Cannabinoid: A Potential Anti-Cancer Agent for Non-Hodgkin Lymphoma

by

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Abstract

The non-Hodgkin lymphomas (NHLs) are a heterogeneous family of lymphoid malignancies and are one of the most commonly diagnosed neoplasm in both dogs and humans. Systemic anticancer chemotherapy is the treatment of choice for human and canine lymphomas. Canine and human lymphoma are generally characterized by a high rate of initial remission following conventional CHOP (cyclophosphamide, hydroxyl-doxorubicin, vincristine, and prednisone) based therapies; however, 95% of dogs and 30% of humans will succumb to drug-resistant relapse and in most cases face severe side effects of chemotherapeutic drugs. Owing to shared molecular, signaling, incidence, and pathologic features, treatment approach and treatment need, studying novel targets for lymphoma treatment in canine can be beneficial for both species.

Cannabis or Marijuana have been used as medicine for centuries. With renewed interest in the cannabinoid (CBs) as a medicine, in the last two decades, cannabinoids have been extensively studied for their anti-cancer effects in various models of cancers including NHL, and have demonstrated promising effects against tumor growth, angiogenesis and metastasis. However, the anti-cancer effects of cannabinoids have never been studied in canine lymphoma and there is very limited literature available on the canine endocannabinoid system. Finding this wide-open area to study in canine lymphoma our aim of this research was to 1. Study the expression of cannabinoid receptors CB1 and CB2, 2. Analyze and compare the anti-cancer effects of cannabinoids in human and canine NHL cell lines and 3. To study the effect of cannabinoids in combination with traditional NLC drugs and compare it with the effect of CBs and NLC drugs alone.

To study the expression of cannabinoid receptors CB1 and CB2, canine B cell type (1771 and CLBL1) and T cell type (CL1) NHL cell lines, canine PMBCs and, human B cell type NHL cell line (Ramos) were used. Cells were cultured in RPMI, and receptor expression was studied using real-time PCR. Our results demonstrated positive expression of cannabinoid receptor CB1 and/or CB2 in both canine and human lymphoma cell lines, with a significantly higher expression of CB1 and CB2 receptors in canine and human B cell lymphoma cell lines, compared to activated PBMCs and canine CL-1 lymphoma cell line. For activated canine PBMCs our results show negative expression of the CB1 receptor gene but significantly higher expression of CB2 receptor gene compared to the canine T cell lymphoma cell line.

After establishing the expression of cannabinoid receptors, anti-cancer effects of cannabinoids [Endocannabinoids (AEA, 2AG), phytocannabinoids (CBD, THC) and synthetic cannabinoid (WIN)] on canine and human NHL cell lines were analyzed using MTT cell viability assay. Cells were treated at concentrations from 0.1µM to 50µM for 24 and 48 hours. Results of the cell viability assay demonstrate a dose-dependent decrease in cancer cell viability as compared to the control (cells treated with vehicle only) with AEA and CBD in both B and T cell type NHL cell lines. Treatment with WIN and THC showed dose-dependent decrease in cell viability in only B-cell NHL cell lines. 2-AG appeared to decrease cell viability in only lymphoma cell lines with higher CB2 receptor expression (Ramos and CLBL-1).

To further confirmed the anti-cancer effects of cannabinoids we selected canine B cell lymphoma cell line 1771 and exposed it to 0 (vehicle only) and 1µM and 50µM concentrations of cannabinoids for 24 h and protein was extracted. Markers of oxidative stress, mitochondrial function and apoptosis analyzed using biochemical spectrophotometric and fluorometric analysis. Results of the biochemical analysis revealed a cannabinoid-induced increase in markers of oxidative stress and apoptosis and a decrease in markers of mitochondrial functions in cells treated with cannabinoids (AEA, CBD, THC and WIN) as compared to the control.

To study the effect of cannabinoids in combination with traditional NHL chemotherapeutic drugs (NLC drugs), canine 1771 lymphoma cells were treated with CBs (AEA, CBD and WIN) and NLC drugs (DOX, CYC, VIN, LOM and PRD) alone and combinations. The cytotoxicity of each drug alone and combinations was analyzed by MTT assay and combination effect was analyzed using combinational index (CI) analysis. Our results demonstrated that the cytotoxic effects of all traditional NHL chemotherapy drugs were synergistically enhanced (interaction with CI <1) by each of the three cannabinoids when added to 1771 canine malignant B type NHL cells.

Taken together, these studies show the anti-cancer potential of cannabinoids against both canine and human NHL and provides a resource for developing therapeutics and testing safety prior to initiating canine and human studies.

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2-AG	2-arachidonoyl-glycerol
AA	arachidonic acid
AEA	anandamide, *N*-arachidonoylethanolamide
AM251	CB1 receptor antagonist/inverse agonist
CBs	cannabinoids
CB1	cannabinoid receptor type 1
CB2	cannabinoid receptor type 2
CBD	cannabidiol
CBR	cannabinoid receptor
CNR1	gene encoding cannabinoid receptor type 1
COX	cyclooxygenase
CYC	cyclophosphamide
DAGL	Diacylglycerol lipase
DOX	Doxorubicin
DMSO	Dimethylsulfoxide
ERK	extracellular signal-regulated kinase
FAAH	fatty acid amide hydrolase
FBS	Fetal Bovine Serum
GPR	G protein-coupled receptor
GSH	Glutathione

H_2O_2 Hydrogen Peroxide

HU 210 CB receptor agonist

LOM lomustine

LOX lipoxygenase

MAGL Monoacylglycerol lipase

MAPK mitogen-activated protein kinase

NADH Nicotinamide Adenine Dinucleotide

NHL Non-Hodgkin Lymphoma

NLC Non-Hodgkin Lymphoma chemotherapeutic

NO Nitric Oxide

PBS Phosphate Buffer Saline

PLC Phospholipase C

PPAR peroxisome-proliferator-activated receptors

PKA protein kinase A

PKB/Akt protein kinase B

PRD prednisolone

ROS reactive oxygen species

THC Δ9 tetrahydrocannabinol

VIN vincristine

WIN55,212-2 CB receptor agonist

1. Literature Review

1.1 Endocannabinoid system

The endocannabinoid system (ECS) is a widespread neuromodulatory network involved in the developing central nervous system as well as plays a major role in tuning many cognitive and physiological processes to control the most vital processes thus creating homeostasis within the organism. ECS consists of a complex network of cannabinoid receptors, endocannabinoid ligands, the enzymatic machinery that drives their biosynthesis, degradation, transport and all cells and neurological pathways that involve endocannabinoid signaling[1, 2] **Fig1, 2.**

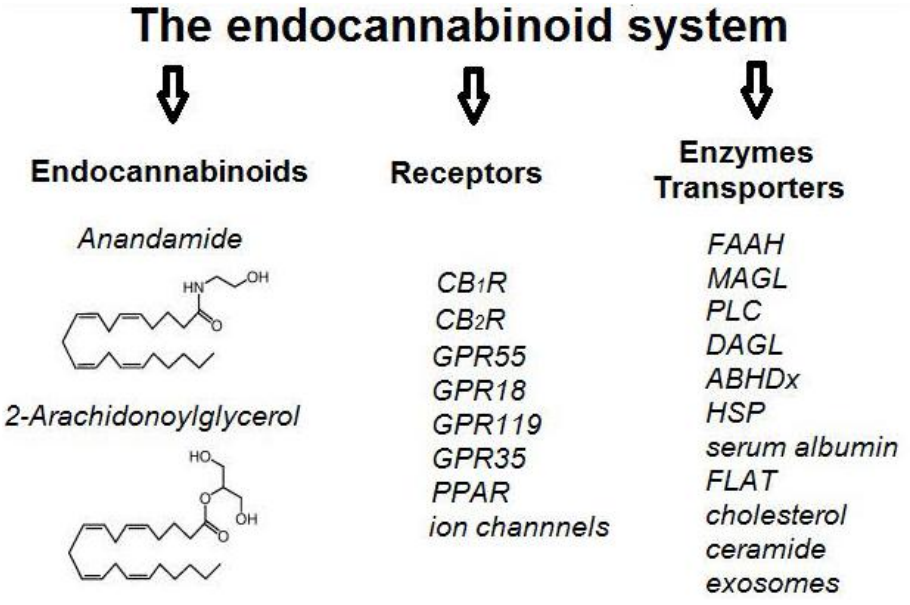

Figure 1-1. A schematic representation of the main components of the endocannabinoid system (ECS).
CBR – cannabinoid receptor, GPR – G protein-coupled receptor, PPAR – peroxisome-proliferator-activated receptors, FAAH – Fatty acid amide hydrolase, MAGL – Monoacylglycerol lipase, PLC – Phospholipase C, DAGL – Diacylglycerol lipase, ABHDx – Alpha-beta hydrolase domain proteins, HSP – heat shock proteins, FLAT – FAAH-like anandamide transporter. Figure from [2]

1.2 Components of Endocannabinoid System

1.2.1 Cannabinoid receptors

Cannabinoid receptors CB1 and CB2 are both G protein-coupled receptors cloned and characterized by mammalian tissue. In addition to these receptors other receptors like transient receptor potential cation channel subfamily V member 1 (TRPV1) and some of orphan G protein-coupled receptors, GPR55, GPR119, and GPR18, have been proposed to act as endocannabinoid receptors [3]. They inhibit adenylyl cyclase and certain voltage-sensitive calcium channels, stimulate MAP (mitogen-activated protein) kinases and inwardly rectifying potassium channels (GIRKs), and recruit beta-arrestins, among other actions [4]. CB1 receptors are highly distributed in the central nervous system (CNS) particularly in discrete areas that are involved in the control of motor behavior (such as the basal ganglia and cerebellum), memory and learning (the cortex and hippocampus), emotions (the amygdala), sensory perception (the thalamus), and autonomic and endocrine functions (the hypothalamus, pons, and medulla). In addition, CB1 receptors are expressed in peripheral nerve terminals and many extra-neural sites including the liver, adipose tissue, and skin. Whereas CB2 receptors are primarily expressed in cells of immune origin [5], including microglia [6, 7], though they may also be expressed in neurons, particularly in pathological states [8], their distribution in CNS is much lower and more restricted [8, 9]. Similar pattern of cannabinoid receptor distribution has been found in canine, Freundt-Revilla J et al studied the spatial distribution of CB1 receptors in the normal canine CNS and PNS using Immunohistochemistry of several regions of the brain, spinal cord and peripheral nerves from a healthy four-week-old puppy, three six-month-old dogs, and one ten-year-old dog revealed strong dot-like immunoreactivity in the neuropil of the cerebral cortex, Cornu Ammonis (CA) and dentate gyrus of the hippocampus, midbrain, cerebellum, medulla oblongata and grey matter of the spinal cord. Dense CB1 expression was found in fibers of the globus pallidus and substantia nigra surrounding immunonegative neurons. Astrocytes were constantly positive in all examined regions. CB1 labeled neurons and satellite cells of the dorsal root ganglia, and myelinating Schwann cells in the PNS. These results demonstrate the spatial distribution of CB1 receptors in the healthy canine CNS and PNS [10]. Andrea Pirone et al assessed CB1 receptors 30-days old canine embryos using immunohistochemistry. CB1R immunoreactivity was mainly epithelial and included most structures of the central and peripheral nervous system, inner ear, olfactory

epithelium, and related structures, eye and thyroid [11]. Giorgia Galiazzo et al. studied the distribution of both CB1 and CB2 receptors in the canine gastrointestinal tract they found CB1 receptor immunoreactivity in the lamina propria and epithelial cells andCB2 receptor immunoreactivity was expressed by lamina propria mast cells and immunocytes, blood vessels, and smooth muscle cells. The faint CB2 receptor immunoreactivity was also observed in neurons and glial cells of the submucosal plexus [12]. Luca Campora et al studied CB1 and CB2 receptors expression in the skin of healthy dogs and dogs with atopic dermatitis. In skin samples of healthy dogs, CB1 and CB2 immunoreactivity were detected in various types of cells in the epidermis and cells in the dermis, including perivascular cells with mast cell morphology, fibroblasts, and endothelial cells. In skin samples of dogs with AD, CB1 and CB2 immunoreactivity was stronger than it was in skin samples of healthy dogs. In positive control tissue samples, CB1 immunoreactivity was detected in all areas of the hippocampus, and CB2 immunoreactivity was detected in B-cell zones of lymphoid follicles [13]. There is limited data available regarding the expression of cannabinoid receptors in canine however studies done so far have found positive expression and similar distribution of cannabinoid receptors in canine as compare to rodents and humans.

Signaling

Both cannabinoid receptors CB_1 and CB_2 primarily couple through inhibitory G proteins ($G_{i/o}$) and activate the pathways associated with $G_{i/o}$ that result in inhibition of adenylate cyclase, thus inhibiting the conversion of ATP to c AMP[14]. cAMP (cyclic adenosine monophosphate) is a secondary messenger, and an important component of signal transduction cascades within the cell, which subsequently activate other kinases to open ion channels or expose the active sites of other proteins. In neurons, calcium-sensitive Adenylyl Cyclases located next to calcium ion channels may play an important role in learning processes. The coupling of cannabinoid receptors with $G_{i/o}$ also results in the activation of mitogen-activated protein kinase (MAPK) [14]. MAPK families play an important role in complex cellular programs like proliferation, differentiation, development, transformation, and apoptosis.

Other than the G protein signaling pathway CB_1 and CB_2 receptors can also signal through an arrestin-dependent pathway[15]. CB1 receptors have been shown to couple ion channels through Gi/o, negatively to N-type and P/Q-type calcium channels and positively to A-type and inwardly

rectifying potassium channels[16]. Under some circumstances, cannabinoid receptor can also couple with $G_{q/11}$ pathways and stimulate cAMP formation [17].

Cannabinoid receptors being GPCRs share the same complexities of this receptor family. Cannabinoid receptor mediate two opposite effects on cyclic AMP production is one of its examples, also natural polymorphisms (heteromers and oligomers) have been identified in both the CB 1 and CB 2 receptors[2]. In addition, alternative splice variants of both receptors have also been identified. Receptor polymorphisms and variants explain the complex nature of cannabinoid receptors. Like other GPCRs cannabinoid receptors also show functional selectivity for different ligands, thus different cannabinoid ligands acting on the same cannabinoid receptor can elicit different signaling cascades, resulting in diverse biological responses [18, 19].

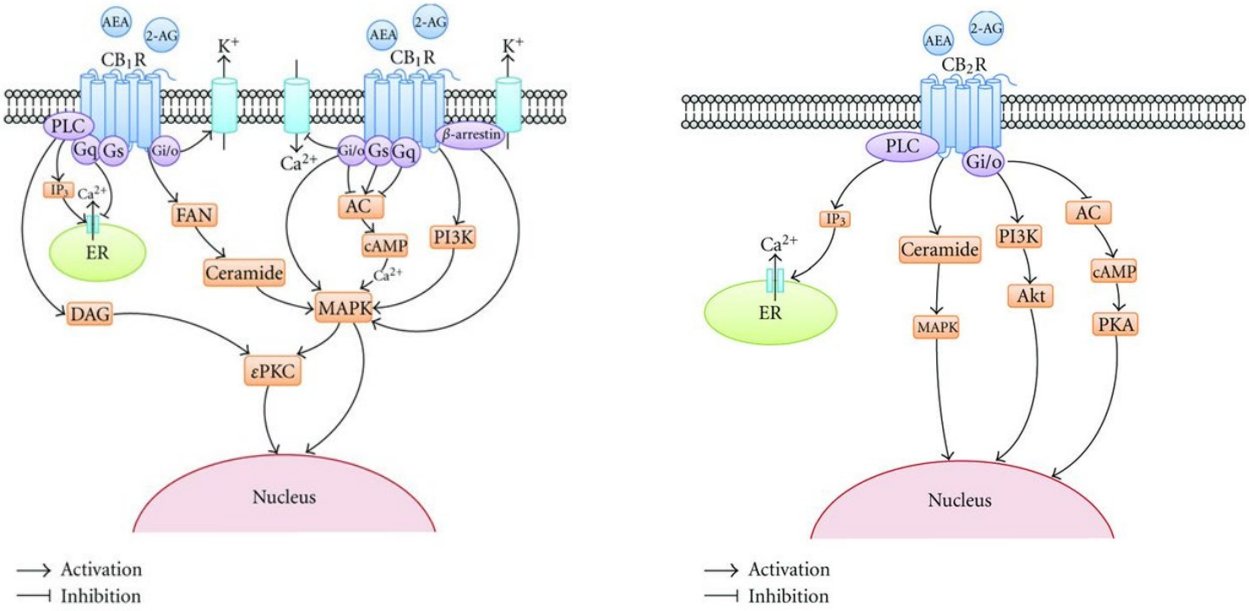

Figure 1-2. CB1 and CB2 receptor intracellular signaling pathways

Figure From [20]

1.2.2 Cannabinoid Ligands

Soon after the characterization of cannabinoid receptors, endogenous ligands for cannabinoid receptors (endocannabinoids) were discovered. These arachidonic acid derivatives are produced from phospholipid precursors. The two major endogenous ligands are anandamide (AEA) and 2-acylglycerol (2AG) **Fig 1**. In addition, a number of other endocannabinoid ligands have been discovered, including N-arachidonoyldopamine, N-arachidonoylglycerolether, and O-arachidonoylethanolamine. Apart from their binding to CB1 and CB2 receptors, endocannabinoids may bind to other receptors. For example, AEA may intracellularly activate the potential vanilloid receptor type 1 (TRPV1) [21]. However, CB1 and CB2 receptors are certainly the most known targets for AEA and 2-AG, which activate them with different affinity. AEA has the highest affinity for both CB1 and CB2, by contrast, 2-AG has the highest efficacy for both the receptors [22]. Felix K Gesell et al assessed endocannabinoids in cerebrospinal fluid of 16 normal, healthy dogs and 40 dogs with epileptic seizure disorder using liquid chromatography with tandem mass spectrometry. According to their results, AEA and total AG were detected and quantified in nearly all the CSF samples. Well in line with previous endocannabinoid measurements in CSF and brain tissue of humans and other mammalians, AEA concentrations were in the picomolar range and the nanomolar range for AG. Animals suffering from idiopathic epilepsy had higher AEA concentrations than control animals. No statistically significant difference was observed for total AG concentration [23].

1.3 Endocannabinoid Biosynthesis

Investigations of the endocannabinoid system in the mature nervous system and heterologous expression systems have led to the concept that endocannabinoid signaling is not a classical example of neurotransmission, as their synthesis is based on “on-demand” principal and their effects are mostly restricted to local sites of their biosynthesis and release. In contrasts to classic neurotransmitters, which are synthesized and stored in vesicles, the principle of on-demand synthesis is that the endocannabinoids in the lipid membrane exist as a precursor and is released by the activation of lipid enzymes that are triggered by a specific signal (e.g., G proteins or elevation of intracellular calcium). This on-demand synthesis of endocannabinoids also strongly contrasts with the administration of exogenous plant-based or synthetic cannabinoid ligands (e.g.

THC and HU-210 respectively) where ligand-receptor binding is indiscriminative and sustained for minutes or longer on the other hand it is only for seconds or less for endocannabinoids. Thus, it is not surprising that the exogenous cannabinoid can take over the endocannabinoid signaling that can result in a range of physiologic effects. Anandamide and 2-arachydonoyl glycerol have very similar chemical structures (**Fig 1**) but the pathways involved in their biosynthesis and degradation are completely different, highlighting their distinct physiological roles. They are both derivatives of arachidonic acid, an essential PUFA however, 2-AG is produced in two steps, in the first step, inositol triphosphate is removed from the arachidonoyl-containing PIP2 (phosphatidylinositol bisphosphate) in a calcium-dependent manner involving phospholipase C (PLC), followed by removal of the acyl group in the 1 position by a DAG (diacylglycerol) lipase on the other hand AEA production is the result of NAPE (*N*-arachidonoyl phosphatidylethanolamine) hydrolysis by a NAPE-PLD (NAPE phospholipase D) [2, 24] **Fig 3**.

1.4 Endocannabinoid Transport

Following the synthesis of endocannabinoids, their transport across the cell membrane is important to reach their site of action followed by their degradation. Due to the hydrophobic nature of endocannabinoids, they cannot simply diffuse through the cell membrane, suggesting the involvement of carrier-mediated facilitated diffusion in their transport. Substantial evidence suggests the involvement of the same carrier proteins in the transport of 2-AG and Anandamide and many transporters such as heat shock proteins, serum albumin and other fatty-acid binding proteins as FAAH-like anandamide transporter (FLAT), cholesterol, ceramides have been implicated in this process depending on the cell type **Fig 3**.

1.5 Endocannabinoid Degradation

Endocannabinoid signaling is terminated by hydrolysis of the arachidonic group from both AEA and 2-AG **Fig 3**. In the case of AEA, the hydrolysis takes place in the endoplasmic reticulum with fatty acid amide hydrolase (FAAH), and 2-AG hydrolysis is primarily carried out in the CNS by MAGL (monoacylglycerol lipase) or ABDH6 (alpha/beta-hydrolase domain containing 6) [25, 26] which is proposed as secondary degraders. Levels of endogenous cannabinoids primarily depend on their uptake and effective transport in the cell, as they are polar and cannot diffuse through the

cytosol and cell membrane passively. Some exogenous cannabinoids, for example, CBD, can interfere with FAAH-mediated breakdown of AEA, raising the levels of available AEA, thus indirectly inducing various non-psychoactive effects [27].

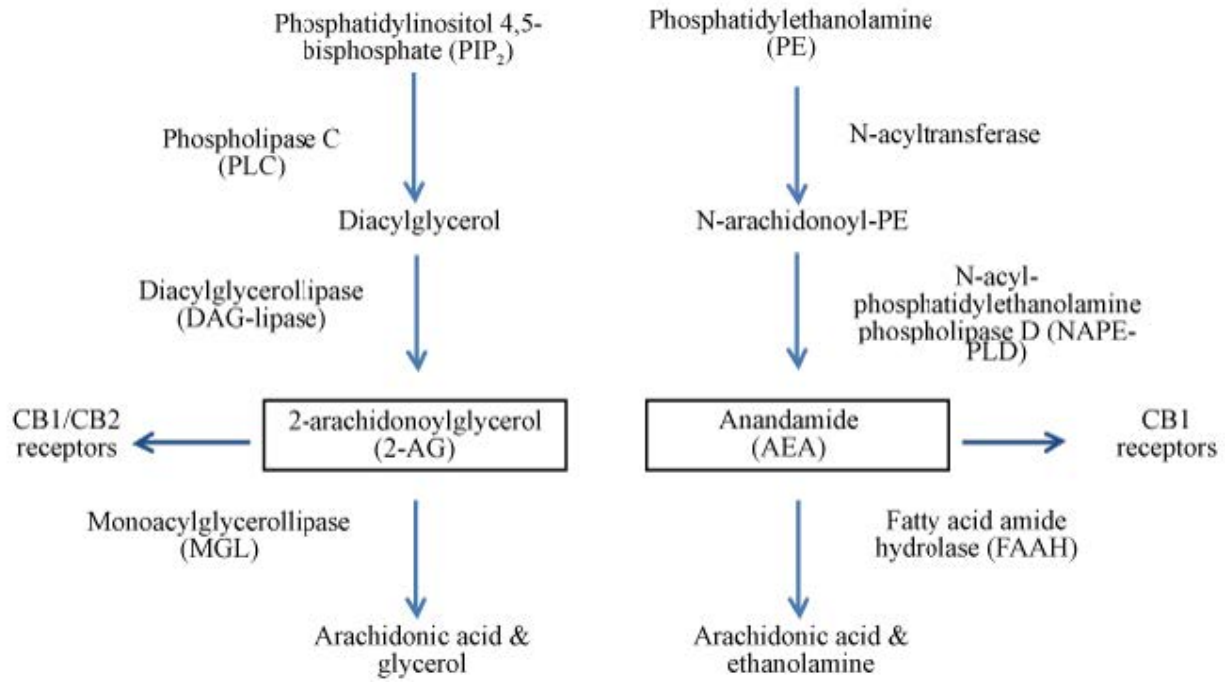

Figure 1-3. Synthesis and degradation of endocannabinoids
Synthesis and degradation of endocannabinoids Major pathways for the synthesis and degradation of 2-AG and anandamide. From Omnics

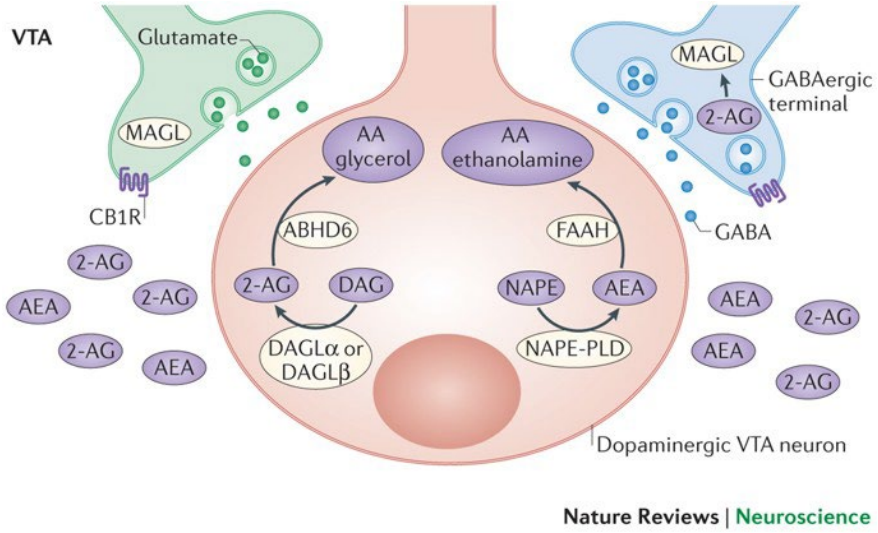

Figure 1-4. Endocannabinoid biosynthesis, signaling, and clearance.
From [28]

1.6 Physiological Functions of Endocannabinoids

Knowing the widespread distribution of cannabinoid receptors in the body it is reasonable to speculate wide-ranging physiological functions of endocannabinoids and to expect pathological outcomes in case of any disturbance in the system [29, 30]. Although a tremendous amount of data is available on the pharmacological actions of endogenous and exogenous (synthetic and plant-based) cannabinoids, there is still limited knowledge available about the physiological functions of the endocannabinoid system in the body and one major reason for that is the very short life of endocannabinoids in the body. In most of the in vivo studies difficulties have been faced due to the lack of sensitivity in detecting the Pico molar amount of endocannabinoids and rapid degradation after the release of the ligand. However, the limited data available indicates, that ECS is a tuning system that finely regulates numerous physiological functions, not only in the CNS but also in the peripheral nervous system and peripheral tissue, including pain, energy metabolism, cardiovascular and reproductive functions, inflammation, glaucoma, cancer, and liver and musculoskeletal disorders [31, 32].

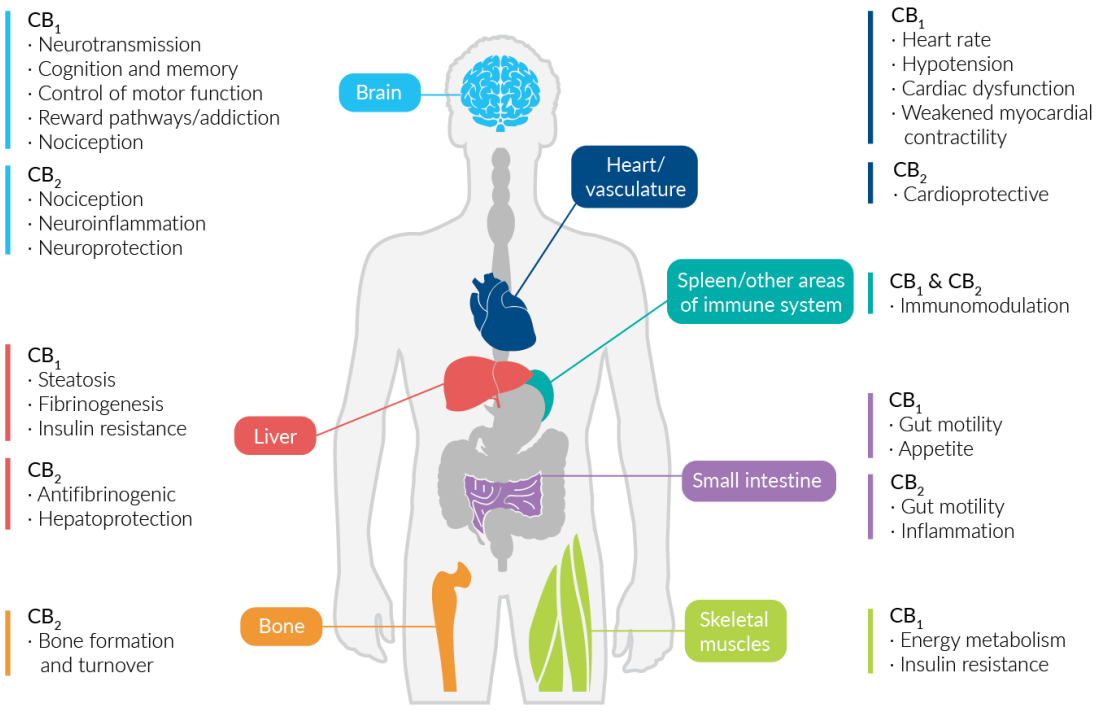

Figure 1-5. Distribution of CB1 and CB2 receptors and their associated functions.

Figure from (Cayman-2020)

1.6.1 Central nervous system

In the central nervous system, CB1R are the most commonly found GPCR, their intraneuronal actions in the CNS indicate their neuromodulatory role in the release of the neurotransmitter and regulating their activities. On activation, CB1R inhibits Ca^{2+} channels and activates K channels that result in delaying the action potential. Also, CB1 receptor-mediated inhibition of adenylate cyclase slows the synthesis and release of neurotransmitters [33]. Studies have found the highest expression of CB1R, Anandamide and its degrading enzymes in the cerebellum, hippocampus, thalamus, cortex, and substantia nigra, and proposed their role in cognitive and motor response [34, 35].

A study conducted on mice demonstrated that AEA impairs the working memory and the memory consolidation in the mice and, the effect was reversed with SR 141716A, the inhibitor of CB1R. The molecular mechanism suggested was inhibition of acetylcholine release by the activation of cannabinoid receptor with synthetic cannabinoid WIN 55, 212-2 and the inhibition canceled with SR 141716A [36, 37].

1.6.2 Hypothalamic functions

Uniform distribution of CB1 receptors in the hypothalamus and anandamide-mediated activation of c-fos in the hypothalamic neurons imply the role of the endocannabinoid system in controlling overall hypothalamic functions and pituitary gland secretions in particular. Endocannabinoid ligands AEA and 2-AG, which binds to CB1 receptor localized to axonal processes, also both ligands and the receptor are expressed in the amygdala [38] [39, 40]. a study has found that, Following intracerebroventricular injection of anandamide, activation of neurosecretory cells within the paraventricular nucleus of the hypothalamus (PVN) followed by the release of corticotropin-releasing hormone that leads to the secretion of adrenocorticotropic hormone from the pituitary, may result in the enhancement of cortisol production from the adrenal gland [40]. Similarly, Anandamide may also inhibit other pituitary gland hormones like prolactin, follicle-stimulating hormone, and growth hormones by acting on the dopaminergic neurons in the hypothalamus. Moreover, studies have suggested the sleep/wake cycle regulation by ECS, by demonstrating prolonged awake time in rats treated with SR 141716A (CB1 receptor inhibitor) and by the fact that the sleep-inducing oleamide may act through anandamide.

Data from recent studies also indicate the negative feedback regulation of the neuroendocrine release by the endocannabinoid system in response to psychological stress [41, 42]. Tissue areas in Amagdala that express AEA and 2-AG were found to be modulated in response to psychological stressors [43, 44] and endocannabinoid signaling within the amygdala is known to modulate both excitatory and inhibitory neurotransmission [45, 46].

1.6.3 Sensory nervous system

Cannabinoid receptors and their endogenous ligands are present at supraspinal, spinal, and peripheral levels. Cannabinoids suppress behavioral responses to noxious stimulation and suppress nociceptive processing through activation of cannabinoid CB1 and CB2 receptor subtypes. ECs acting at CB_1 negatively regulate neurotransmission throughout the nervous system, whilst those acting at CB_2 regulate the activity of CNS immune cells. Signaling through both of these receptor subtypes has a role in normal nociceptive processing and also in the development resolution of acute pain states [47].

A study conducted on inflammatory pain, in which an inflammatory substance applied to the rodent hind paw elicited an oedemic response and measurable nociceptive behavior. Peripheral administration of AEA in the formalin model temporarily reduced the nociceptive behavior in a CB_1-sensitive manner [48]. Conversely, increased nociceptive responses were recorded on blocking CB_1 and/or CB_2 receptors, suggesting an intrinsic role for EC in inflammatory pain. Another interesting rodent study showed that anandamide inhibits carrageenan-induced thermal hyperalgesia in rats and capsaicin-evoked release of calcitonin gene-related peptide from isolated rat hind paw skin.

Studies also indicate a role for the spinal EC system in nociceptive transmission. Spinal ECs are elevated in animal models of acute and chronic pain [49]. At the level of the spinal cord, exogenous application of EC has an anti-nociceptive effect [48] Conversely, intrathecal administration of a CB_1 receptor antagonist produces hyperalgesia in mice [50], enhancing nociception-evoked firing of wide dynamic range neurons in the dorsal horn of the spinal cord [50].

1.6.4 Autonomic nervous system and vasculature

Cannabinoid receptors, their endogenous ligands, as well as enzymes conferring their synthesis and degradation, exhibit overlapping distributions in the autonomic nervous system and vasculatures [51]. Endocannabinoids exert their effects on the cardiovascular system by acting on neurotransmitter release at the central nervous system (CNS) and sympathetic nerve terminals or locally by modulating vascular smooth muscle cells (VSMCs), inflammatory cells, and endothelial function [52]. CB1-mediated inhibitory action on the release of acetylcholine from autonomic fibers has been implied. Anandamide was shown to inhibit guinea pig small intestine and mouse urinary bladder and vas deferens contractions by a pre-synaptic, CB1-mediated inhibitory. Hypotension and bradycardia induced by anandamide were also shown to be due, at least in part, to a pre-synaptic action on CB1 receptors and the subsequent inhibition of noradrenaline release from post-ganglionic sympathetic nerve terminals innervating the heart and vasculature. Derivatives of anandamide hydrolysis, arachidonic acid, and eicosanoids may also be responsible for the hypotensive effect of anandamide. As the involvement of epoxyeicosatetraenoic acids and prostacyclin has been suggested in a bovine coronary artery [53].

1.6.5 Immune system and cytoprotection

Endocannabinoids play role in immune function in a mode that, primarily, is linked to the CB2 receptor. The activation of cannabinoid seven-transmembranal, G protein-coupled receptors results in series of signal transductional events that end in transcribing proteins which are responsible for regulating cell migration and the production of cytokines and chemokines. In the light of data available, it is now clear that the endocannabinoid system plays a major role in hematopoietic stem cells and progenitor cells migration and according to the studies the proposed mechanism of their immune system regulation is, binding of endocannabinoids to CB1 and CB2 receptors, followed by the inhibition of cAMP formation or the activation of MAPK and phospholipase A_2, that may act as chemical signals between different immune cells or between sensory fibers and blood cells. Activated basophils and mast cells by IgE receptor cross-linking, may release *N*-palmitoylethanolamine and anandamide, together with serotonin, histamine, and leukotrienes may produce vascular permeability and chemotaxis, *N*-palmitoylethanolamine has shown to acts as an anti-inflammatory mediator in vivo and in vitro studies [54, 55], acting as an autacoid signal on basophils and mast cells and inhibiting their degranulation. However,

Anandamide, which can also be produced by macrophages, was found to counteract this effect, and activate basophil/mast cell and macrophage release, thus potentially leading to the formation of leukotrienes and prostaglandin E_2 which may have opposing actions on immune cell functionality [33, 56, 57]. Moreover, 'endocannabinoid' might directly down-regulate the immune response by inhibiting lymphocyte proliferation. The study conducted by Kose et al. has shown a higher concentration of 2-AG in the blood plasma compared to bone marrow in healthy individuals and they also demonstrated that stimulation of cannabinoid receptor with endocannabinoids can stimulate migration of human hematopoietic stem cells [58]. Some recent studies demonstrate CB1/CB2 receptor-mediated decrease in CXCR4 in bone marrow and inhibition of lymphocyte recovery after bone marrow transplant [59]. The effects of AEA and palmitoylethanolamide, as well as Δ9- THC, on the production of tumor necrosis factor (TNF)- α, IL-4, IL-6, IL-8, IL-10, IFNγ, p55, and p75 TNF-α soluble receptors have been examined [60]. AEA was shown to diminish the production of IL-6 and IL-8 at low nanomolar concentrations and to inhibit that of TNF-α, IFNγ, IL-4, and p75 TNF-α soluble receptors at micromolar concentrations. Palmitoylethanolamide, at concentrations similar to those of AEA, inhibited the synthesis of IL-4, IL-6, and IL-8 and the production of p75 TNF-α soluble receptors [61]. In contrast to AEA, 2-AG has been associated primarily with the augmentation of immune responses. 2-AG was most often found to modulate functions related to leukocyte recruitment, such as chemokine release, adhesion to fibronectin, and migration. 2-AG is the main pro-inflammatory effect of endocannabinoids or cannabinoids in vitro that has been reported. AEA, on the other hand, was found to down-regulate leukocyte functions, such as pro-inflammatory cytokine release and nitric oxide production [62]. In vivo and in vitro studies showing mixed results in table 4 (12).

