillover between FITC and PI (FL1 and FL3 channels respectively) was accounted for and compensated. Early apoptosis was defined as Annexin V positivity with PI negativity, late apoptosis was defined as Annexin V and PI positivity and dead cell fractions as Annexin V negativity with PI positivity. Experiments were run in triplicate for each cell line.

2.6.2 Autophagy assays

Flow cytometry was used to quantify autophagy through the detection of autophagic vesicles via a 488 nm-excitable green, fluorescent detection reagent (Abcam, Cambridge, UK).

Adherent cells in culture, from MDCK and all UC cell lines, were harvested using 0.25% trypsin/1mM EDTA and counted manually with a hemocytometer. Cells were seeded into 6-well plates at 3.5 x 10^5 cells per well and incubated for 24 hours. Cells were then treated with a control or CBD (5 μM) for 24 hours in serum-free media. Positive controls included Rapamycin (300 nM) and Chloroquine (20 μM) in serum-free media. CBD vehicle (5% ethanol, 5% Tween, and 90% PBS) alone in serum-free media was used as a negative control. Additionally, one treatment group was incubated with media containing FBS, as cell starvation is a well-described inducer of autophagy.

After 24 hours, cells were rinsed with PBS, lifted using 0.25% trypsin/1mM EDTA, spun (1000rpm for 5 min) and resuspended in assay buffer provided by the manufacturer. The spinning step was subsequently repeated, and cells were resuspended in indicator-free media with green-fluorescent detection reagent added to the solution to detect autophagic vacuoles via flow cytometry. Evaluation of cell fractions was performed on a BD Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, New Jersey) at 5 x 10^4 events per group. The intensities of fluorescence, associated with the presence of autophagic vacuoles and positively correlated with autophagy levels, was then compared to control readings.

2.7 Statistical Analysis

Statistical analysis was performed using GraphPad Prism v.9 (GraphPad Software Inc, San Diego CA). Half-maximal inhibitory concentrations curves for CBD were generated using a non-linear regression model and Hill's equation with the assumption

of Gaussian distribution. Comparison of groups for assessment of apoptosis, autophagy and the influence of cannabinoid receptor antagonists was performed using one-way ANOVA and two-way ANOVA with post-hoc analysis using Dunnett's multiple comparison test. Statistical significance was set to $P < 0.05$ (*<0.05, **<0.01, ***<0.001) and all means were reported with standard deviation (SD) or range. FlowJo V10 (FlowJo LLC, Ashland Oregon) was used for flow cytometry analyses and to create density plots or histograms depicting flow cytometric data.

3 Results

3.1 Cannabinoid receptor expression

Cannabinoid receptor protein expression was evaluated in all cell lines. Immunoblotting revealed a major band for all 5 cannabinoid receptors (CB1, CB2, PPARγ, GPR55 and TRPV1) in all UC cell lines investigated (ORIG, AXA, SH), and in both the negative (MDCK) and positive (MB-231) controls [**Figure 3**]. For CB1, Western blot analysis repeatedly revealed a band at ~75 kDa in all cell lines, despite a predicted molecular weight of 53 kDa for this protein. For all other receptors, in all cell lines, a band appeared near the theoretical molecular weight of the protein of interest. For CB2, analysis revealed a band of ~40 kDa, for PPARγ ~57 kDa, for GPR55 ~37 kDa, and for TRPV1 ~95 kDa, confirming endogenous expression of these proteins. Higher levels of CB2 were present in all 3 UC cell lines compared with either the canine non-neoplastic or the human cell lines across all replicates. Differential expression of PPARγ was also seen between the UC cell lines and other cells lines, however this finding was inconsistent between replicates.

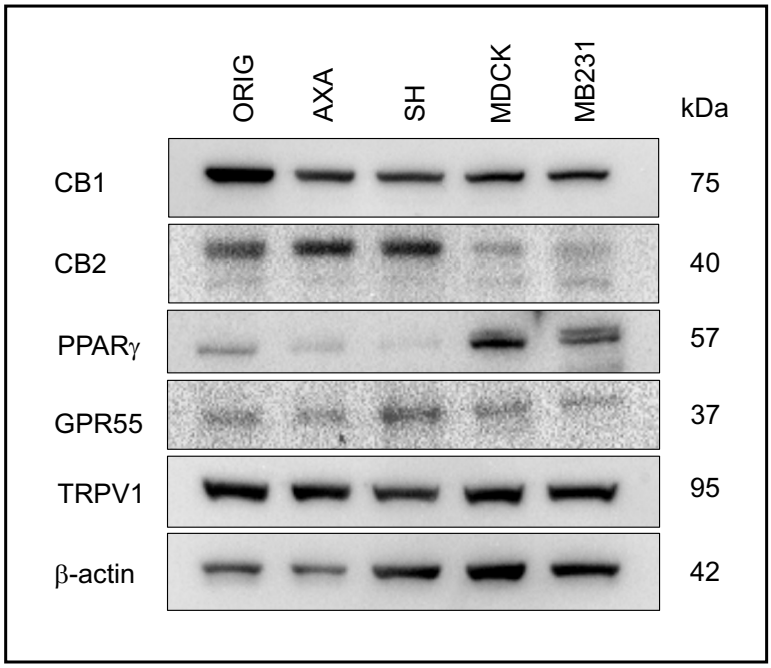


Figure 3. Cannabinoid receptor expression in canine UC cell lines (Orig, AXA, SH) and non-neoplastic immortalized canine kidney epithelial cell line (MDCK).

Representative images of Western blot analysis showing the specificity of the primary antibodies: rabbit anti-cannabinoid receptor 1 (CB1), rabbit anti-cannabinoid receptor 2 (CB2), rabbit anti-nuclear peroxisome proliferator-activated receptor gamma (PPARγ), rabbit transient receptor potential vanilloid type 1 (TRPV1) and rabbit anti-G protein-coupled receptor 55 (GPR55). Each antibody showed a major band, with most showing near the theoretical molecular weight (as listed) in all cell lines assessed. Human breast adenocarcinoma epithelial cell line (MB231) used as positive control. B-Actin used as loading control.

3.2 Effect of CBD on canine UC cell viability

Viability of each cell line was evaluated following treatment with increasing concentrations of CBD. A dose dependent reduction in cell viability was observed in all cell lines and treatment yielded IC50 values in the low micromolar range for all canine UC cell lines (ORIG = 2.021 μM; AXA = 1.563 μM, SH = 2.196 μM) and for the canine renal epithelial cell line (MDCK = 2.267 μM) [**Figure 4.**].

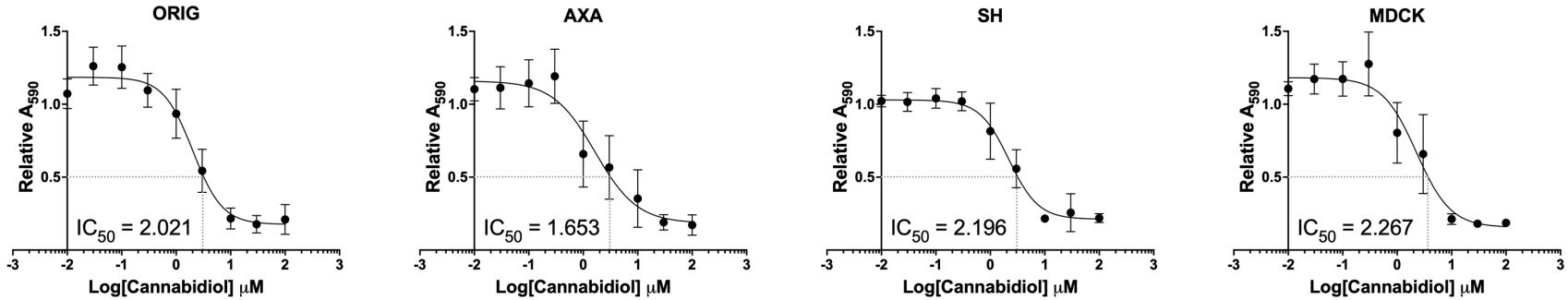


Figure 4. Dose-dependent decrease in cell viability of canine UC cells and renal epithelial cells treated with CBD.

Representative IC50 curves of cells treated with increasing concentrations (0.1μM – 100μM) of CBD for 24 hours. Graphs represent mean ± standard deviation of 3 technical replicates with 3 biological replicates from each cell line.

3.3 Cannabinoid receptor antagonists: assessment of toxicity

Cell viability was assessed following treatment with known antagonists to the various cannabinoid receptors of interest to determine a dose for use in subsequent assays and to rule out significant toxicity of these drugs as single agents at selected doses.