Table 1-1. CB2 mediated effects of endocannabinoids on immune cell functions.
Adapted from [63]

Cell type	Species	Endocannabinoid	Effects	Ref
Anti-inflammatory effect				
T84 intestinal epithelial cells	Human	AEA	↓Neutrophils	[64]
Astrocytes	Rat	AEA	↓TNF-α	[65]
Dendritic cells	Human	AEA	↓ IL-6, IL-12 and IFN-α	[66]
Microglia	Mouse (BV-2 cell line)	AEA	↓ Nitric oxide	[67]
	Mouse	AEA	↑ IL-10	[68]
			↑ IL-10 ↓ IL-12p70 and IL-23	[69]
	Rat	AEA	LPS-induced nitric oxide release	[70]
Neutrophils	Human	2-AG	↓ fMLP-induced migration	[71]
Splenocytes	Human	AEA	↓ Primary and secondary antibody formation	[72]
T cells (not separated)	Human	AEA	↓ Cell proliferation	[73]
		2-AG	↓ SDF-1-induced migration	[74]
CD4+ T cells	Human	AEA	↓ IL-17, IFN-γ and TNF-α	[73]
CD8 + T cells	Human	AEA	↓ IFN-γ and TNF-α	[73]
	Human	AEA	↓ SDF-1-induced migration	[75]
Pro-inflammatory effect				

B cells	Human	2-AG	↑ Migration	[76]
	Mouse	2-AG	↑ Migration	[77, 78]
Dendritic cells	Human	2-AG	↑ Migration	[79]
Eosinophils	Human	2-AG	↑ Migration	[80]
	Human	2-AG	↑ Migration ↑ LTC_4 and EXC_4 synthesis	[62]
Macrophages	Mouse (peritoneal)	2-AG	↑ Zymosan phagocytosis	[81]
	Human (HL-60)	2-AG	↑ Actin polymerization ↑ Adhesion to fibronectin	[82]
			↑ MCP-1 and IL-8	[83]
Microglia	Mouse (BV-2 cell line)	2-AG	↑ Migration	[84]
Monocytes	Human	2-AG	↑ Adhesion to fibronectin	[85]
			↑ Migration	[82]
NK cells	Human	2-AG	↑ Migration	[86]
T cells	Human (Jurkat)	2-AG	↑ L- and P-selectin ↑ Adhesion and transmigration	[87]

1.6.6 Reproductive system

The presence of the ECS has been demonstrated in numerous cell types that are involved in male and female reproduction. As previously mentioned, endocannabinoids and cannabinoid receptors have shown to be present in the uterus, ovarian, and testicular tissue, as well as ovum and spermatozoa in various species from invertebrates to mammals [88]. Both endogenous and exogenous cannabinoids have also been associated with the regulation of reproductive events. It was furthermore localized in areas of the hypothalamus responsible for the production of gonadotrophic releasing hormone (GnRH) and can thus also exert a role to control number of reproductive events via the hypothalamus-pituitary-gonadal (HPG) axis. It is therefore clear that the ECS is strongly involved in the control of the male and female reproductive system and endocrine function[89] [90]. The ECS has been suggested to mediate chemical communication between the embryo and the uterus as CB1 and CB2 receptors are present in the embryo from the very early stages of development. Also, anandamide synthesis by the uterus has been suggested to direct the timing and the implantation of embryo as a higher level of anandamide synthase has been detected when uterus is least receptive to embryonic implantation. Administration of exogenous cannabinoids has also been shown to affect male and female reproductive hormones and reproductive functions, chronic exposure to cannabinoids in the female has been shown to delay sexual maturation, cause menstrual cycle disruption, depress ovarian follicular maturation, and reduce serum concentrations of LH and sex hormones. In male rodents and humans, chronic exposure to cannabinoids has been shown to result in reduced sperm count, serum testosterone levels, and serum luteinizing hormone (LH) [91, 92].

1.7 Cannabinoids as a Medicine

Few plant species have been the subject of so much scientific, clinical, and social debate as *Cannabis*. Preparations from this plant have been used for many centuries both medicinally and recreationally. Cannabis which is also known as marijuana originated in Central Asia but is grown worldwide today. One of the major resin produced from the *Cannabis* plant is called *cannabinoids* that are 21-carbon terpene phenolic compounds, in addition, other compounds found in cannabis, such as terpenes and flavonoids. The highest concentration of cannabinoids is found in the female flowers of the plant [93].

1.7.1 History

The use of *Cannabis* for medicinal purposes dates back at least 3,000 years [94]. It was first introduced into western medicine by a surgeon, W.B. O'Shaughnessy in 1839. He learned the medicinal properties of cannabis while working in India for the British East India Company. Its use was promoted for reported analgesic, sedative, anti-inflammatory, anticonvulsant and antispasmodic effects. However, the chemical structures of cannabinoids active components were not established until the 1960s, three decades later molecular action of cannabinoids were established, which resulted in an impressive expansion of basic cannabinoid research and began a new era in the study of the therapeutic effects of cannabinoids in various fields, including oncology [95].

1.7.2 Cannabis species

Considering so many chemical and physical characteristics of cannabis, it was initially thought that there are several species of cannabis and certain cannabis plants were called with names as *cannabis sativa*, *cannabis indica* and *cannabis ruderalis,* etc. these names were never consistent and changed with different philosophies and political interest. Today all cannabis are recognized to belong from *Cannabis Sativa* species. However, breeders of cannabis still use the term Cannabis Indica and Sativa. Breeders of cannabis differentiate cannabis indicia and sativa based on THC level and their origin. Cannabis indica plant is defined as cannabis with a moderate level of THC with CBD and THC in 1: 1 ratio and originated in the Middle East, places such as Afghanistan and Northwest Pakistan and India. On the other hand, Cannabis sativa is considered to have a high

THC level and originated from southern Asia and South America [96]. Due to multitude use of cannabis today, they are usually bred in two distinct forms, Hemp and Marijuana. Hemp is a non-intoxicating type of cannabis plant with less than 0.3% THC. It is traditionally bred for food and fiber. In contrast, Marijuana is an intoxicating plant that contains more than 0.3% THC and is mainly used for medicinal or recreational purposes. Although Hemp and Marijuana plants differ in physical and chemical characteristics, there is not enough difference in the two to categorize them as separate species [97].

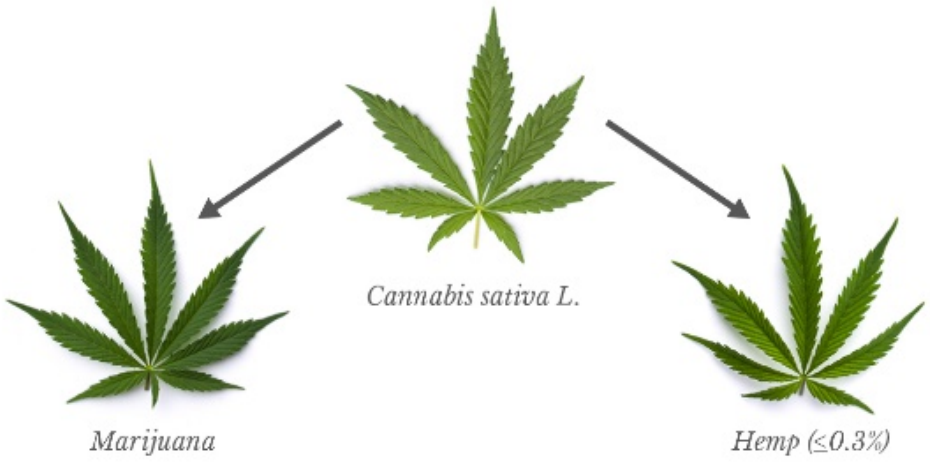

Figure 1-6. Difference between Hemp and Marijuana plant.

From intro-cannabis

1.8 Classification of cannabinoids

Cannabinoids are a diverse group of chemical compounds that interact with cannabinoid receptors in the mammalian body. These compounds can be classified in several ways. On the basis of their interaction with cannabinoid receptors, they can be classified as1. Agonists or cannabimimetics (they exhibit cannabinoid activity). 2. Partial agonists (they exhibit partial cannabinoid activity) and 3. Antagonists (they block the cannabinoid receptors and do not produce cannabinoid effect). Based on their chemical structure cannabinoid are conventionally classified as 1. Classical cannabinoids: dibenzopyran derivatives (THC, its isomers, and structurally related synthetic analogs such as, e.g., HU210). 2. Nonclassical cannabinoids: synthetic cyclohexyl phenol derivatives (3arylcyclohexanols such as, e.g., CP 47497 and CP 55940). 3. Hybrid cannabinoids

combining the structural features of classical and nonclassical cannabinoids. 4. Aminoalkylindoles: a large class of synthetic cannabinoids subdivided, according to the current classification, into naphthoylindoles, phenylacetylindoles, benzo ylindoles, and naphthylmethylindoles. 5. Eicosanoids: endocannabinoids (such as e.g., anandamide) or their synthetic analogs. 6. Others: such compounds as diarylpyrazoles (e.g., SR141716A and SR144528), naphthoylpyrroles, naphthylmethylindenes, and other cannabinoids constituting no classes in their own right [98]. As cannabinoids can be produced from different sources they are also classified as 1. Endocannabinoids, produced by the neurons and immune cells in the form of 2AG and AEA. 2. Phytocannabinoids are extracted from a plant source, e.g. CBD and THC. 3. Synthetic cannabinoids, manufactured and produced as pharmacological targets e.g. WIN 55-212 22 and HU-210.

Cannabinoid Classifications	
Classification based on interaction with cannabinoid receptors	Agonists
	Partial agonists
	Antagonists
	Inverse agonists
Classification based on chemical structure	Classical cannabinoids - dibenzopyran derivatives
	Non-classical cannabinoids - synthetic cyclohexyl phenol derivatives
	Hybrid cannabinoids – combination of classical and non-classical derivatives
	Aminoalkylindoles
	Eicosanoids
	Miscellaneous cannabinoids
Classification based on the source of production	Endocannabinoids
	Phytocannabinoids
	Synthetic cannabinoids

Table 1-2. Classifications of cannabinoids.
Table adapted from [98]

1.9 Therapeutic potential of cannabinoids

Cannabis preparations exert numerous therapeutic effects. They have antispastic, analgesic, antiemetic, neuroprotective, and anti-inflammatory actions, and are also effective against certain psychiatric diseases. Currently, in the United States, 36 states and 4 territories allow for the medical use of cannabis products (NCLS-2021). Of the states that allow for some access to cannabis compounds, cancer, HIV/AIDS, multiple sclerosis, glaucoma, seizures/epilepsy, and pain are among the most recognized qualifying ailments [99, 100] (NCLS-2021). However, only one cannabis extract is approved for use. It contains THC and CBD in a 1:1 ratio and was licensed in 2011 for treatment of moderate to severe refractory spasticity in multiple sclerosis (MS).

Table 1-3. Overview of controlled trials of cannabis medications for established indications. Table adapted from [101]

Indication	Number of randomized controlled trials	Positive studies	Negative studies
Spasticity	n = 12 (dronabinol and cannabis) in multiple sclerosis	n = 9	n = 3
	n = 3 (dronabinol and nabilone in paraplegia)	n = 3	–
Nausea and vomiting due to cytostatics	n = 41 (dronabinol, cannabis cigarettes, cannabis extract, nabilone and levonantradol)	n = 40	n = 1
Loss of appetite/weight loss	n = 7 (dronabinol and cannabis cigarettes) in HIV/Aids	n = 7	–
	n = 4 (dronabinol and cannabis extract) in various tumor diseases	n = 3	n = 1
	n = 1 (dronabinol) in Alzheimer's disease	n = 1	–
Chronic pain	n = 14 (dronabinol, nabilone, cannabis extract, cannabis cigarettes, CT3 (ajulemic acid) in neuropathic pain or pain in MS	n = 12	n = 2
	n = 12 (dronabinol, NIB, benzopyranoperidine, cannabis extract, nabilone, cannabis cigarettes) in chronic pain (cancer, rheumatism, fibromyalgia)	n = 11 (cannabis extract)	n = 2 (dronabinol)

2. Cannabinoids: Potential Anticancer Agents for Canine Cancer

Cannabinoids have been shown to provide relief of pain and nausea in cancer patients. Furthermore, various in vitro and in vivo studies have shown that cannabinoids can also reduce tumor growth and progression. Cancer is a leading cause of canine death and morbidity with an unmet medical need; hence there is a need to study the endocannabinoid system and anti-tumor effects of cannabinoids in canine cancers. The impact of cannabinoids on endocannabinoid system in a body and cancer is highly complex and much is unknown. This review article discusses the endocannabinoid system and reviews the known and potential anti-cancer effects of cannabinoids. Such knowledge will help in understanding the endocannabinoid system and how the anti-cancer effects of cannabinoids can be translated into canine oncology.

2.1 Introduction

Cannabinoids are diverse group of chemicals that act on cannabinoid receptors in the body. These compounds can be classified into three categories based on sources they produce from (their availability in nature) (Fig 7); endocannabinoids, produce in the post synaptic neurons in form of Anandamide and 2Acylglecerol, synthetic cannabinoids, commercially produced in the labs and phytocannabinoids, compounds derived from the Cannabis Sativa (Marijuana) and other species of the cannabis plant. The extract of cannabis plant has been used as a medicinal drug for centuries. The era of cannabinoid research began after the chemical structure and the molecular action of cannabinoids have discovered in 1960. In the last two decades, cannabinoids have been studied extensively for their potential use in various fields of medicine including oncology. Today some of the cannabinoids are approved for the treatment of chemotherapy-induced side effects however, studies are showing their effect against tumor growth and progression as well [102, 103]. The purpose of this review is to summarize these observations and evaluate the potential use of cannabinoids as anticancer drugs in canine cancer treatment.

2.2 Endocannabinoid System

The endocannabinoid system is composed of cannabinoid receptors and intrinsic ligands. These cannabinoid receptors include CB1, CB2, TRPV1, and some of the orphan G protein coupled receptors[3]. The intrinsic ligands of the receptors include N-arachidonoylethanolamide (anandamide AEA) and the 2-arachidonoylglycerol (2-AG). Furthermore, biosynthetic and degradative enzymes are involved (e.g. Fatty acid amide hydrolase FAAH, Monoacylglycerol lipase MAGL). The Endocannabinoid system aside from its pivotal neuromodulatory activity[104], also exerts other regulatory functions in the body, such as the control of cardiovascular tone, energy metabolism, immunity, and reproduction[31, 105]. This miscellaneous activity makes the pharmacological manipulation of the endocannabinoid system a promising strategy for the management of many different diseases. The Endocannabinoid system in small animals has not been explored as such yet. However, the limited data currently available regarding the expression of cannabinoid receptors shows positive expression and similar distribution of cannabinoid receptors in canine as compare to rodents and humans. (Table 4)

2.3 Endocannabinoid System in Cancer

The role of the endocannabinoid system in cancer physiology is currently a matter of active debate however generally endocannabinoid system is upregulated in cancer tissues compared with non-tumor tissue which suggests the pro-tumorigenic nature of the system. In this context poor prognosis of cancer patients has been associated with high expression of CB1 receptors in malignant tissue, such as pancreatic cancer [106]. Glioblastoma studies have also demonstrated, CB1 receptors to be highly expressed in high-grade gliomas compared to low-grade tumors and healthy brain samples [107]. The upregulation of CB2 receptors is also observed in a variety of cancer types [108]. With respect to endogenous ligands of cannabinoid receptors, the level of 2AG found to be upregulated in meningiomas, gliomas, and prostate cancer [109, 110]. In human meningiomas, AEA, but not 2-AG, was up-regulated [111], whereas a converse regulation with decreased AEA and increased 2-AG was observed in blood analyses of circulating endocannabinoids from patients suffering from different kinds of cancers [112] including glioma tissues [107]. Increased endocannabinoid level in some tumors suggests inhibition of enzymes

responsible for these ligands degradation (e.g. FAAH, MAGL). In glioma, the expression and the activity of degrading enzymes were reduced, compared with normal brain tissue [107]. However, in some studies, in line with evidence that the pharmacological activation of cannabinoid receptors reduces tumor growth [102, 103], the upregulation of endocannabinoid-degrading enzymes has been observed. For example, in breast ductal carcinomas, MAGL expression was increased [113], and in prostate tumors, high levels of AEA and its major degradative enzyme were detected compared to normal prostate tissue [114]. Similarly, FAAH overexpression has been associated with cancer invasion and disease outcomes [115], indicating that endocannabinoid signaling can also have a tumor-suppressive role. In support of this, the deletion of CB1 receptors was found to accelerate intestinal tumor growth in a genetic mouse model of colon cancer [116]; increased endocannabinoid levels diminished azoxymethane-induced precancerous lesions in the mouse colon, and a reduction in the expression of the endocannabinoid-degrading enzyme monoacylglycerol lipase reduced tumor growth in xenografted mice[117]. Therefore, further studies will be required to understand the precise signaling mechanisms that regulate cannabinoid-induced cell death or cell proliferation. Such information is needed to determine when this system acts as a guardian, or, as an inducer of tumorigenesis or tumor progression.

2.4 Cannabinoids and Antitumor activity

In the last two decades, extensive research has been done to demonstrate the antitumor effects of cannabinoids in various experimental models of cancer from cancer cell lines to mice models of human cancers. Studies have used different types of cannabinoids (endocannabinoid, phytocannabinoids, and synthetic cannabinoids) and found in most cases antitumorigenic effects however some of the compounds show higher affinity to receptors compared to the others. Nonetheless, some reports have proposed tumor-promoting effects of cannabinoids at lower concentrations.

Cannabinoids exhibit antitumor activity at different levels of tumor growth and progression. One of the major antitumor activity that cannabinoid demonstrate is inhibition of cancer cell proliferation and induction of apoptosis (Fig.8). In addition, studies have also demonstrated that cannabinoids impair tumor angiogenesis and inhibit cancer cell invasion and metastasis (Table 5).

2.5 Mechanisms of Antitumor effects

2.5.1 Anti-proliferative and apoptotic effects

The antitumor effects of cannabinoids were first investigated in the 1970s by Munson et al, who demonstrated tumor growth inhibition of Lewis lung adenocarcinoma by the oral administration of Δ9-tetrahydrocannabinol (Δ9-THC), Δ8-tetrahydrocannabinol (Δ8-THC), and cannabinol (CBN) in an animal model [118]. Since then extensive additional research has been conducted investigating the anti-tumor effects of endocannabinoids, phytocannabinoids, and synthetic cannabinoids. There are mixed findings regarding the action of cannabinoids through cannabinoid receptors and the molecular pathway involved in the antiproliferative and apoptotic effect (Fig 9). In the late 1990s, De Petrocelliset al. addressed the role of cannabinoid receptors in the growth inhibitory action of several cannabinoid compounds and reported endocannabinoid AEA and synthetic cannabinoid HU-210 reduce the proliferation of human breast cancer cell lines via CB1 receptor-dependent mechanism [119]. Extensive research on human glioma also found receptor-mediated anti-tumor effects of cannabinoids. Carracedo et al. demonstrated glioma cell death in response to THC treatment. Furthermore, the proposed mechanism responsible was CB2 receptor-mediated up-regulation of the stress-associated transcriptional co-activator p8, a pro-apoptotic transcriptional factor that results in activating transcription factor (ATF)-4 and the pseudokinase tribbles homolog(TRB)3 [120]. A contribution of p8 induction to cannabinoid-induced apoptosis was later substantiated for THC and HU-210 in rhabdomyosarcoma cells by Oeshch et al. in 2009. In that study, cannabinoid-induced apoptosis was associated with inhibition of Akt signaling and, as shown for HU-210, restored by a CB1 receptor antagonist [121]. Another study conducted by Caffarel et al. reported THC and the selective CB2 receptor agonist, JWH-133, to inhibit the growth of highly aggressive ErbB2-positive breast cancers, associated with inhibition of the pro-tumorigenic Akt pathway [122]. The direct involvement of p8 mediated pathway in the anti-tumor action of cannabinoids in glioma as well as in breast, pancreatic, hepatic, and rhabdomyosarcoma cancer cells suggests that ER stress-related AKT inhibition pathway is a general molecular mechanism by which activation of cannabinoid receptors results in cancer cell death.

A group of studies on the other hand also reported impaired cancer cell viability with cannabinoids without cannabinoid receptor involvement. For example, CP55940, JW015, and the FAAH inhibitor, N-arachidonoyl serotonin(AA-5HT), inhibited proliferation of rat glioma cells

independently of both CB receptors and TRPV1 channel activation [123]. In the same study, however, AEA and 2-AG exerted antiproliferative receptor-dependent and TRPV1-dependent oxidative stress and calpain activation. Furthermore, R (+)-methanandamide induced a cannabinoid receptor and TRPV1-independent apoptosis in human neuroglioma cells by de novo synthesis of ceramide demonstrated by Hinz et al.[124]. In the latter type of cells, the proapoptotic mechanism of R (+)-methanandamide was based on a ceramide-dependent up-regulation of COX-2 expression [125] and increased synthesis of proapoptotic PGE2 [124]. The mechanism by which cannabinoids apoptotic death of cancer cells independent of cannabinoid receptor is not clearly known however to some extent it seems to rely on its ability to enhance reactive oxygen species in cancer cells.

2.5.2 Inhibition of angiogenesis

Various in vitro and animal studies have found that cannabinoids block the activation of vascular endothelial growth factor (VEGF) pathways that play a key role in angiogenesis[126]. Cosanova et al. showed in an animal study that cannabinoids inhibit tumor vascularization by downregulating proangiogenic factors like VEGF, angiotensin-2 and placental growth factor [127]. Other studies of cannabinoid treatment on skin carcinoma, thyroid carcinoma and glioma have also reported downregulation of active forms of vascular endothelial growth factors (VEGF1 and VEGF2). More recently Picardi et al. found AEA inhibited endothelial cell proliferation in breast cancer cells by inhibiting angiogenesis-related factors: VEGF, leptin, interferon-γ, and thrombopoietin [128]. However, some in vitro studies have found no such effects or opposite effects on endothelial cells with low concentrations of CDB and THC. Kogan et al found no antiangiogenic effect of endocannabinoids and even proangiogenic effects on using low concentrations of THC and CBD [129]. These findings were further confirmed by Ramer et al. hence some cannabinoids at lower concentrations may induce rather than inhibit angiogenesis [130].

2.5.3 Inhibition of tumor invasion and metastasis

The first finding of the cannabinoid anti-invasive effect was found by Nithipatikon et al. in a prostate cancer cell study [109]. Later, several other studies observed cannabinoids to inhibit adhesion, migration, and invasion of glioma, breast, lung, and cervical cancer cell cultures. The shared observation from these studies was downregulation of extracellular proteases (such as

matrix metalloproteinases2 MMP2). Studies have also found down-regulation of Id-1(inhibitor of DNA binding 1 protein) to be involved in inhibiting cancer cell invasion in CBD treated breast cancer cells [131, 132]. Another investigation on glioblastoma cells also found that Id-1 plays a critical role in modulating the invasiveness of glioblastoma cell lines and primary glioblastoma cells. They also demonstrated that cannabidiol significantly downregulates Id-1 gene expression and associated glioma cell invasiveness and self-renewal. In addition, cannabidiol significantly inhibits the invasion of glioblastoma cells through an organotypic brain slice and glioma progression in vivo[133]. Studies have also found inhibition of ceramide biosynthesis and knocking down p8 expression results in inhibition of anti-tumor, antiangiogenic and anti-invasive effects of cannabinoids [134], indicating the p8-regulated pathway plays a general role in inti-tumor actions exhibited by cannabinoids.

2.5.4 Selectivity of cannabinoids for cancer cells

For antitumor compounds to be effective it should be selective as well. Cannabinoids have been found to selectively kill or suppress the growth of cancer cells but do not affect their healthy counterparts. The molecular mechanism of this differential effect of cannabinoids is not known but studies hypothesize the selectivity to be due to differences in ceramide synthesis of the tumor and non-tumor cells following cannabinoid receptor activation [135]. The glioma cells are exemplary for this phenomenon. In glioma cells, cannabinoids trigger ceramide synthesis followed by apoptosis via inhibition of the AKT-mTORC1 pathway, whereas in normal astrocytes cannabinoids activate AKT and prevent ceramide-induced AKT inhibition [136]. A similar effect was seen in other studies where effects of cannabinoids were compared between non-transformed and transformed cells such as in primary embryonic fibroblasts. Thyroid epithelioma [137] and skin cancer [127] are other reported examples of cannabinoid selective anti-tumor effects. By contrast, cells with high proliferative tendency, such as immune cells and endothelial vascular cells, were found to undergo apoptosis in response to cannabinoids [138]. However, at lower concentrations cannabinoids enhances lymphocytes and myeloid cell growth [139]. Thus, for cannabinoids to be established as an anticancer drug it is crucial to monitor its immunosuppressive effects.

2.6 Conclusion and Future directions

A huge amount of literature is available on cannabinoid therapeutic effects in general and anti-tumor effects of cannabinoids in particular. In this review, we have attempted to summarize the research done on the endocannabinoid system and focused on the anti-tumor effects of cannabinoids and their mechanism of anti-tumor effects. Based on the limited data available for the canine endocannabinoid system, there appears to be similar expression and distribution of cannabinoid receptors and ligands compared to humans. Based on current research, cannabinoids appear to be capable of modulating tumor growth, angiogenesis and invasion in various in vitro and in vivo cancer models. Importantly, these anti-cancer effects appear to depend on the cancer type, drug dose, and the cannabinoid.

Cancer is one of the leading causes of canine mortality and there is no data available on the anti-cancer effects of cannabinoids in companion animals. This review will help veterinary scientists in understanding the anti-cancer effects of cannabinoids and implying that knowledge to veterinary oncology that is urgently required. It not only provides potential better cancer treatment to the animal but it will also help scientists to gather facts concerning the risks and benefits of cannabinoids involved in cancer treatment otherwise non-scientific media will create its own facts. Since the therapeutic effects of cannabinoids are subject to discussion in numerous internet forums and such debate could result in treatment attempts using cannabinoids without taking necessary safety precautions.

Table 2-1. CB1 and CB2 receptors distribution in canine tissues.

Tissue	CB1 Receptor	CB2 Receptor	References
Olfactory bulb	✓		[140] [141]
Cerebrum	✓		[140]
Cerebellum	✓		[140]
Hippocampus	✓		[140] [141]
Basal ganglia	✓		[140]
Midbrain	✓		[140] [141]
Medulla oblongata	✓		[140] [141]
Pons	✓		[140] [141]
3^{rd} and 4^{th} ventricle	✓		[140]
Spinal cord	✓		[140] [141]
Inner ear	✓		[141]
Primordium of eye	✓		[141]
Primordia of nasal cavities	✓		[141]
Thyroid	✓		[141]
Small intestine	✓	✓	[12]
Large intestine	✓	✓	[12]
Immune cells	✓	✓	[12] [13]
Lymph nodes	✓	✓	[12] [13]
Blood vessels	✓	✓	[12] [13]
Smooth muscles		✓	[12] [13]
Skin epidermis	✓		[13]
Hair follicle	✓	✓	[13]
Sebaceous glands	✓		[13]
Sweat glands	✓	✓	[13]

Table 2-2. Effect of cannabinoids in different types of cancer.

Cancer	Receptor involved	Cannabinoid used	Effect	Reference
Glioma	CB1 CB2	CBD	↓Tumor growth; apoptosis; ↓tumor invasion; ↓tumor angiogenesis	[131] [43]
Breast cancer	CB2 TRPV1	CBD AEA	Cell cycle arrest; apoptosis; ↓ tumor invasion; ↓angiogenesis	32 [137, 142]
Pancreatic cancer	CB2	THC WIN55,212-2	↓cell viability; apoptosis	[143, 144]
Hepatocellular carcinoma	CB2	THC JWH-051	↓Tumor growth; apoptosis	[145]
Lung cancer	CB1 CB2 TRPV1	THC	↓Tumor growth; ↓Tumor invasion; apoptosis	[146]
Colon cancer	CB1 TRPV1	CBD	↓Proliferation; ↓tumor growth	[130]
Thyroid carcinoma	CB1	AEA	↓Tumor growth; apoptosis; cell cycle arrest	[147, 148]
Leukemia	CB2	THC HU210 AEA JWH-051	Apoptosis	[149, 150]
Mental cell lymphoma	CB1 CB2	AEA WIN55,212-2	↓Tumor growth; apoptosis	[151]
Non- Hodgkin lymphoma	CB1 CB2	AEA	↓Tumor growth; ↓Tumor invasion; apoptosis	[152]
Melanoma	CB2	JWH-133 THC WIN55,212-2	Apoptosis	[153]
Rhabdomyosarcoma	CB1	HU210 THC	Apoptosis	[121]
Canine osteosarcoma	N.D	WIN55,212-2	↓Tumor angiogenesis	[154]
Skin carcinoma	CB1 CB2	WIN55,212-2 JWH-133	↓Tumor growth; apoptosis; ↓Tumor invasion; ↓angiogenesis	[127]
Uterus carcinoma	N.D	THC	↓Cell growth	[155, 156]
Prostate carcinoma	CB2	AEA JWH-051	Apoptosis;	[157]

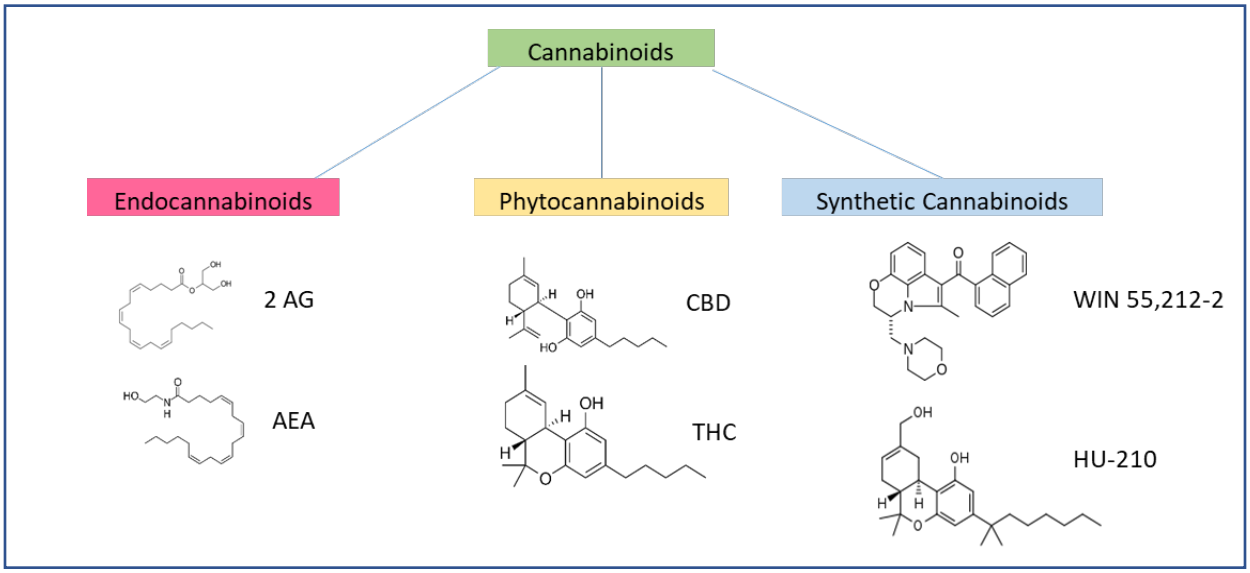

Figure 2-1. Three major types of cannabinoids.

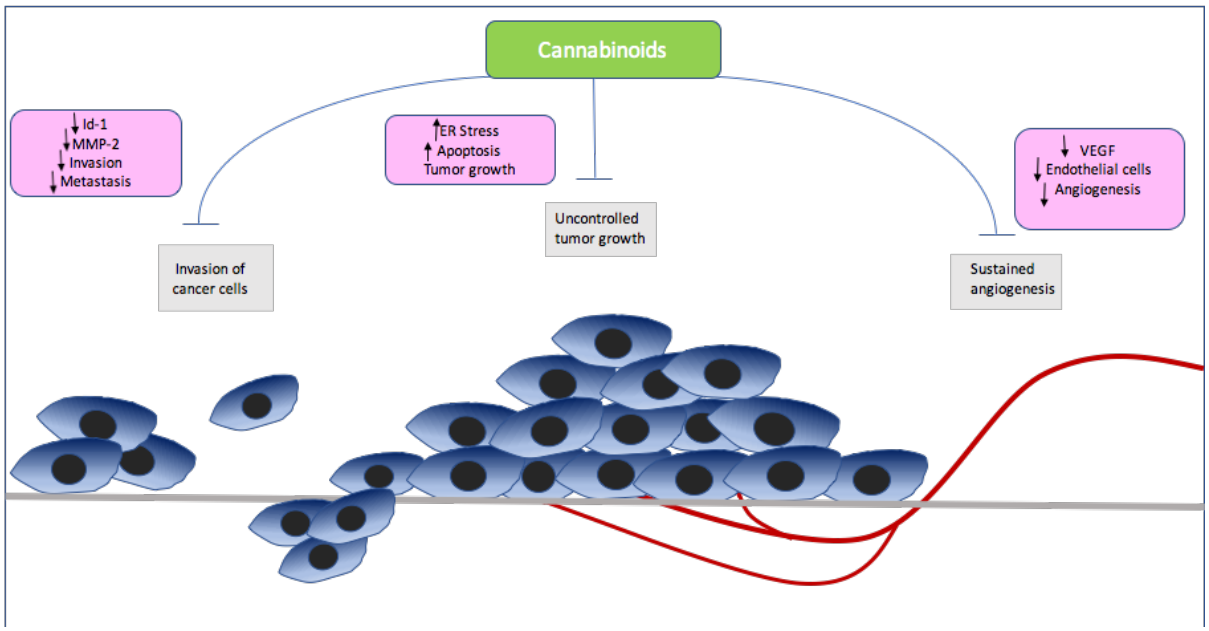

Figure 2-2. Mechanisms of anti-cancer effects of cannabinoids.

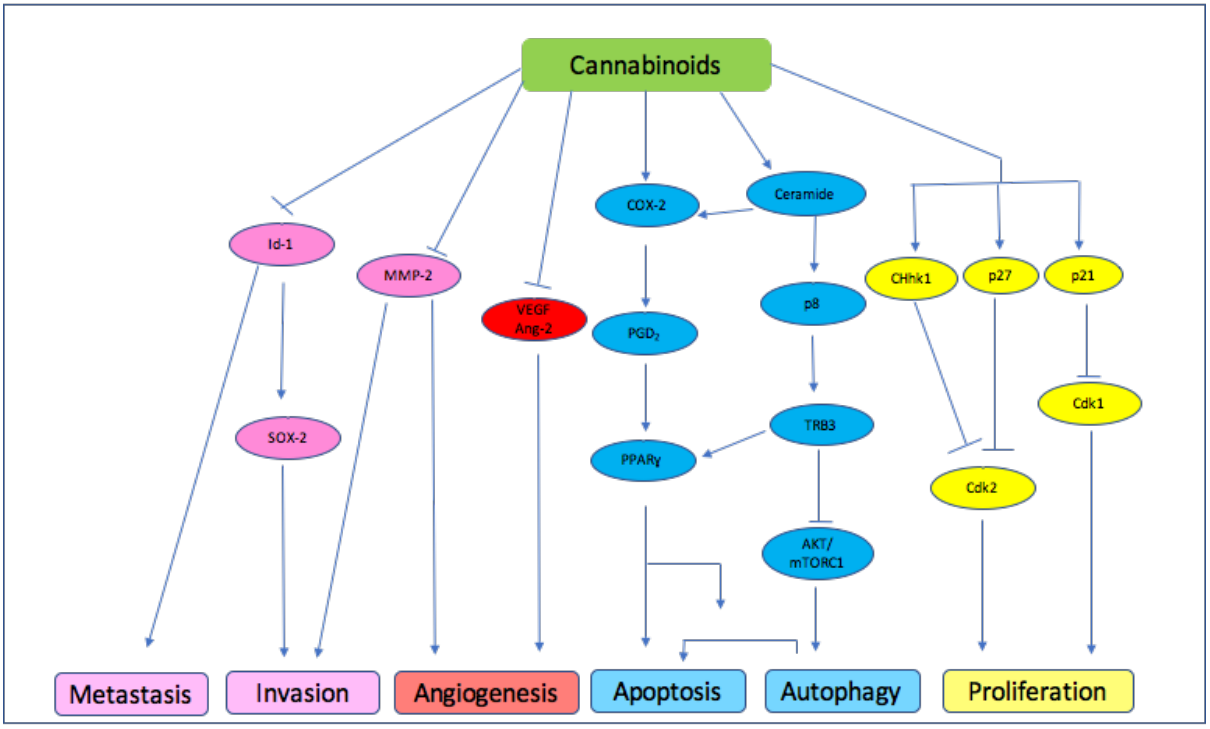

Figure 2-3. Molecular pathways involved in anti-cancer effects of cannabinoids.

3. Expression of Cannabinoid Receptors and Effect of Endocannabinoids on Non-Hodgkin Lymphoma cell lines

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3.1 Abstract

The role of the endocannabinoid system in cancer physiology is currently a matter of active debate however, generally, the endocannabinoid system is upregulated in cancer tissues compared with non-tumor tissues which suggests the pro-tumorigenic nature of the system. In this context poor prognosis of cancer patients has been associated with high expression of cannabinoid receptors in the malignant cells, increased level of endocannabinoids, and deficiency of endocannabinoid degrading enzymes in cancer patients. On the other hand, in the last two decades, many in vitro and in vivo studies have been shown to induce cancer cell death on activation of cannabinoid receptors with endogenous and exogenous cannabinoids. These contrasting observations indicate further need for investigations to understand the exact role of the endocannabinoid system in cancer if it is one of the antitumor effects, the precise mechanisms by which cannabinoids accomplish this. The purpose of this research was to explore, for the first time, the effects of cannabinoids on canine non-Hodgkin lymphoma (NHL) cell lines and activated PBMCs in addition to the human NHL cell line. Specifically, the goal was to study in detail the effects of endocannabinoids, AEA, and 2AG, on canine and human NHL cell viability. Non-Hodgkin lymphoma is one of the leading causes of cancer-associated mortality in humans and canines. Canine and human NHL share similar biology, histology, and genetic expression. As such, the commonly encountered canine cancer should be suitable for exploring the effects of cannabinoids in both species. In this study, we have used canine B cell (1771 and CLBL1) and T cell type (CL1) NHL cell lines, canine PMBCs and, human B cell type NHL cell line (Ramos). Cells were cultured in RPMI, and the expression of endocannabinoid receptors (CB1 and CB2) was studied using real-time PCR. To study the effects of AEA and 2-AG, cells were treated at concentrations from 0.1µM to 50µM for 24 and 48 hours. The vehicle was used as a control. Cell viability was assessed using colorimetric MTT assay (3-(4,5-Dimethylthiazol-2-yl)-2,5 Diphenyltetrazolium Bromide) and markers of oxidative stress, inflammation, mitochondrial function, and apoptotic cell death were assessed using spectrophotometric and fluorometric analysis. Data were analyzed using one-way ANOVA, student t-test, and regression analysis with Prism® and SAS® statistical software. Our results demonstrate positive expression of cannabinoid receptor CB1 and/or CB2 in both canine and human lymphoma cell lines, with a significantly higher expression of CB1 and CB2 receptors

in canine and human B cell lymphoma cell lines, compare to activated PBMCs and canine CL-1 lymphoma cell line. For activated canine PBMCs our results show negative expression of the CB1 receptor gene but significantly higher expression of CB2 receptor gene compared to the canine T cell lymphoma cell line. Results of the cell viability assay demonstrate a dose-dependent decrease in cancer cell viability with AEA in 1771, CLBL-1, Ramos, and CL1. 2-AG appears to decrease cell viability in cell lines with higher CB2 receptor expression (Ramos and CLBL-1) and does not affect the cell viability significantly in cell lines with lower CB2 expression (1771 and CL-1). Results of the biochemical analysis revealed a significant increase in markers of oxidative stress, inflammation, and apoptosis and a decrease in markers of mitochondrial function in treated 1771 B cell lymphoma cells as compared to control untreated cells. Our results suggest that canine B cell lymphoma shows higher expression of cannabinoid receptors compare to canine T cell lymphoma and healthy canine PBMCs and endocannabinoids have an anti-proliferative and pro-apoptotic effect on canine and human NHL cells. These results support the need for further studies providing evidence of cannabinoid receptor expression and efficacy of endocannabinoids against both human and canine lymphomas.

3.2 Introduction

The endocannabinoid system primarily comprised of the two ″classical″ endocannabinoids, N arachidonoylethanolamine (anandamide, AEA) and 2-arachidonoylglycerol (2-AG), and the endocannabinoid receptors CB1 and CB2. CB1 is the most common GPCR in the brain where it regulates neurotransmission and neural networks [8]. CB2 is the predominant cannabinoid receptor in the immune system [158] studies have found, mice lacking CB2 have reduced numbers of marginal zone B cells, CD4+ T cells, and NK cells [159]. In recent years, some new endocannabinoid ligands, receptors, and enzymes have been identified that have expanded the original definition of the endocannabinoid system. These newly discovered endogenous cannabinoid receptor ligands are, 2-arachidonoyl glyceryl ether (noladin ether, 2-AGE), O-arachidonoylethanolamine (virodhamine), N-arachidonoyldopamine (NADA), oleic acid amide (oleamide, OA) new receptor targets identified are: G protein-coupled receptor (GPR) 55 and peroxisome proliferatoractivated receptors (PPARs) [160](Iannotti et al., 2016). Moreover, AEA

has been found to activate the cation channel transient receptor potential vanilloid 1 (TRPV1) in addition to CB1 and CB2 receptors. [161, 162]

Studies have shown important functions of the cannabinoid receptors and endocannabinoid system overall in several types of cancer (glioma, astrocytoma, breast-, prostate-, colon-, pancreatic- and hepatocellular- cancer and also mantle and non-Hodgkin lymphomas) [95, 163]. Therefore gene expression profiling of cannabinoid receptors can be a valuable tool to investigate similarities and differences between lymphoma and non-malignant lymphoid tissue. In general, malignant tissues express higher levels of cannabinoid receptors than the non-malignant counterparts and the endocannabinoid system is therefore considered as a potential novel therapeutic target in cancer therapy. [95, 164, 165]

Like several other cancers research conducted on mantle cell lymphoma (MCL) found higher expression cannabinoid receptors in MCL than reactive lymphoid tissue or purified B cell subsets. In the light of this observation, it was initially hypothesized that cannabinoid receptor could have a pro-survival function. However, further studies on MCL did not give evidence of the pro-survival function of cannabinoid receptors, rather studies have shown an anti-proliferative and apoptotic effect of cannabinoids in lymphoma cells. However, the pro-survival action of cannabinoid receptors can yet not be excluded.[165, 166]

In the present study, we analyzed CB1 and CB2 expression by both conventional and quantitative real time PCR in a panel of non-Hodgkin lymphomas of B and T cell type, including canine malignant B-cell lymphoma cell lines (1771 and CLBL-1), human malignant Burkett's B-cell lymphoma (Ramos), canine T-cell lymphoblastoid malignant lymphoma cell line (CL-1) and canine activated normal lymphocytes (PBMCs). Our results demonstrate that both malignant B and T cell lymphomas and normal canine PBMCs express CB1 and/or CB2 but at a highly variable level. Treatment with the anandamide induced apoptosis in both B and T malignant lymphoma cell lines(only at the highest concentration used), the effect of anandamide was most prominent in canine and human B cell lymphoma cell lines, expressing higher levels of CB1 and CB2 receptors than canine T cell lymphoma cell line. 2AG induced no significant effect on canine B cell lymphoma cell line 1771 and T cell lymphoma cell line CL-1. However, 2AG reduced the cell viability in canine and human B cell lymphoma CLBL-1 and Ramos respectively, both with much higher expression of CB2 receptor compared to other cell lines. Our results were further confirmed with the biochemical analysis of cytotoxic and apoptotic markers in 1771 B cell lymphoma cell

line treated with AEA and 2AG compared with control untreated 1771 cells. The anti-proliferative and pro-apoptotic effects of cannabinoids make the endocannabinoid system a potential new therapeutic target for individualized therapy in lymphomas that overexpress cannabinoid receptors.

3.3 Materials and methods

3.3.1 Chemicals

Endocannabinoids Anandamide and 2-Acylglycerol were purchased from Sigma (St. Louis, MO).

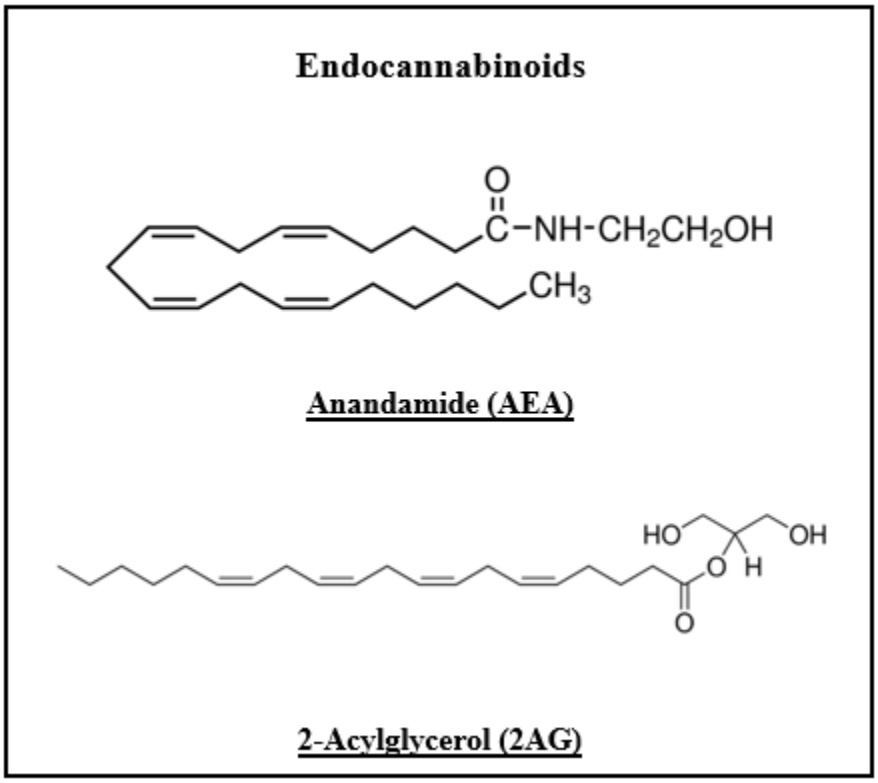


Figure 3-1 Chemical structures of Endocannabinoids: Anandamide and 2-Acylglycerol.

3.3.2 Reagents

Thiazolyl Blue Tetrazolium Bromide (MTT) was purchased from American Type Culture Collection. Penicillin-Streptomycin solution was purchased from Thermofisher Scientific (Waltham, MA). RPMI 1640 Medium, ES Cell Qualified Fetal Bovine Serum (FBS) and L-Glutamine Solution were purchased from EMD Millipore (Burlington, MA). Phosphate buffer saline (PBS), dimethylsulfoxide (DMSO), nicotinamide adenine dinucleotide (NADH), 2′, 7-dichlorofluorescindiacetate (DCF-DA), pyrogallol, hydrogen peroxide (H202), phosphoric acid, 1-methyl-4-phenylpyridinium (MPP+), O-phthalaldehyde (OPT), L-glutathione reduced, trichloroacetic acid, thiobarbituric acid and [henylmethanesulfonyl fluoride (PMSF) were purchased from Sigma Aldrich (St. Louis, MO). Glutamate was purchased from Alfa Aesar (Haverhill, MA). Thermo Scientific Pierce 660 nm Protein Assay reagent kit was purchased (Pierce, Rockford, IL) for protein quantification. The caspase substrates, AC-YVAD-AMC (Caspase-1/ICE-1 substrate), AC-DEVD-AMC (Caspase-3 substrate), Ac-VETD-AMC (Caspase-8 substrate) and Ac-LEHD-pNa (Caspase-9 substrate) were purchased from Sigma Aldrich (St. Louis, MO).

3.3.3 Cell Lines

Canine B-cell lymphoma cell lines 1771 and CLBL-1 and T-cell lymphoma cell line CL-1 were a kind gift from Dr. Steven Suter's Lab (North Carolina State University). Human B-cell lymphoma cell line RAMOS and activated canine PBMCs were generously shared by Dr. Bruce Smith's Lab (Auburn University). All cells were cultured in RPMI 1640 Medium, supplemented with fetal bovine serum (10%), penicillin-streptomycin (1%), and L-Glutamine (1%). For the cell viability assay, all canine and human lymphoma suspension cells were grown and harvested via centrifuge. Cells were then seeded into 96 well plates at a density of 1×10^4 cells/well. Lymphoma cells were incubated under standard conditions at 37 °C and supplemented with 5% CO_2.

3.3.4 RT-qPCR

Total RNA was isolated from PBMCs and lymphoma cells using the RNeasy kit according to the vendor's protocol. The ratio of optical densities of RNA samples at 260 and 280 nm was consistently >1.8. cDNA was synthesized by reverse transcription of 1 µg of extracted RNA with 200 units of Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) in the

presence of oligo dT and deoxynucleotide triphosphate (Promega). The reaction mixture was kept frozen at −80°C until enzymatic amplification. Quantitative real-time PCR assays were performed using TaqMan Gene Expression Assays (Applied Biosystems) to quantify mRNA levels for CB1 receptor (Cf02722816_u1) and CB2 receptor (Cf02696139_s1). In all cases, we used GAPDH expression (Cf04419463_gH) as an endogenous control gene for normalization. The PCR assay was performed using the 7300 Fast Real-Time PCR System (Applied Biosystems) and the threshold cycle (Ct) was calculated by the instrument's software (7300 Fast System, Applied Biosystems). Values were normalized as percentages over the control group. CB1 and CB2 receptors were amplified using a Quanstudio real-time PCR system obtained from Thermos Fisher Scientific. The cycler was programmed with the following conditions (a) initial denaturation at 94ºC for 2 minutes, followed by 35 cycles of (b) 94ºC for 40 seconds, (c) annealing of the primer-template at 58ºC for 40 seconds, and (d) extension at 72ºC for 40 seconds.

Table 3-1 CB1 and CB2 primers

Gene	Primer	Source
CBR1	Cf02722816_u1	TaqMan Gene Expression Assays (Applied Biosystems)
CBR2	Cf02696139_s1	TaqMan Gene Expression Assays (Applied Biosystems)

3.3.5 Treatment design for evaluating the lymphoma cells viability and mechanisms of endocannabinoid induced cytotoxicity

AEA and 2AG dissolved in ethanol and acetonitrile respectively were used to treat cells. To evaluate the cytotoxicity, different concentrations of AEA and 2AG were obtained by diluting in a serum-enriched, fresh culture medium. With regard to the control, lymphoma cells were treated with a vehicle for respective drugs. Cells were exposed to 0.1-50µM concentrations of AEA and 2AG for 24 and 48 h. However, to establish the cytotoxicity of AEA and 2AG on, oxidative stress and apoptosis, the canine 1771 lymphoma cells were exposed to 1µM and 50µM concentrations for 24 h. Lower (1 µM) and higher dose (50µM) were used to analyze the change in biochemical marker's expression with increasing dose and compared to the control untreated group.

3.3.6 Cell Viability

The effect of AEA and 2AG on the viability of cells was determined by (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) (MTT) assay. The cells were plated at 1 10^4 cells per well in 100µl of complete culture medium containing 0.1- 50.0 µM concentrations of AEA and 2AG for 24 and 48 hours at 37ºC in a humidified chamber. After incubation for specified times at 37ºC in a humidified incubator, MTT reagent (10µl) was added to each well and incubated for 4 hours followed by adding (100µl) solubilization solution in each well to dissolve formazan crystals. Absorbance was recorded on a microplate reader at 570 nm wavelength. The effect of AEA and 2AG on cell viability was assessed as the percentage of inhibition in cell viability where vehicle-treated cells were taken as 100% viable.

3.3.7 Protein quantification

Protein was quantified using a Protein Assay reagent kit (Thermo Scientific Pierce 660 nm, Pierce, Rockford, IL). Bovine serum albumin (BSA) was used as a standard for protein measurement.

3.3.8 Biochemical analysis

Hydrogen peroxide content

The content of hydrogen peroxide in control/ AEA and 2 AG treated 1771 canine B-cell lymphoma cells was measured using the Abcam Hydrogen Peroxide Assay procedure. A standard curve for hydrogen peroxide was obtained using fluorimetric plate reader (BioTek Synergy HT plate reader, BioTek, VT, USA) at 335nm (excitation wavelength) and 390nm (emission wavelength). Hydrogen peroxide content in control and drugs treated supernatant was calculated from slope obtained from standard curve. Results were expressed as hydrogen peroxide uM/mg protein [167].

Reactive oxygen species generation

The generation of reactive oxygen species in the control/AEA and 2AG treated 1771 canine B-cell lymphoma cells was estimated via spectrofluorometry by measuring the conversion of non-fluorescent chloromethyl-DCF-DA (2′, 7- dichlorofluorescindiacetate, DCF-DA) to fluorescent DCF using an excitation wavelength of 492 nm and an emission wavelength of 527 nm. [168-170]

Nitrite content

The final products of nitric oxide oxidation pathways are nitrite and nitrate, which are used as an expression of nitric oxide production. Nitrite content in the control/ AEA/ 2AG treated 1771 canine B-cell lymphoma cells was measured using Griess reagent. NO2 reacts with sulfanilamide under acidic conditions leading to the production of diazonium ion. This diazonium ion association with N-(1-naphthyl) ethylenediamine to form 36 chromophoric azo product which can be measured spectrophotometrically at 545 nm.[168, 171]

Glutathione content

GSH content in the control/ AEA and 2AG treated 1771 canine B-cell lymphoma cells was measured via spectrofluorometry (327 nm excitation and 423 nm emission) using O-phthalaldehyde (OPT). GSH measured was normalized to total protein content and reported as relative GSH content (μM)/mg protein. [168, 172, 173]

NADH content

A standard curve for NADH was obtained using a spectrophotometric plate reader (BioTek Synergy HT plate reader, BioTek, VT, USA) and was measured at 340nm. NADH content was calculated from the slope obtained from the standard curve. The results were expressed as NADH uM/mg protein [174]

Mitochondrial complex-I activity

NADH oxidation to NAD+ is catalyzed by mitochondrial Complex-I (NADH dehydrogenase). Cell homogenate obtained from control/AEA/2AG treated 1771 canine B-cell lymphoma cells were added to PBS and conversion of NADH to NAD+ was measured spectrophotometrically at 340 nm. [168, 173, 175]

Interleukin converting enzyme-I activity

Spectrofluorimetric method using the plate reader (BioTek Synergy HT plate reader, BioTek, VT, USA) was to measure the ICE-1 activity. AC-YVAD-AMC (10uM) was used a substrate and the product was measured at 360nm/460nm. The ICE-1 activity in the control and drugs treated supernatant were expressed as relative fluorescence intensity (RFU)/mg protein [176].

Cyclooxygenase activity

Cyclooxygenase activity was quantified spectrophotometrically (calorimetrically). TMPD substrate was used as the substrate for measuring the COX activity in the control and drugs treated supernatant. The product formed from cleavage of TMPD by cyclooxygenase was measured at 600nm. COX activity in the control and drugs treated supernatant were expressed as formation of product/mg protein. [177]

Caspases (3, 8, and 9) activity

Spectrofluorimetric method using the plate reader (BioTek Synergy HT plate reader, BioTek, VT, USA) was used to measure the caspase activity. Appropriate DEVD-AMC (10uM) was used as a substrate for measuring caspases activities and the product formed was measured at 360nm/460nm. The ICE-1 activity in the control and drugs treated supernatant were expressed as relative fluorescence intensity (RFU)/mg protein [168, 178] .

3.4 Statistics

For the statistical analysis of data, the range of the measured variable, means, standard deviations (SD) and standard error (SE) were calculated, using statistical software (SAS® and Prism-V La Jolla, CA, USA). The data are presented as median and range or mean ± SD values. The differences between sample populations were evaluated with Student's t-test and non-parametric ANOVA followed by Dunnet's multiple comparisons test; P values less than 0.05 were considered statistically significant.

3.5 Results

3.5.1 Expression and quantification of CB1 and CB2 mRNA in non-Hodgkin lymphomas of B and T cell type

In the present study, we have analyzed CB1 and CB2 expression in non-Hodgkin lymphomas of the B and T-cell types. Using RT-qPCR, we found that both canine B and T cell lymphoma and human Ramos B cell lymphoma cell lines express CB1 and CB2 receptors. In contrast, only CB2 receptors were expressed in canine activated PBMCs. In addition, results of RT-qPCR showed

higher expression levels of CB1 receptor gene in canine B cell lymphoma cell lines compared to T cell lymphoma cell line. The order of expression of CB1 receptor was 1771 > CLBL-1 and CL-1. The relative order of expression of CB2 receptors was CLBL-1 > activated PBMCs > 1771 > CL-1. Importantly, while one lymphoma cell line expressed lower levels of cannabinoid receptors than the other, no lymphoma entity uniformly lacked expression of either cannabinoid receptor. Since we have found no expression of the CB1 receptor gene in reactivated PBMCs and we have found the CL-1 cell line to be non-responsive to the endocannabinoid, we used T cell lymphoma cells CL-1 as the control to analyze the relative expression of CB1 and CB2 receptor genes in q-RT PCR. Comparison between human and canine lymphoma cell lines was not made since the human lymphoma cell line was not compatible with the canine housekeeping gene used in the experiment.

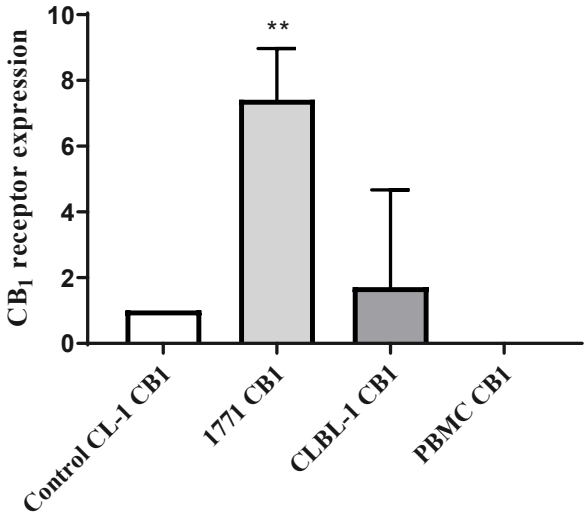


Figure 3-2 Relative expression of CB1 receptor in canine reactivated PBMCs and B and T cell lymphoma cell lines.

Results of real-time PCR found a higher expression level of CB1 receptor gene in B cell lymphoma compared to T cell lymphoma cells and no expression of CB1 receptor gene in reactivated canine PBMCs.

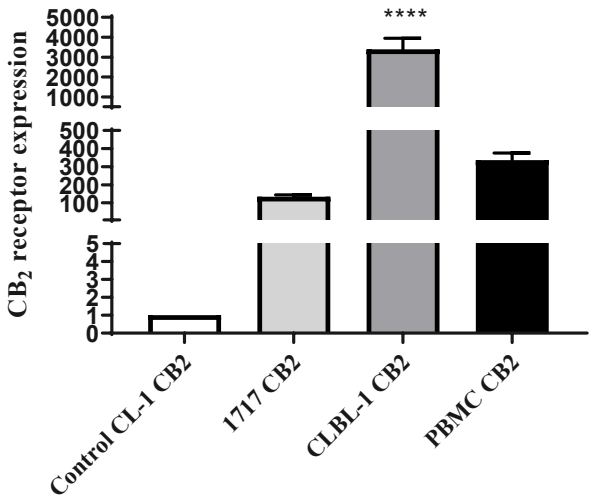


Figure 3-3 Relative expression of CB2 receptor in canine reactivated PBMCs and B and T cell lymphoma cell lines.

Positive expression of CB2 receptor gene found in all lymphoma cell lines and reactivated PBMCs. However, CB2 receptors expressed thousands of folds higher in B cell lymphoma cell line CLBL-1 compared to other cell lines.

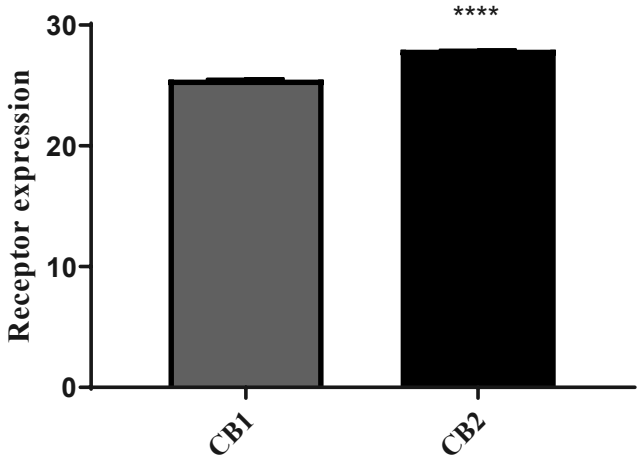


Figure 3-4 Expression of CB1 and CB2 receptors in human B cell lymphoma cell line Ramos.

Positive expression of CB1 and CB2 receptor gene found in human B cell lymphoma cell line. The expression level of CB2 receptor was significantly higher than the CB1 receptor.

3.5.2 Cell Viability Assay

AEA caused a dose-dependent decrease in lymphoma cell viability compared to the control (treated with the vehicle) in all lymphoma cell lines. (Fig. 2a, n=12, $p < 0.0001$) at the dose of 1-50 μM. In CL-1 AEA decreased cell viability only at the highest concentration of 50 μM. 2AG had no significant time or dose-dependent effect on 1771 and CL-1 canine B and T cell lymphoma cells viability. However, 2AG induced dose and time-dependent decrease in cell viability on Ramos and dose-dependent effect on CLBL-1 human and canine B cell lymphoma cell lines respectively.

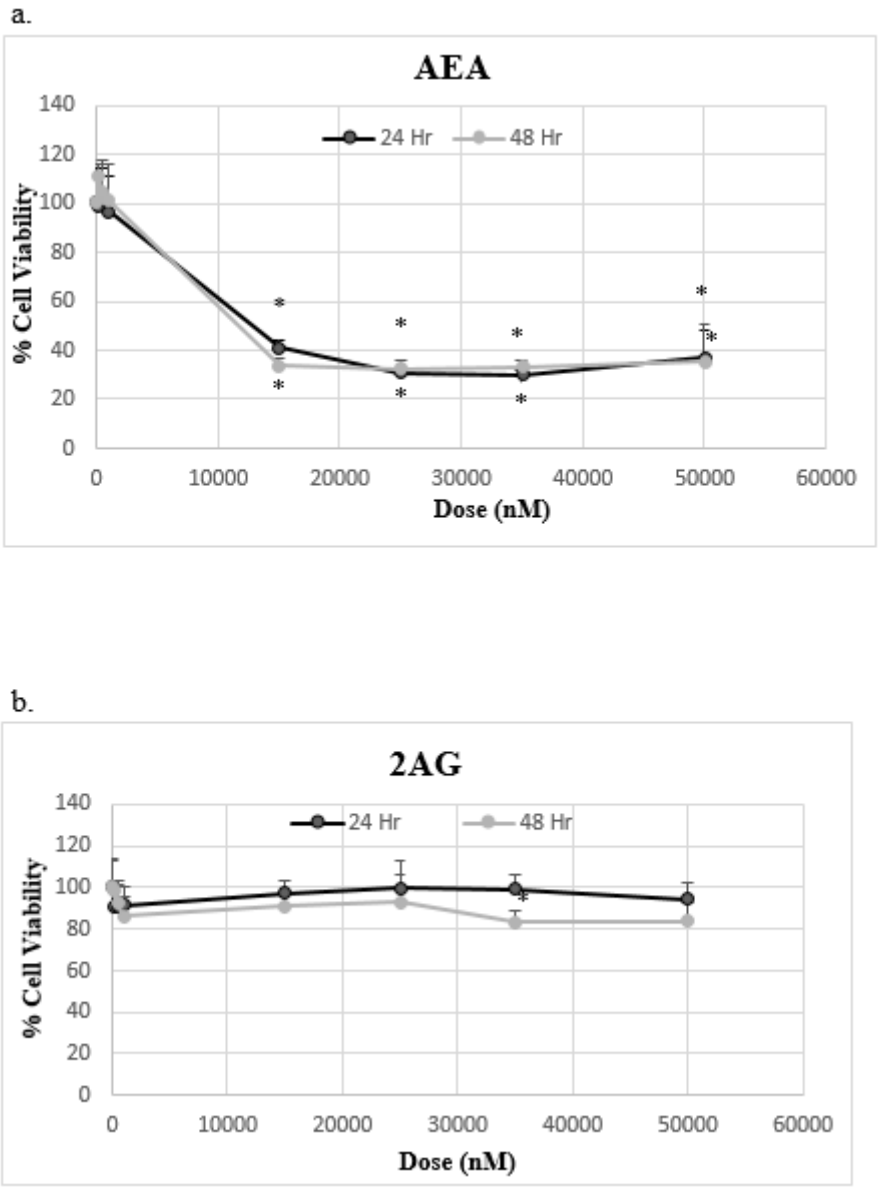
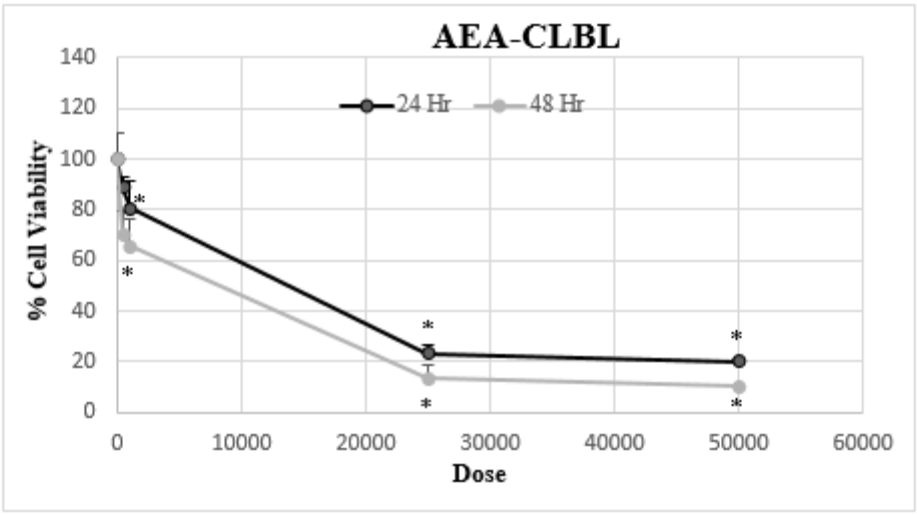


Figure 3-5. Effect of AEA and 2AG on canine 1771 B-cell lymphoma cells viability

Canine 1771 lymphoma cells were treated with 0.1-50µM concentrations of endocannabinoids, AEA and 2AG for 24 h and 48 h. MTT assay was used to evaluate the lymphoma cell viability. Results are expressed as (%) change as compared to the control, Mean ± SD. **a**: AEA dose-dependently decreased the lymphoma cell viability significantly as compared to the control (n=12, $p < 0.0001$). **b**: 2AG did not show a significant dose-dependent effect on 1771 canine B-cell lymphoma cells viability.

a.



b.

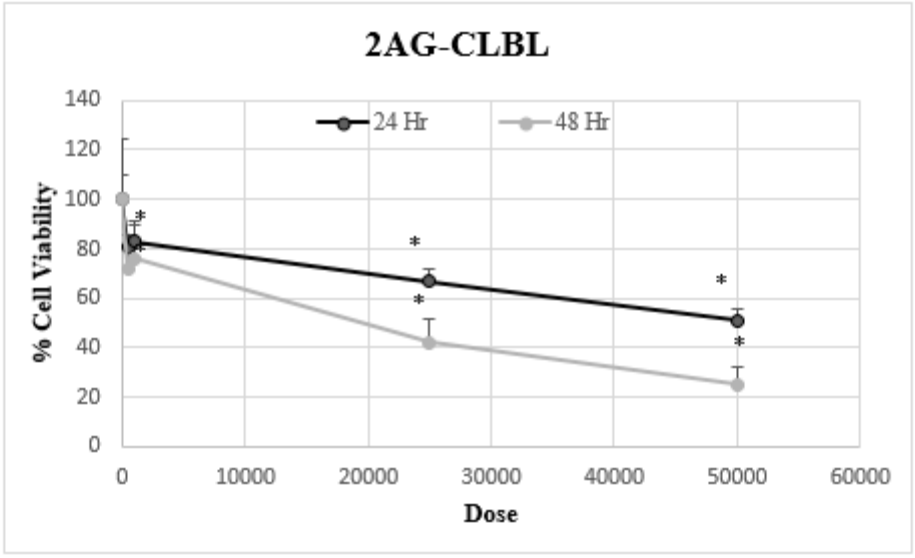
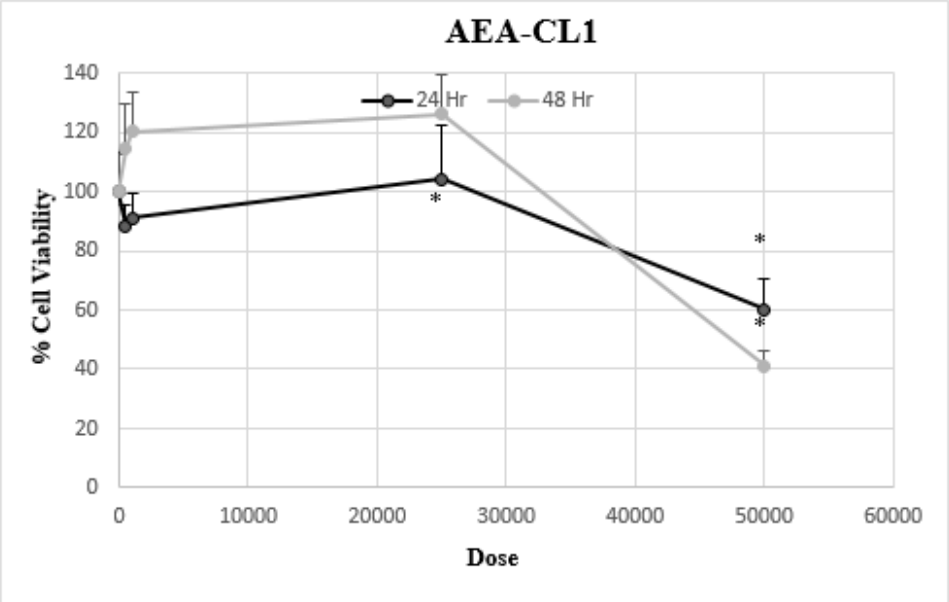


Figure 3-6 Effect of AEA and 2AG on canine CLBL-1 B-cell lymphoma cells viability

Results are expressed as (%) change as compared to the control, Mean ± SD. **a:** AEA and **b:** 2AG dose-and time dependently decreased the lymphoma cell viability significantly as compared to the control (n=12, $p < 0.0001$).

a.



b.

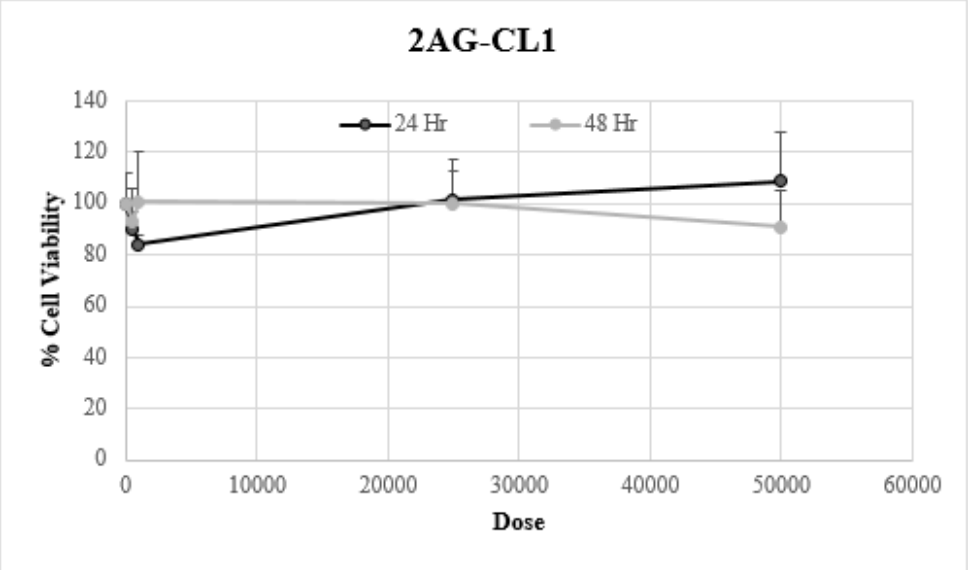
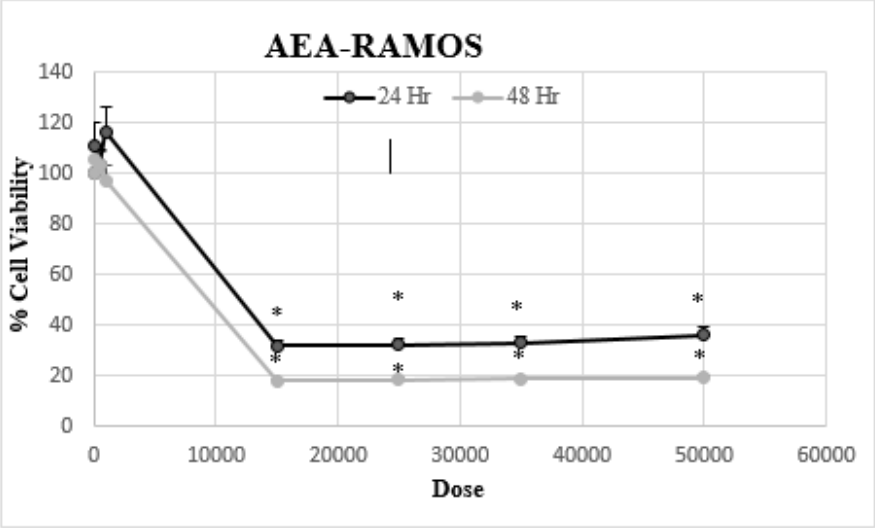


Figure 3-7 Effect of AEA and 2AG on canine CL-1 T-cell lymphoma cells viability

Results are expressed as (%) change as compared to the control, Mean ± SEM. **a:** AEA dose-dependently decreased the lymphoma cell viability significantly as compared to the control (n=12, $p < 0.0001$) **b:** 2AG did not show significant dose or time-dependent effect on CL-1 canine T-cell lymphoma cells viability.

a.



b.

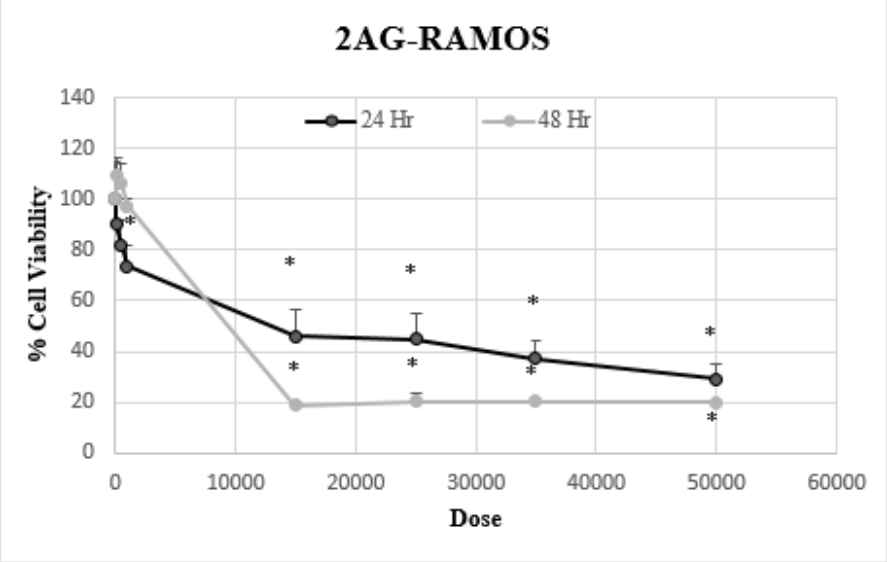


Figure 3-8 Effect of AEA and 2AG on human Ramos B-cell lymphoma cells viability

Results are expressed as (%) change as compared to the control, Mean ± SEM. **a:** AEA dose-dependently and **b:** 2AG both dose and time-dependently decreased the lymphoma cell viability significantly as compared to the control ($n=12$, $p < 0.0001$).

3.5.3 AEA induces oxidative stress

AEA induced dose-dependent upsurge in oxidative stress as seen by the significant increase in the generation of hydrogen peroxide, reactive oxygen species (Fig. 10a, 13a, n=5, $p < 0.05$). However, glutathione (Fig. 11a, n=5, $p < 0.05$), NADH (Fig. 12a, n=5, $p < 0.05$) and nitrite content (Fig. 14, n=5, $p < 0.05$) decreased, which can be an indication of a cellular defense mechanism against increased oxidative stress. 2AG on the other hand no significant effect on oxidative stress in 1771 lymphoma cells (Fig. 11b-14b).