Following treatment for 24 hours with each cannabinoid receptor antagonist (AM 251, SCH 336, T0070907, ML 193 and capsazepine), a significant reduction in cell viability was observed for AM 251 in ORIG and SH cell lines at the two highest doses assessed (5μM and 10μM). For ORIG, cell viability was also significantly reduced using 10 μM of T0070907 and capsazepine, and at all doses of ML 193. In the SH cell line, treatment with the 10μM dose of ML 193 also resulted in significant reduction in cell viability. No difference in cell viability was observed in AXA and MDCK cell lines at any evaluated dose [**Figure 5]**. Based on this information, a dose of 1 μM was considered the highest dose likely to produce the least reduction in cell viability in all cell lines for all drugs and this dose was used in subsequent assays. A dose having minimal impact on cell viability was selected to minimize potential confounding effects between the toxicity of cannabinoid receptor antagonists with that of CBD, and to more clearly highlight any potential impacts of these antagonists on the negative effect of CBD on cell viability.

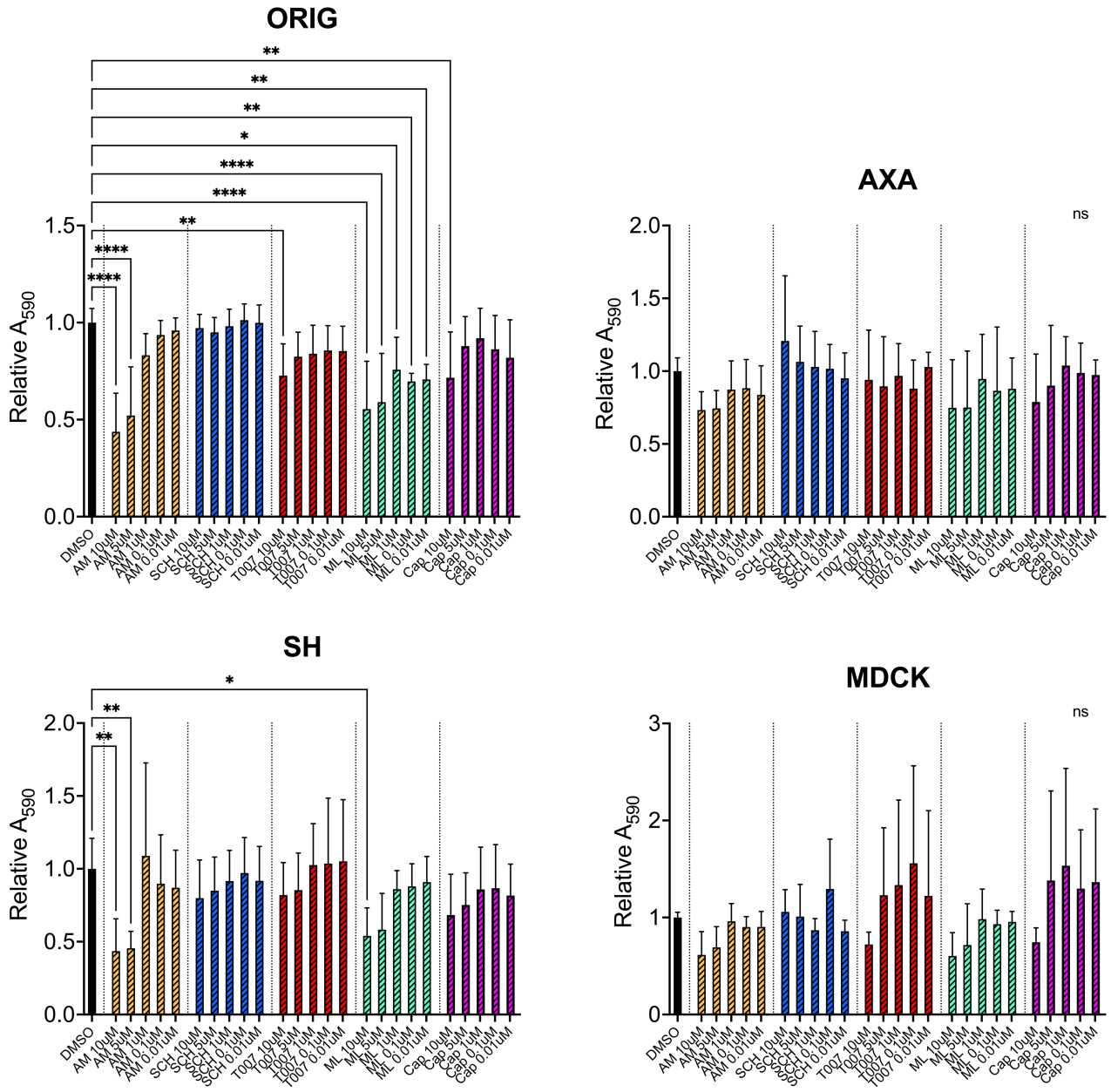


Figure 5. Cell viability of CB1 receptor antagonist (AM 251), CB2 receptor antagonist (SCH 336), PPARγ receptor antagonists (T0070907), GPR55 receptor antagonist (ML 193) and TRPV1 receptor antagonist (capsazepine) treated cells.

Urothelial cell carcinoma and immortalized canine renal epithelial cells from each cell line were seeded for 24 hours before treatment and incubated for 1 hour with the

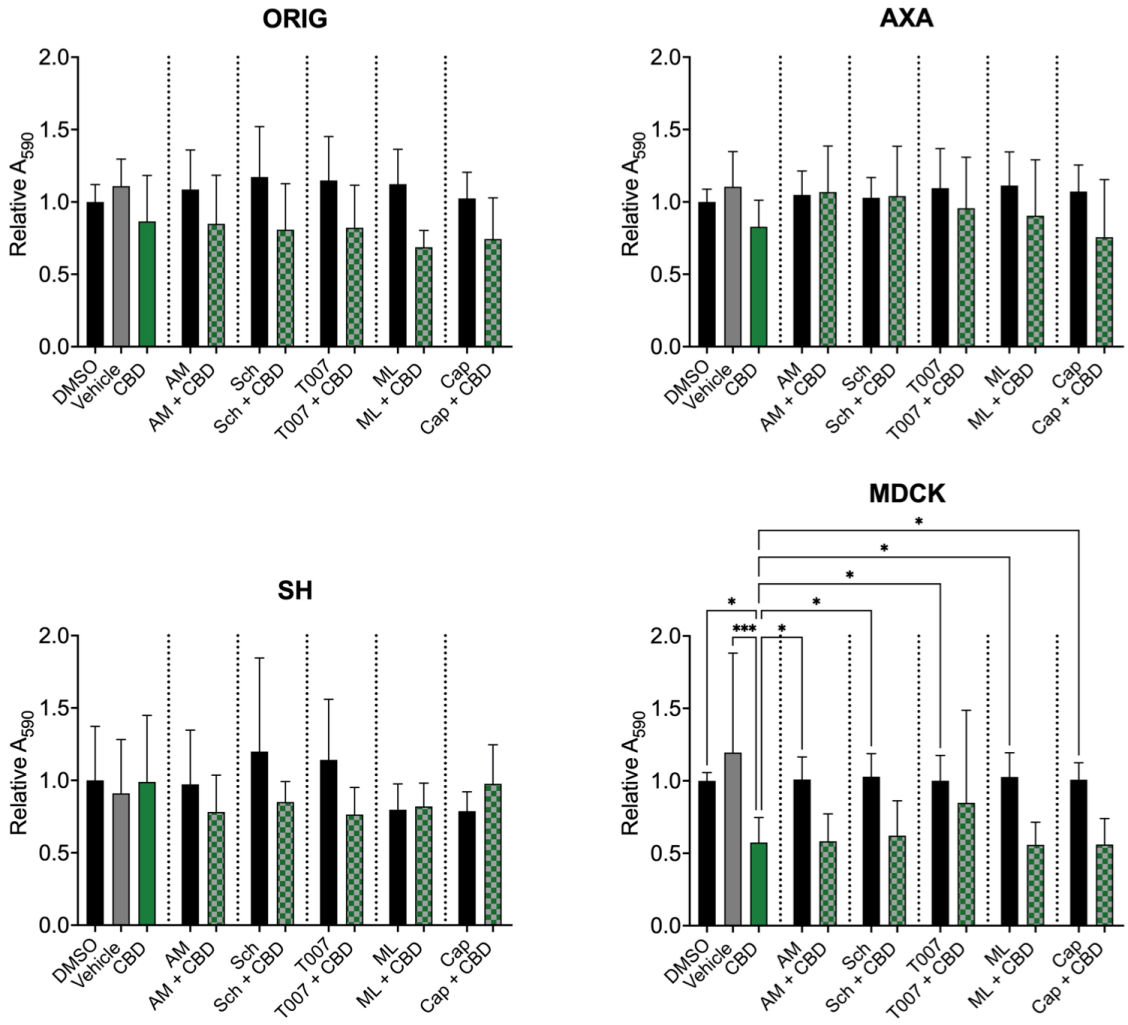
antagonists. A crystal violet assay was used to evaluate cell viability after 24 hours. Data is expressed as a percentage of the vehicle control (DMSO) and the mean and standard deviation of three technical replicates consisting of 2 biological replicates from each cell line. One-way ANOVA was used to compare all treatments with DMSO control and significance was reported at *P< 0.05, **P< 0.01, ****P <0.0001, ns no significant difference.