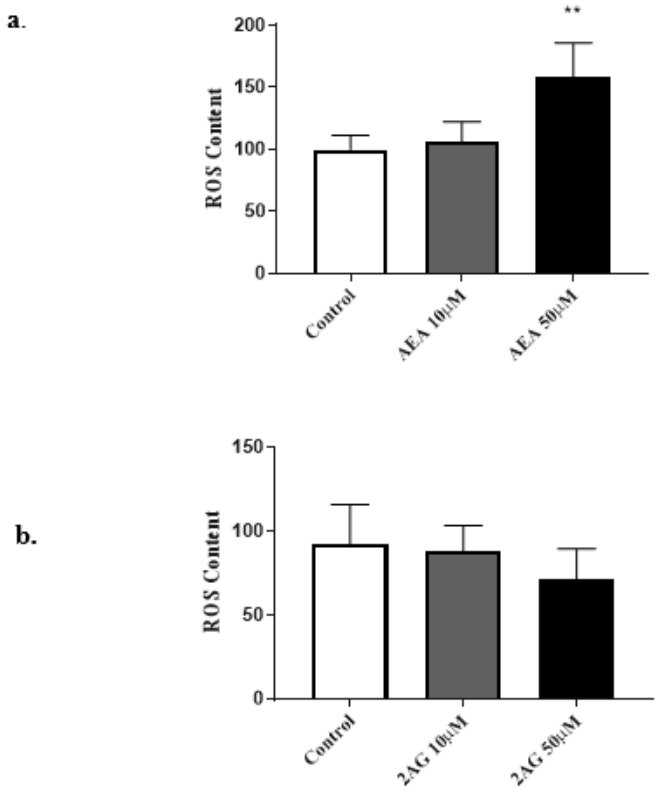
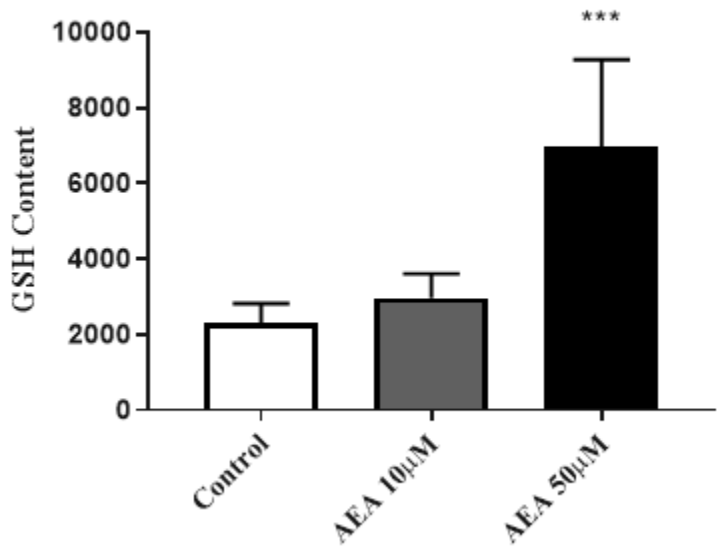


Figure 3-9 Effect of AEA and 2AG on canine 1771 B-cell lymphoma cells reactive oxygen species generation

Reactive oxygen species was measured spectrofluorimetrically. **a.** AEA showed a significant dose-dependent increase in reactive oxygen species generation as compared to the control (*$p < 0.0001$, n=5). **b.** 2AG showed a dose-dependent decrease in reactive oxygen species generation as compared to the control. Vehicle was used as a control. Results are expressed in absolute scale compared to the control, Mean ± SD.

a.



b.

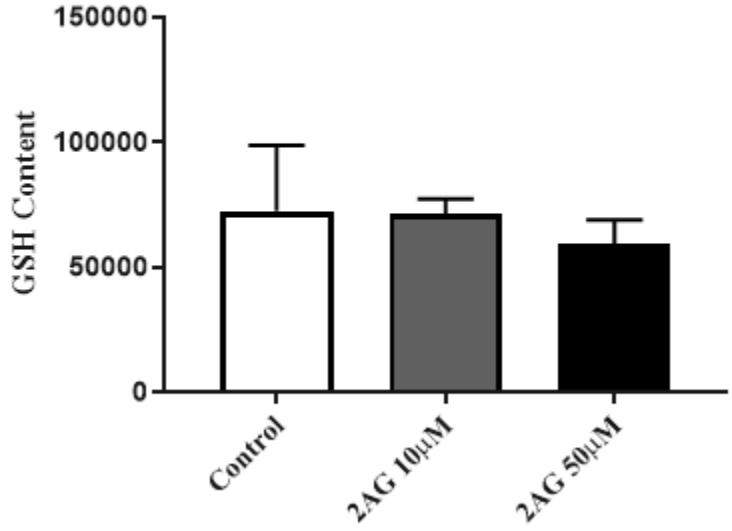
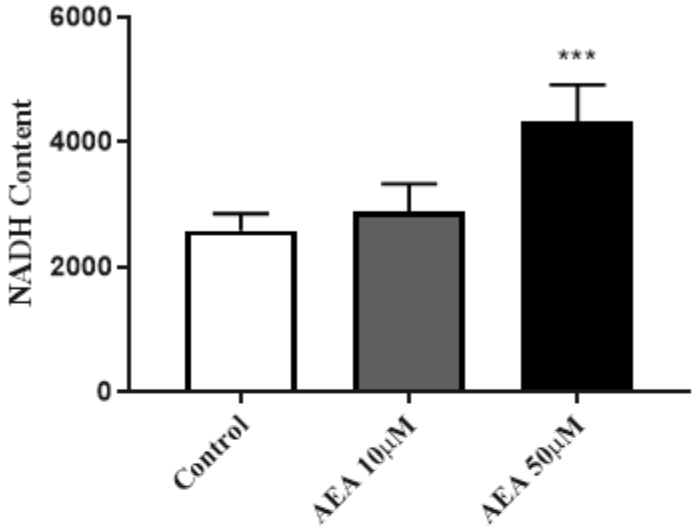


Figure 3-10 Effect of AEA and 2AG on canine 1771 B-cell lymphoma cells glutathione content

Glutathione content was measured spectrophotometrically. **a.** AEA induced a significant dose-dependent increase in GSH content ($*p < 0.0001$, n=5). **b.** 2AG showed no significant effect on GSH content. Vehicle (50 μM) used as a control did not affect. Results are expressed in absolute scale as compared to the control, Mean ± SD.

a.



b.

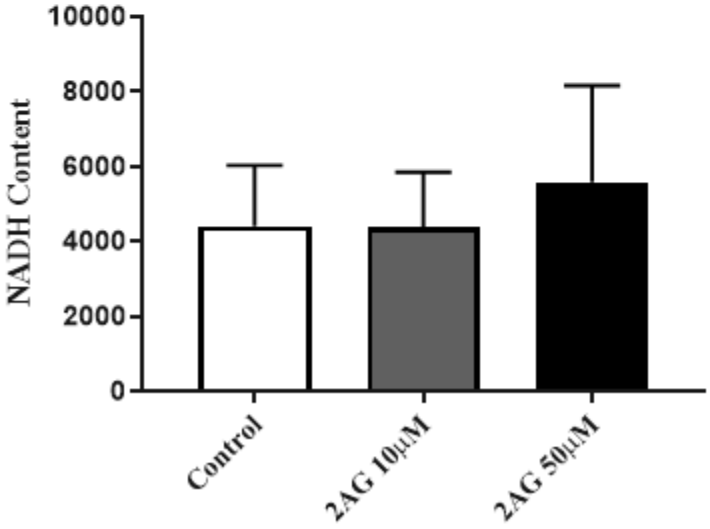
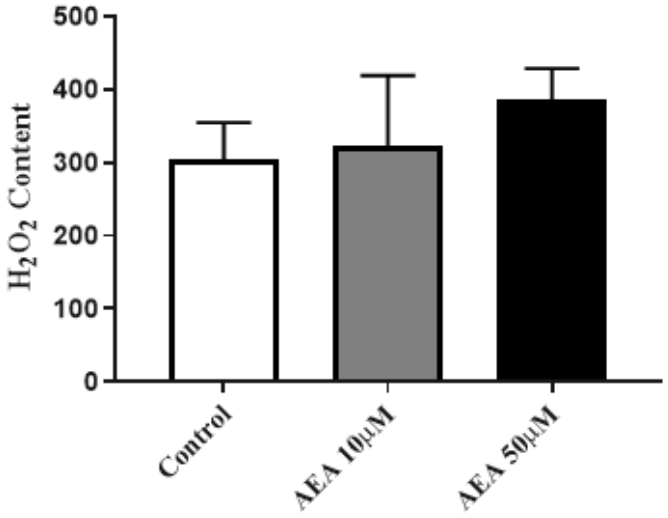


Figure 3-11 Effect of AEA and 2AG on canine 1771 B-cell lymphoma cells NADH content

NADH content was measured spectrophotometrically. **a.** AEA induced a significant dose-dependent increase in NADH content ($*p < 0.0001$, n=5) as compared to the control. **b.** 2AG showed no significant effect in the NADH content. Vehicle (50 μM) used as a control did not affect. Results are expressed in absolute scale as compared to the control, Mean ± SD.

a.



b.

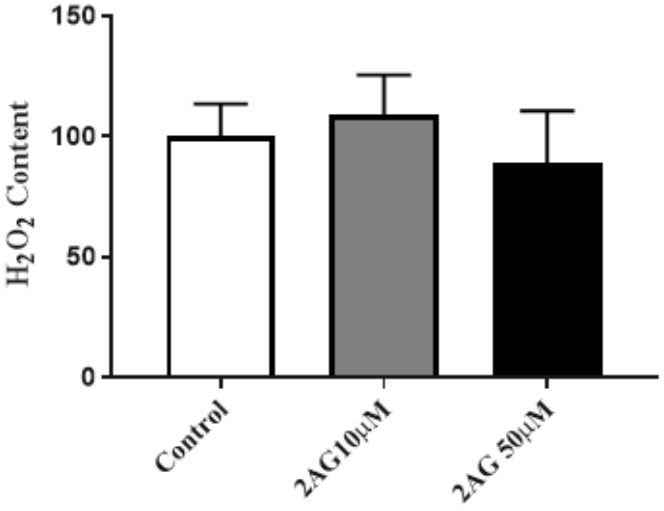
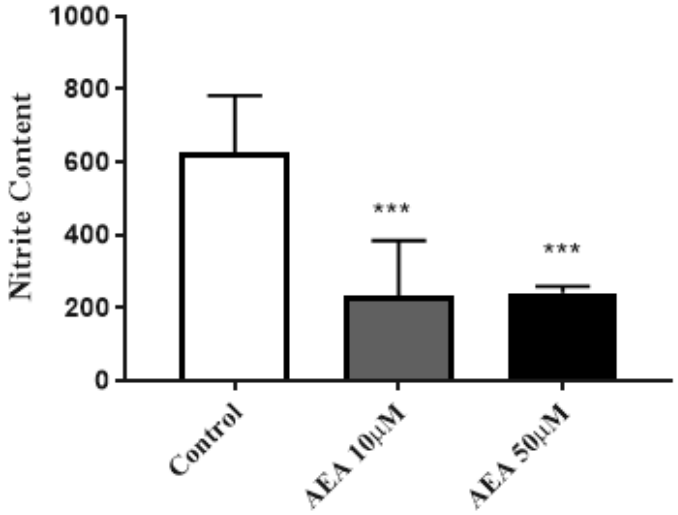


Figure 3-12 Effect of AEA and 2AG on canine 1771 B-cell lymphoma cells Hydrogen peroxide content

Hydrogen peroxide content was measured spectrophotometrically. **a.** AEA induced a dose-dependent increase in H_2O_2 content. **b.** 2AG induced no significant effect on H_2O_2 content. Vehicle (50 μM) used as positive control did not affect. Results are expressed in absolute scale as compared to the control, Mean ± SD

a.



b.

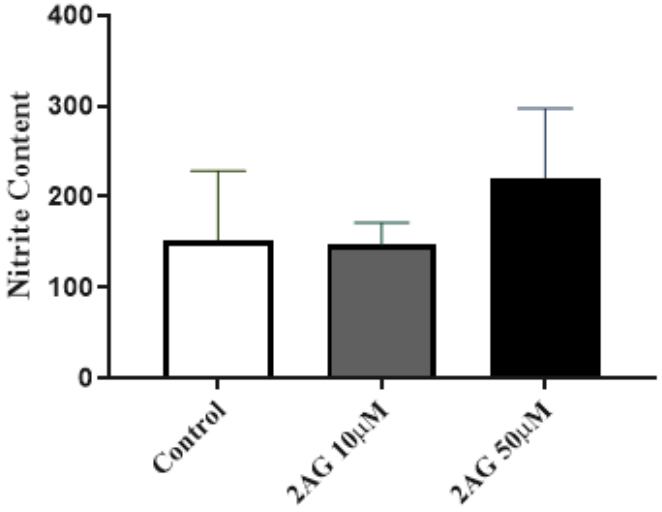


Figure 3-13 Effect of AEA and 2AG on canine 1771 B-cell lymphoma cells nitrite content

Nitrite content was measured spectrophotometrically. **a.** AEA showed a significant dose-dependent decrease in nitrite content as compared to the control ($*p < 0.0001$, n=5). **b.** A significant effect could not be demonstrated as compared to the control with 2AG. Vehicle (50 μM) was used as a control. Results are expressed in absolute scale as compared to the control, Mean ± SD.

3.5.4 AEA induce Inflammation

AEA induced pyroptosis (a highly inflammatory form of lytic programmed cell death) as seen by the changes in the activity of inflammatory markers ICE-1 and COX. AEA induced a significant dose-dependent increase in the activities of ICE-1 and COX (Fig. 15a and 16a, n=5, $p < 0.05$). 2AG showed no significant effect on ICE-1 activity.

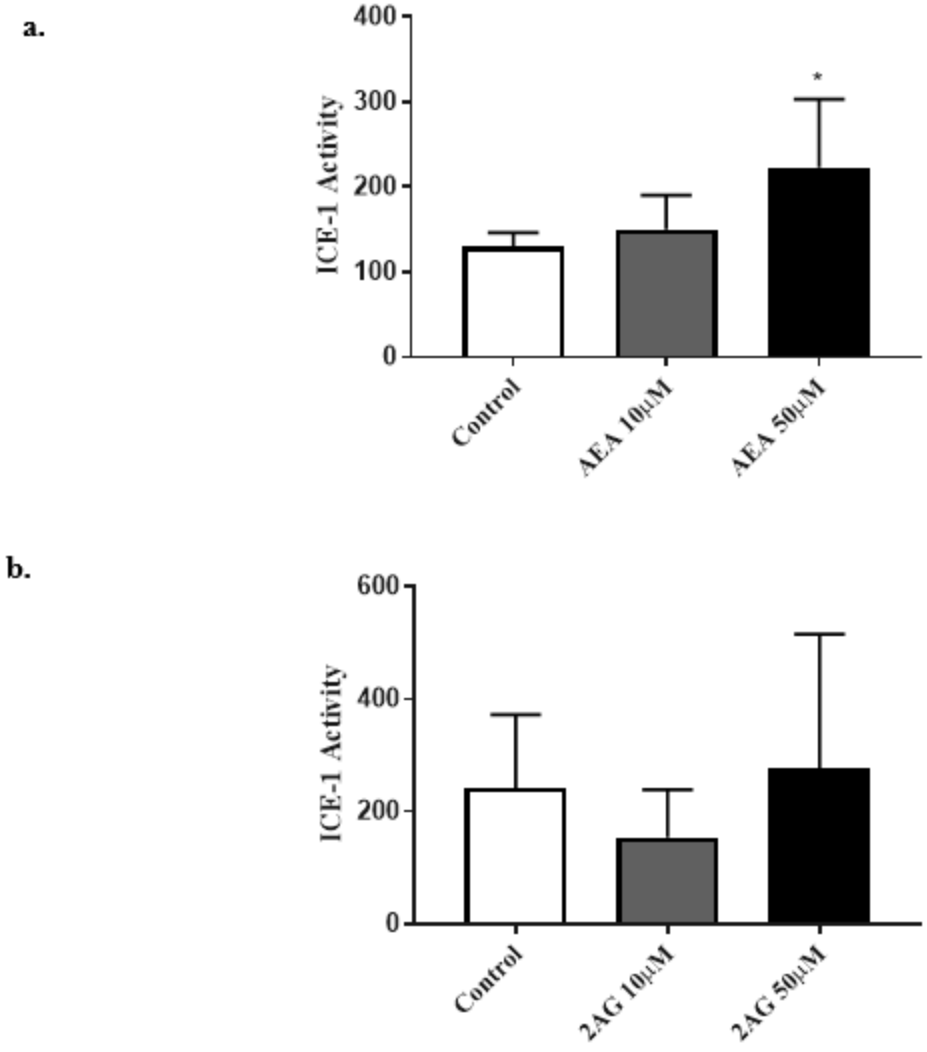
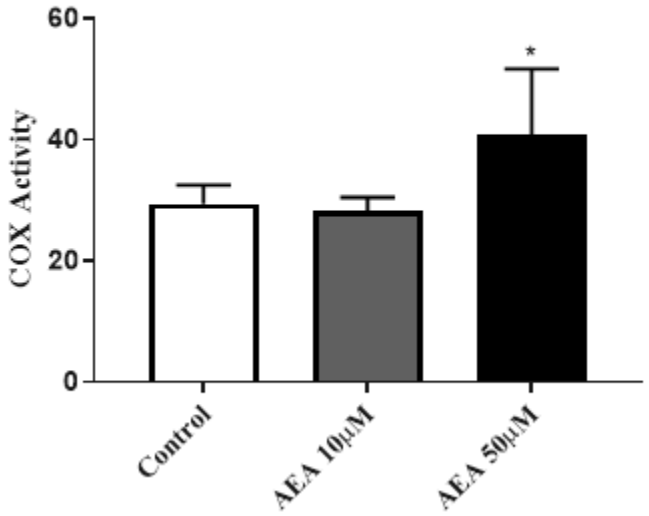


Figure 3-14 Effect of AEA and 2AG on canine 1771 B-cell lymphoma cells ICE-1 activity

ICE-1 activity was measured spectrofluorimetrically using AC-YVAD-AMC as substrate. **a.** AEA significantly and dose-dependently increased Caspase-1 activity ($*p < 0.05$, n=5). **b.** 2AG could not demonstrate a significant effect on ICE activity as compared to the control. Results are expressed in absolute scale as compared to the control, Mean ± SD.

a.



b.

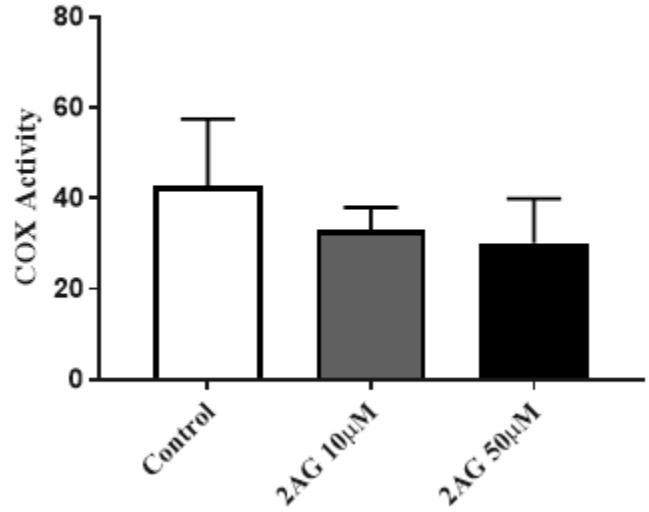


Figure 3-15 Effect of AEA and 2AG on canine 1771 B-cell lymphoma cells Cyclooxygenase

Cyclooxygenase was quantified calorimetrically by using N, N, N', N'-tetramethyl-p-phenylenediamine (TMPD) as a substrate. **a.** AEA significantly increased cyclooxygenase ($*p < 0.05$, n=5). **b.** 2AG induced dose-dependent decreased in cyclooxygenase activity as compared to the control. Vehicle (50 μM) was used as a control. Results are expressed in absolute scale as compared to the control, Mean ± SD.

3.5.5 AEA induces apoptosis

AEA induced apoptosis as seen by the changes in the activity of various apoptotic markers. AEA increased the activity of caspase-3, caspase-8 and caspase-9, however, not to a significant level (Fig. 17a, 18a, 19a). 2AG had either no significant effect or decreased the activity of caspases (Fig. 17b, 18b, 19b).

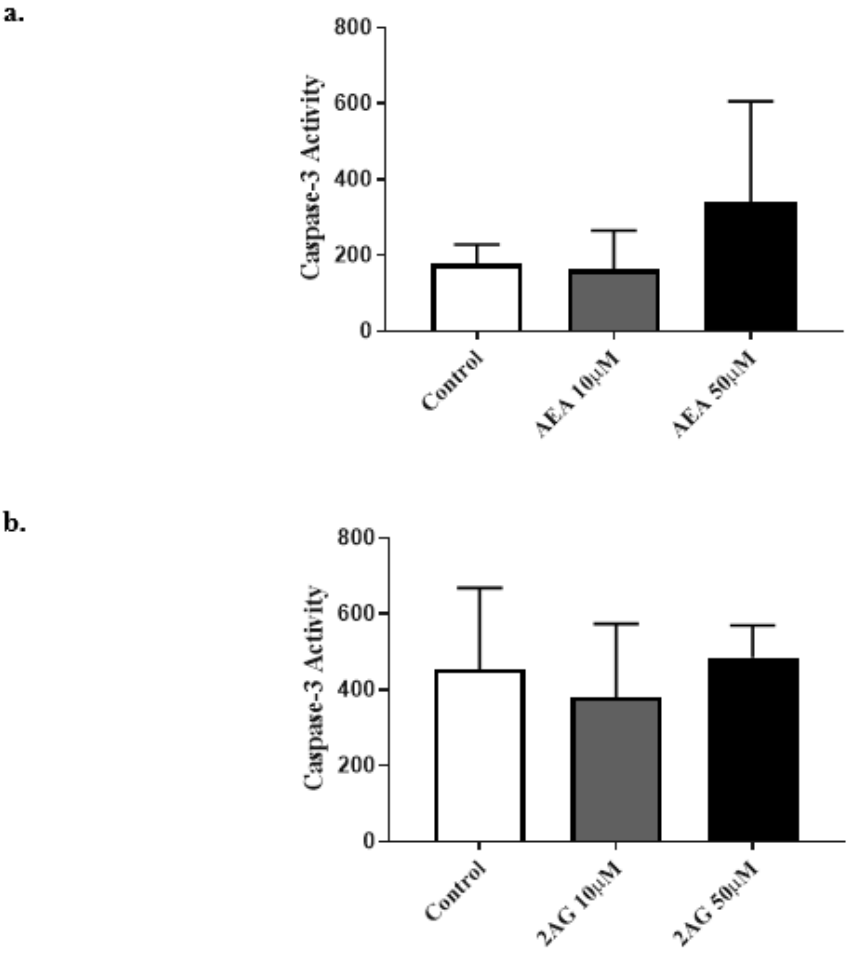
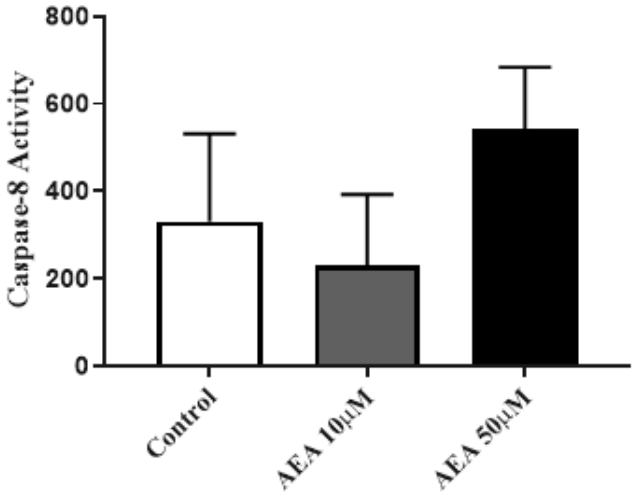


Figure 3-16 Effect of AEA and 2AG on canine 1771 B-cell lymphoma cells Caspase-3 activity

Caspase-3 activity was measured spectrofluorimetrically using AC-DEVD-AMC as substrate. **a.** AEA dose-dependently increased Caspase-3 activity. **b.** Significant dose-dependent effect could not be demonstrated with 2AG as compared to the control. Results are expressed in absolute scale as compared to the control, Mean ± SD.

a.



b.

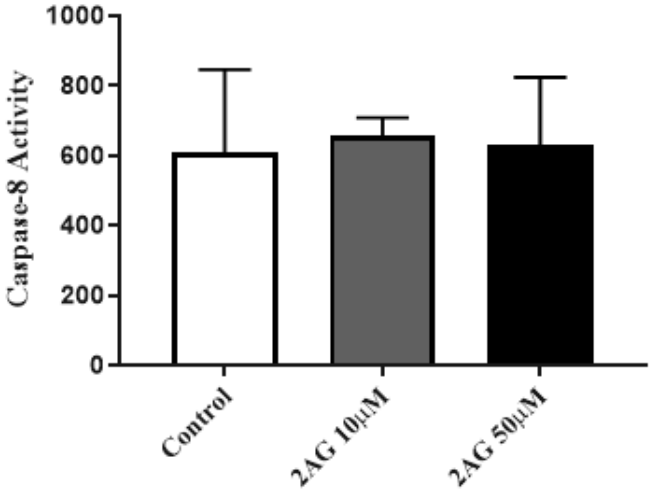
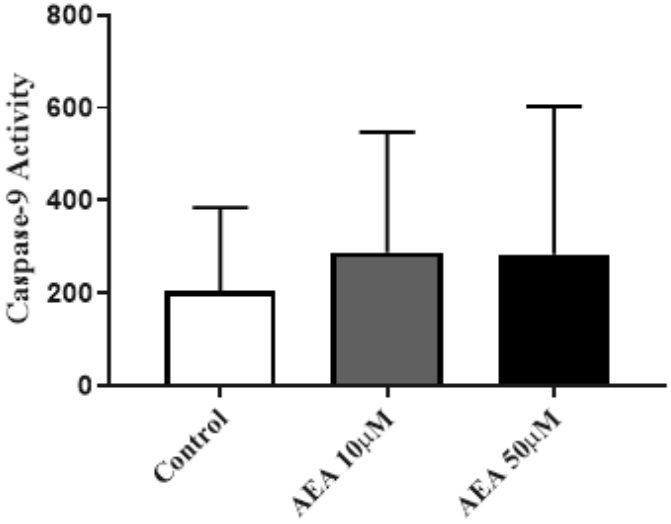


Figure 3-17 Effect of AEA and 2AG on canine 1771 B-cell lymphoma cells Caspase-8 activity

Caspase-8 activity was measured spectrofluorimetrically using Ac-VETD-AMC as substrate. **a.** AEA increased Caspase-8 activity at 50 μM concentration. **b.** with 2AG significant effect on the caspase-8 activity could not be demonstrated as compared to the control. Results are expressed in absolute scale as compared to the control, Mean ± SD.

a.



b.

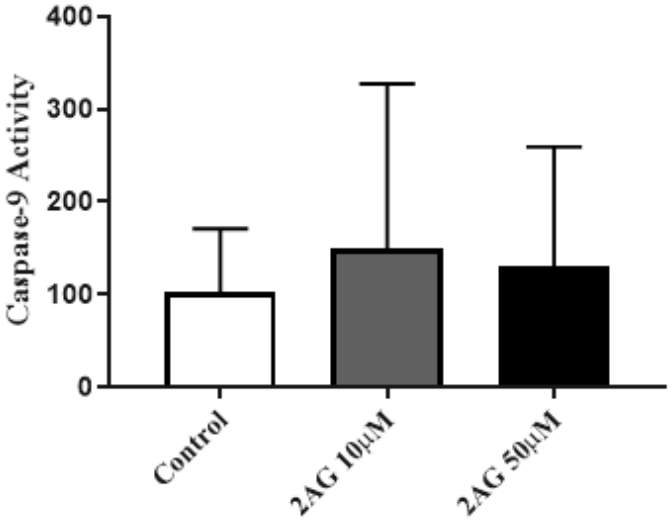


Figure 3-18 Effect of AEA and 2AG on canine 1771 B-cell lymphoma cells Caspase-9 activity

Caspase-9 activity was measured spectrofluorimetrically using Ac-LEHD-pNa as substrate. **a.** AEA and **b.** 2AG increased Caspase 9 activity, however, not a significant level as compared to the control. Results are expressed in absolute scale as compared to the control, Mean ± SD.

3.5.6 AEA inhibit mitochondrial function

The main role of mitochondria in the cells is energy production (ATP) through respiration. Thus, the mitochondria play vital role in regulating cancer cell survival. AEA inhibited the Complex-I activity in a dose-dependent manner when compared to the control (n=5, $p < 0.05$; Fig. 19a). 2AG did not affect the activity of complex-I (n=5, Fig. 19b).

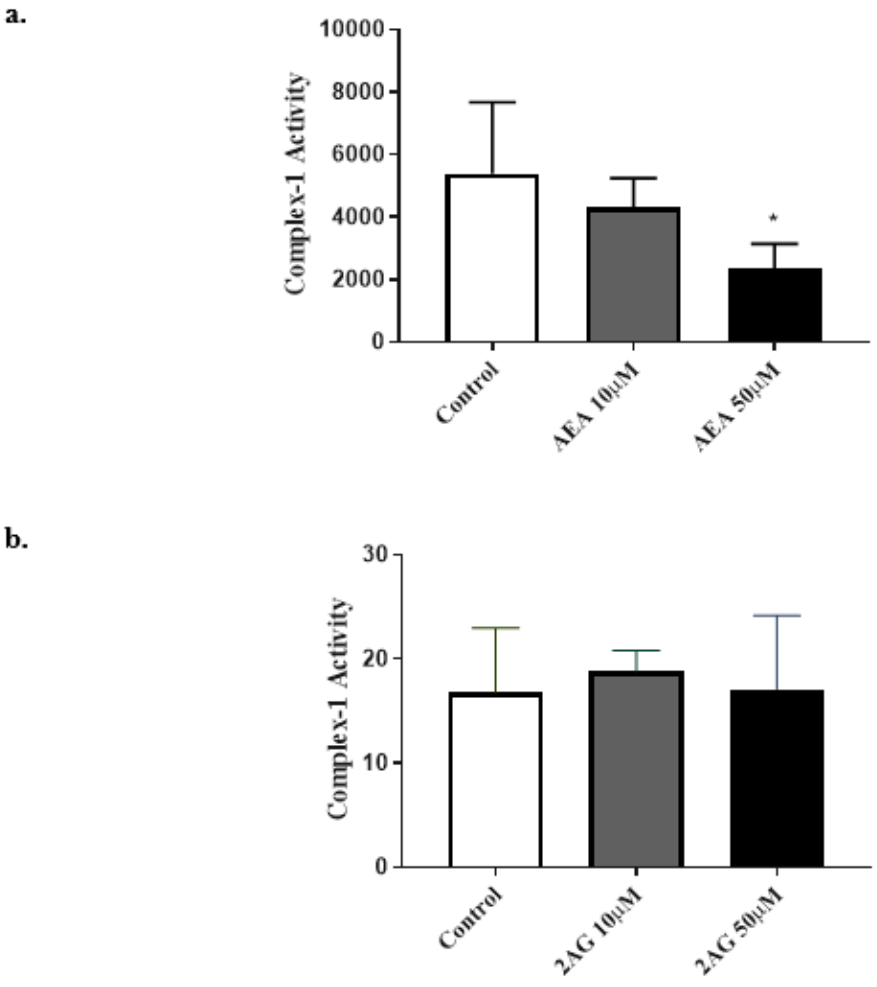


Figure 3-19 Effect of AEA and 2AG on canine 1771 B-cell lymphoma cells Complex-I activity

Complex-I activity was measured spectrophotometrically using NADH as substrate. **a.** Endocannabinoid AEA showed a significant dose-dependent decrease in Complex-I activity ($*p < 0.05$, n=5). **b.** Significant effect on Complex-1activity activity as compared to the control could not be demonstrated with 2AG. Results are expressed in absolute scale as compared to the control, Mean ± SD.

3.6 Discussion

Multiple studies have also demonstrated anti-cancer effects of cannabinoids in multiple models of human cancers and most but not all of the anti-cancer effects of endocannabinoids and synthetic cannabinoids have been associated with cannabinoid receptors [95, 152, 166]. Deregulation of endocannabinoid receptors has been identified in various malignant cancers with generally higher expression of endocannabinoid receptors found in malignant tissues compared to the non-malignant and normal surrounding tissues. Gene expression profiling of cannabinoid receptor genes can be useful in cancer diagnostics and prognosis [162, 163]. To investigate the potential anti-cancer effects of cannabinoids in canine and human non-Hodgkin lymphoma we have analyzed the expression of endocannabinoid receptors CB1 and CB2, our results indicate positive expression of CB1 and CB2 in both canine and human lymphoma cell lines, with much higher expression of cannabinoid receptors in B cell compared to T cell lymphoma cell line. Our results are in parallel with the findings of Gustafsson et.al (2008) and Wasik et.al (2014) regarding the expression of cannabinoid receptor in non-Hodgkin and Mantle cell lymphoma [152, 165]. In addition, to study the expression of cannabinoid receptors in canine lymphoma we have also analyzed their expression in activated canine PBMCs, where we have found positive expression of CB2 receptor with expression level higher than in T cell lymphoma and 1771 B cell lymphoma cell line. However, we have not found the expression of CB1 receptor gene in the activated canine PBMCs. Previous studies on human PBMCs have also found much lower expression of CB1 receptors compared to CB2 [152, 179]. Endocannabinoid receptor expression in canine lymphomas and PBMCs have never been studied before, our findings will build the foundation for future research.

The inhibitory effect of endocannabinoids on cancer cell viability has been well established in multiple types of cancers including Non-Hodgkin lymphoma [95, 162]. Our results also demonstrate a significant dose and/or time-dependent decrease in lymphoma cell viability with endocannabinoids AEA and 2AG. However, 2AG had no significant effect on CL-1 canine T cell lymphoma and 1771 canine B cell lymphoma cell line. Interestingly another common observation in 1771 and CL-1 canine lymphoma cell lines was very low expression of CB2 receptor compared to other lymphoma cell lines studied. And the effect of 2AG and AEA on lymphoma cell viability was more pronounced on Ramos and CLBL-1 that expressed higher CB2 receptors compared to

the other two cell lines. These results indicate the potential involvement of CB2 receptor in 2AG mediated effect on lymphoma cell viability. Significant dose-dependent effect of AEA on 1771 B cell lymphoma cell line but not on T cell lymphoma cell line CL-1 indicates involvement of CB1 and/ or other receptors in AEA mediated effect. Lower expression of both CB1 and CB2 receptors in CL-1 potentially explains the resistance of T cell lymphoma cells to AEA and 2AG induced anti-proliferative effect. Zhang et.al studied the effect of 2AG on diffuse large B cell lymphoma (DLBCL) cell lines and found the proliferative effect of 2AG [180].

The results provide a new insight into the relationship between cannabinoid receptor expression and the effect of endocannabinoids on lymphoma cell viability which should be taken into account while selecting cannabinoids to study on lymphomas.

To determine whether treatment with endocannabinoid would lead to induction of apoptosis/cell death. 1771 canine B cell lymphoma cells were analyzed for biochemical markers of oxidative stress, mitochondrial function, inflammation, and apoptosis.

ROS homeostasis is essential to sustain cell function. Toxicity agents induced the over-production of ROS and the subsequent oxidative stress may cause severe cell toxicity leading to cell death. In this study, we found a dose-dependent increase in reactive oxygen species ROS and H_2O_2 indicating enhanced oxidative stress in 1771 lymphoma cells treated with AEA. However, we also found an increase in GSH and NADH content at that time point and dose that indicates a cellular defense mechanism against oxidative stress. On the other hand, 2AG had no significant effect on oxidative stress.

Two markers of inflammation were studied in this study, COX, and ICE-1. COX-2 induction or overexpression is associated with increased production of PGE2, one of the major products of COX-2 which is known to modulate cell proliferation, cell death, and tumor invasion in many types of cancers including colon, breast, and lung [181]. Our results demonstrate a significant increase in cyclooxygenase activity in cells treated with AEA and a decrease in COX activity in cells treated with 2AG. Our results for AEA follow the results of Pastos et al. (2010) and Adenolfi et al. (2013) [182, 183]. ICE-1 initiates a pro-inflammatory response through the cleavage and thus activation of inflammatory cytokines as well as pyroptosis, a programmed lytic cell death pathway. Our results demonstrate a dose-dependent increase in ICE-1 activity in cells treated with AEA but no significant change in ICE-1 activity with 2AG. Similar effect of AEA on endothelial cells have also been reported and linked to cause apoptosis [184].

The decrease in mitochondrial oxygen consumption has been observed with cannabinoids including AEA, being inhibitors of the mitochondrial respiratory chain [185]. Earlier studies also reported that a variety of cannabinoids when administered to rats *in vivo* caused differential decreases in the synthesis of RNA, DNA, and protein synthesis [186]. Our results showed a significant dose-dependent decrease in mitochondrial complex-1 activity in cells treated with AEA. However, we also found an increase in NADH content at that point and dose, which can be a mitochondrial reaction to decrease in its function. 2AG showed no significant effect on mitochondrial function.

Caspases have been implicated in apoptosis, necroptosis, and autophagy. Studies carried on various cancer models including lymphomas have demonstrated cannabinoid-induced increased caspase activity and apoptosis of cancer cells [95, 151, 162, 163]. In our study, we have found AEA induced increase in caspase 3, 8, and 9 activity, however, not to a significant level. We did not found an increase in any caspase activity in cells treated with 2AG at that time point and dose.

3.7 Conclusion

In summary, our study for the first time demonstrated the endocannabinoid receptor expression and anti-cancer effects of endocannabinoids in canine non-Hodgkin lymphoma cell lines in addition to human non-Hodgkin lymphoma cell line. Our results show that CB1 and CB2 express in both canine and human entities of non-Hodgkin lymphomas of B and T cell type with higher expression of endocannabinoid receptors in B cell lymphoma cell lines compared to T cell lymphoma. The highly variable expression within well-defined lymphoma entities suggests that cannabinoid receptors may be potential targets for individualized therapeutic interventions. Treatment with the endocannabinoid AEA inhibits cancer cell viability in both human and canine non-Hodgkin lymphoma cell lines, AEA treated canine 1771 B cell lymphoma also express markers of oxidative stress and apoptosis. Treatment with 2 acyl glycerol inhibits cell viability in only Ramos and CLBL-1 human and canine B cell lymphoma cell lines that also express much higher expression of CB2 receptors compared to other lymphoma cell lines studied, 1771 and CL-1. 2AG treated 1771 cells do not express markers of cell death at that time point and dose. Our results are similar to previous cannabinoid studies done on various models of human cancers including non-Hodgkin lymphoma. These cumulative data suggest that the endocannabinoid system and anti-cancer effects of endocannabinoids are quite similar in canine and human non-

Hodgkin lymphomas and targeting of the endocannabinoid system could possibly be part of future therapy for certain malignant lymphomas in human and canine as has been suggested for other forms of cancer.

4. Effect of Exogenous Cannabinoids on Canine and Human Non-Hodgkin Lymphoma Cell Lines

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4.1 Abstract

Non-Hodgkin's lymphoma is the fifth leading cause of human cancer death, and one of the most commonly encountered neoplasms in canine. Approximately 30% of human and 95% of canine lymphoma tumors develop chemotherapy resistance, hence, novel strategies that might be effective against therapy-resistant lymphoma are needed for both species. Canine lymphoma shows striking similarities to human Non-Hodgkin's lymphoma (NHL), in cancer biology, histology, and gene expression, which makes canine the best model to study novel treatment of lymphoma for both species. The anti-cancer effects of cannabinoids (CB) have been studied extensively in the last two decades with evidence of potential efficacy demonstrated using various models of human cancer. In our previous study, we demonstrated overexpression of CB1 and CB2 cannabinoid receptors and anti-proliferative effect of endocannabinoids on canine and human Non-Hodgkin lymphoma cell lines compared to cells treated with vehicle. The purpose of this study was to demonstrate the anticancer effects of the phytocannabinoids CBD and THC and the synthetic cannabinoid WIN 55-212-22 (WIN), in canine and human lymphoma cell lines and to compare their impact to that of endocannabinoids which we previously have reported (Chapter 3). We have used malignant canine B cell type (1771 and CLBL1) and T cell type (CL1) NHL cell lines, canine PBMCs and, human B cell type NHL cell line (RAMOS). All cell lines were cultured in RPMI and treated with all cannabinoids individually at concentrations from 0.1μM to 50μM for 24 and 48 hours. Canine PBMCs and vehicle were used as a control. Cell viability was assessed using the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5 Diphenyltetrazolium Bromide) cell proliferation assay and markers of oxidative stress, mitochondrial function, inflammation and apoptosis were assessed using spectrophotometric and fluorometric analysis. Data were analyzed using one-way ANOVA multiple comparison (Prism®) and SAS to analyze the IC50 of each drug. Our results demonstrate, compared to controls, a significant dose-dependent decrease in cancer viability with both phytocannabinoids and synthetic cannabinoids in all tumor cell lines. Demonstrable indicators of cytotoxicity were limited to CBD in canine T-cell lymphoma lines. Overall, based on IC50 values CBD was found to be the most potent phytocannabinoid we studied. Previously we demonstrated AEA to be more potent than 2-AG, suggesting that CBD and AEA be targeted for future studies for cannabinoid therapies that might contribute to reduction in tumor burden in malignant NHL of canines and humans.

4.2 Introduction

Non-Hodgkin lymphoma (NHL) is the fifth leading cause of human cancer death and is the second fastest growing cancer concerning mortality in people [187]. Likewise, lymphoma is one of the most common types of neoplasm in dogs. It accounts for about 20% of all canine cancers and about 85% of blood cancers, with an incidence rate of 20-100 cases per 100,000 dogs and in many respects comparable to NHL in humans [188]. Canine and human lymphoma are generally characterized by a high rate of initial remission following conventional CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) based chemotherapies; however, 95% of dogs and 30% of humans will succumb to drug-resistant relapse [189-191]. To date, lymphoma is remains a serious condition, indicating the need for novel strategies to improve the outcome of patients suffering from aggressive or therapy-resistant lymphoma.

Cannabinoids have been used in human patients with cancer for their palliative effects (e.g., inhibition of chemotherapy-induced nausea, vomiting, apatite stimulation and pain) since the early 1970s. However, in addition to palliative interventions, in the past decade cannabinoids have drawn renewed attention by demonstrating diverse pharmacologic activities such as cell growth inhibition, anti-inflammatory effects, and tumor regression on various xenograft animal models of cancer [102, 192, 193]. Using in vitro methods, cannabinoids have been shown to induce apoptosis in glioblastomas [194], PC-12 pheochromocytoma [195], CHP 100 neuroblastoma [196], breast [197], lung [198], prostate [199], and colon [182]cancer in vitro and/or in vivo. In most cases, these diversified effects of cannabinoids have been attributed to the ability of these compounds to activate specific G protein-coupled receptors CB1 and CB2. These cannabinoid receptors are normally bound by a family of endogenous ligands, the endocannabinoids anandamide (AEA), and 2 acyl-glycerol (2AG), or the transient receptor potential (TRP) vanilloid type-1 (TRPV1) (as in case of anandamide) [161, 162, 200, 201]. In the previous study, we have shown the expression level of CB1 and CB2 receptors in canine and human lymphoma and the anti-proliferative and apoptotic effect of endocannabinoids, on canine and human NHL cell lines. In the present study, we demonstrate the inhibition of lymphoma cell growth with selected exogenous (phytocannabinoids [CBD, THC] and synthetic [WIN] cannabinoid) cannabinoids and compare it with endogenous cannabinoids results.

4.3 Materials and methods

4.3.1 Chemicals

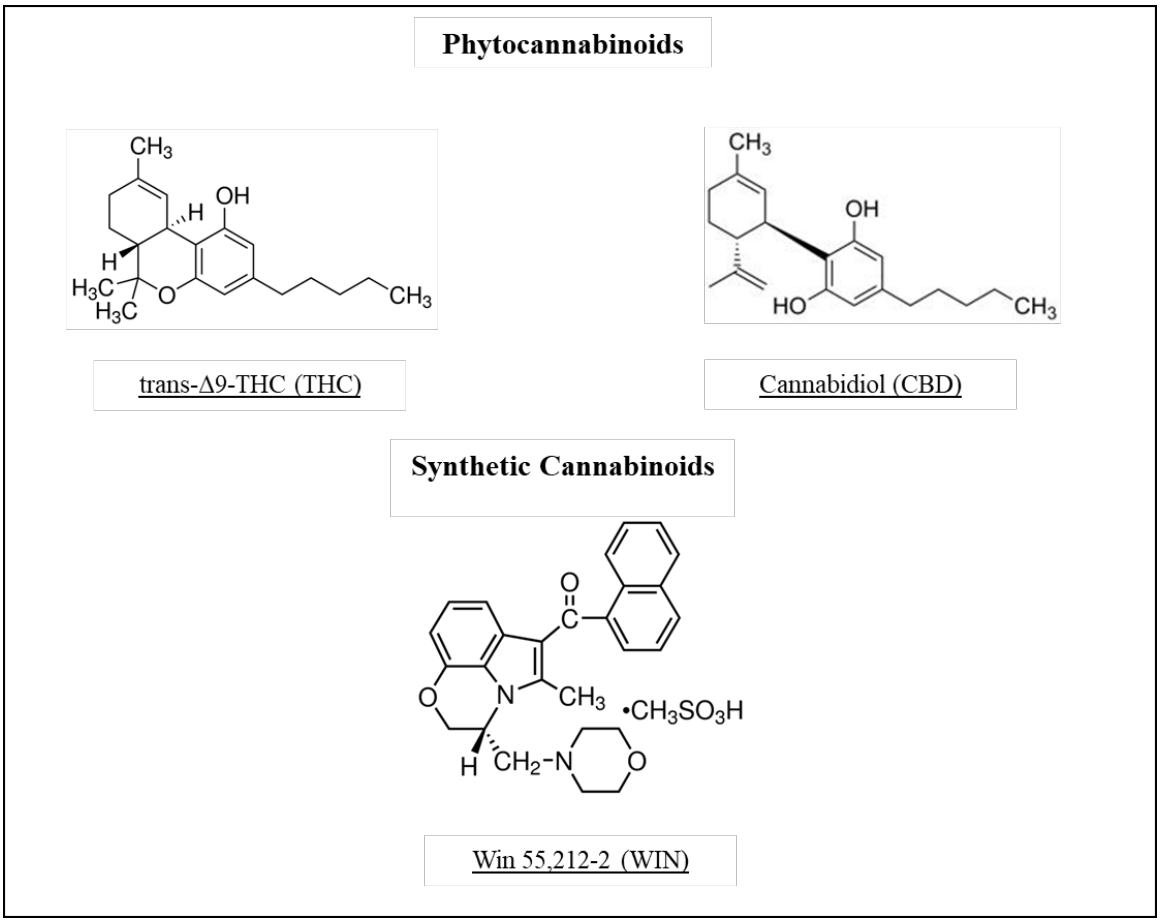


Figure 4-1 Chemical structures of Endocannabinoids

Anandamide and 2-Acylglycerol; Phytocannabinoids: trans-Δ9-THC and Cannabidiol; Synthetic Cannabinoid: Win 55,212-2.

Table 4-1 Binding affinities of cannabinoids to cannabinoid receptors CB1 and CB2

Cannabinoid	Description	Reported binding activity Ki (nM)
Anandamide-AEA	Partial Agonist	CB1: 61-543 CB2: 279-1940
2-acylglecerol-2AG	Agonist	CB1: 58.3, 472 CB2: 145, 1400
Cannabidiol-CBD	Partial Agonist/ Inverse Agonist	CB1: 4350 - >10,000 CB2: 2399 - >10,000
Delta 9 tetrahydrocannabinol-THC	Partial Agonist	CB1: 5.05-80.3 CB2: 3.13-75.3
WIN-55 212-2	Agonist	CB1: 1.89-124 CB2: 0.28-16.2

Table adapted from [202, 203]

4.3.2 Reagents

All the cannabinoids (CBD, THC and WIN Fig. 1) were purchased from Sigma Aldrich (St. Louis, MO). Thiazolyl Blue Tetrazolium Bromide (MTT) was purchased from American Type Culture Collection. Penicillin-Streptomycin solution was purchased from Thermofisher Scientific (Waltham, MA). RPMI 1640 Medium, ES Cell Qualified Fetal Bovine Serum (FBS) and L-Glutamine Solution were purchased from EMD Millipore (Burlington, MA). Phosphate buffer saline (PBS), dimethylsulfoxide (DMSO), nicotinamide adenine dinucleotide (NADH), 2′, 7-dichlorofluorescindiacetate (DCF-DA), pyrogallol, hydrogen peroxide (H202), phosphoric acid, 1-methyl-4-phenylpyridinium (MPP+), O-phthalaldehyde (OPT), L-glutathione reduced, trichloroacetic acid, thiobarbituric acid and [henylmethanesulfonyl fluoride (PMSF) were purchased from Sigma Aldrich (St. Louis, MO). Glutamate was purchased from Alfa Aesar (Haverhill, MA). Thermo Scientific Pierce 660 nm Protein Assay reagent kit was purchased (Pierce, Rockford, IL) for protein quantification. The caspase substrates, AC-YVAD-AMC (Caspase-1/ICE-1 substrate), AC-DEVD-AMC (Caspase-3 substrate), Ac-VETD-AMC (Caspase-8 substrate) and Ac-LEHD-pNa (Caspase-9 substrate) were purchased from Sigma Aldrich (St. Louis, MO).

4.3.3 Cell Lines

Canine B-cell lymphoma cell lines 1771 and CLBL-1 and T-cell lymphoma cell line CL-1 were a kind gift from Dr. Steven Suter's Lab (North Carolina State University). Human B-cell lymphoma cell line RAMOS, activated and unprocessed canine PBMCs were generously shared by Dr. Bruce Smith's Lab (Auburn University) (Table 1, 2). All cells were cultured in RPMI 1640 Medium, supplemented with fetal bovine serum (10%), penicillin-streptomycin (1%), and L-Glutamine (1%). For the cell viability assay, all canine and human lymphoma suspension cells were grown and harvested via centrifuge. Cells were then seeded into 96 well plates at a density of 1×10^4 cells/well. Cells were incubated under standard conditions at 37 °C and supplemented with 5% CO_2.

Table 4-2 List of NHL cell lines used in the study and their description.

NHL Cell lines	Description
1771	Canine B cell lymphoma cell line
CLBL-1	Canine B cell lymphoma cell line
CL-1	Canine T cell lymphoma cell line
Ramos	Human B cell lymphoma cell line
Canine PBMCs	Canine peripheral blood mononuclear cells

4.3.4 Treatment design for evaluating the lymphoma cells viability and mechanisms of exogenous cannabinoid- induced cytotoxicity

All drugs were dissolved in their recommended vehicles. CBD and THC were dissolved in ethanol, and WIN in DMSO. To determine dose-cytotoxicity relationships, different concentrations of drugs were obtained by diluting each cannabinoid in a serum-enriched, fresh culture medium. For controls for each cannabinoid, cells were treated with the vehicle only for that cannabinoid. Cells were exposed to each drug in the range of 0.1-50µM concentrations for 24 and 48 h. To study the cytotoxicity of drugs, the canine 1771 lymphoma cells were exposed to 0 (vehicle only) and 1µM and 50µM concentrations for 24 h. Lower (1 µM) and higher dose (50µM) were used to analyze the change in biochemical markers of oxidative stress, apoptosis and mitochondrial function with

increasing dose and compared to the control untreated group. The vehicle of each drug has used a control.

4.3.5 Cell Viability

The effect of cannabinoids on the viability of cells was determined by (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) (MTT) assay. The cells were plated at $1x10^4$ cells per well in 100µl of complete culture medium at 0.1- 50.0 µM of cannabinoid for 24 and 48 hours at 37ºC in a humidified chamber. At either time point, MTT reagent (10µl) was added to each well and incubated for 4 hours. At that time, solubilization solution (100µl) was added to each well to dissolve formazan crystals. Absorbance was immediately recorded on a microplate reader at 570 nm wavelength. The effect of drugs on cell viability was assessed as the percentage of inhibition in cell viability where vehicle-treated cells were taken as 100% viable.

4.3.6 Protein quantification

Protein was quantified using a Protein Assay reagent kit (Thermo Scientific Pierce 660 nm, Pierce, Rockford, IL). Bovine serum albumin (BSA) was used as a standard for protein measurement.

4.3.7 Biochemical analysis of cytotoxicity markers

Hydrogen peroxide content

The content of hydrogen peroxide in control and treated 1771 canine B-cell lymphoma cells was measured using the Abcam Hydrogen Peroxide Assay procedure. A standard curve for hydrogen peroxide was obtained using a fluorimetric plate reader (BioTek Synergy HT plate reader, BioTek, VT, USA) at 335nm (excitation wavelength) and 390nm (emission wavelength). Hydrogen peroxide content in control and drugs treated supernatant was calculated from slope obtained from a standard curve. Results were expressed as hydrogen peroxide uM/mg protein [167].

Lipid peroxidation

Lipid peroxide content in the control and treated 1771 canine B-cell lymphoma cells was measured via colorimetry by measuring the malondialdehyde (MDA) content in the form of Thiobarbituric acid-reactive substances (TBARS) [168, 204, 205].

Reactive oxygen species generation

The generation of reactive oxygen species in the control and treated 1771 canine B-cell lymphoma cells was estimated via spectrofluorometry by measuring the conversion of non-fluorescent chloromethyl-DCF-DA (2′, 7- dichlorofluorescindiacetate, DCF-DA) to fluorescent DCF using an excitation wavelength of 492 nm and an emission wavelength of 527 nm. [168-170]

Nitrite content

The final products of nitric oxide oxidation pathways are nitrite and nitrate, which are used as an expression of nitric oxide production. Nitrite content in the control and treated 1771 canine B-cell lymphoma cells was measured using Griess reagent. NO2 reacts with sulfanilamide under acidic conditions leading to the production of diazonium ion. This diazonium ion association with N-(1-naphthyl) ethylenediamine to form 36 chromophoric azo product which can be measured spectrophotometrically at 545 nm.[168, 171]

Glutathione content

GSH content in the control/treated 1771 canine B-cell lymphoma cells was measured via spectrofluorometry (327 nm excitation and 423 nm emission) using O-phthalaldehyde (OPT). GSH measured was normalized to total protein content and reported as relative GSH content

NADH content

A standard curve for NADH was obtained using a spectrophotometric plate reader (BioTek Synergy HT plate reader, BioTek, VT, USA) and was measured at 340nm. NADH content was calculated from the slope obtained from the standard curve. The results were expressed as NADH uM/mg protein [174]

Mitochondrial complex-I activity

NADH oxidation to NAD+ is catalyzed by mitochondrial Complex-I (NADH dehydrogenase). Cell homogenate obtained from control and treated 1771 canine B-cell lymphoma cells were added to PBS and conversion of NADH to NAD+ was measured spectrophotometrically at 340 nm. [168, 173, 175]

Interleukin converting enzyme-I activity

Spectrofluorimetric method using the plate reader (BioTek Synergy HT plate reader, BioTek, VT, USA) was to measure the ICE-1 activity. AC-YVAD-AMC (10uM) was used a substrate and the product was measured at 360nm/460nm. The ICE-1 activity in the control and drugs treated supernatant were expressed as relative fluorescence intensity (RFU)/mg protein [176].

Cyclooxygenase activity

Cyclooxygenase activity was quantified spectrophotometrically (calorimetrically). TMPD substrate was used as the substrate for measuring the COX activity in the control and drugs treated supernatant. The product formed from the cleavage of TMPD by cyclooxygenase was measured at 600nm. COX activity in the control and drugs treated supernatant were expressed as the formation of product/mg protein. [177]

Caspases (3, 8, and 9) activity

Spectrofluorimetric method using the plate reader (BioTek Synergy HT plate reader, BioTek, VT, USA) was used to measure the caspase activity. Appropriate DEVD-AMC (10uM) was used as a substrate for measuring caspases activities and the product formed was measured at 360nm/460nm. The ICE-1 activity in the control and drugs treated supernatant were expressed as relative fluorescence intensity (RFU)/mg protein [168, 178].

4.3.8 Statistics

For the statistical analysis of data, the range of the measured variable, means, standard deviations (SD) were calculated, using statistical software (SAS® and Prism-V La Jolla, CA, USA). The data are presented as median and range or mean ± SD values. The differences between sample populations were evaluated with Student's t-test and non-parametric ANOVA followed by Dunnet's multiple comparisons test; P values less than 0.05 were considered statistically significant.

4.4 Results

4.4.1 Effect of Exogenous cannabinoids on NHL cell viability

Phytocannabinoids

Results of the cell viability assay demonstrated a slight stimulatory effect at lower doses followed by a significant dose-dependent decrease in cell viability with phytocannabinoids, CBD and THC in canine B and T cell and human B-cell NHL cell lines compared to the vehicle control (Fig. 2a, $n=12$, $p < 0.0001$) at the dose of 1-50 μM. However, we could not demonstrate significant effect of THC on CL-1 viability. Overall CBD demonstrated to be more potent against B cell lymphoma cell viability as compared to the T cell lymphoma. We could not demonstrate the significant time-dependent effect of phytocannabinoids on any NHL cell lines used in the study.

Synthetic cannabinoid

NHL cells except for CL-1, treated with synthetic cannabinoid agonist, WIN demonstrated a significant dose-dependent decrease in cell viability, compared to the control (Fig. 2a, $n=12$, $p < 0.0001$) at the dose of 1-50 μM. We could not demonstrate the significant time-dependent effect of WIN on cell viability.

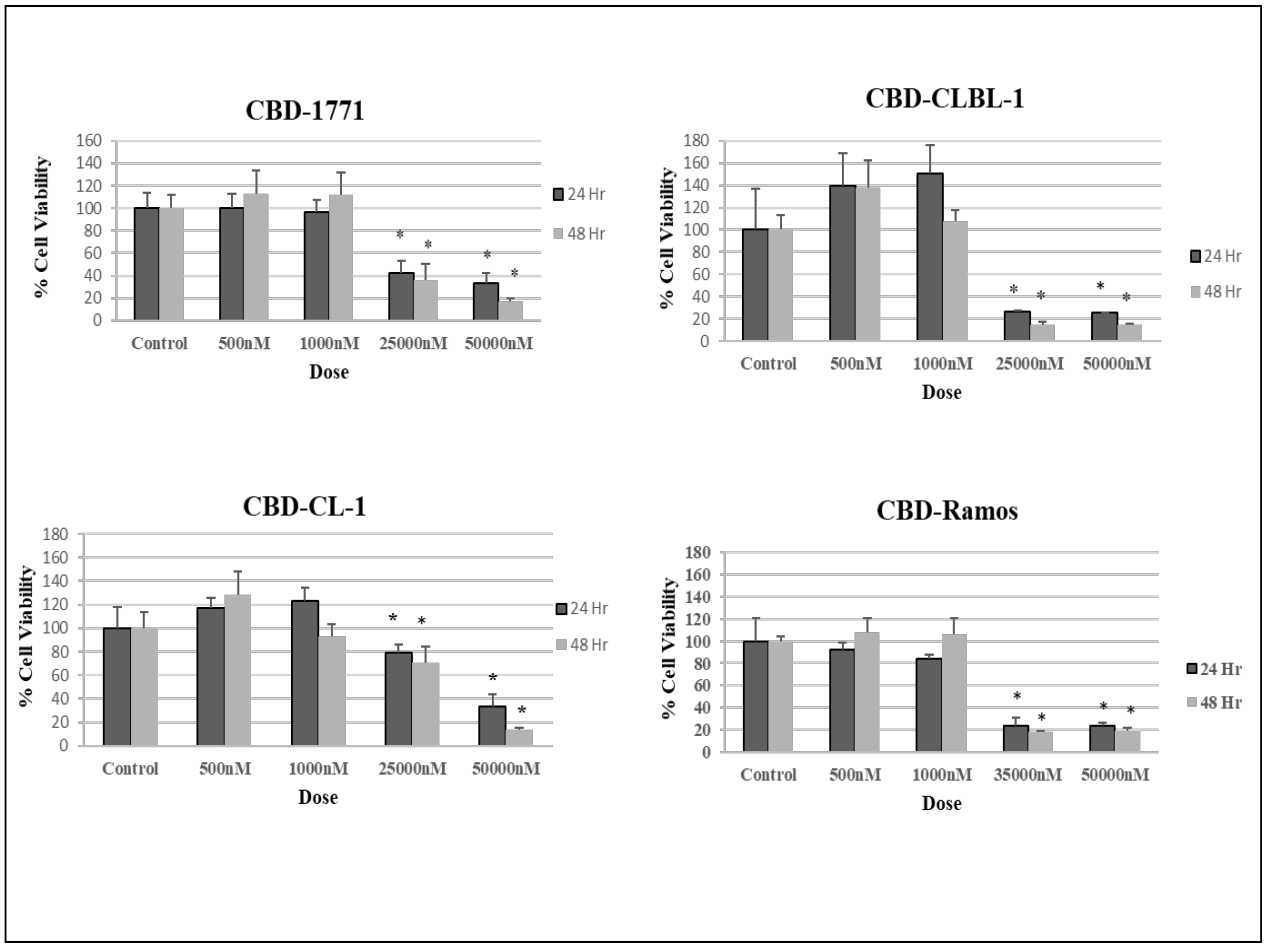


Figure 4-2 Effect of CBD on canine and human NHL cells viability

Canine and human lymphoma cells were treated with CBD at 0.1-50µM concentration, for 24 h and 48 h. MTT assay was used to evaluate the lymphoma cell viability. Results are expressed as (%) change as compared to the control, Mean ± SD. **a**: CBD dose-dependently decreased cell viability significantly as compared to the control ($n=12$, $p < 0.0001$) in all NHL cell lines. No significant time-dependent effect could be demonstrated.

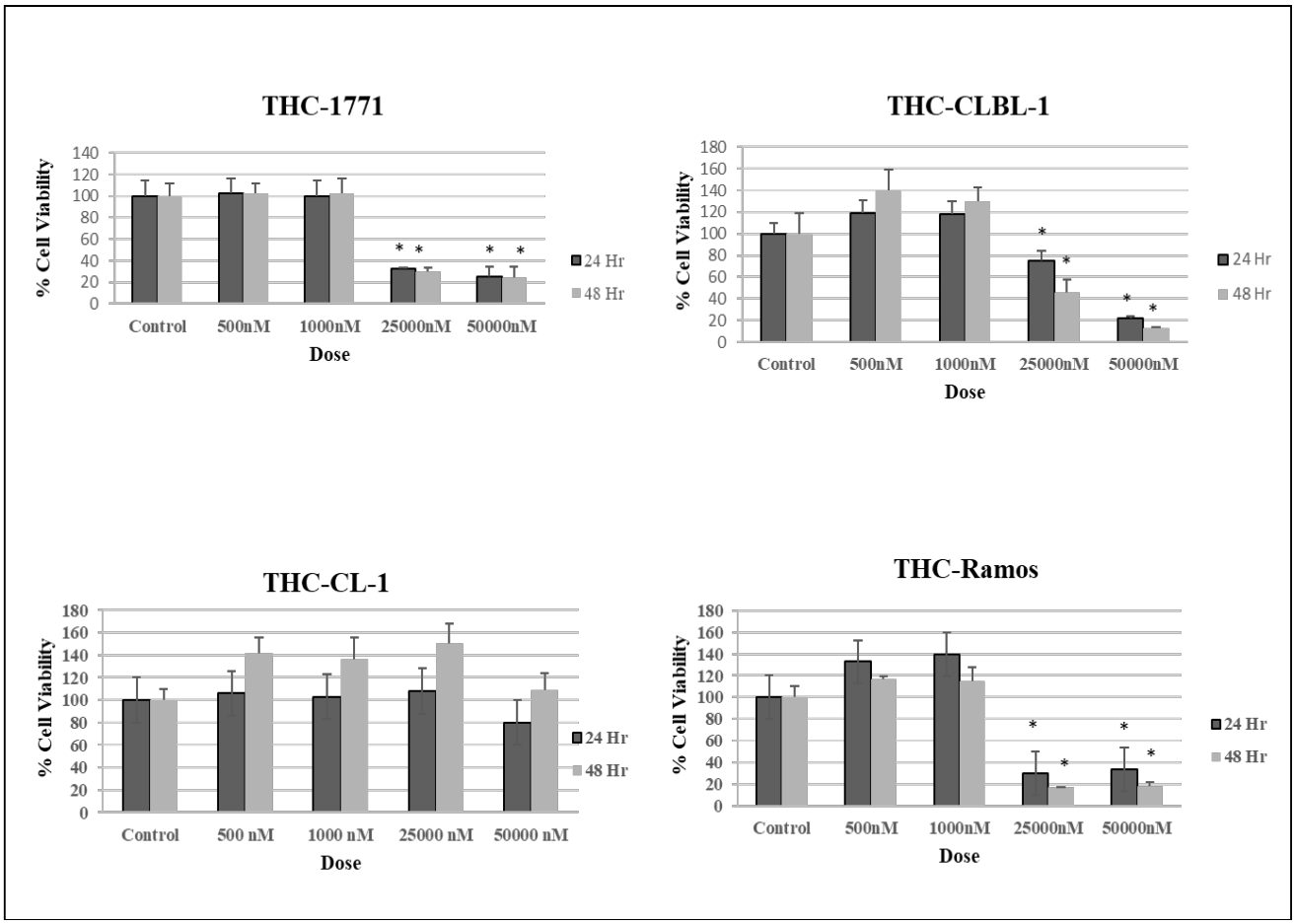
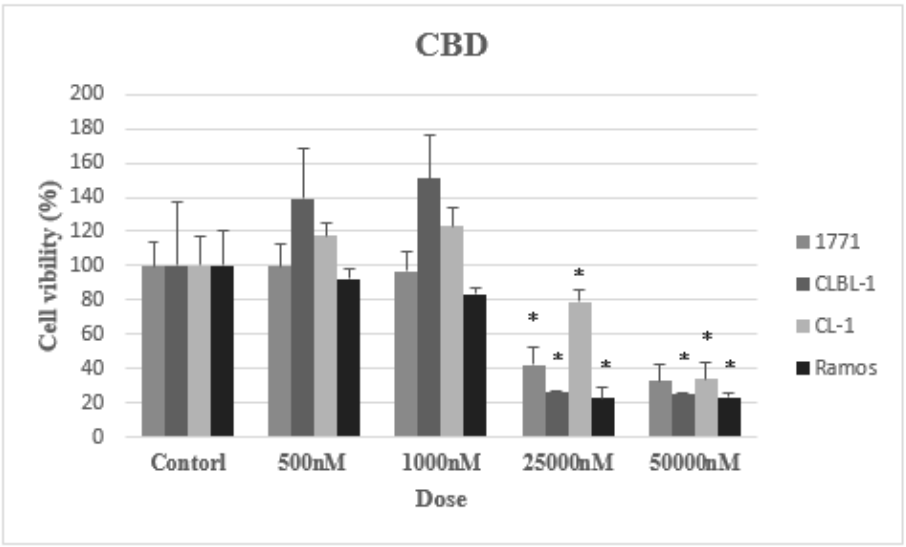


Figure 4-3 Effect of THC on canine and human NHL cells viability

Results are expressed as (%) change as compared to the control, Mean ± SD. **a:** THC stimulated cell viability at lower drug concentrations followed by dose-dependent decrease in cell viability in 1771, CLBL-1 and Ramos as compared to the control ($n=12$, $p < 0.0001$). Significant dose or time-dependent effect on cell viability could not be demonstrated on CL-1 cell line treated with THC.

a.



b.

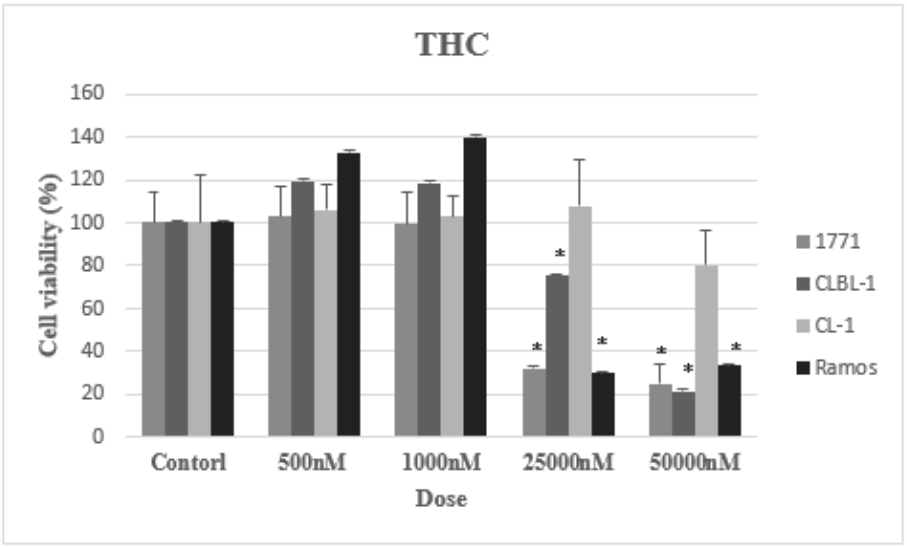


Figure 4-4 Effect of Phytocannabinoids on B and T cell lymphoma cells at 24 Hour

a. b. Both phytocannabinoids at 24 hours demonstrated a stimulatory effect on cell viability at lower doses followed by a decrease in cell viability from 1-50µM as compared to the control (n=12, $p < 0.0001$). However, no significant effect of THC could be demonstrated on CL-1. Results are expressed as (%) change as compared to the control, Mean ± SD.

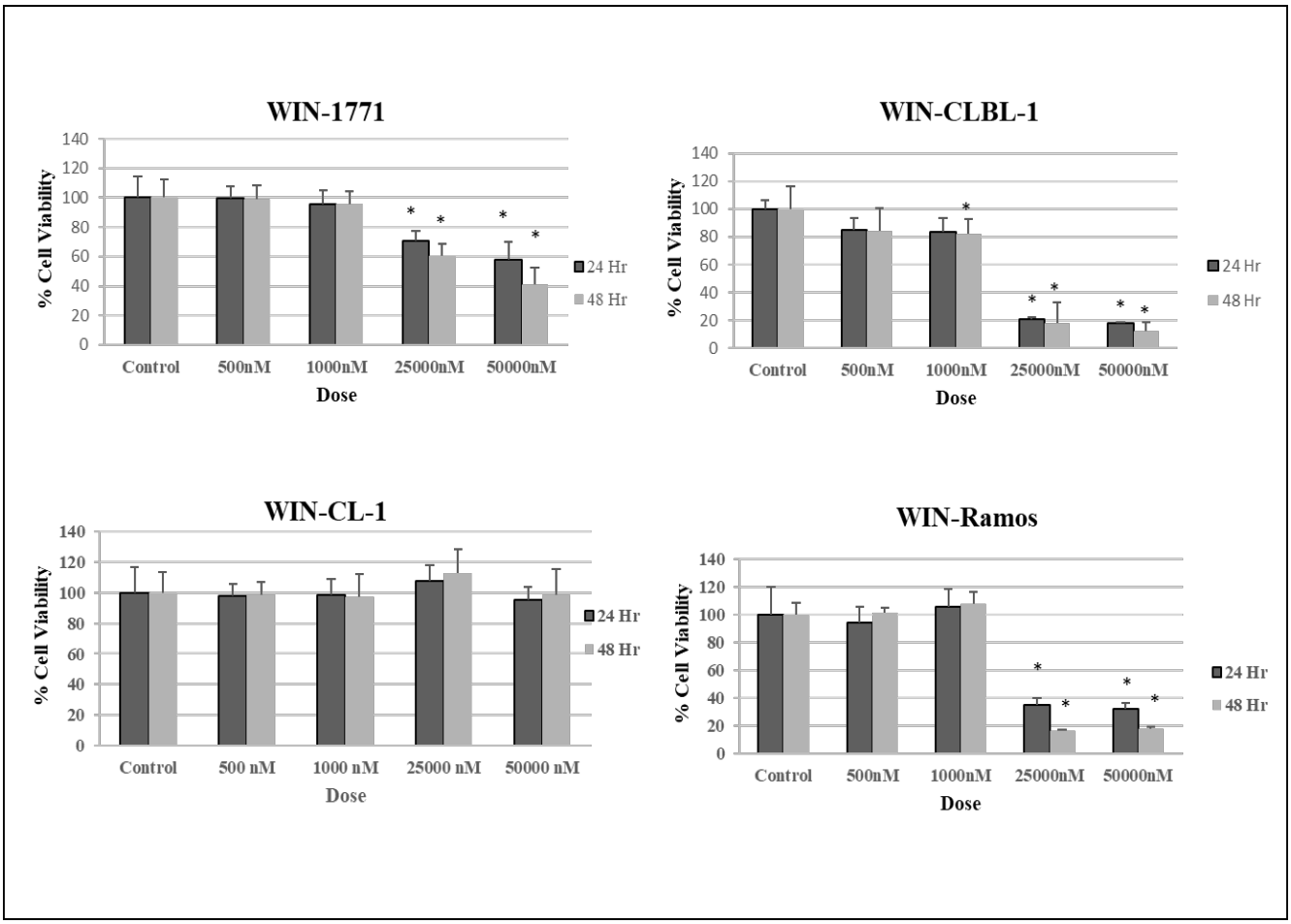


Figure 4-5 Effect of WIN on canine and human NHL cells viability

Canine and human lymphoma cells were treated with WIN at 0.1-50µM concentration, for 24 h and 48 h. MTT assay was used to evaluate the lymphoma cell viability. Results are expressed as (%) change as compared to the control, Mean ± SD. **a**: WIN dose-dependently decreased cell viability significantly as compared to the control ($n=12$, $p < 0.0001$) in all NHL cell lines except for CL-1. No significant time-dependent effect could be demonstrated.

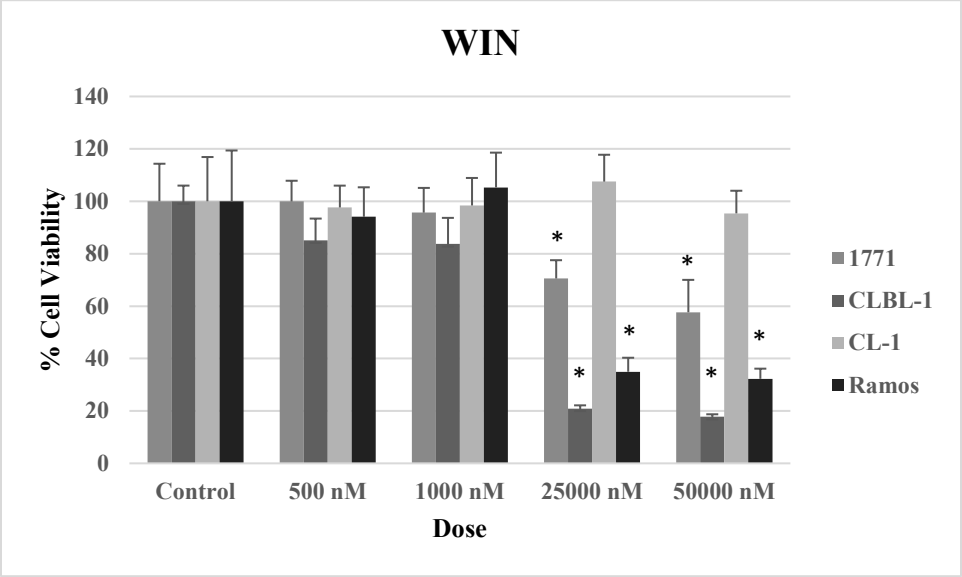


Figure 4-6 Effect of WIN on B and T cell lymphoma cells at 24 Hour

Results are expressed as (%) change as compared to the control, Mean ± SD. **a**: WIN dose-dependently decreased cell viability significantly as compared to the control ($n=12$, $p < 0.0001$) in all NHL cell lines except for CL-1.