3.4 Interaction of CBD and cannabinoid receptor antagonists

To elucidate the mechanisms by which CBD induces its effects on cell viability, treatment with CBD in combination with a selective receptor antagonist (AM 251, SCH 336, T0070907, ML 193 or capsazepine) for each cannabinoid receptor of interest (CB1, CB2, PPARγ, GPR55 or TRPV-1) was performed.

Comparing the relative viabilities of cells treated with CBD alone with those of cells pre-treated with a cannabinoid receptor antagonist, it was found that selective CB1, CB2, PPARγ, GPR55 or TRPV-1 antagonists did not alter cell viability at either 5μM [**Figure 6. A**] or 10μM [**Figure 6. B**], suggesting that cytotoxicity may occur through either cannabinoid receptor-independent mechanisms or through interactions between multiple receptors.

(A)


ORIG
AXA
SH
MDCK
Relative A_{590}
2.0
1.5
1.0
0.5
0.0
DMSO
Vehicle
CBD
AM
AM + CBD
Sch
Sch + CBD
T007
T007 + CBD
ML
ML + CBD
Cap
Cap + CBD
*
***

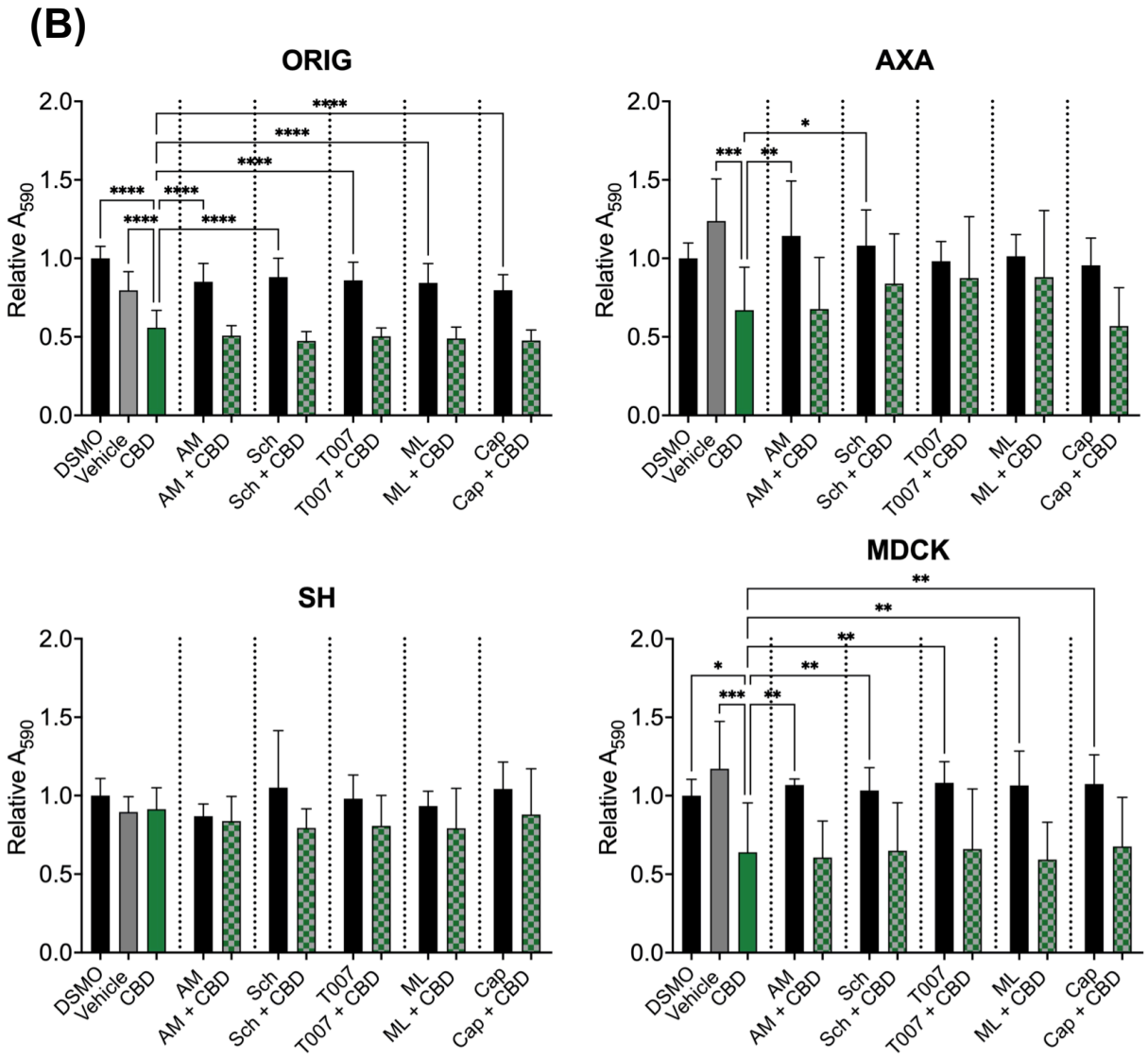


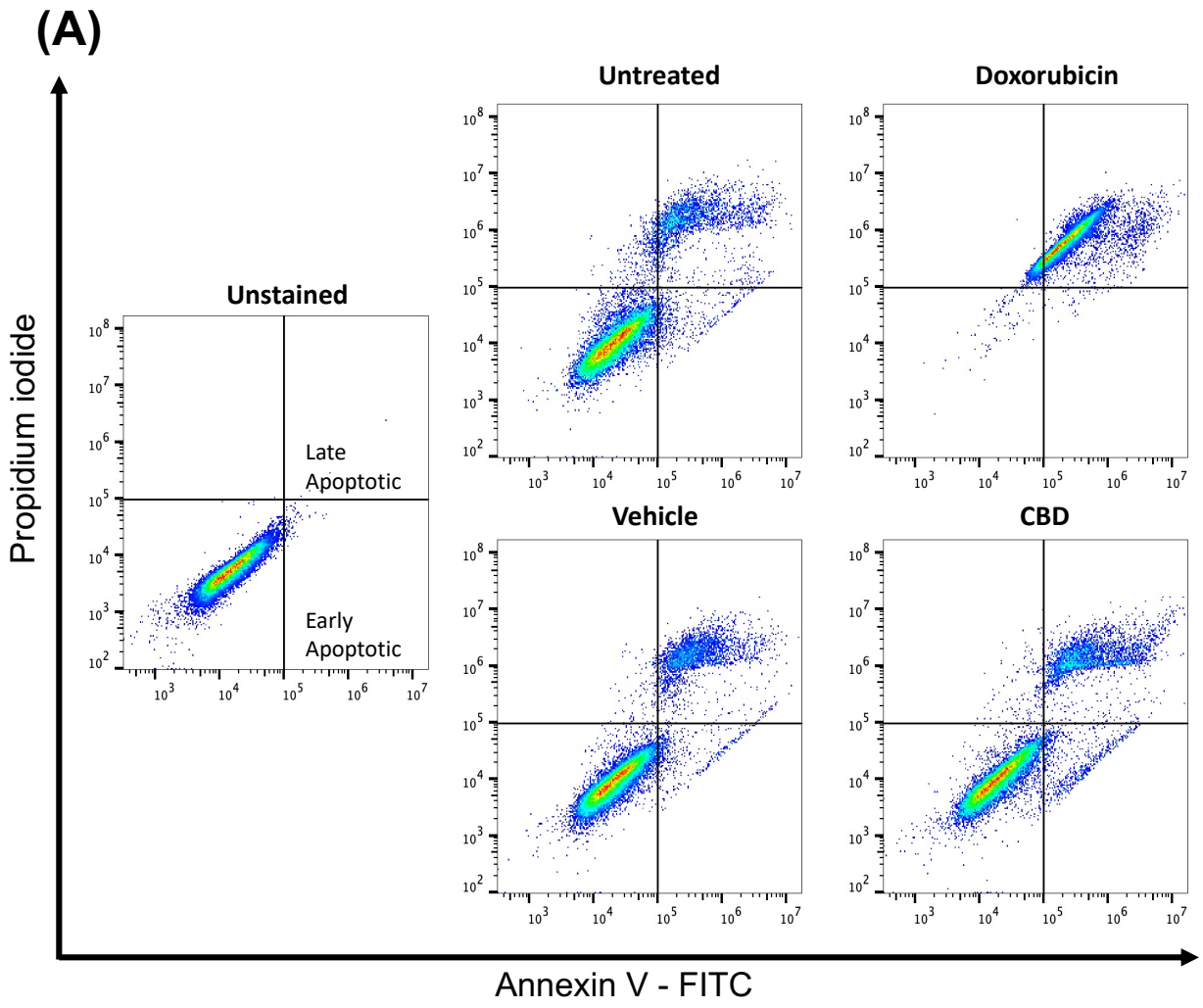
Figure 6. Cell viability of canine UC and renal epithelial cells treated with a combination of CB1 receptor antagonist AM251 (AM), CB2 receptor antagonist SCH336 (Sch), PPARγ receptor antagonist T0070907 (T007), GPR55 receptor antagonist ML193 (ML) or TRPV1 receptor antagonist capsazepine (Cap) with CBD.

(A) CBD 5μM (B) CBD 10μM. Cells were seeded for 24 hours prior to pre-treatment for 1 hour with 1 μM of antagonist followed by CBD or treatment with CBD alone. CBD vehicle control and DMSO control (vehicle for antagonists) were also used.

Cell viability was assessed 24 hours after treatment with CBD. Data is expressed as a percentage of the relative absorbance of DMSO control. Mean and standard deviation of three technical replicates consisting of 3 biological replicates from each cell line is shown. One-way ANOVA was used to compare all treatment groups with CBD alone and significance was reported at *P< .05, **P< .01, ***P <0.001. ****P <0.0001.

3.5 Apoptosis

To evaluate whether CBD affects cell viability via apoptosis, annexin V and PI were measured via flow cytometry in all 3 UC cell lines and in MDCK following treatment. Significant changes in early or late apoptosis were not observed between CBD treated cells and the vehicle control after 8-hour drug treatment with 5 μM CBD. [**Figure 7. A and B**].


(A)
Propidium iodide
Annexin V - FITC
Unstained
Late Apoptotic
Early Apoptotic
Untreated
Doxorubicin
Vehicle
CBD

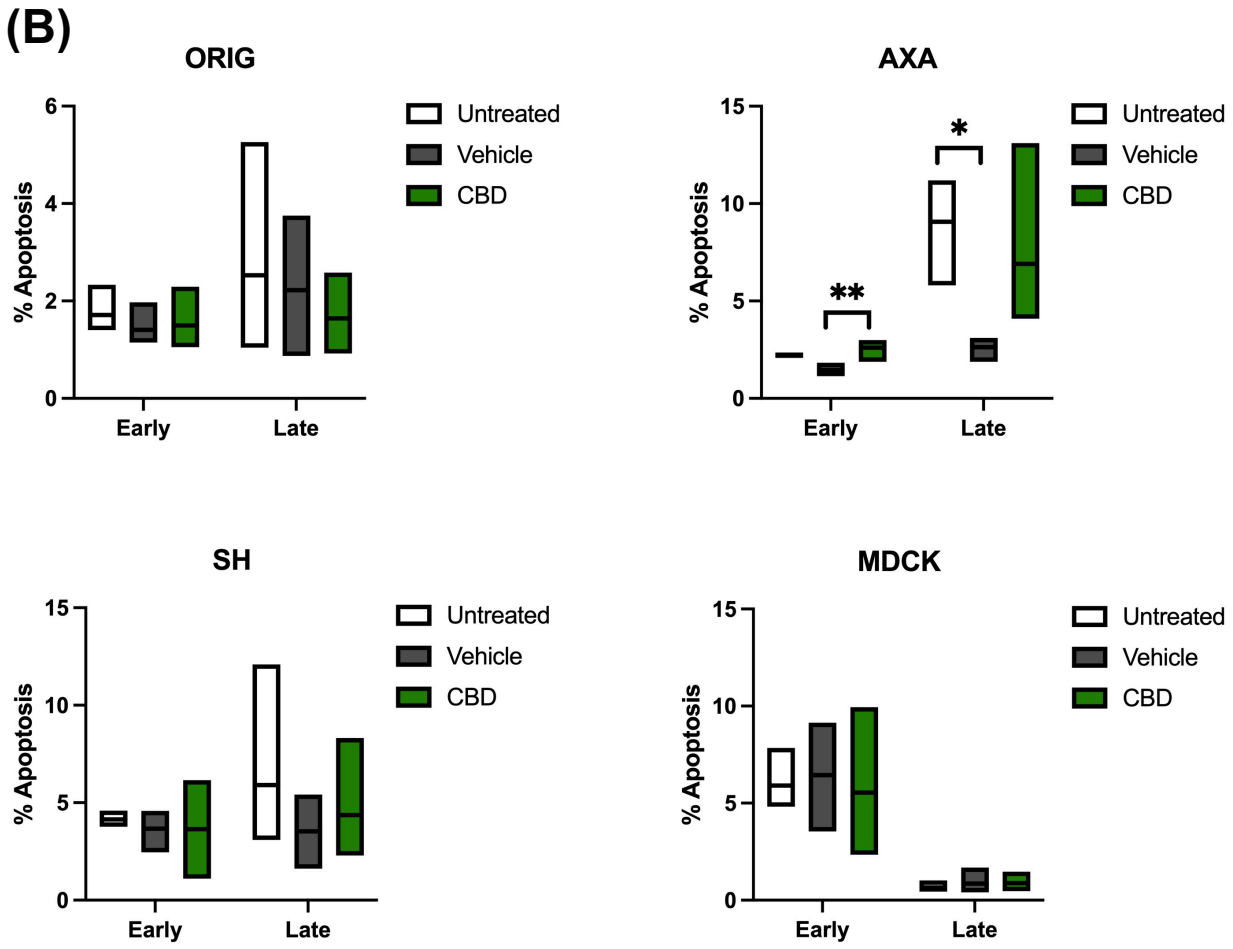
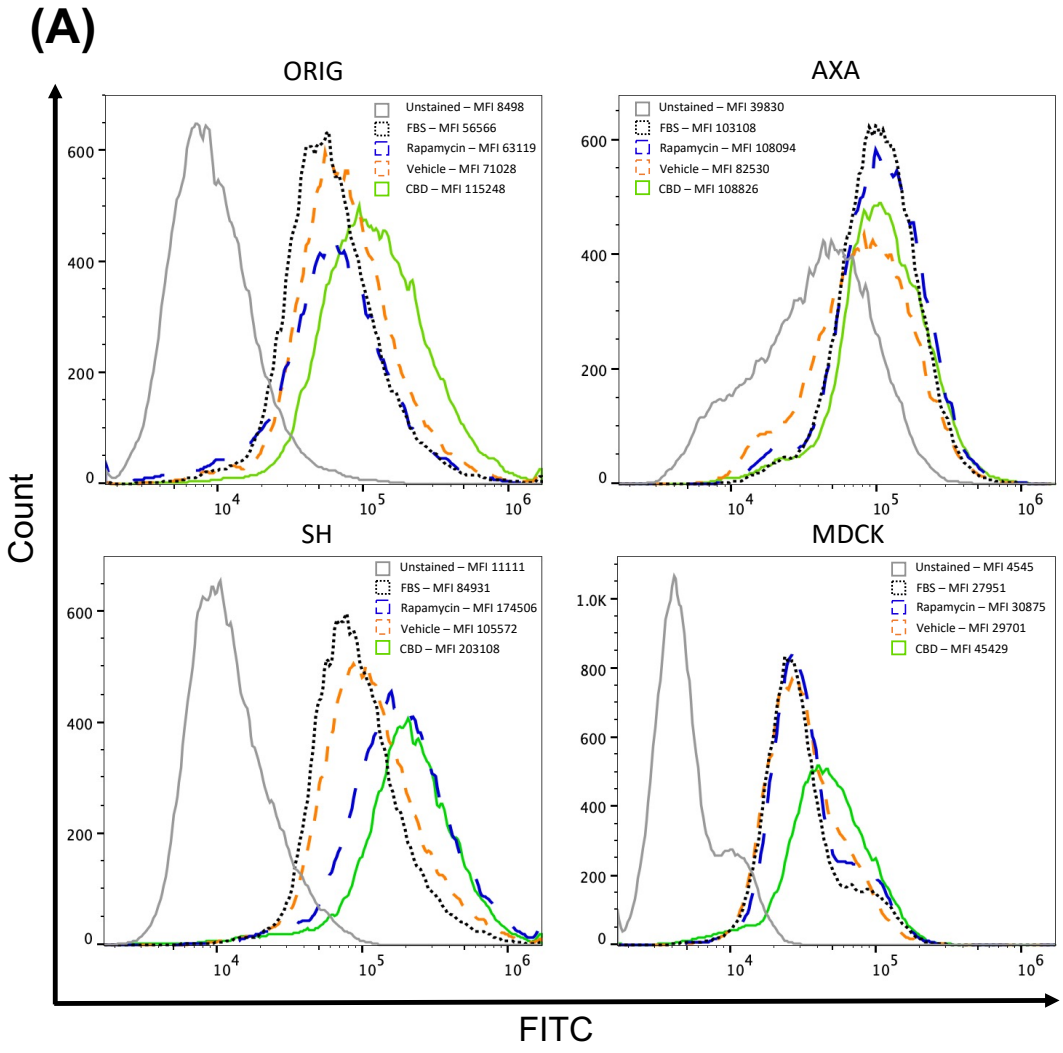