Comparison of endogenous and exogenous cannabinoids effect on canine and human NHL cell viability

To compare the effects of exogenous cannabinoids used in this study with endogenous cannabinoid used in our previous study on NHL cell lines, we analyzed the dose-response curve for each cannabinoid and calculated the lowest dose at which 50% of lymphoma cell viability reduced (IC50) using the following formula:

$$\mathrm{IC50} = \frac{50 - Intercept\ estimate}{Concentration\ estimate}$$

According to our results of LD50 comparison, among exogenous cannabinoids, CBD is the most potent exogenous cannabinoid and AEA is the most potent endogenous cannabinoid against NHL cells used in this study. Both endogenous and exogenous cannabinoids are more effective against B cell lymphoma cell lines compared to the T cell lymphoma cell line. Among B cell lymphoma cell line cannabinoids are more effective against CLBL-1 and Ramos cell lines compared to the 1771 cell line. A similar pattern was observed when the effect of all cannabinoids was compared among all NHL cell lines at the highest dose used in the study.

Table 4-3 IC50 of Endo, Phyto and Synthetic cannabinoids in NHL cell lines.

Cell Line	Cannabinoid	IC50
1771	AEA	34 µM
	2AG	36 µM
	CBD	30 µM
	THC	40 µM
	WIN	53 µM
CLBL-1	AEA	25 µM
	2AG	48 µM
	CBD	35 µM
	THC	37 µM
	WIN	24 µM
CL-1	AEA	79 µM
	2AG	878 µM
	CBD	35 µM
	THC	135 µM
	WIN	161 µM
Ramos	AEA	29 µM
	2AG	27 µM
	CBD	23 µM
	THC	32 µM
	WIN	28 µM

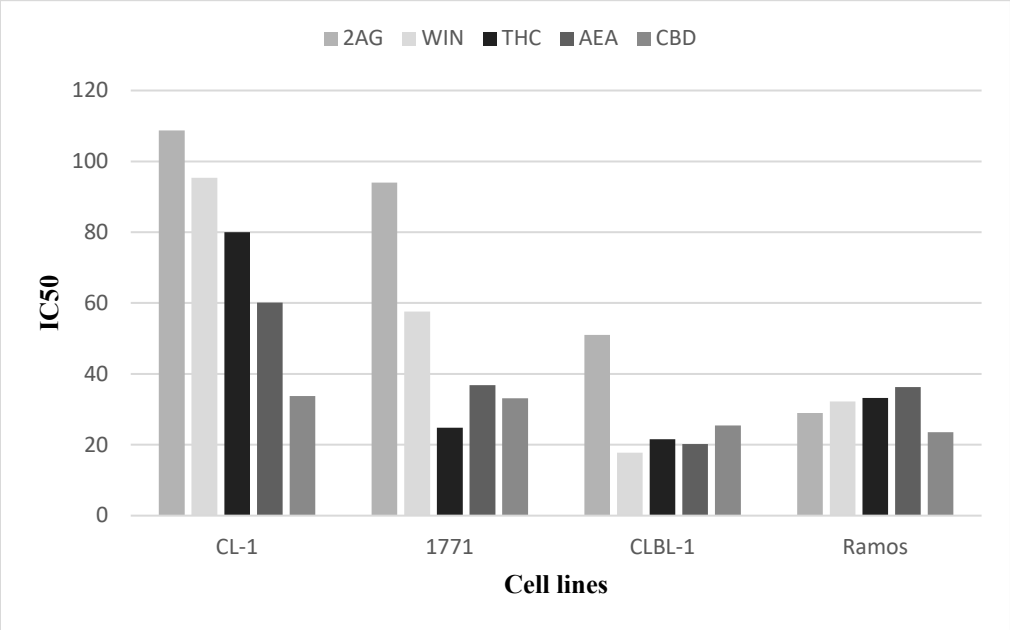


Figure 4-7 IC50 values of endocannabinoids, phytocannabinoids and synthetic cannabinoids in NHL cell lines.

Effect of exogenous cannabinoids on oxidative stress

Treatment with exogenous cannabinoids increased oxidative stress as seen by the significant increase in the generation of reactive oxygen species, NADH, H_2O_2 and a decrease in glutathione content (Fig. 10a, 13a, n=5, $p < 0.05$). However, the variable effect of CBD and THC observed on other markers of oxidative stress analyzed, THC significantly increased H_2O_2 however, we could not demonstrate a significant effect of CBD on H_2O_2. A significant increase in Nitrite content observed with CBD but opposite effect observed with THC (Fig. 11a, n=5, $p < 0.05$).

Exogenous cannabinoids induce inflammation in 1771 lymphoma cell line

Treatment of lymphoma cells with phytocannabinoids induced inflammatory form of programmed cell death as seen by the changes in the activity of inflammatory markers ICE-1 and COX. THC induced a significant dose-dependent increase in the activities of ICE-1 and COX (Fig. 15a and 16a, n=5, $p < 0.05$). CBD also caused an increase in ICE-1 and COX activity however, we could not demonstrate increase in ICE-1 activity at the level of significance.

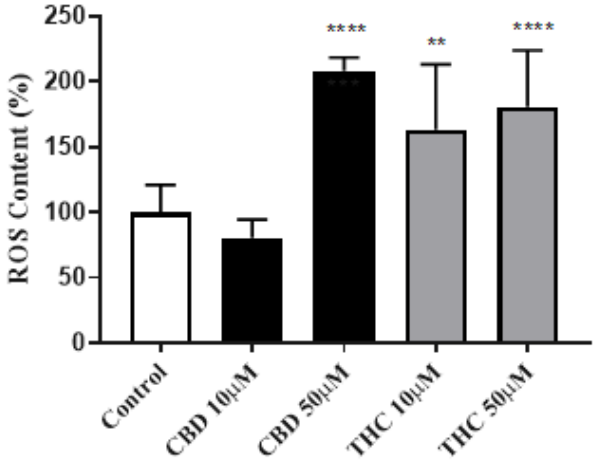
Phytocannabinoids *induce apoptosis*

Changes in the activity of various apoptotic markers indicated the apoptotic effect of phytocannabinoids against all lymphoma cell lines. Treatment with THC caused a significant increase in all 3 of the caspases analyzed. Similarly, CBD dose-dependently increased activitycaspase-8, and caspase-9. The impact on caspase-3 as a numerical, but not statistic increase in activity. (Fig. 17b, 18b, 19b).

Effect of exogenous cannabinoids on mitochondrial function

In cells treated with THC no significant effect on complex-1 activity could be demonstrated. This is in contrast to treatment with with either CBD or WIN, for which a a s complex-1 activity was significantly decreased when compared to the control (n=5, $p < 0.05$; Fig. 19a).

a.



b.

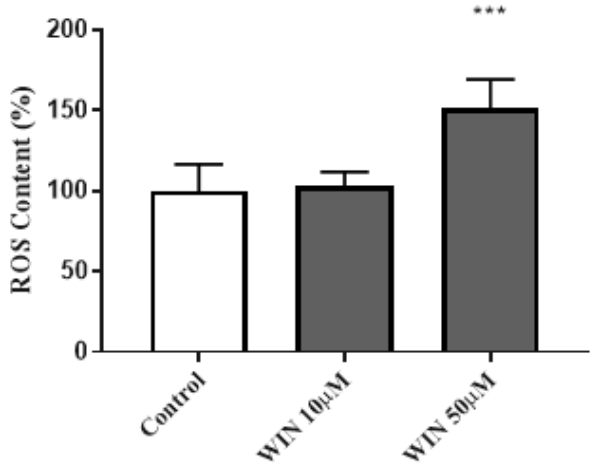
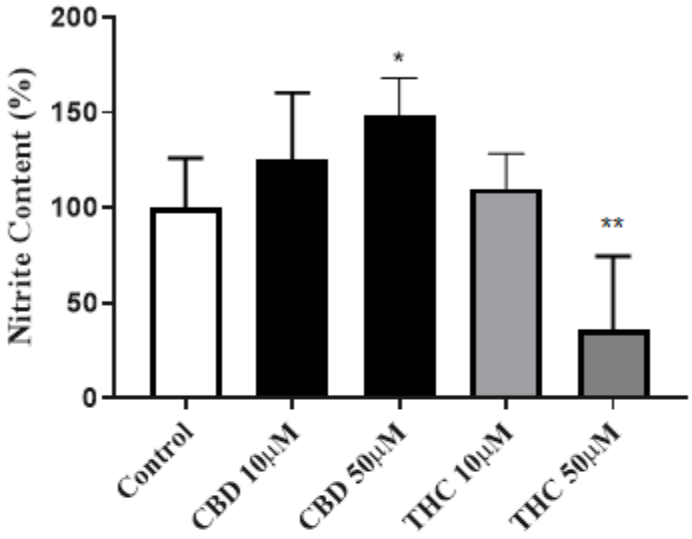


Figure 4-8 Effect of exogenous cannabinoids on canine 1771 B-cell lymphoma cells reactive oxygen species generation

Reactive oxygen species was measured spectrofluorimetrically. **a.** phytocannabnoids **(**CBD and THC) and **b**. synthetic cannabinoid (WIN) showed a significant dose-dependent increase in reactive oxygen species generation as compared to the control (*$p < 0.0001$, n=5). Vehicle was used as a control. Results are expressed as % change as compared to the control, Mean ± SD.

a.



b.

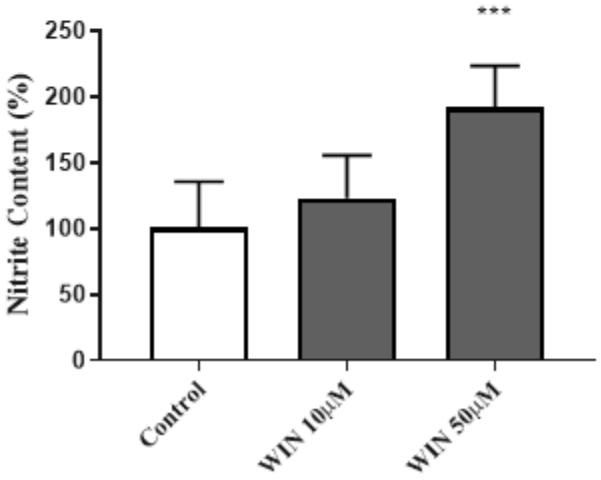


Figure 4-9 Effect of exogenous cannabinoids on canine 1771 B-cell lymphoma cells nitrite content

Nitrite content was measured spectrophotometrically. **a.** CBD showed a significant dose-dependent increase and THC caused significant dose-dependent decrease in nitrite content as compared to the control ($*p < 0.0001$, n=5). **b.** WIN induced a significant dose-dependent increase in nitrite content. Vehicle (50 µM) was used as a control. Results are expressed as % change as compared to the control, Mean ± SD.

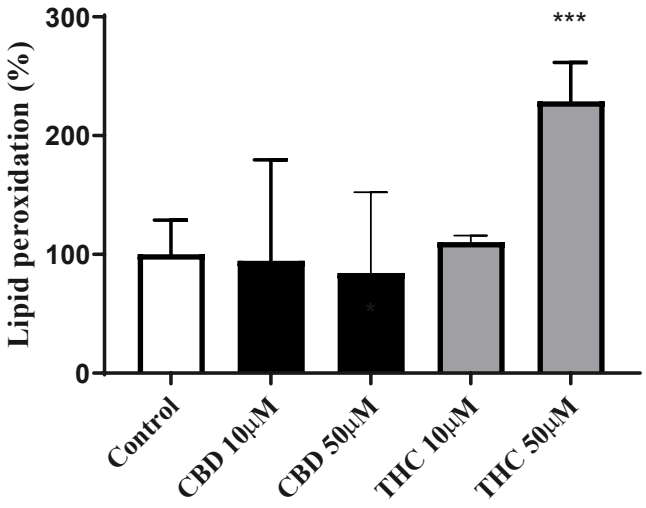


Figure 4-10 Effect of CBD and THC on canine 1771 B-cell lymphoma cells lipid peroxide formation

Lipid peroxide was measured spectrophotometrically. Due to the increased reactive oxygen species generation THC induced a significant dose-dependent formation of lipid peroxide ($*p < 0.05$, n=5). No significant lipid peroxide formation could be demonstrated in cells treated with CBD. Vehicle (50 μM) was used as a control. Results are expressed as % change as compared to the control, Mean ± SD.

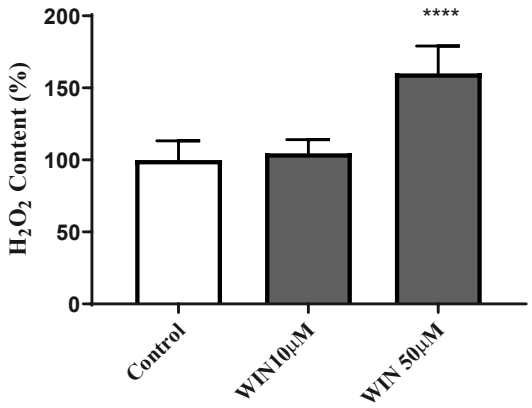
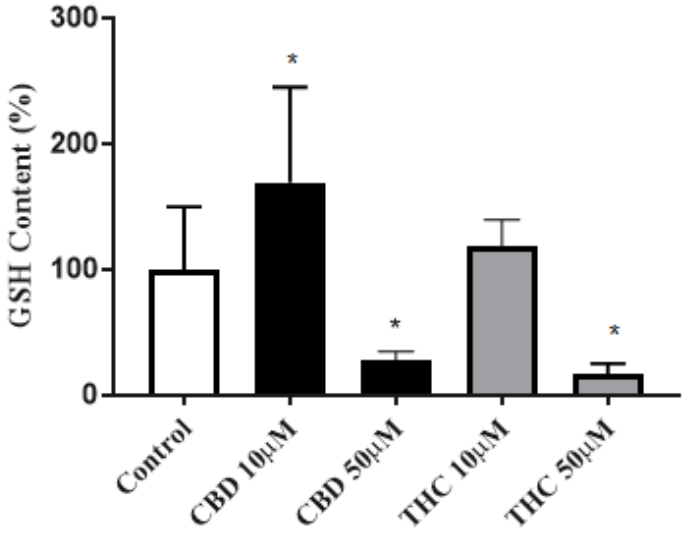


Figure 4-11 Effect of WIN on canine 1771 B-cell lymphoma cells Hydrogen peroxide content

Hydrogen peroxide content was measured spectrophotometrically. **a.** WIN induced a dose-dependent increase in H_2O_2 content as compared to the control. Vehicle (50 μM) used as positive control Vehicle (50 μM) was used as a control. Results are expressed as % change as compared to the control, Mean ± SD.

a.



b.

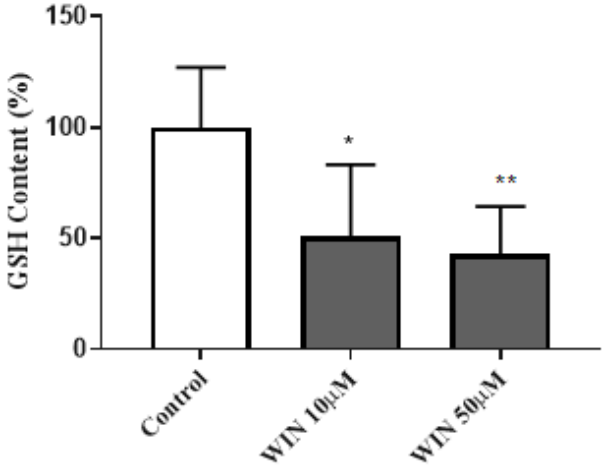
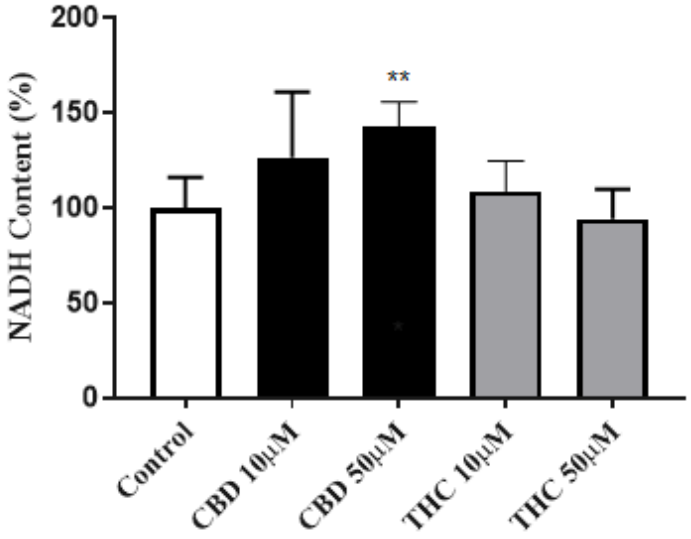


Figure 4-12 Effect of exogenous cannabinoids on canine 1771 B-cell lymphoma cells glutathione content

Glutathione content was measured spectrophotometrically. **a.** Phytocannabinoids and **b.** Synthetic cannabinoid agonist induced a significant dose-dependent depletion in GSH content ($*p < 0.05$, n=5). Vehicle (50 μM) was used as a control. Results are expressed as % change as compared to the control, Mean ± SD.

a.



b.

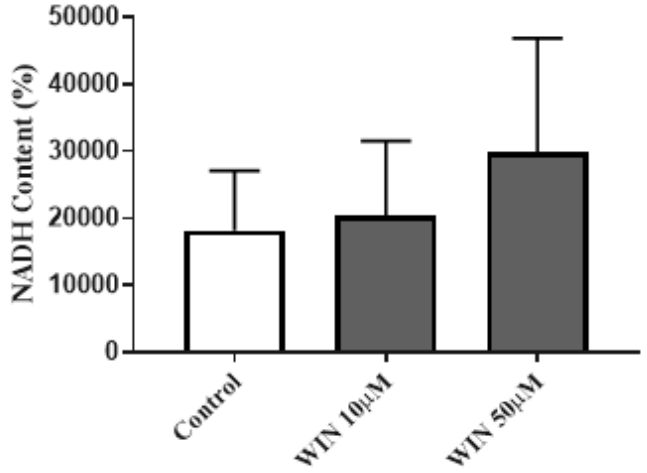
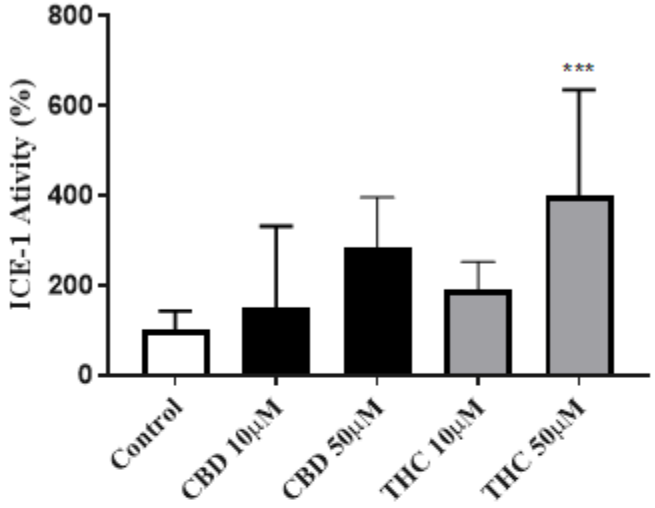


Figure 4-13 Effect of exogenous cannabinoids on canine 1771 B-cell lymphoma cells NADH content

NADH content was measured spectrophotometrically. **a.** CBD induced a significant dose-dependent increase in NADH content ($*p < 0.0001$, n=5) as compared to the control. Cells treated with THC and **b.** WIN increased NADH content, however not at the level of significance at this time point and dose. Vehicle (50 μM) used as a control did not affect. Results are expressed as % change as compared to the control, Mean ± SD.

a.



b.

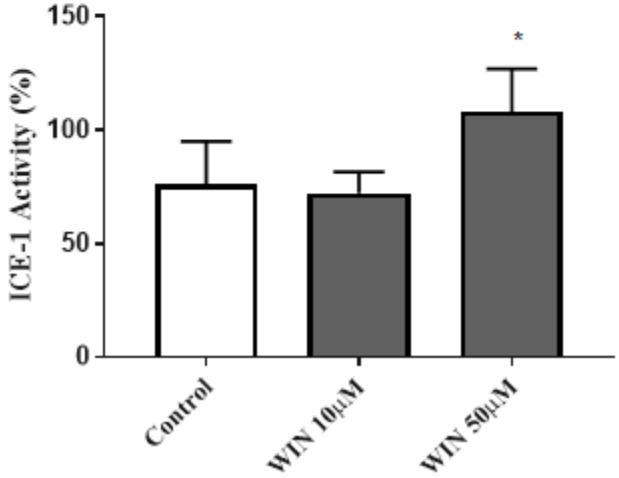
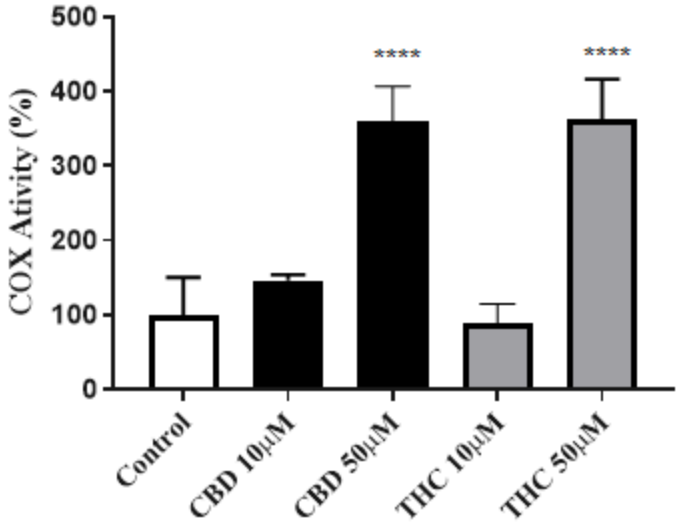


Figure 4-14 Effect of exogenous cannabinoids on canine 1771 B-cell lymphoma cells ICE-1 activity

ICE-1 activity was measured spectrofluorimetrically using AC-YVAD-AMC as substrate. **a.** Lymphoma cells treated with THC demonstrated significant dose-dependent increased in ICE-1 activity ($*p < 0.05$, n=5). CBD and **b.** WIN also increased ICE-1 activity but not significantly as compared to the control. Vehicle (50 μM) used as a control did not affect. Results are expressed as % change as compared to the control, Mean ± SD.

a.



b.

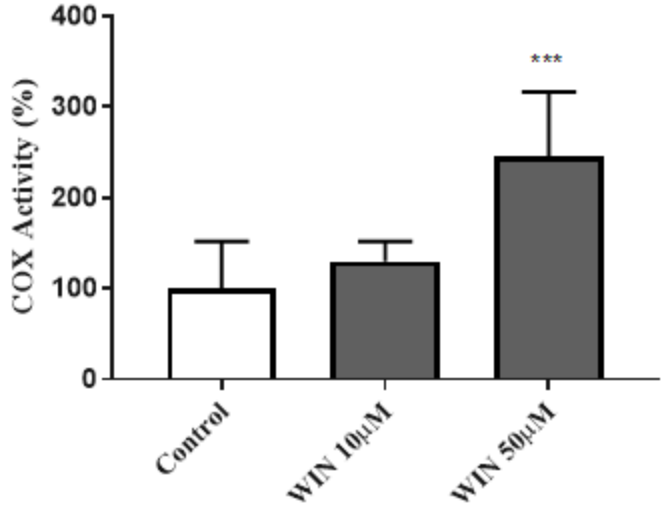
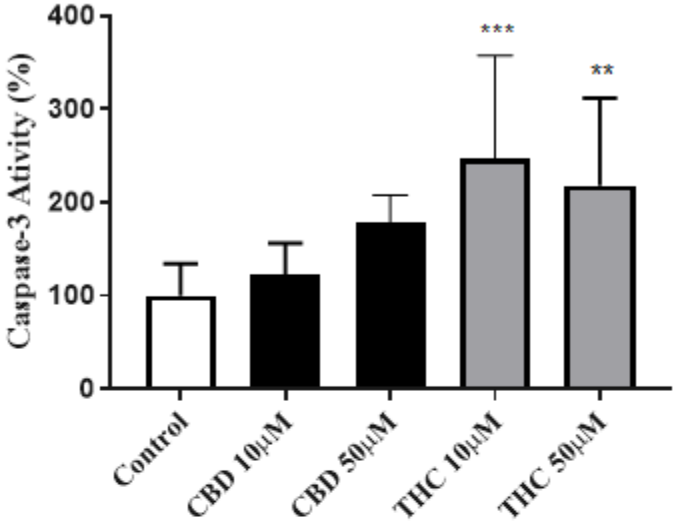


Figure 4-15 Effect of exogenous cannabinoids on canine 1771 B-cell lymphoma cells Cyclooxygenase activity

Cyclooxygenase was quantified calorimetrically by using N, N, N', N'-tetramethyl-p-phenylenediamine (TMPD) as a substrate. **a.** Phytocannabinoids and **b.** WIN significantly increased cyclooxygenase activity ($*p < 0.05$, n=5) as compared to the control. Vehicle (50 μM) was used as a control. Results are expressed as % change as compared to the control, Mean ± SD.

a.



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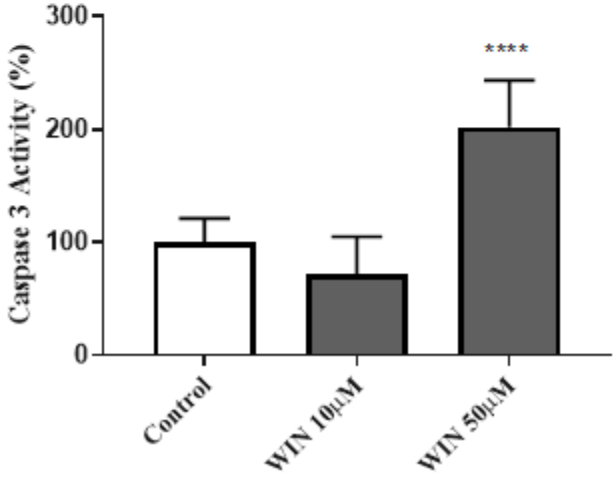
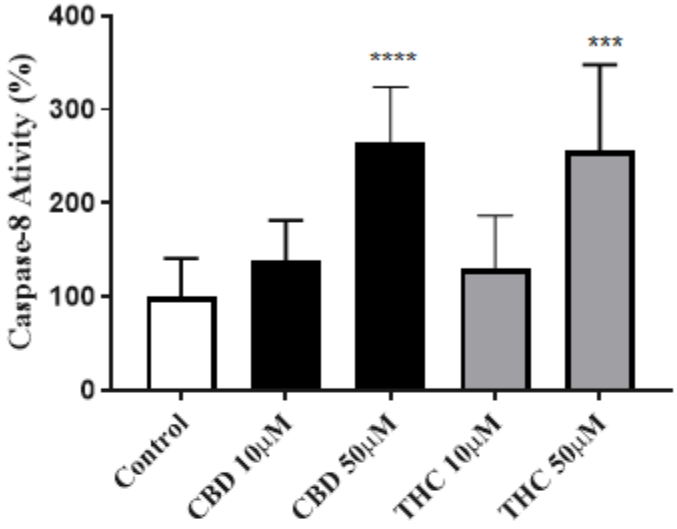


Figure 4-16 Effect of exogenous cannabinoids on canine 1771 B-cell lymphoma cells Caspase-3 activity

Caspase-3 activity was measured spectrofluorimetrically using AC-DEVD-AMC as substrate. **a.** CBD and THC dose-dependently increased Caspase-3 activity. Significant dose-dependent effects could not be demonstrated with CBD as compared to the control. **b.** Significant increase in caspase-3 activity demonstrated in cells treated with WIN. Results are expressed as % change as compared to the control, Mean ± SD.

a.



b.

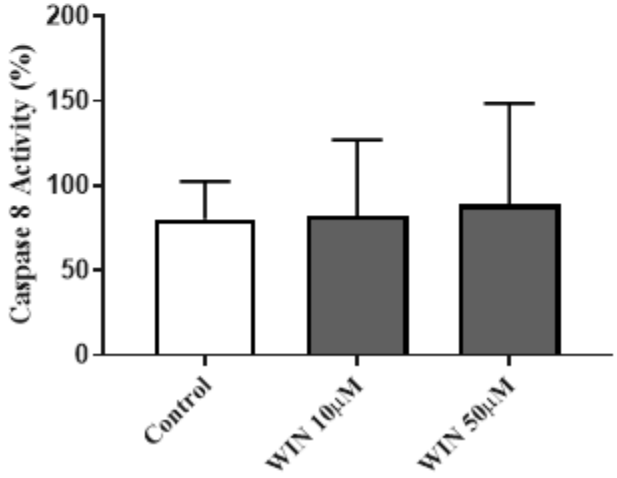
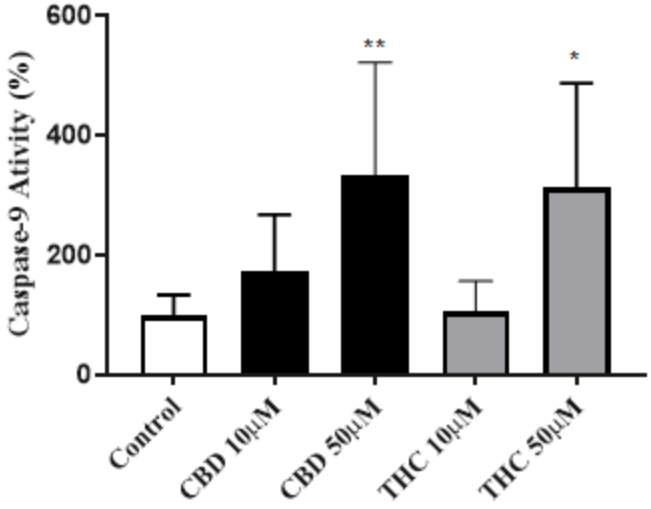


Figure 4-17 Effect of AEA and 2AG on canine 1771 B-cell lymphoma cells Caspase-8 activity

Caspase-8 activity was measured spectrofluorimetrically using Ac-VETD-AMC as substrate. **a.** phytocannabinoids significantly increased Caspase-8 activity dose-dependently. **b.** with WIN significant effect on the caspase-8 activity could not be demonstrated as compared to the control. Results are expressed as % change as compared to the control, Mean ± SD.

a.



b.

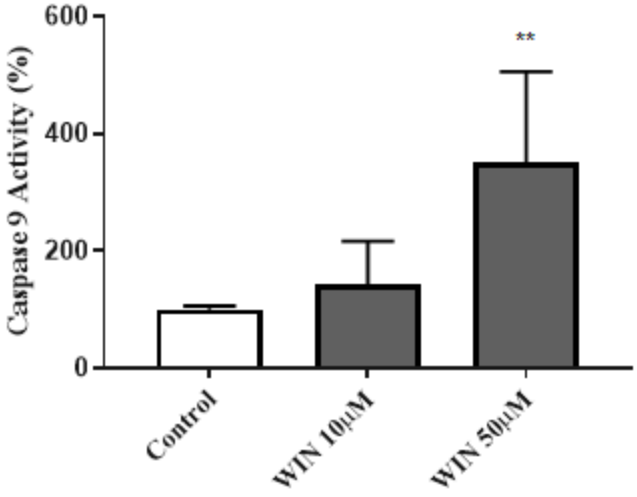
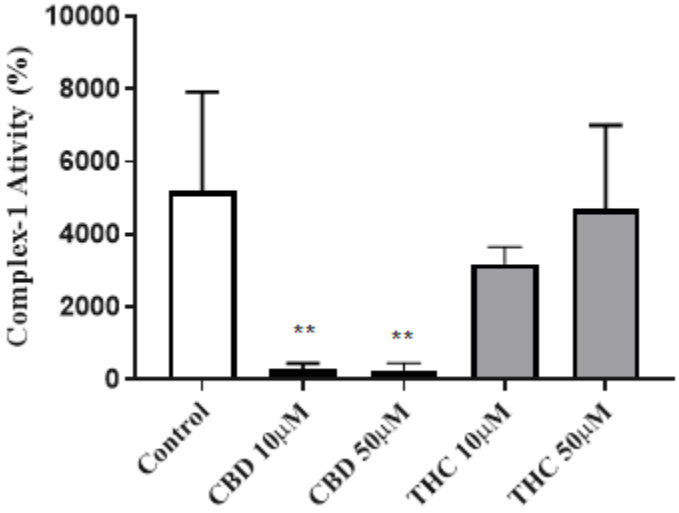


Figure 4-18 Effect of exogenous cannabinoids on canine 1771 B-cell lymphoma cells Caspase-9 activity

Caspase-9 activity was measured spectrofluorimetrically using Ac-LEHD-pNa as substrate. **a.** Both phytocannabinoids and **b.** the synthetic cannabinoid, WIN significantly increased Caspase 9 activity as compared to the control. Results are expressed as % change as compared to the control, Mean ± SD.

a.



b.

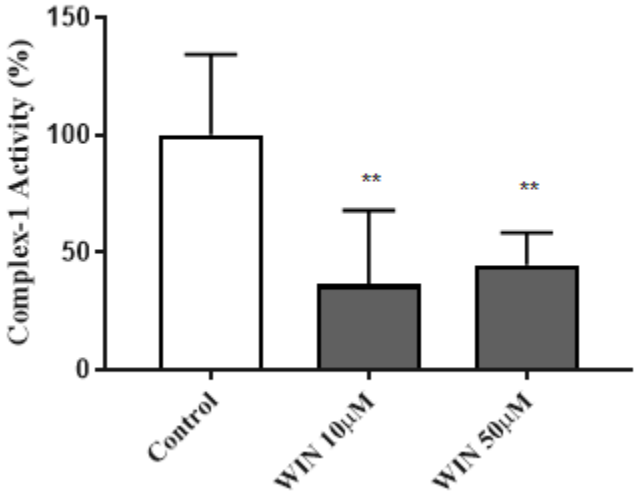


Figure 4-19 Effect of exogenous cannabinoids on canine 1771 B-cell lymphoma cells Complex-I activity

Complex-I activity was measured spectrophotometrically using NADH as substrate. **a.** CBD and **b.** WIN showed a significant decrease in Complex-I activity ($*p < 0.05$, n=5) as compared to the control. Significant effect on Complex-1activity activity could not be demonstrated with THC. Results are expressed as % change as compared to the control, Mean ± SD.

Table 4-4 Summary of Biochemical analysis results.

Cellular response to cytotoxicity		**Lymphoma cells response to cannabinoid-induced cytotoxicity**		
		CBD	THC	WIN
Markers of oxidative stress				
ROS	↑	↑	↑	↑
GSH	↓	↓	↓	↓
NADH	↑	↑	↔	↔
H_2O_2	↑	ND	ND	↑
Nitrite	↑	↑	↓	↑
Lipid peroxidation (LP)	↑	↔	↑	ND
Markers of inflammation				
ICE-1	↑	↔	↑	↑
COX	↑	↑	↑	↑
Markers of apoptosis				
Caspase-3	↑	↔	↑	↑
Caspase-8	↑	↑	↑	↔
Caspase-9	↑	↑	↑	↑
Markers of mitochondrial function				
Complex-1	↓	↓	↔	↓

[↑ =increased, ↓ =decreased, ↔ = no significant effect, ND = not determined]

4.5 Discussion

It is now well accepted that uncontrolled cellular growth, which may be a result of a defect in the cell cycle and apoptotic pathway, is one of the leading causes for the development of cancers including lymphoma. Therefore the agents which can modulate apoptosis in cancer cells may be able to reduce tumor load and may be useful in cancer treatment. One of the most important and promising areas of the current cannabinoid research is the ability of these compounds to control cell survival/death decision.

We have previously studied the endocannabinoid receptors and the effects of endocannabinoids in using canine and human NHL cell lines. Our results demonstrated positive expression of cannabinoid receptors, CB1 and CB2 in both canine and human NHL with significant higher expression in B cell lymphoma compared to the T cell. Also, we have demonstrated the anti-proliferative and apoptotic effect of endocannabinoids in NHL. In the current study, we expanded our research from endogenous to exogenous cannabinoids and for the first time, we have demonstrated **1.** The effects of phytocannabinoids and synthetic cannabinoids on canine NHL cell lines; **2.** Compared the effect of endogenous and exogenous cannabinoids on canine B versus T cell lymphoma and **3.** Compared the effects of cannabinoids between canine and human NHL cell lines.

Our results of cell viability assay demonstrated the stimulation of cell growth at lower concentrations and a dose-dependent decrease in cell viability at higher concentrations of phytocannabinoids. CBD decreased cell viability in both B and T cell lymphoma cell lines on the other hand THC and synthetic cannabinoid agonist, WIN, decreased cell viability in only B cell lymphoma cell lines significantly. Lower expression of CB1 and CB2 receptors in canine T cell lymphoma cells as compared to the B cell lymphoma as reported in our previous study can be the reason for the non-responsive behavior of CL-1 against THC and WIN, selective cannabinoid receptor agonists. CBD-induced cytotoxicity in CL-1 can potentially be through receptors other than CB1 and CB2, as CBD has shown to activate the transient receptor potential of vanilloid subtype 1 (TRPV1), G-protein coupled receptor GPR55, the 5-HT1a receptor and the α3 and α1 glycine receptors. [206] Several other lymphoma studies have also demonstrated similar effects of exogenous cannabinoids on cell viability [152, 166, 207, 208].

To study the mechanism of the cytotoxic effect of cannabinoids, markers of oxidative stress, inflammation, apoptosis and mitochondrial function were analyzed in the 1771 cell line. Our results of the biochemical analysis revealed increase in ROS, H_2O_2, nitrite, lipid peroxidation and a decrease in GSH content in cells treated with phyto or synthetic cannabinoids. Various cannabinoid studies have also demonstrated increased in oxidative stress with exogenous and endogenous cannabinoids in multiple cancer types [209] [210, 211].

Cyclooxygenase and ICE-1 are known to initiate pro-inflammatory mechanisms leading to cell death. Overexpression of COX and ICE-1 in cells treated with CBD, THC and WIN indicates the possibility of induction of an inflammatory pathway involved in cannabinoid induced decrease in lymphoma cell viability. Similar effect of CBD has been demonstrated in glioblastoma [212, 213].

Several cancer studies have demonstrated cannabinoid induced apoptosis and associated it with increased caspase activity. We have also demonstrated increase in the activity of caspases in lymphoma cells treated with phyto or synthetic cannabinoids [95, 151, 209, 210].

The inhibitory effect of cannabinoids on the mitochondrial respiratory chain has been demonstrated in past studies. Similarly, we have found a decrease in complex-1 activity with WIN and THC as compared to the control. However, we could not demonstrate significant effect of CBD on complex-1 activity. [214]

In this study, we have also compared the effect of endo, phyto, and synthetic cannabinoids in lymphomas by calculating their LD_{50} values for each cell line. We found CBD and AEA as a more potent phytocannabinoid and endocannabinoid as compared to THC and 2-AG respectively. Overall lymphomas with higher expression of cannabinoid receptors responded more to the cannabinoid treatment compared to one with lower expression, Ramos > CLBL-1 > 1771 > CL-1.