Figure 7. Analysis of apoptosis in canine UC and renal epithelial cells treated for 8 hours with CBD (5 µM).

(A) Representative density plots obtained after compensation for spillover by the BD Accuri C6 flow cytometer. Untreated cells, vehicle-only and doxorubicin (5 µM) used as a negative and positive controls. (B) Combined analysis of early (Annexin V+/PI-) and late (Annexin V+/PI+) apoptotic fractions compared to the vehicle control and to untreated cells. Mean ± range from 3-4 independent replicates is shown. Significance was reported at *P< .05, **P< .01.

3.6 Autophagy

To evaluate the role of autophagy in CBD's effects on cell viability, a fluorescent marker of autophagic vacuoles was measured via flow cytometry in all 3 UC cell lines and MDCK, 24 hours after treatment with 5 μM CBD. Autophagy was significantly increased following treatment with CBD in a single cell line (SH) [**Figure 8. A and B**]. However, no difference in autophagic activity between CBD-treated cells and vehicle treated cells was apparent in SH cells. Additionally, no change in autophagy was observed in other UC cell lines or in MDCK.


(A)
ORIG
Unstained – MFI 8498
FBS – MFI 56566
Rapamycin – MFI 63119
Vehicle – MFI 71028
CBD – MFI 115248
AXA
Unstained – MFI 39830
FBS – MFI 103108
Rapamycin – MFI 108094
Vehicle – MFI 82530
CBD – MFI 108826
SH
Unstained – MFI 11111
FBS – MFI 84931
Rapamycin – MFI 174506
Vehicle – MFI 105572
CBD – MFI 203108
MDCK
Unstained – MFI 4545
FBS – MFI 27951
Rapamycin – MFI 30875
Vehicle – MFI 29701
CBD – MFI 45429
Count
FITC

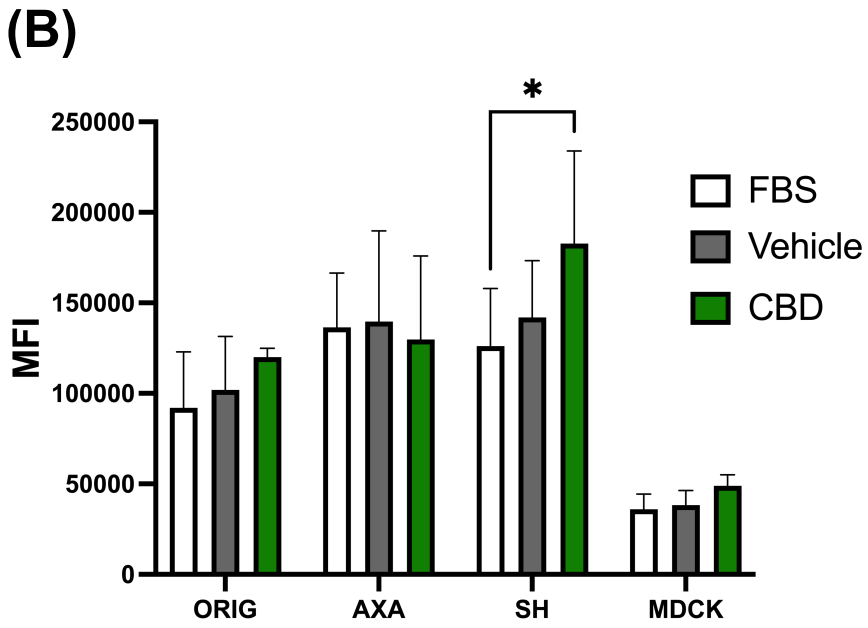


Figure 8. Analysis of autophagy in canine UC and renal epithelial cells treated for 24 hours with CBD (5μM).

(A) Representative histogram overlays of isotype unstained controls, cells exposed to fetal bovine serum (FBS), vehicle-only, rapamycin (300nM) positive control, and CBD and stained with 488 nm-excitable green, fluorescent marker for autophagic vacuoles. Median fluorescent intensity (MFI) values are listed. (B) Combined analysis of MFI of vehicle control and CBD-treated cells compared to untreated controls exposed to FBS. Mean and standard deviation from 3-4 independent replicates is shown. Significance was reported at *P< .05.

4 Discussion

Canine urothelial carcinoma represents a persistent clinical challenge, primarily as a result of its aggressive local behaviour, disappointing responses to existing anti-neoplastic therapies and a lack of promising new treatment modalities developed in recent years to treat this devastating disease.

The aim of this study was to evaluate and better characterize the effects of CBD, a novel treatment modality, on canine UC *in vitro*.

4.1 Cannabinoid receptor expression

Through qualitative protein detection using western blot, we demonstrated a major band at the expected molecular weight for most cannabinoid receptors of interest, namely, CB2, PPARγ, GPR55 and TRPV-1, in a small number of established UC cell lines harvested from different canine UC tumours and an immortalized renal epithelial cell line. A 5th receptor of interest, and a canonical cannabinoid receptor, CB1, repeatably demonstrated a major band, however, it was located at a considerably higher molecular weight (~75kDa) than expected for this protein (~53 kDa) [**Figure 3**].

There are various potential theories to explain this effect. In humans, numerous CB1 isoforms have been isolated.[168,169] Although several different canine CB1 isoforms may also exist, this phenomenon would not be expected to result in such a marked shift in molecular weight as most human isoforms for this protein are of similar molecular

weights.[168,169] This theory also does not account for the presence of a major band at 75kDa in the human MB-231 cell line, seen here.

Post-translational modifications, including glycosylation, may result in differences in protein molecular weights. N-linked glycosylation of CB1 has been reported in rat brain tissues, with immunoblotting resulting in 64kDa bands.[170] In that report, glycosylated forms represented 65-80% of CB1 receptors, while non-glycosylated forms accounted for only 20-35%. Similarly, in a previous study of CB1 receptor expression in ferrets, an 81kDa minor band was also observed via immunoblotting and attributed to protein glycosylation.[171] In that study however, a major band was also observed near the predicted 53kDa. Our results differ from these findings, in the absence of a band at or near the predicted 53kDa weight of CB1.

Dimerization represents another mechanism which may produce a higher-than-expected molecular weight band during western blot analysis. This phenomenon is reported for CB1; however, it would be expected to produce a considerably higher molecular weight band, most likely at least doubling the expected 53kDa molecular weight, which was not observed in this study.[172] The 75kDa band seen here could however, theoretically, be attributable to heteromerization with an alternate, smaller protein and it is interesting to note that CB1 interactions with GPR55, a 37kDa protein, have previously been reported.[107]

The canine CB1 protein sequence is homologous (98.3%) with the human CB1 protein sequence, suggesting that a human antibody would be reactive with the canine

protein.[57] Based on this homology, reactivity with canine tissues is predicted by the manufacturer of the antibody used in this study. However, despite this homology, cross-reactivity of the human CB1 antibody with an alternate canine protein bearing the same epitope remains possible. Additionally, CB1 expression is not ubiquitous in all tissues and is expected to primarily occur in the CNS.[37,38,49] It is therefore possible that CB1 is simply not expressed in canine UC. However contrary to this theory, CB1 expression has been confirmed in human prostate cancer, breast cancer and non-small cell lung cancer, among other neoplasms outside of the CNS.[173,174] In human breast cancer cell lines, CB1 expression is often low while CB2 expression is high and is positively correlated with tumour aggressiveness and negatively correlated with estrogen and progesterone hormone expression.[138,175,176] Another demonstration of CB1 receptor expression outside of CNS, is the confirmed immunoreactivity to CB1 within canine gastrointestinal tissue and dorsal root ganglion using IHC.[57,145] The same antibody product was used in our work as in one of these previous reports, in which a major band was observed at 52 kDa.[57]

Further investigation of CB1 expression is therefore warranted to characterize the expression patterns of this receptor more thoroughly in canine UC. This additional exploration would involve performing a de-glycosylation step prior to immunoblotting, IHC assays to verify the membranous cellular localization of the proteins being detected by the present antibody, PCR-based gene expression analysis of these cell lines with

CB1 specific primers, and/or assessment of these tissues with alternate CB1 antibody products.