4.6 Conclusion

Overall our study demonstrated inhibitory effect phyto and synthetic cannabinoids on NHL cell growth with CBD as the most potent exogenous cannabinoid. We could not show the inhibitory effect with THC and WIN on canine T cell lymphoma cell line that expresses very low expression of cannabinoid receptors as compared to B cell lymphoma cell lines, as observed in our previous study. Our results also revealed that exogenous cannabinoids decrease lymphoma cell viability as a result of increased oxidative stress leading to apoptosis.

5. In Vitro Cytotoxicity of Cannabinoids in Combination with Traditional Lymphoma Chemotherapeutic Drugs against Non-Hodgkin Lymphoma

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5.1 Abstract

Cannabinoids (CBs) are known for their palliative effects against chemotherapy-induced side effects (e.g. pain, nausea, vomiting, and loss of appetite) and some of the CBs are currently FDA approved for the treatment of chemotherapy-induced side effects in cancer treatment. However, in the last two decades, various in vitro and in vivo studies have also demonstrated their effect against tumor growth and progression in many models of human cancers including non-Hodgkin lymphoma (NHL) and mantle cell lymphoma. Previously, we demonstrated the cytotoxic effect of endogenous and exogenous cannabinoids on human and canine B and T cell type NHL cell lines. The purpose of this study was to demonstrate the cytotoxic effect of cannabinoids in combination with the components of CHOP, a traditional NHL chemotherapy regimen. (cyclophosphamide, doxorubicin, vincristine, prednisone, lomustine). We hypothesized that the synergistic combination of cannabinoids and components of NHL chemotherapy (NLC) drugs might be more effective against cancer cells compared to their alone treatments. For this study, three cannabinoids were studied, one from each of the three major categories of cannabinoids (endocannabinoid AEA, Phytocannabinoid CBD, and synthetic cannabinoid WIN-55 212 22). Each cannabinoid was selected based on potency as determined in our previous experiments. For the combination, we have used five NHL chemotherapy drugs. We used canine malignant B type NHL cell line 1771, cells were cultured in RPMI. The cytotoxicity of each drug alone and combinations was analyzed by colorimetric MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5 Diphenyl tetrazolium Bromide) cell proliferation assay and Combination index (CI) analyses based on the Chou-Talalay method was used to study the combinational effect. Our results demonstrate that the cytotoxic effects of all traditional NHL chemotherapy drugs are synergistically enhanced (interaction with CI <1) by each of the three cannabinoids when added to 1771 canine malignant B type NHL cells. This work provides proof-of-concept for using cannabinoids and traditional NHL drugs in combination to reduce the dose, and therefore the toxicity, of chemotherapeutic drugs and possibly increasing the survival benefit in lymphoma clinical translation studies.

5.2 Introduction

Cancer is one of the most common causes of death. As a major global health concern, it is characterized by a persistently t high incidence and mortality rate in both humans and canines [215]. Therapeutic options and chemotherapeutic regimens vary depending on the type of cancer. The majority of chemotherapeutic drugs target genes or proteins associated with cancer cell proliferation or survival pathways [216]. However, most of these drugs cause severe side effects due to their cytotoxicity on normal non-target cells. In addition, cancer cells also develop drug resistance through multiple mechanisms, including increased efflux [217]. Combination therapy has long been adopted as the standard first-line treatment of many malignancies in order to induce synergistic drug actions, deter the onset of drug resistance, and improve the clinical outcome [218-220]. However, such combinations increase the risk of host toxicity and while not totally resolving other factors contributing to therapeutic failure, such as drug resistance. [221]. Therefore, a novel combination of chemotherapeutic drugs is required to address the current chemotherapy-related issues [222].

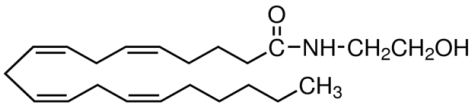
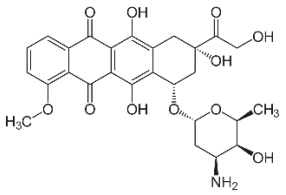
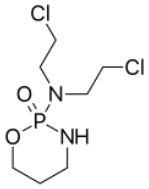
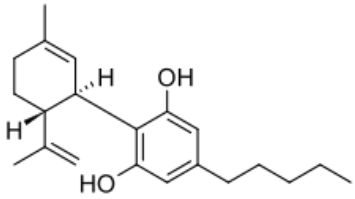
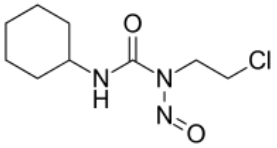
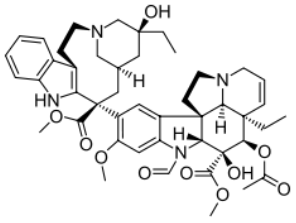
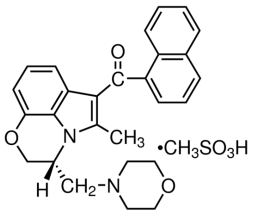
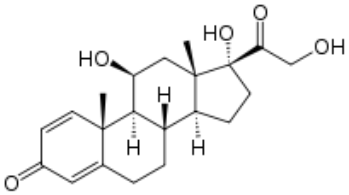
Canine lymphoma (cL) is a common type of neoplasia in dogs with an estimated incidence rate of 20-100 cases per 100,000 dogs and is in many respects comparable to non-Hodgkin lymphoma in humans. Canine and human lymphoma are generally characterized by a high rate of initial remission following conventional CHOP (cyclophosphamide, (hydroxy)doxorubicin, vincristine, and prednisone) based therapies; however, 95% of dogs and 30% of humans will succumb to drug-resistant relapse [223, 224]. In humans, new therapies have improved response to the treatment with standard chemotherapy. However, there is still a relapsed or refractory group with poor prognosis and treatment of canine lymphoma has not benefitted from these human treatments. [188, 225, 226]. For this reason, there is an essential need to find new therapeutic approaches for both human and canine drug-resistant NHL.

In the past two decades, cannabinoids have been extensively studied for their anti-cancer effects in various models of cancers and have demonstrated promising effects against tumor growth and metastasis [102, 227, 228]. In our previous studies, we have also demonstrated the anti-cancer effects of endogenous and exogenous cannabinoids in canine and human NHL cell lines. In the present study, we aimed to analyze the effects of cannabinoids in combination with traditional lymphoma chemotherapeutic drugs in canine B-cell lymphoma.

5.3 Materials and methods

5.3.1 Chemicals

Table 5-1 Chemical structures and abbreviations of cannabinoids and chemotherapeutic drugs used.

Cannabinoids (CBs) Panel	NHL Chemotherapy drugs panel (NLC)
Anandamide (AEA)-Endocannabinoid 	Doxorubicin-(DOX) 
	Cyclophosphamide-(CYC) 
Cannabidiol (CBD)-Phytocannabinoid 	Lomustine-(LOM) 
	Vincristine-(VIN) 
WIN 55-212 22 (WIN))-Synthetic Cannabinoid 	Prednisolone-(PRD) 

5.3.2 Reagents

Thiazolyl Blue Tetrazolium Bromide (MTT) was purchased from American Type Culture Collection. Penicillin-Streptomycin solution was purchased from Thermofisher Scientific (Waltham, MA). RPMI 1640 Medium, ES Cell Qualified Fetal Bovine Serum (FBS) and L-Glutamine Solution were purchased from EMD Millipore (Burlington, MA). Phosphate buffer saline (PBS), dimethylsulfoxide (DMSO) were purchased from Sigma Aldrich (St. Louis, MO). All the cannabinoids (AEA, CBD and WIN Table.1) and chemotherapeutic drugs (DOX, VIN, PRD and LOM Table.1) were purchased from Sigma Aldrich (St. Louis, MO).

5.3.3 Cell maintenance

Canine B-cell lymphoma cell line 1771 was generously shared by Dr. Steven Suter's Lab (North Carolina State University). All cells were cultured in RPMI 1640 Medium, supplemented with fetal bovine serum (10%), penicillin-streptomycin (1%), and L-Glutamine (1%). For the cell viability assay, lymphoma suspension cells were grown and harvested via centrifuge. Cells were then seeded into 96 well plates at a density of 1×10^4 cells/well. Cells were incubated under standard conditions at 37 °C and supplemented with 5% CO_2.

5.3.4 IC_{50} calculation

To calculate the IC50 of each cannabinoid and chemotherapeutic drug, each drug was dissolved in its respective vehicle and then further dissolved in the media to desired concentrations. Cells were treated with the individual drug for 24 or 48 hours depending on the drug. Dose-response curve generated using SAS and GraphPad Prism®. The IC50 value for each drug calculated using the following formula;

$$\mathrm{IC50} = \frac{50 - Intercept\ estimate}{Concentration\ estimate}$$

Where intercept estimate and concentration estimate were calculated using linear regression analysis [229] in SAS statistical package [230].

5.3.5 Drug combination

All drugs were dissolved in their respective vehicle and further diluted in RPMI-1640 media to the desired concentrations. Drug mixtures for calculation of CI values were based on the median effect

analysis method [231]. Two-fold serial dilutions of working concentrations were prepared in RPMI-1640 with three concentrations above and three concentrations below the calculated IC50 for each drug in each cell line. These ratios corresponded to 0.12, 0.25, 0.5, 1, 2, 4, 8 times the IC50 for each drug. Cannabinoid/chemotherapeutic drug mixtures were made using those ratios for each drug. Each concentration was tested in 8 replicates for individual drug and combinations and the results were confirmed in at least three independent experiments.

5.3.6 Cell Viability

The effect of the drug combinations on the viability of cells was determined by (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) (MTT) assay. The cells were plated at $1x10^4$ cells per well in 100µl of complete culture medium containing the appropriate drugs for 24 or 48 hours (depending on the drug) at 37ºC in a humidified chamber. After incubation for specified times at 37ºC in a humidified incubator, MTT reagent (10µl) was added to each well and incubated for 4 hours followed by adding (100µl) solubilization solution in each well to dissolve formazan crystals. Color absorbance (OD) was recorded on Appliskan® microplate reader at 570 nm wavelength. The effect of drugs on cell viability was assessed as the percentage of inhibition in cell viability where vehicle-treated cells were taken as 100% viable. Each experiment was repeated three times.

5.3.7 Data analyses

Data from MTT viability assays were expressed as the mean ± SD. MTT data were analyzed by GraphPad Prism® to produce dose-response curves for cannabinoids and chemotherapeutic drugs using nonlinear analysis. To calculate drug effect, the mean OD values for each drug concentration were subtracted from the mean OD values of cells treated with vehicle only and the resulting fractions (between 0-100%) were plotted against drug concentrations in a logarithmic scale. combination index (CI) values were determined by the third generation "CompuSyn" software written by Nick Mart of MIT using the median-effect method[232], derived from the mass-action law principle, which is the unified theory that provides the common link between single entity and multiple entities, and first order and higher order dynamics. This general equation encompasses the Michaelis-Menten, Hill, Henderson-Hasselbalch, and Scatchard equations in biochemistry and biophysics [231].

$$CI = \frac{CA, X}{ICX, A} + \frac{CB, X}{ICX, B}$$

Where CA, X and CB, X are the concentrations of cannabinoids and chemotherapeutic drugs used in combination to achieve X% drug effect. The ICX, A and ICX, B are the concentrations for single drugs (cannabinoids or chemotherapeutic) that achieve the same effect. The sum of CA, X/ICX, A and CB, X/ICX, B is defined as the combination index at effect X as indicated by the CI equation above. Synergy is defined as CI <1; additivity is defined as CI = 1, and antagonism is defined as CI> 1.

5.4 Results

5.4.1 Determination of IC50 for Cannabinoids and NLC drugs in 1771 lymphoma cells

To identify the cannabinoids: NLC drugs interaction as synergism, additive effect, or antagonism, we calculated the dose-effect curves for cannabinoids and NLC drugs applied singly to canine B cell lymphoma cells. This step generates the IC50--the drug concentration causing 50% cell growth inhibition for each drug--that is requisite for the CI calculation. CI<1 indicates synergism, CI=1 indicates additive effect, and CI>1 indicates antagonism. Two-fold serial dilutions of each drug were used in the *in vitro* MTT experiments and data showed that CBs or NLC treatments inhibited the proliferation of 1771 cells with variable IC50 values. CBs IC50s were 14, 23 and 69 μM for AEA, CBD and WIN, respectively (Fig 1). NLC drug values were 0.73, 31, 0.25, 27 and 44 μM respectively for DOX, VIN, CYC, LOM and PRD respectively (Fig 2).

5.4.2 Combination of CBs and NLC drugs synergistically caused death of 1771 B lymphoma cells

To determine if CBs augment NLC drugs-induced cell death in a synergistic or additive manner, we performed dose-effect MTT experiments using 1771 lymphoma cells. Isobologram[233] and CI analyses showed that the CI for drug combinations was <1 in all combinations. However, some synergistic data points were simulated at low Fa > 0.5 (Fa = Fraction affected) which means less relevant to therapy. In the case of CBs combination with CYC all data points showing synergistic relationship were simulated at Fa > 0.5 (Table 2 and Fig 3-7). These observations demonstrated

that at low doses combination of CBs and NLC drugs caused significantly greater cell growth inhibition as compared to treatment with any drug alone.

5.4.3 CBs combined with low doses of NLC drugs synergistically inhibited 1771 B-cell lymphoma cell growth

We conducted cell viability assays on 1771 cells using the IC50 concentrations for each drug singly or in combination. The IC50 concentration of CBs or NLC drugs caused approximately 50% cell death whereas CBs combined with NLC drugs at the IC50 concentration for each caused a significant increase in cell death (Fig 8, 10, 11). However, we could not demonstrate a significant synergistic effect of any CBs on CYC and AEA on DOX when combined at IC50 concentrations for each (Fig 9).

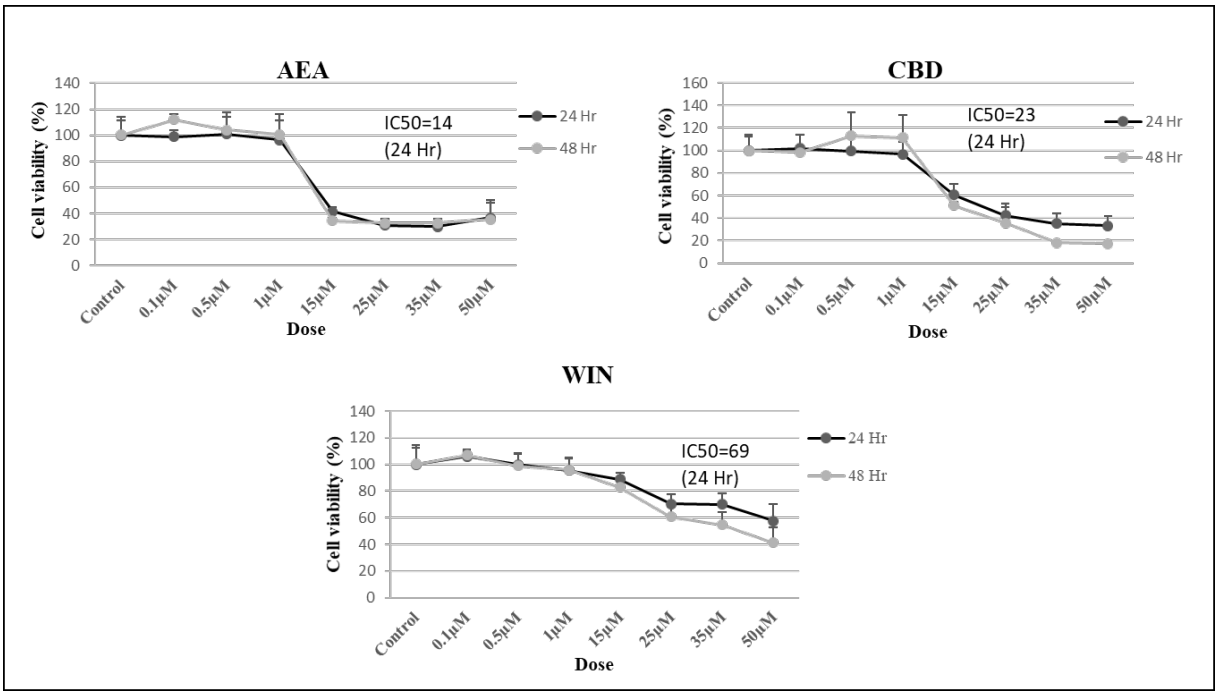


Figure 5-1 IC50 of Cannabinoids (AEA, CBD and WIN)

Calculation of IC50 for Cannabinoids (AEA, CBD and WIN) using MTT dose-response curves expressed as the drug concentration vs viability/proliferation of 1771 lymphoma cells. CBs IC50s were 14, 23 and 69 µM for AEA, CBD and WIN respectively in 1771 cells.

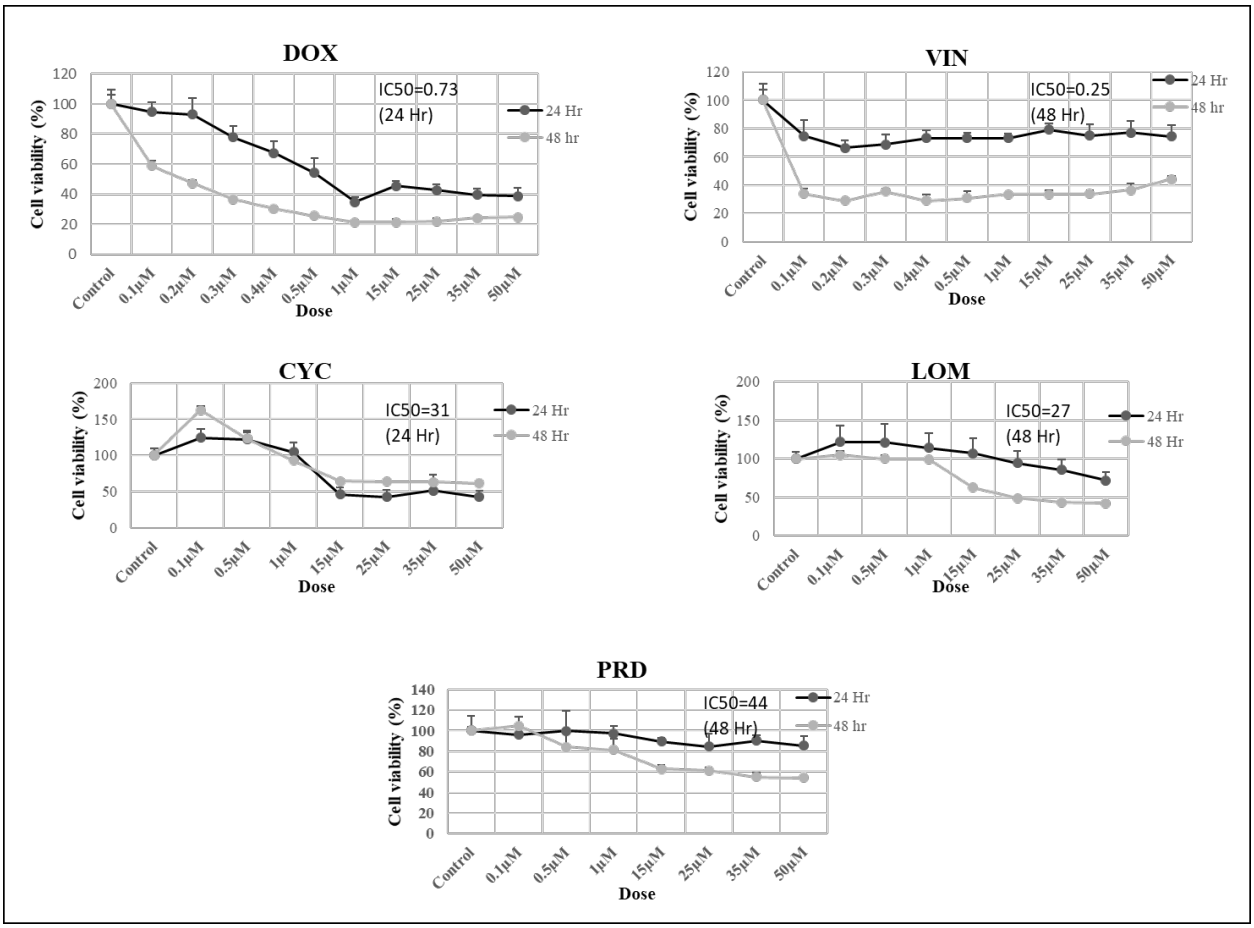


Figure 5-2 IC50 of NLC drugs (DOX, CYC, VIN, LOM and PRD)

Calculation of IC50 for CHOP drugs (DOX, CYC, VIN, LOM and PRD) using MTT-dose response curves expressed as the drug concentration vs viability/proliferation of 1771 lymphoma cells. CHOP drugs IC50s were 0.73, 31, 0.25, 27 and 44 μM for DOX, CYC, VIN, LOM and PRD respectively in 1771 cells.

Table 5-2 Combination index (CI) at 50-95% effect

	Combination index (CI) at 50-95% effect			
Drug combination	**ED50**	**ED75**	**ED90**	**ED95**
AEA/DOX	0.78232	0.34562	0.17554	0.11614
CBD/DOX	0.62255	0.28710	0.13356	0.07974
WIN/DOX	0.45266	0.21730	0.10565	0.06518
AEA/CYC	< 0.001	< 0.001	< 0.001	< 0.001
CBD/CYC	< 0.001	< 0.001	< 0.001	< 0.001
WIN/CYC	< 0.001	< 0.001	< 0.001	< 0.001
AEA/VIN	0.66737	0.52721	0.41795	0.35757
CBD/VIN	0.66684	0.43908	0.28932	0.21794
WIN/VIN	0.46094	0.28730	0.18033	0.13186
AEA/LOM	0.98415	0.75361	0.57993	0.48661
CBD/LOM	0.92988	0.58054	0.36441	0.26628
WIN/LOM	0.92655	0.78793	0.67335	0.60671
AEA/PRD	0.41611	34.6162	10311.2	497964.
CBD/PRD	0.46768	0.89025	4.48310	14.9966
WIN/PRD	0.26785	0.59590	6.96326	42.1908

[CI < 1 indicates synergism; CI > 1 indicates antagonism; and CI = 1 indicates additive effect. CI is obtained by dividing CBs IC50/ NLC drugs IC50]

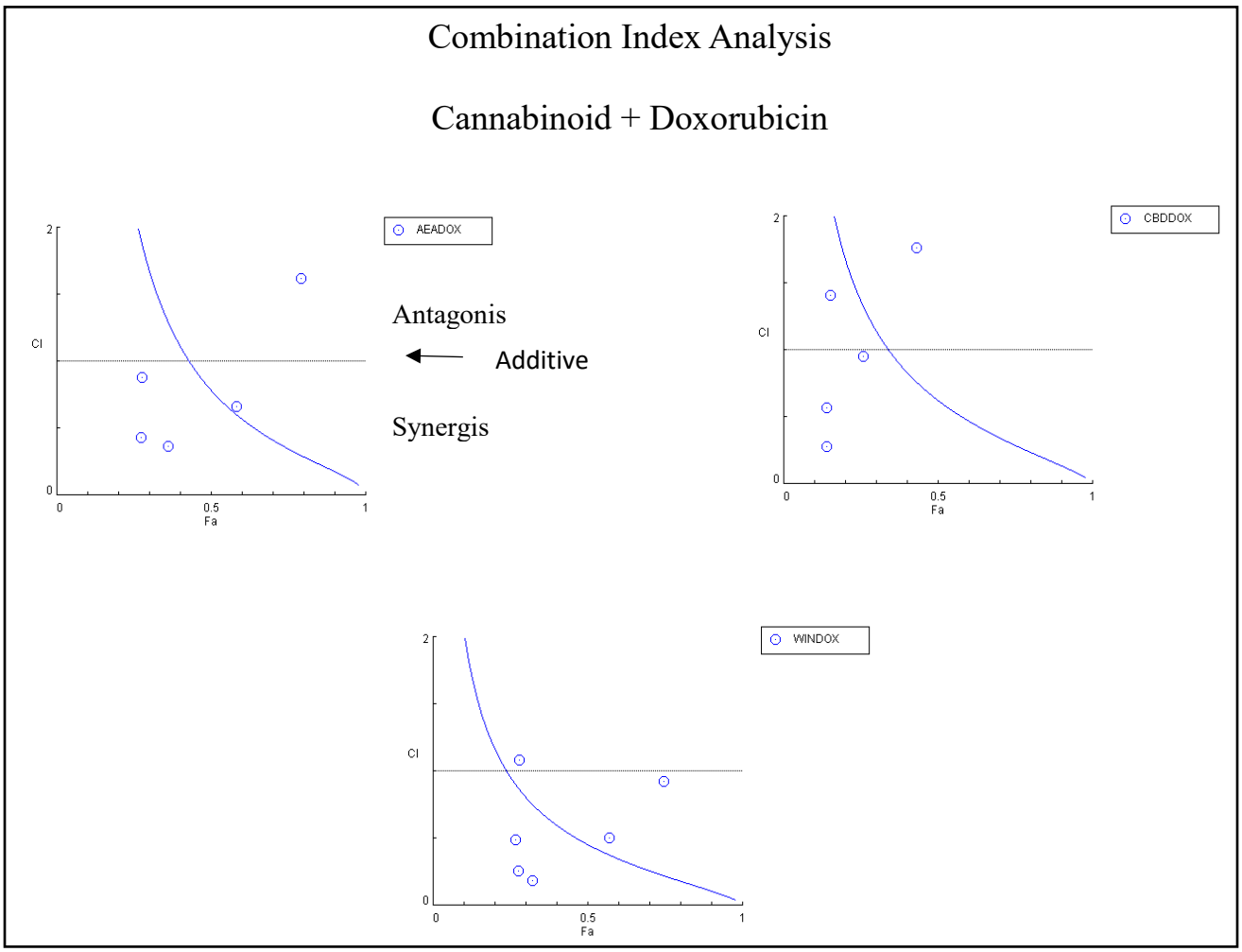


Figure 5-3 Combination index plot CBs+DOX

The *Combination index plot* A (AEADOX) represents 5 combination data points, 4 of which demonstrate a synergistic relationship (CI < 1) and one data point describes an antagonistic relationship. Plot B (CBDDOX) represents 5 combination data points, 3 of which demonstrate a synergistic relationship (CI < 1) and the other two antagonistic. Plot C (WINDOX) represents 6 combination data points, 5 of which demonstrate a synergistic relationship (CI < 1) and one data point describes an additive relationship. Simulations at low Fa > 0.5 show less relevance to therapy since cancer cell death in small numbers is less useful.

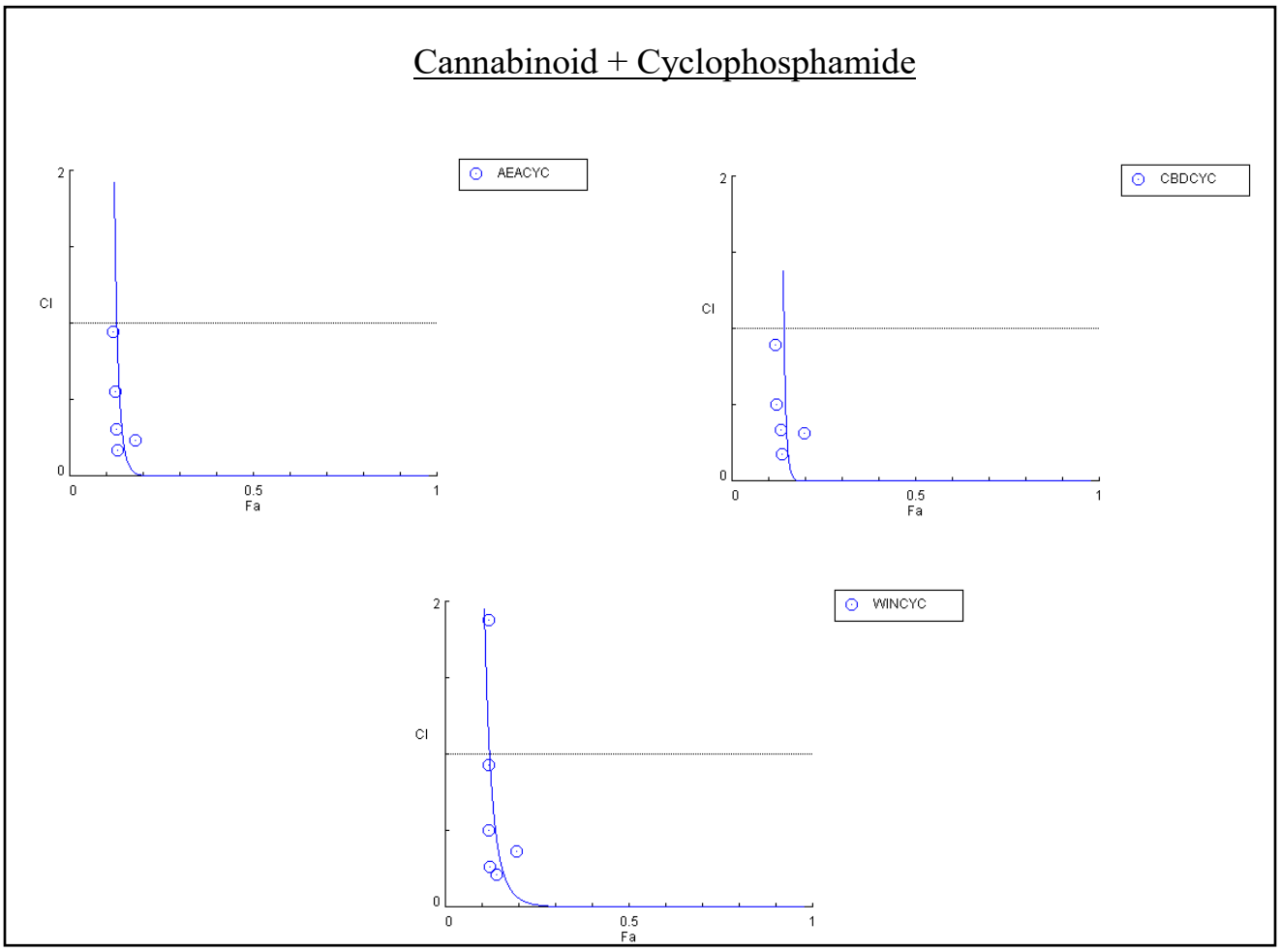


Figure 5-4 Combination index plot CBs+CYC

The *Combination index plot* AEACYC and CBDCYC represents 5 combination data points, all demonstrate synergistic relationship (CI < 1) however, at Fa > 0.5. Plot WINCYC represents 6 combination data points, 5 of which demonstrate a synergistic relationship (CI < 1) and one data point describes an antagonistic relationship, all data points simulated at Fa > 0.5. Simulations at low Fa > 0.5 show less relevance to therapy since cancer cell death in small numbers is less useful.

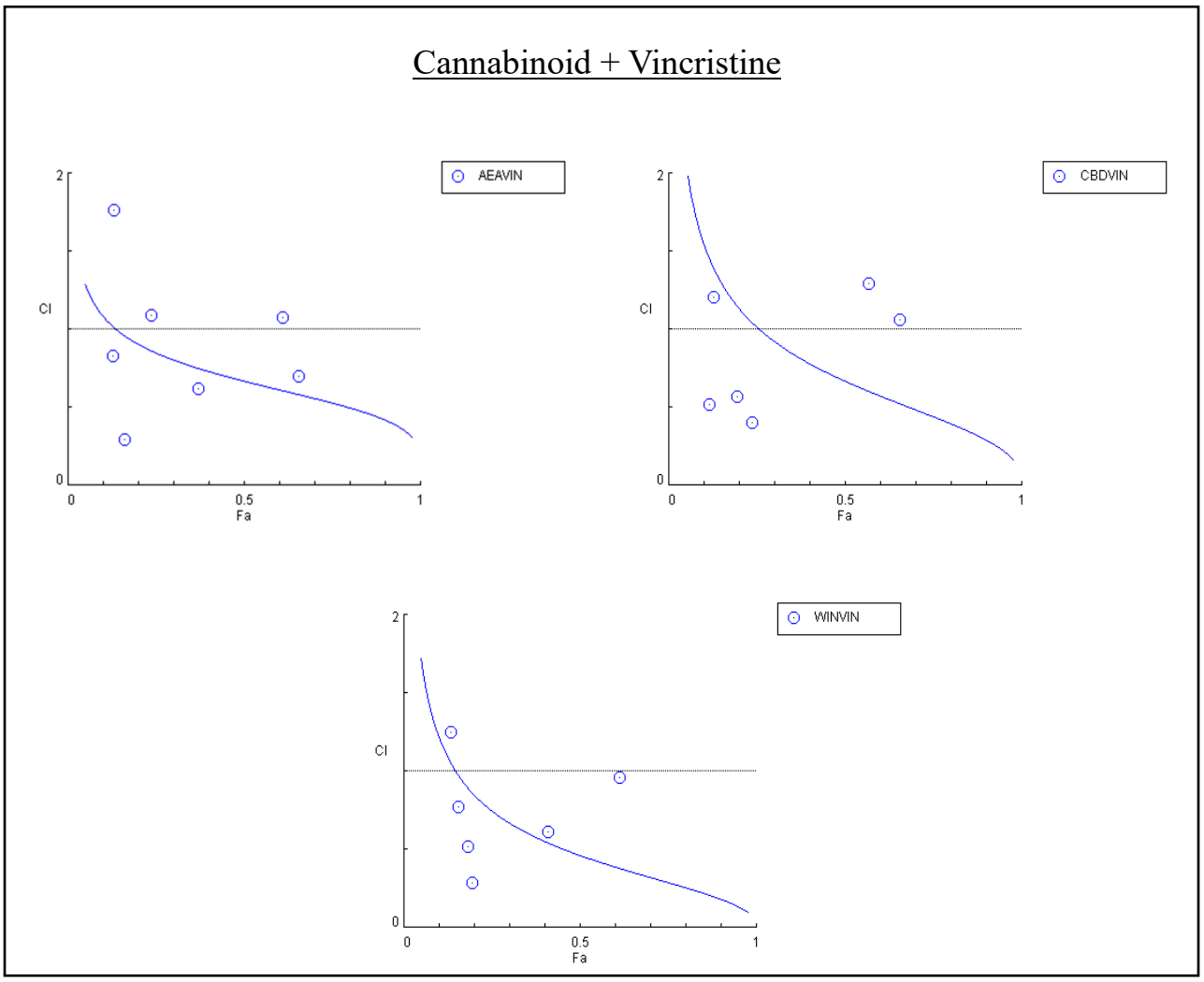


Figure 5-5 Combination index plot CBs+VIN

The *Combination index plot* AEAVIN represents 7 combination data points, 4 of which demonstrate a synergistic relationship (CI < 1) and 2 describe an additive and one data point shows an antagonistic relationship. Plot CBDVIN represents 6 combination data points, 3 of which demonstrate a synergistic relationship (CI < 1), one additive, and the other two describe the antagonistic relationship. Plot WINVIN represents 6 combination data points, 5 of which demonstrate a synergistic relationship (CI < 1) and one data point describes an antagonistic relationship. Simulations at low Fa > 0.5 show less relevance to therapy since cancer cell death in small numbers is less useful.

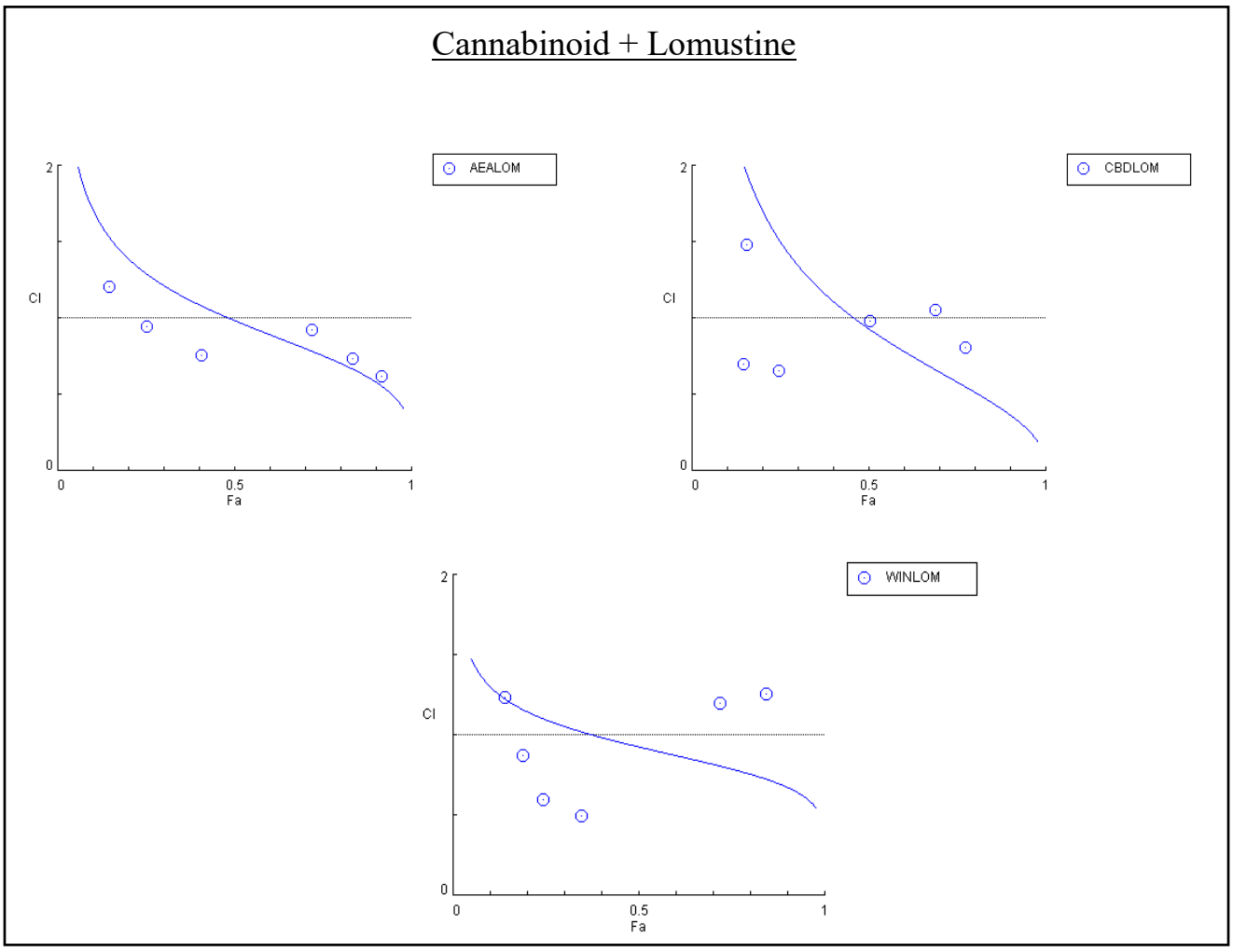


Figure 5-6 Combination index plot CBs+LOM

The *Combination index plot* AEALOM represents 6 combination data points, 5 of which demonstrate a synergistic relationship (CI < 1) and one describes an antagonistic relationship however at a low Fa value. Plot CBDLOM represents 6 combination data points, 4 of which demonstrate a synergistic relationship (CI < 1), one additive and one describes an antagonistic relationship. Plot WINLOM represents 6 combination data points, 3 of which demonstrate a synergistic relationship (CI < 1) and the other 3 describe an antagonistic relationship. Simulations at low Fa > 0.5 show less relevance to therapy since cancer cell death in small numbers is less useful.