Expanding on the subject of protein homology raised above, canine CB2 shares between 76 and 82% homology with rat, mouse and human CB2 receptors.[177] Canine TRPV1 shares between 83 and 89% sequence homology with rat, mouse and human receptors.[178] Canine PPARγ also shares 98.2% homology with the human amino acid sequence.[179] Finally, to this author's knowledge, canine GPR-55 does not yet appear to have been cloned and shows only low sequence homology with other G-protein coupled cannabinoid receptors, like CB1 and CB2, in other species.[94]

For each of these cannabinoid receptors, a major band was present at the expected molecular weight in immunoblotting assays. Most of these cannabinoid receptors have previously been demonstrated in various canine tissues via both western blot and immunohistochemical analysis.[57,145–150] However, to date, this represents the first demonstration of cannabinoid receptor expression in canine neoplastic tissues.

A limitation in the present study, is the lack of unquestionable specificity of these antibodies in canine tissues. Two of the specific antibodies used here have been previously employed in published literature investigating canine tissues.[57,145] The CB1 antibody (Orb10430, Biorbyt) and the GPR55 antibody (NLS6817 Novus) have both been used in studies examining cannabinoid receptor expression in canine dorsal root ganglia and the gastrointestinal tract.[57,145] As above, to strengthen these findings and

further validate these antibodies for continued use in canine tissues, additional molecular analysis involving IHC-based assays are warranted. These experiments would confirm the cellular localization of these proteins and would strengthen the assertion that the antibodies used here are recognizing the protein of interest in their expected membranous locations for CB1, CB2, GPR-55 and TRPV1 or nuclear location for PPARγ.

4.2 The effects of CBD on cell viability of canine UC and of non-neoplastic canine renal epithelial cells

In support of previous work performed in this research group, this study demonstrates a dose-dependent reduction in the cell viability of canine UC, induced by CBD under serum-free conditions.[133] IC50s also approach those found to be achievable *in vivo*. Contrary to our hypothesis, and to *in vitro* findings in several human tumour types, this reduction in cell viability did not differ from that seen in a canine non-neoplastic renal epithelial tissues.[125,139,144]

In a report by Shrivastana et al., CBD decreased the viability of both human breast cancer cells and a nontumorigenic, human, mammary epithelial cell line in a concentration dependent manner. However, in that report, significantly more cells in the non-tumorigenic cell line were found to survive CBD treatment.[125] Ligresti et al. also investigated the effects of CBD on human breast carcinoma and demonstrated

significantly lower potency of CBD in non-cancerous human, rat and mouse cell lines when compared to neoplastic MB-231 (human breast carcinoma) cell lines.[139]

In studies of human UC, the effects of CBD on cell viability also differed between a well-differentiated and a poorly-differentiated UC cell line.[144] In that report, CBD-induced cell death was attributed to apoptosis caused by continuous influx of calcium through TRPV2.[144] Expression of this protein also differed between the two evaluated cell lines with poorly differentiated, more CBD-sensitive, cell lines expressing higher levels of TRPV2.[144] In our study, TRPV1 was evaluated rather than TRPV2, however expression of this vanilloid receptor was found across both neoplastic and non-neoplastic cell lines. These expression patterns may explain the similar responses to CBD seen between MDCK and the UC cell lines. However, our study did not quantify protein expression and therefore, to support this assertion, further evaluation using RT-PCR to quantify cannabinoid receptor mRNA expression would be warranted.

4.3 The effects of cannabinoid receptor antagonism on treatment with CBD

In this study, five specific cannabinoid receptor antagonists to CB1, CB2, PPARγ, GPR55 and TRPV1 respectively, were used in combination with CBD in an effort to elucidate the mechanisms of action of this drug. The antagonism of a cannabinoid receptor that is critical to the cell-killing effects of CBD would be expected to result in increased cell viability following combination therapy, when compared to

treatment with CBD alone. Ultimately, the cannabinoid receptor antagonists used here did not attenuate the negative effect of CBD on cell viability [**Figure 6. B**].

Cell viability assays in this experiment were first performed using a dose of 5μM CBD [**Figure 6. A**], which is greater than twice the IC50s demonstrated in these cell lines [**Figure 4**]. Despite this, treatment with CBD in these cell viability assays did not result in a significant reduction in relative absorbance in any of the UC cell lines treated with CBD alone or in combination with the cannabinoid receptor antagonists. Interestingly, this dose did produce a significant reduction in cell viability for the non-neoplastic cell line, MDCK, despite similar IC50s to UC cell lines in earlier experiments. Ultimately, this outcome in the UC cell lines prevented any evaluation of the consequences of treatment with cannabinoid receptor antagonists and therefore the experiment was repeated using a higher dose of CBD.

Reasons for this deviation from the expected effect, at doses well above the IC50s, are largely unknown though may be related to the stability, shelf-life, and storage conditions of CBD. An early investigation into the stability of several pure cannabinoids in varied formulations and stored under varied conditions, led to the conclusion that herbal or resin cannabis extracts are stable for up to 2 years when stored in the dark and at room temperature.[180] Air oxidation in particular was found to lead to degradation of the product.[180] This early exploration into stability testing of cannabinoids was however primarily carried out on plant extracts and did not focus on the specific stability of CBD in solution.

A more recent investigation, exploring the stability of CBD in solution, used high-performance LC-MS for analysis of CBD in ethanol.[181] In that report, temperature was found to be one of the most critical factors affecting product stability, with CBD demonstrating relative stability for at least 12 months when stored under refrigerated conditions at 5°C, compared with high instability at room temperature.[181] Both oxidation and exposure to light also contributed to degradation of this cannabinoid. Finally, the nature of the solvent also affected stability with ethanol favoured over an aqueous medium. Of note, was that in simulated physiologic conditions of pH 7.4 and 37°C, 10% of CBD was degraded within the first 24 hours.[181] A second recent report also demonstrated an approximately 13% loss of CBD content after one year of storage in darkness at room temperature.[182]

In our experiments, the same stock CBD solution was used for the duration of the study in assays performed over an approximately 2-year period. This stock solution was stored in the dark and at room temperature for the duration of this period. Based on the above findings, some degree of CBD degradation of the stock solution would therefore have been expected to occur over that two-year period.

From the stock product, CBD was diluted in a vehicle made up of 5% ethanol, 5% Tween, and 90% PBS, prior to use in the various experimental assays. Once prepared, diluted CBD was stored in aliquots at -20°C and used within 4 months of preparation. Further questions arise therefore, as to the stability and shelf-life of CBD under frozen conditions.

This stability question was explored in a recent technical report released by Ascension Sciences.[183] In that report, Δ^9-THC and CBD content was evaluated through LC-MS after storage for 2 months at various temperatures. This report identified a reduction in both Δ^9-THC and CBD content over that 2-month period at all temperatures however that reduction was minimized through storage at lower temperatures (4°C and -20°C). Over that timeframe, CBD content decreased by approximately 43% in the samples refrigerated or frozen in darkness, while it decreased by over 70% when stored at room temperature or in light. In contrast, the CBN content, an oxidation product of Δ^9-THC increased over that time frame.[183]

To assess whether the apparent reduction in the effects of CBD seen over the duration of this study is due to degradation of the product, re-evaluation of the CBD stock product and frozen stored solubilized aliquots of CBD via methods such as LC-MS are therefore warranted. This was not performed in the present study and therefore represents a limitation to the interpretation of these results.

Despite these unexpected findings at 5μM CBD, when UC and MDCK cell lines were treated with a dose of 10μM CBD, either alone or in combination with each antagonist, a significant reduction in relative absorption, compared with untreated cells, was seen in most cell lines. However, cell viability did not differ between cells treated with these combinations and those treated with CBD alone. This effect was found across all cannabinoid receptor antagonists, suggesting that the cell-killing effect of CBD is not mediated by individual cannabinoid receptors in canine UC.

The mechanism of action of the anti-neoplastic effects of CBD remains unclear. Given the low affinity for CB1 and CB2 receptors, it would be unsurprising for the effects of CBD to occur independently of these receptors.[54,68,69] In addition, while literature exists to directly support the induction of apoptosis via TRPV2-mediated calcium-gated channels,[144] yet other reports have found the CBD-dependent induction of apoptosis to be cannabinoid and vanilloid-receptor independent.[125]

Our findings agree with a previous study by Shrivastana et al, in which the effects of CBD on cell-viability appear independent of both canonical cannabinoid receptors and vanilloid receptors. That report used cannabinoid receptor antagonists AM 251 (selective for CB1), AM 630 (selective for CB2) and capsazepine (selective for TRPV1), in combination with CBD and found viability levels to be equivalent among neoplastic cells treated with CBD alone and those treated with CBD in combinations with each antagonist, suggesting cannabinoid receptor independent mechanisms. Rather, CBD-induced apoptosis and autophagy of breast cancer cells were proposed to be mediated by the generation of reactive oxygen species in this study.[125]

Although one explanation for the lack of apparent effect of individual cannabinoid receptor antagonists on treatment with CBD may be that this action is not receptor mediated, the result may also be explained by interactions between multiple cannabinoid receptors. This theory is supported by evidence of complex interactions between GPR-55 and both CB1 and CB2.[107–109] Further confounding matters, CBD is itself reported to be an antagonist of GPR-55 receptor activity, which may provide

challenges in interpreting the results of assays such as this one, in which GPR-55 specific receptor antagonists are used to alter and examine the effects of CBD.[92]

It is interesting to note that in one cell line, AXA, a numerical increase in the relative absorbance was seen following treatment with CBD in combination with three of the selective antagonists compared with CBD alone, although this effect was not found to be significant. While this finding may not be meaningful, it was observed for selective antagonists to CB2 (Sch 336), PPARγ (T0070907) and GPR-55 (ML 193) but was not observed for the TRPV1 antagonist, capsazepine. This is noteworthy, as vanilloid receptors have the strongest evidence for direct implication in the cell-killing effects of CBD, as previously discussed, suggesting that their inhibition in particular would have been most likely to reduce the cell-killing effects of CBD.[144]

The response to CBD of another UC cell line, namely SH, also differed from the others. For SH, no significant reduction in cell viability occurred in any of the treatment combinations, when compared to vehicle controls. This effect was seen even at the high (10μM) dose level of CBD. The underlying mechanism behind this disparate response is unknown. As discussed above, this effect may be attributable to degradation of the CBD product over time, however this theory would not explain the responses seen in the remaining UC cells lines.

Alternatively, the difference in sensitivity to CBD may be attributable to differential receptor expression. Though not cannabinoid receptors, differences in the expression of COX-2, pRB and p53, between SH and both ORIG and AXA have been

documented.[36] Further, in mouse xenograft studies, SH was the only one found to produce metastasis to regional lymph nodes, suggesting a more biologically aggressive behaviour.[36]

Additional differences between SH and ORIG or AXA have arisen in previous work performed by our research group. In this work, SH demonstrated the greatest expression of MAGL, an enzyme involved in the hydrolysis of endocannabinoids, as assessed by western blotting techniques. In addition, MAGL expression appeared positively correlated with cell migration capacity, which was also found to be higher in SH compared to both ORIG and AXA cell lines. These results led to the conclusion that differences in MAGL expression may correlate with a more behaviorally aggressive phenotype (submitted, under review).

Despite such differences between SH and the other UC cell lines evaluated here, our qualitative assessment of cannabinoid receptor expression did not yield differences in expression between UC cell lines. To determine whether such differences exist and explore this theory further, quantitative assays in the form of RT-PCR would be of value.

Another limitation, which may have impacted the results of selective cannabinoid receptor antagonist assays, likely arose from the experimental design. Here we pre-treated cells with cannabinoid receptor antagonists for 1 hour prior to treatment with CBD. This is consistent with the experimental design of a similar study investigating the treatment of human breast cancer cell lines with CBD.[125] In our experimental design after treatment of cell lines for 1 hour, media was removed and replaced to allow

incubation with CBD. Different results from those described here may have been achieved by allowing more prolonged exposure to the antagonist or simply by adding CBD to the pre-existing media, thus allowing for concurrent exposure to both the selective antagonists and CBD.

4.4 The effects of CBD on cellular apoptosis

To assess whether CBD induces apoptosis in canine UC, Annexin V and PI flow cytometric assays were used. In our study, treatment of UC or MDCK cell lines with 5μM CBD for 8 hours did not result in significant induction of apoptosis compared to vehicle controls [**Figure 7. B**]. In contrast, Doxorubicin produced a strong effect in all cell lines [**Figure 7. A**]. This result contrasted with our hypothesis, in which CBD was expected to induce apoptosis in canine UC cells.

This lack of apoptosis also deviates from previous *in vitro* reports for various human neoplasms including glioma, bladder carcinoma, prostate carcinoma and breast carcinoma.[125,126,129,139,144] Our findings also differ from those of a previous study evaluating the effects of CBD on canine B-cell lymphoma, mammary carcinoma and osteosarcoma cell lines, in which apoptosis and autophagy were both observed as a result of exposure to CBD.[134] The reason for this divergence is unknown; however, various factors may play a role, including the time point selected to evaluate for early and late apoptosis, the dose and stability of the product (as previously discussed), or an inherent resistance of canine UC to the potential pro-apoptotic effects of single-agent CBD.

Regarding CBD dose, it is possible that 5μM CBD, as was used in these assays, was insufficient to induce apoptotic signals, particularly in the context of the previous discussions of the stability of CBD. This theory is also supported by slightly higher IC50s seen by both Inkol et al. and Henry et al. We therefore propose additional experiments using a 10μM dose of CBD, as with the cannabinoid receptor antagonist assays above, and using a fresh CBD stock product.

It is also possible that the 8-hour incubation period evaluated here is too early to detect a CBD-induced apoptotic response. This timing was selected based on previous literature, which suggested peak apoptotic activity between 4-8 hours after treatment with CBD in several cell lines of various other canine neoplasms.[134] Nevertheless, it is to be expected that different cell lines derived from different tumour types would experience different sensitivity and time to response to the same agents. In other previous studies evaluating CBD-induced apoptosis in human breast cancer and glioma cells *in vitro*, significant apoptosis was observed 24 hours following treatment with CBD, as assessed by flow cytometry and staining for Annexin V, PI or both.[125,126] In human bladder cancer, significant apoptosis was identified via similar assays, following incubation for 15 hours with CBD.[144]

While it may be tempting to suggest repeating these assays and evaluating for early and late apoptosis after a 24 hour incubation period with CBD, previous work performed by this research group also observed no significant apoptotic activity following exposure of the same UC cell lines to CBD for 24 hours.[133] As mentioned

previously, that study examined the combined effects of CBD and various commonly used chemotherapeutic agents in canine UC. While CBD alone did not induce significant apoptosis after 24 hours, a synergism was observed between CBD and both mitoxantrone and vinblastine, while an antagonistic interaction occurred through combination with carboplatin.[133] Synergism has also been observed between vincristine and CBD in canine lymphoma, mammary carcinoma and osteosarcoma cells, while the combination of doxorubicin and CBD on the same cell lines was less clear cut and was also dependent on the dose of CBD.[134] Finally, synergism has also been demonstrated between CBD and docetaxel in human prostatic carcinoma and between CBD and bortezomib, a proteosome inhibitor, in multiple myeloma cell lines *in vitro*.[129,184] From these findings, it can be postulated that the primary role of CBD in the treatment of cancer may arise from its ability to potentiate the toxic effects of standard chemotherapeutic agents, rather than as a single agent therapy.

4.5 The effects of CBD on the induction of autophagy

To assess whether CBD induces autophagy in canine UC, this project also employed flow cytometry to measure the presence of green, fluorescent markers targeting autophagic vacuoles. Autophagy is a process in which membrane-enclosed vesicles, termed autophagosomes, are synthesized within cells to subsequently engulf, consume, and often recycle, various cellular constituents.[185] Autophagy is a well-described method of cell survival, particularly in the face of nutrient starvation, however its role in programmed cell death is also becoming more apparent.[185,186] Moreover,

there is growing literature to suggest a form of “cross-talk” between this method of programmed cell death and apoptosis.[185] This is highlighted by the existence of multiple regulatory and mechanistic genes and proteins, whose functions are shared across both pathways including p53, p19ARF, Atg5 and Bcl-2 family proteins.[185]

Results of our experiments found no significant increase in autophagic activity in either UC or MDCK cell lines compared to vehicle controls when treated with 5μM CBD for 24 hours [**Figure 8. B**]. Given earlier discussions regarding the doses of CBD used across all experiments and the potential impact of drug degradation on our experimental results, it is worth noting that during an early replicate, one treatment group was incubated with 10μM CBD. This dose led to cell death in a very large proportion of the cell population (data not shown) so further experimentation at that higher dose was not pursued.

Furthermore, only a limited increase in MFI was observed in cells treated with an inducer of autophagy, rapamycin, as a positive control [**Figure 8. A**]. In one single cell line, SH, cells treated with CBD differed significantly from those exposed to FBS, however, no difference between CBD-treated cells and vehicle-treated cells was found. This result may suggest that this finding represents a type I error.

The lack of a significant increase of the MFI in rapamycin-treated cells is notable and suggests that our cells may not be undergoing the expected autophagic response. Henry et al. found peak autophagy to occur rapidly in various canine neoplastic cell

lines, at 2-8 hours following treatment.[134] In sharp contrast, autophagy was measured 24 hours after incubation with CBD in the present study.

It is possible that both the timing and methods of evaluation used here impair the accurate recognition of autophagic activity. Alternative mechanisms exist for the measurement of autophagy and should be pursued prior to concluding that CBD does not produce an autophagic response in canine UC. Some such methods quantify or monitor the presence of autophagosomes, as in the method used here, while others instead measure changes that occur over the course of progression through the autophagic process.[187,188] A previous extensive review of techniques used in the investigation of autophagy in eukaryotes proposes that the steady state measurement of autophagosomes is insufficient and should be replaced by methods that account for autophagic flux.[187] Autophagic flux refers to the complete process of autophagy including the delivery of cellular cargo to lysosomes via fusion with autophagosomes, and its subsequent breakdown and recycling.[187] The evaluation of autophagic flux can be achieved by measuring turnover of LC3-II via immunoblotting techniques or fluorescent microscopy.[188] During autophagy, LC3-I is recruited to the autophagosome membrane, attaches to autophagic vacuoles, and is converted into LC3-II. The amount of LC3-II therefore reflects the number of autophagosomes present at a given moment and can be followed, along with LC3-I, over time. Although insufficient when used as the solitary marker of autophagic flux, degradation of p62, an autophagic cargo protein, is

also widely used in conjunction with measurement of LC3 as this protein directly binds to LC3 and is selectively degraded during autophagy.[188]

Autophagy has also been demonstrated to have a role in the anti-neoplastic effects of CBD in human breast cancer.[125] In one report, various methods were used to confirm autophagic activity including flow-cytometric assays measuring for acridine orange, measurement of LC3-I and LC3-II and the documentation of autophagic vacuoles using electron microscopy. Another previous study implicating cannabinoids in the induction of the autophagic process, involved treatment of human and mouse glioma cells with Δ^9-THC.[130] In that report, autophagy was measured through immunostaining for LC3 and time-course analysis of LC3-I and LC3-II using immunoblotting techniques. This analysis led to the conclusion that Δ^9-THC produces an ER stress response promoting autophagy via the AKT/mTORC1 axis.[130]

Therefore, further investigation of the impact of CBD on the autophagic process in canine UC would be of value and should include time course analysis of LC3-I, LC3-II and p62, rather than the exclusive measurement of autophagic vacuoles at a single time-point, as was performed here.

Several previous reports have proposed a mechanism of cross-talk between the autophagic and apoptotic responses, in which autophagy appears to precede apoptosis.[130,134,136,185] This publication record includes results by Henry et al. investigating the treatment of canine neoplasms with CBD, which led to the conclusions that autophagy precedes apoptosis, and that a relationship exists between autophagy

and the MAP Kinase signaling pathway, via ERK and JNK.[134] If this is indeed the case, and CBD treated cells enter autophagy prior to the stimulus to undergo apoptosis, this also implies that future research into the role of CBD in the induction of programmed cell death of canine UC should examine the apoptotic response concurrently and at similar timepoints to autophagy.

Finally, another confounding factor in this study may arise from the use of serum deprivation. Stressful environmental and intracellular stimuli, including cellular starvation in particular, are important triggers for the autophagic response.[136] It is therefore possible that all cells in these experiments are undergoing a baseline level of autophagic activity regardless of their treatment with either rapamycin or CBD, thus preventing any appreciation of a difference between cells treated with a vehicle control, positive control and drug. In an effort to overcome this limitation, one treatment group was not serum-starved, and cells were untreated but exposed to FBS. Nonetheless, no difference was observed between these serum-fed cells and any of the other treatment groups. Another possibility is that despite the inclusion of a population of cells which were not starved for comparison, that the process of flow cytometry itself, including lifting cells from the basement membrane to create a cell suspension, and various spinning, washing and incubation steps, may also be sufficient to induce a baseline level of autophagic activity. These lingering questions all support the case for further research using an alternate methodology to more accurately and comprehensively explore the effects of CBD on programmed cell death in canine UC.

4.6 Additional limitations

Several additional limitations are inherent to the current study. First, this includes the use of serum deprivation in all assays. The decision to treat cells with CBD under serum-starved conditions is based on previous findings in which cannabinoids, including CBD in particular, demonstrated a higher potency and efficacy in the absence of serum proteins.[129] Despite this convention for *in vitro* studies exploring the effects of CBD, it likely does not accurately recapitulate the conditions of the tumour microenvironment as it exists *in vivo*.

Another broader limitation of this study arises from its *in vitro* design. Similar to the serum-free assays discussed above, *in vitro* experimentation in general does not precisely reproduce the complexity of the tumour microenvironment. In addition, extrapolation of results from *in vitro* studies may not accurately represent tumour response, account for drug metabolism, or provide information on clinically relevant drug doses and interactions *in vivo*.

Drug doses used in this study approach or slightly exceed those achievable in canines *in vivo*. Several previous pharmacokinetic studies have examined plasma levels of CBD in dogs exposed to various formulations, dosages and delivery methods.[152,153,156–158] In general oil-based formulations, administered with food, have the most favourable pharmacokinetic profiles and highest bioavailability, resulting in plasma concentrations ranging from 347.0 ng/mL (1.1μM)[156], to 903.68 ng/mL (2.87μM).[158] In the present study, dose-response curve experiments did produce IC50

values within this range, however doses used experimentally (5 μM and 10μM) exceeded these values. As previously discussed, the requirement for the higher doses used in this study appeared to arise with time and may relate to the storage and stability of the product. However, these doses were also based on previous literature investigating the use of CBD in canine neoplastic cell lines in which similar or higher doses were used (5-47μM), exceeding the C_{max} achieved in canine pharmacokinetic studies.[133,134]

Another important limitation is the lack of consistency of time points across experimental assays. This inconsistency precludes any definitive conclusions as to the existence of a relationship between CBD and the various mechanisms of programmed cell death examined here. As mentioned above, from these results, it may be tempting to suggest that CBD does not induce either autophagic or apoptotic programmed cell death in canine UC. However, prior to arriving at these conclusions, further work is critical and should explore whether such activity may be occurring either earlier or later than the times investigated here. This should ideally involve a time course analysis over a range of incubation times, using several markers of apoptosis and autophagy including caspase 3/7, cleaved caspases, LC3-1, LC3-II and p62.

5 Conclusions

This study demonstrated the expression of both canonical and non-canonical cannabinoid receptors in canine urothelial cell carcinoma *in vitro*. It also reinforces previous findings to confirm that treatment with CBD produces a reduction of canine UC cell viability at near-physiologic doses. However, similar reductions in cell viability were also seen in non-neoplastic canine renal epithelial cells *in vitro*.

Selective CB1, CB2, PPARγ, GPR55 or TRPV-1 antagonists did not alter cell viability compared to CBD alone, suggesting that cytotoxicity may occur through more complex interactions between receptors or through cannabinoid receptor-independent mechanisms. Finally, at the evaluated time points, CBD also did not induce significant apoptosis or autophagy in canine UC when compared to untreated cells.

This report marks the first demonstration of cannabinoid receptor expression in canine neoplastic tissues. Despite the negative results observed in various other aspects of this work, it represents another step in the foundation of our knowledge of the effects of CBD in neoplasia and raises additional questions, acting as a springboard for further work investigating the role of CBD in the treatment of canine UC.

6 References

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