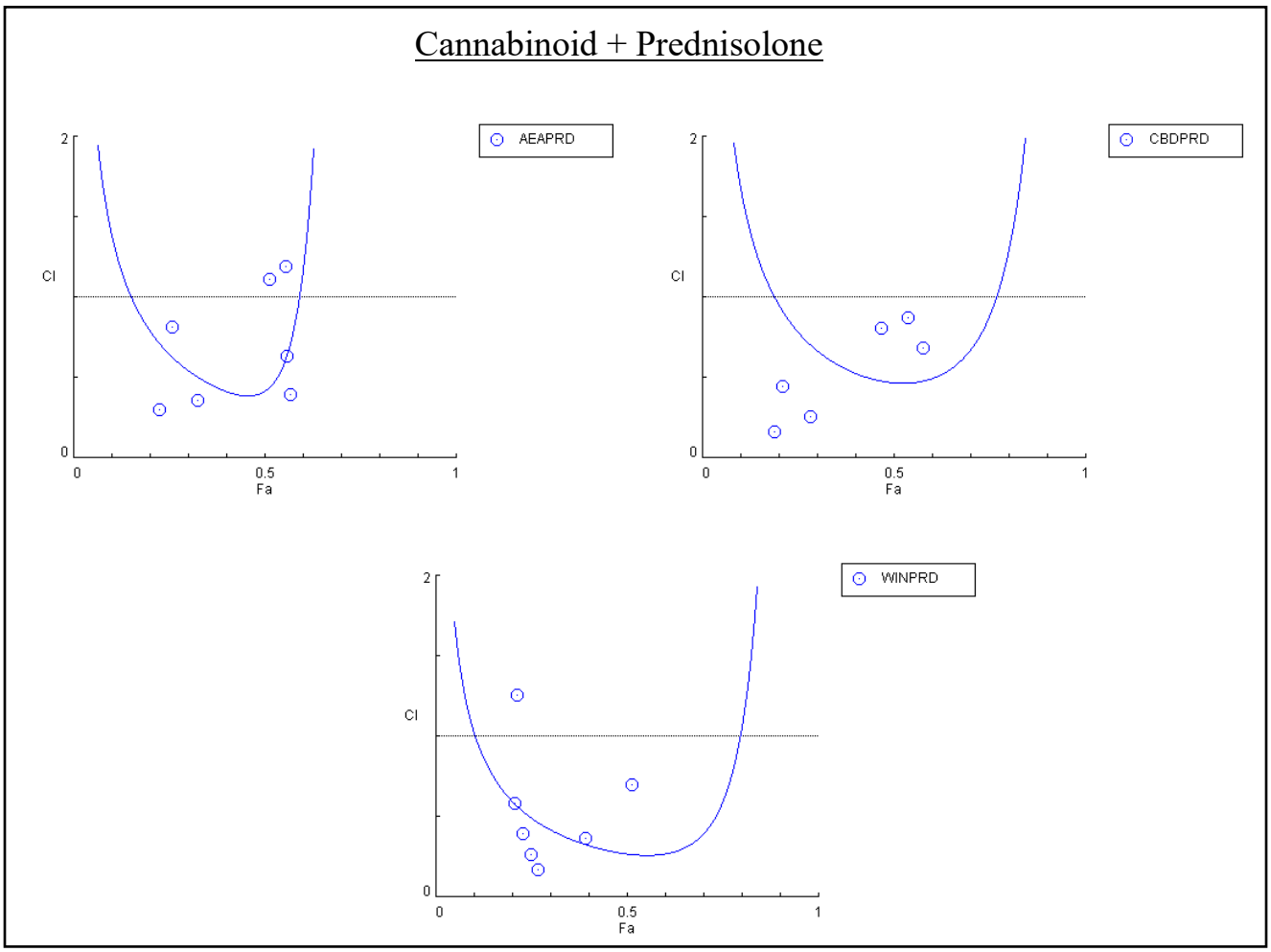


Figure 5-7 Combination index plot CBs+PRD

The *Combination index plot* AEAPRD represents 7 combination data points, 5 of which demonstrate a synergistic relationship (CI < 1) and the other two describe an additive relationship. Plot CBDPRD represents 6 combination data points, all demonstrate a synergistic relationship (CI < 1). Plot WINPRD represents 7 combination data points, 6 of which demonstrate a synergistic relationship (CI < 1) and one describes an antagonistic relationship. Simulations at low Fa > 0.5 show less relevance to therapy since cancer cell death in small numbers is less useful.

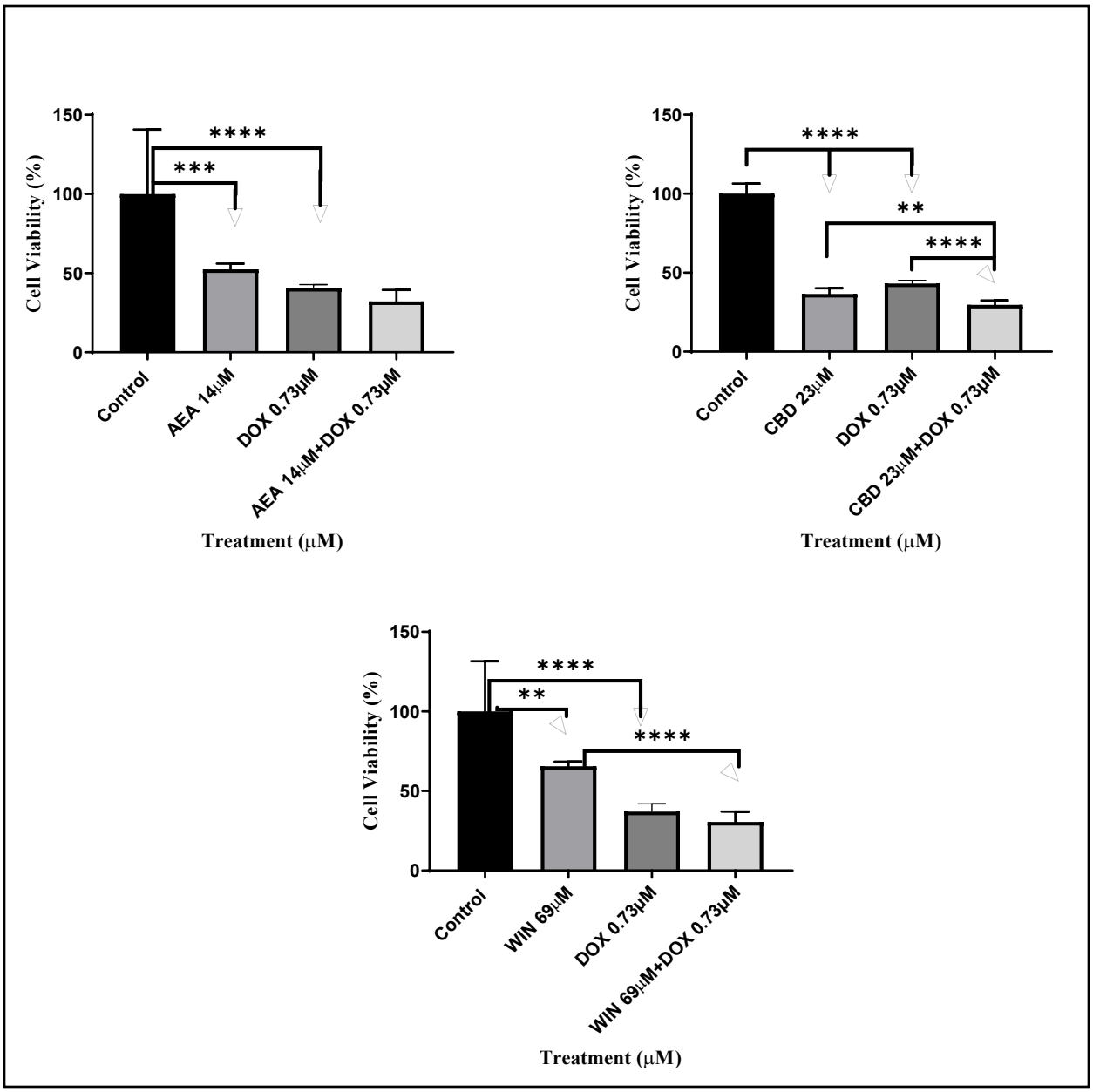


Figure 5-8 Effect of CBs and DOX combination at IC50

MTT cell proliferation assays showed that CBD potentiated DOX inhibition of 1771 cell growth. CBD synergistically enhanced DOX's cytotoxicity to lymphoma cells when the two drugs were combined at the IC50 concentration equivalent for each drug. We could not demonstrate the synergistic effect of AEA and WIN on DOX at that time and dose. Results are expressed as (%) change as compared to the control, Mean ± SD.

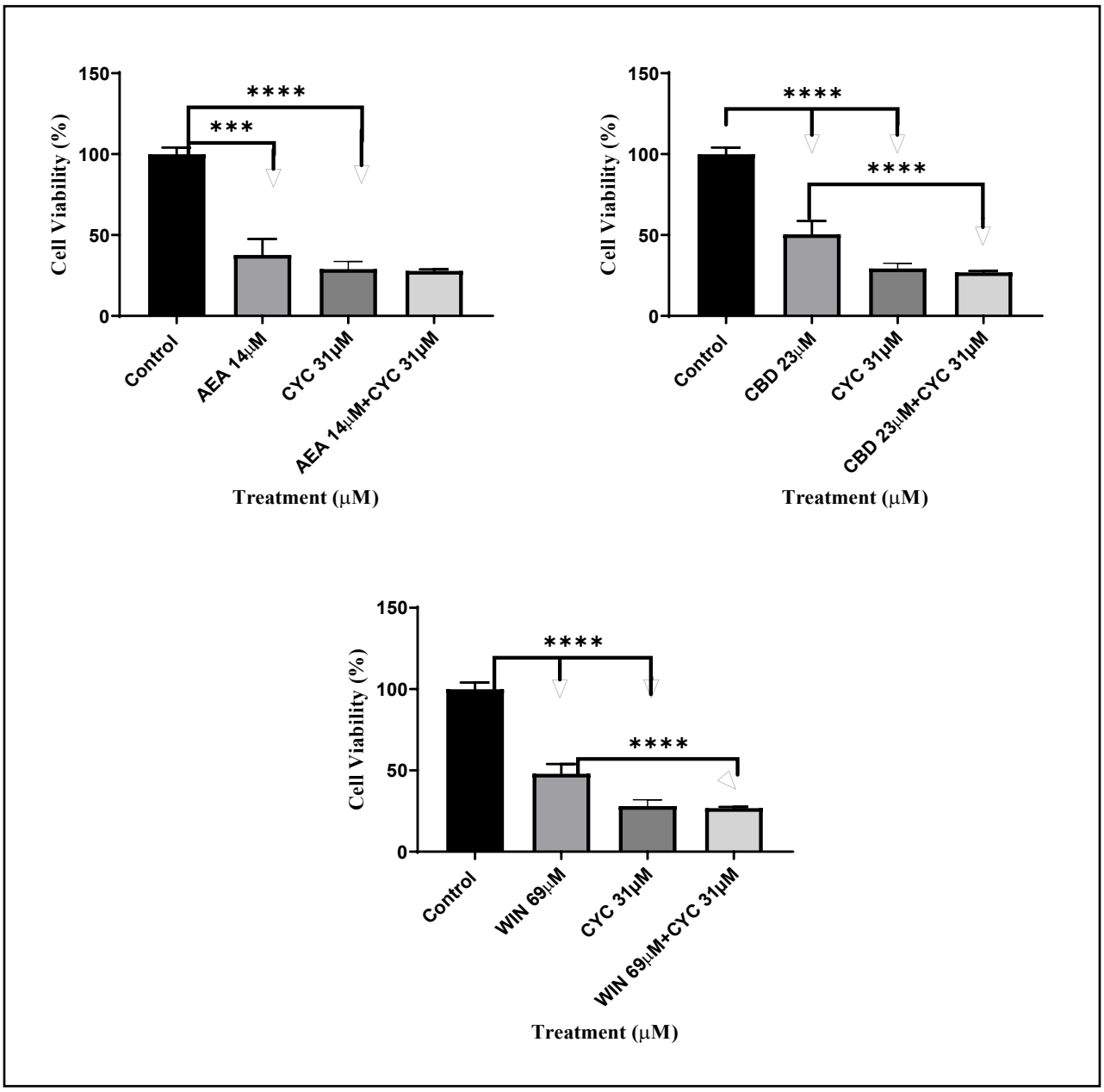


Figure 5-9 Effect of CBs and CYC combination at IC50

MTT cell proliferation assays showed no significant potentiating effect of CNBs on CYC-induced cytotoxicity in lymphoma cells when combined at the IC50 concentration equivalent for each drug. Results are expressed as (%) change as compared to the control, Mean ± SD.

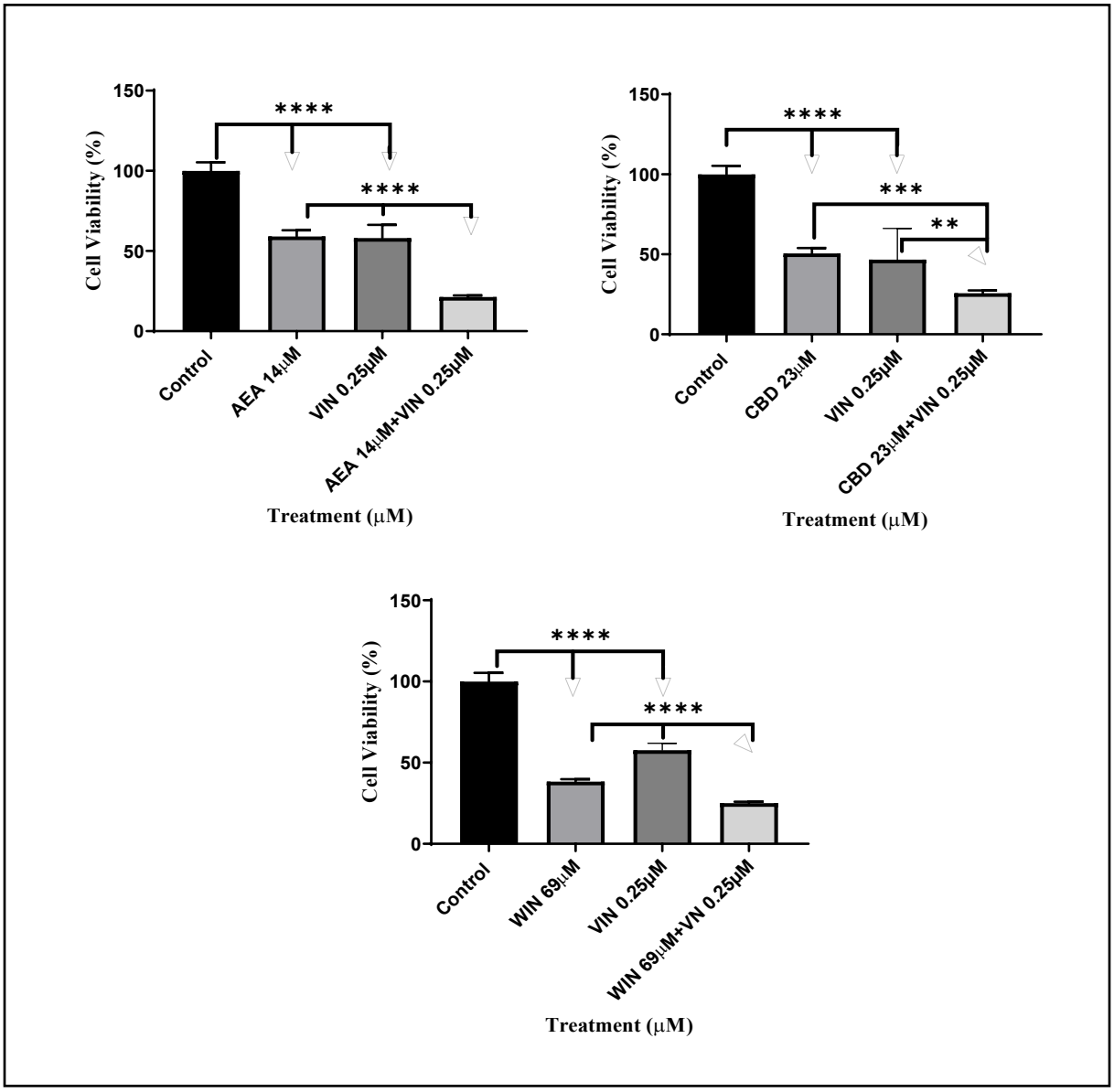


Figure 5-10 Effect of CBs and VIN combination at IC50

MTT cell proliferation assays demonstrated that all three CBs significantly potentiated VIN inhibition of 1771 cell growth. AEA, CBD and WIN synergistically enhanced DOX's cytotoxicity to lymphoma cells when the two drugs were combined at the IC50 concentration equivalent for each drug. Results are expressed as (%) change as compared to the control, Mean ± SD.

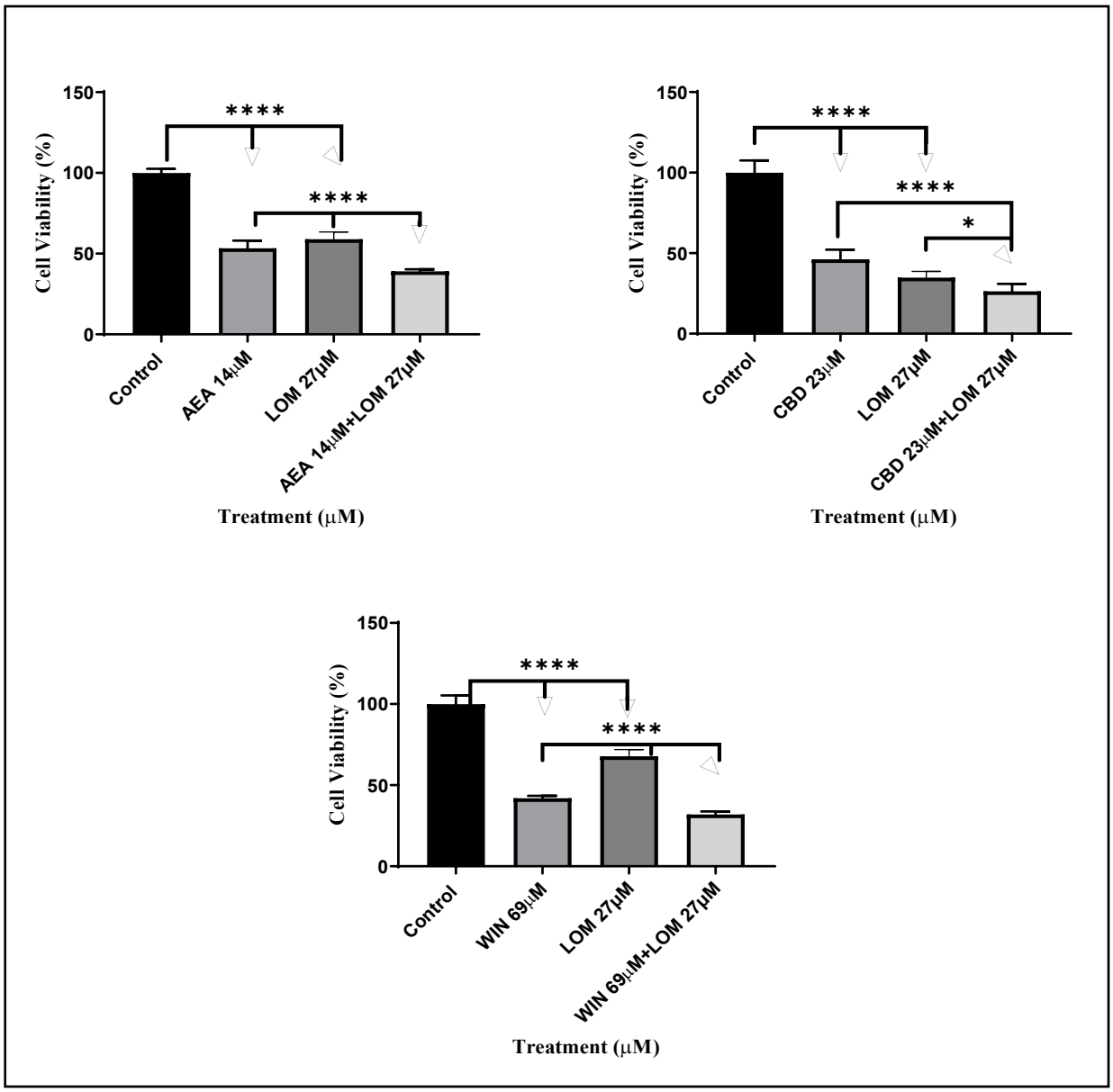


Figure 5-11 Effect of CBs and LOM combination at IC50

MTT cell proliferation assays demonstrated that all three CBs significantly potentiated LOM's induced inhibition of 1771 cell growth. AEA, CBD and WIN synergistically enhanced DOX's cytotoxicity to lymphoma cells when the two drugs were combined at the IC50 concentration equivalent for each drug. Results are expressed as (%) change as compared to the control, Mean ± SD.

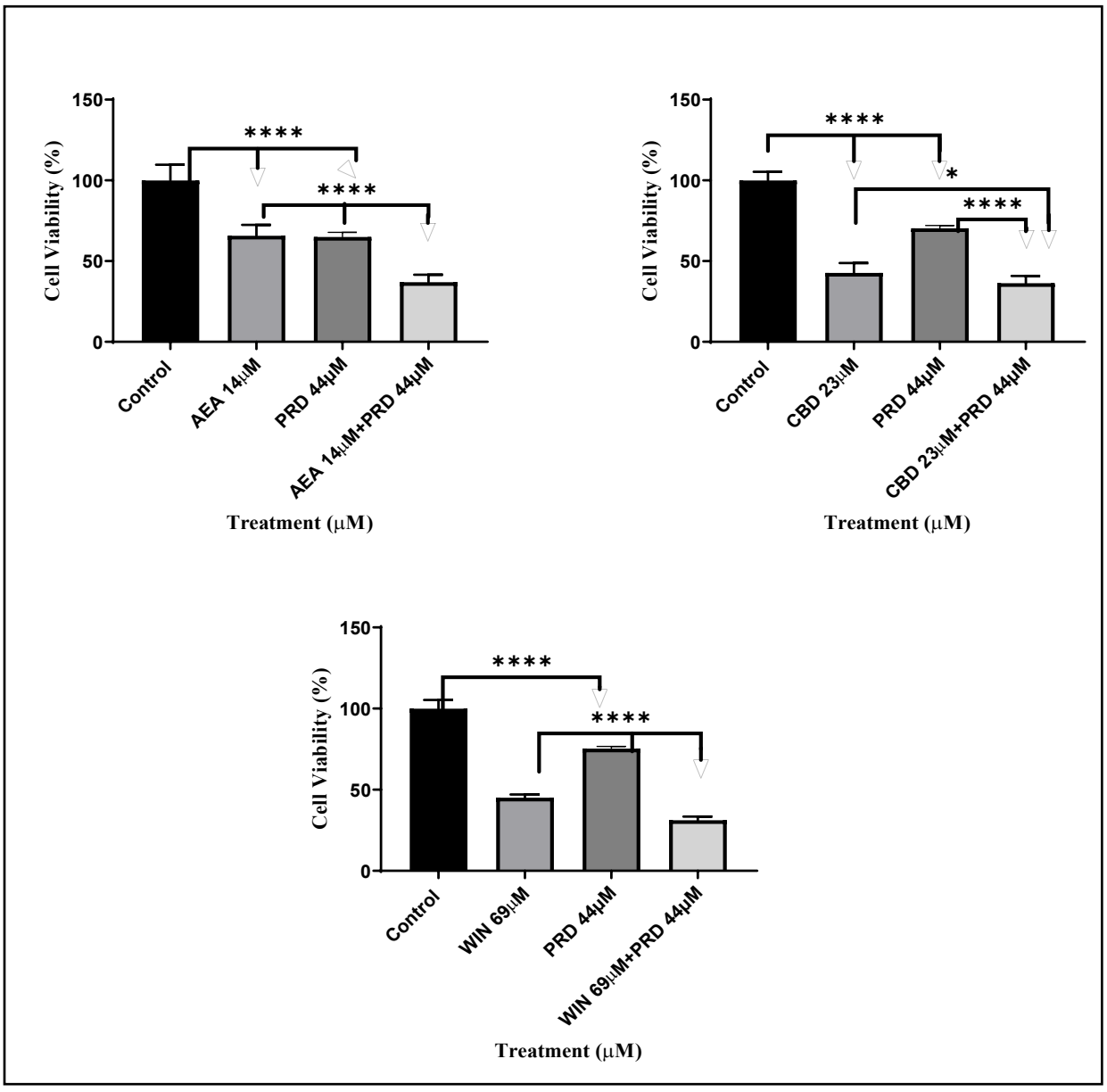


Figure 5-12 Effect of CBs and PRD combination at IC50

MTT cell proliferation assays demonstrated that all three CBs significantly increased PRD's induced inhibition of 1771 cell growth. AEA, CBD and WIN synergistically enhanced DOX's cytotoxicity to lymphoma cells when the two drugs were combined at the IC50 concentration equivalent for each drug. Results are expressed as (%) change as compared to the control, Mean ± SD.

5.5 Discussion

After studying the cannabinoid receptors and demonstrating the inhibitory and apoptotic effects of endogenous and exogenous cannabinoids on human and canine NHL cell lines, in the present study, we demonstrated that CBs in combination with traditional lymphoma chemotherapeutic drugs potentiated their anti-cancer effects synergistically in 1771 lymphoma cells.

We treated 1771 canine lymphoma cells with CBs and NLC drugs, singly or in combination to document the effect of these drugs on lymphoma cells. The isobologram and CI analysis showed that the CI for CBs combination with NLC drugs was <1 or =1 in low doses indicating synergistic and additive relationship respectively; however, at higher doses, we also found antagonistic relation CI >1 of the combinations, which can be the response of cancer cells against excessive drug exposure. We also observed some synergistic and antagonistic simulations at Fa <1 that indicate those combinations are less relevant therapeutically since cancer cell death in small numbers is less useful. To further validate our results of CI analysis we analyzed the viability of 1771 cells treated with IC50 concentration of each CB and NLC drug individually and compared it with the viability of cells treated with the combination at IC50 concentration and control untreated cells. Our results demonstrated the potentiated effect of CBs on NLC drug's cytotoxicity. However, at IC50 concentrations we could not demonstrate the synergistic effect of AEA on DOX and any cannabinoid used in this study on cyclophosphamide-induced inhibition of 1771 cell growth.

Gustafsson et.al. Studied the effect of endogenous and synthetic CBs in combination with chemotherapeutic drug 5-fluorouracil in colorectal carcinoma cells. They also demonstrated the synergistic effect of synthetic cannabinoids on the chemotherapeutic drug but not with endocannabinoid AEA [234]. Our results of the IC50 combination of CBD with CYC are similar to the results of Andradas et.al in vitro study in which they analyzed the effect of CBs in combination with cyclophosphamide in medulloblastoma cells [235]. Strong et al. also showed the synergistic effect of cannabidiol with other traditional lymphoma chemotherapeutic drugs such as ibrutinib and proteasome inhibitors such as carfilzomib [236].[233]

5.6 Conclusion

In summary, findings from this study showed that a combination of CBs and traditional lymphoma chemotherapeutic drugs have a synergistic effect on 1771 lymphoma cells. Cannabinoids could be combined with traditional lymphoma chemotherapeutic drugs to reduce the dose necessary to inhibit cancer cell growth and thus unspecific toxicity associated with it. In keeping with the results of our previous studies, the synergistic effect of CBs with lymphoma chemotherapeutic drugs is a further indication of targeting the cannabinoid pathway for the treatment of malignant lymphoma.

6. Chapter 4

Summary and Future Directions

In the past two decades, various in vitro and in vivo studies have demonstrated the anti-cancer effects of cannabinoids in multiple models of human cancer [102, 227, 228]. In addition, studies have demonstrated the upregulation of the endocannabinoid system in malignant tissues as compared to the non-malignant counterpart [95, 228]. These findings indicate that the endocannabinoid system can be a potential indicator and therapeutic target in the treatment of cancer. To extend this research to the clinical trial phase there is a need for substantial pre-clinical data to prove the therapeutic safety. In this research, there were two reasons to use canine NHL cell lines: **1.** Striking similarities between canine and human NHL in terms of cancer incidence, molecular mechanism, histological characteristics, treatment approach in addition to the advantage of a spontaneous tumor, chronology of disease adapted to the life span and a need of alternative treatment approach in canine as in human [187, 188]. **2.** Endocannabinoid system and anti-cancer effects of cannabinoids have never been studied in canine NHLs and there is very limited data available on endocannabinoid system in canine cancer in general. Therefore studying the anti-cancer effects of cannabinoids in canine will potentially benefit both the species.

In chapter one, we have studied the expression of endocannabinoid receptors (CB1 and CB2) in human and canine NHL cell lines and canine normal PBMCs using real-time PCR. In our results, we have found positive expression of cannabinoid receptor CB1 and CB2 in both canine and human NHL cell lines, with a significantly higher expression of CB1 and CB2 receptors in canine and human B cell NHL cell lines, compared to activated canine PBMCs and T-cell NHL cell line. For activated canine PBMCs our results show negative expression of the CB1 receptor gene but significantly higher expression of CB2 receptor gene compared to the canine T cell lymphoma cell line. Since several cannabinoid studies have associated anti-cancer effects of endogenous and exogenous cannabinoids with cannabinoid receptors [95, 152, 166] studying the cannabinoid receptors was the first essential step in this research.

Considering the finding of previous studies and our results above, we expected to see cannabinoids to be more effective against the B-cell NHL cell line as compared the T-cell. And as we expected, on treatment of NHL cell lines with endogenous and exogenous cannabinoids in chapter two and three we have found significant dose-dependent decrease in cancer cell viability in B cell NHL

cell lines. Only, CBD and AEA demonstrated significant decrease in cell viability in T cell lymphoma cell line and that can possibly through receptors other than CB1 and CB2 [196, 206]. Endocannabinoid 2AG seems to mediate its action through CB2 receptors primarily since it showed significant decrease in cell viability in cell lines with higher expression of CB2 receptor (Ramos and CLBL-1 cell lines) only[180, 196]. These results provide a new insight into the relationship between cannabinoid receptor expression and the effect of cannabinoids on lymphoma cell viability which should be taken into account while selecting cannabinoids to study on lymphomas.

To further confirm our results of cell viability assay we analyzed biochemical markers of oxidative stress, mitochondrial function and apoptosis in 1771 canine B cell NHL cell line treated with CBs and we found our result in alignment with the results of cell viability assay. All endogenous and exogenous cannabinoids significantly increased the markers of oxidative stress, apoptosis and decreased the markers of mitochondrial function except for 1771 cells treated with 2AG.

After finding the inhibitory effect of endogenous and exogenous cannabinoids CBs on NHL cell viability our third goal was to study the effect of CBs in combination with traditional NHL chemotherapeutic drugs (NLC drugs), for that we used Combination index (CI) analyses based on the Chou-Talalay method [231]. In this study we used 1771 cell line, we selected three CBs based on their IC50 values and 5 NLC drugs. IC50 values of each cannabinoid and NLC drug calculated after treating 1771 cells with each drug alone for 24 and 48 hours. To study drugs in combination two-fold serial dilution of each CB and NLC drug prepared three concentrations above and below the IC50 value of each drug. CB/NLC drug mixtures were made using 1:1 ratio for each drug. 1771 cells were treated with individual and combinations in 8 replicas and effect of drugs on the cell viability was determined by MTT assay. Results were confirmed in at least three independent experiments. Combination index (CI) values for combinations were determined using "CompuSyn" software [232]. CI<1 indicates synergism, CI=1 indicates additive effect, and CI>1 indicates antagonism. Results of CI analysis and isobologram generated by the software showed that the CI for all drug combinations was <1. However, in the case of CBs combination with CYC all data points showing synergistic relationship were simulated at Fa > 0.5, which means the synergistic effect of the combination on a small fraction of cancer cells, indicating less therapeutically relevant drug combination. To further validate CI analysis results, we analyzed the effect of each CB and NLC drug individually and in combination at IC50 concentration of each

drug and compared with control cells treated with vehicle only. In our results, we found that adding CBs to NLC drugs significantly enhanced the effect of NLC drugs alone. However, in this study using IC50 concentrations, we could not demonstrate the synergistic effect of AEA on DOX and any cannabinoid used in this study on cyclophosphamide.

In summation, this study for the first time demonstrates the expression of cannabinoid receptors and anticancer effects of endogenous and exogenous cannabinoids in canine NHL in addition to human NHL. Also, this research provides proof-of-concept for using cannabinoids in combination with traditional NHL drugs to reduce the dose, and therefore the toxicity, of chemotherapeutic drugs and possibly increasing the survival benefit in future lymphoma clinical translation studies.

Bibliography

1. Lu, H.-C. and K. Mackie, *Review of the endocannabinoid system.* Biological Psychiatry: Cognitive Neuroscience and Neuroimaging, 2020.
2. Moreno, E., et al., *The endocannabinoid system as a target in cancer diseases: are we there yet?* Frontiers in pharmacology, 2019. **10**: p. 339.
3. Pertwee, R.G., et al., *International Union of Basic and Clinical Pharmacology. LXXIX. Cannabinoid receptors and their ligands: beyond CB1 and CB2.* Pharmacological reviews, 2010. **62**(4): p. 588-631.
4. Howlett, A., et al., *International Union of Pharmacology. XXVII. Classification of cannabinoid receptors.* Pharmacological reviews, 2002. **54**(2): p. 161-202.
5. Munro, S., K.L. Thomas, and M. Abu-Shaar, *Molecular characterization of a peripheral receptor for cannabinoids.* Nature, 1993. **365**(6441): p. 61-65.
6. Cabral, G.A., G.A. Ferreira, and M.J. Jamerson, *Endocannabinoids and the immune system in health and disease.* Endocannabinoids, 2015: p. 185-211.
7. Stella, N., *Cannabinoid and cannabinoid-like receptors in microglia, astrocytes, and astrocytomas.* Glia, 2010. **58**(9): p. 1017-1030.
8. Atwood, B.K. and K. Mackie, *CB2: a cannabinoid receptor with an identity crisis.* British journal of pharmacology, 2010. **160**(3): p. 467-479.
9. Fernández-Ruiz, J., et al., *Cannabinoid CB2 receptor: a new target for controlling neural cell survival?* Trends in pharmacological sciences, 2007. **28**(1): p. 39-45.
10. Freundt-Revilla, J., et al., *Spatial distribution of cannabinoid receptor type 1 (CB1) in normal canine central and peripheral nervous system.* PloS one, 2017. **12**(7).
11. Pirone, A., et al., *Preferential epithelial expression of type-1 cannabinoid receptor (CB1R) in the developing canine embryo.* SpringerPlus, 2015. **4**(1): p. 1-7.
12. Galiazzo, G., et al., *Localization of cannabinoid receptors CB1, CB2, GPR55, and PPARα in the canine gastrointestinal tract.* Histochemistry and cell biology, 2018. **150**(2): p. 187-205.
13. Campora, L., et al., *Cannabinoid receptor type 1 and 2 expression in the skin of healthy dogs and dogs with atopic dermatitis.* American journal of veterinary research, 2012. **73**(7): p. 988-995.
14. Howlett, A.C. and J.-Y. Shim, *Cannabinoid receptors and signal transduction*, in *Madame Curie Bioscience Database [Internet]*. 2013, Landes Bioscience.
15. Piscitelli, F. and H.B. Bradshaw, *Endocannabinoid analytical methodologies: Techniques that drive discoveries that drive techniques.* Advances in Pharmacology, 2017. **80**: p. 1-30.
16. Hampson, R.E., et al., *Role of cyclic AMP dependent protein kinase in cannabinoid receptor modulation of potassium "A-current" in cultured rat hippocampal neurons.* Life sciences, 1995. **56**(23-24): p. 2081-2088.
17. Lauckner, J.E., B. Hille, and K. Mackie, *The cannabinoid agonist WIN55, 212-2 increases intracellular calcium via CB1 receptor coupling to Gq/11 G proteins.* Proceedings of the National Academy of Sciences, 2005. **102**(52): p. 19144-19149.

18. Kenakin, T., *Biased receptor signaling in drug discovery.* Pharmacological reviews, 2019. **71**(2): p. 267-315.
19. Kenakin, T., *A scale of agonism and allosteric modulation for assessment of selectivity, bias, and receptor mutation.* Molecular pharmacology, 2017. **92**(4): p. 414-424.
20. Howlett, A., *Cannabinoid receptor signaling.* Cannabinoids, 2005: p. 53-79.
21. Ross, R.A., *Anandamide and vanilloid TRPV1 receptors.* British journal of pharmacology, 2003. **140**(5): p. 790-801.
22. McPartland, J.M., R.W. Norris, and C.W. Kilpatrick, *Coevolution between cannabinoid receptors and endocannabinoid ligands.* Gene, 2007. **397**(1-2): p. 126-135.
23. Gesell, F.K., et al., *Alterations of endocannabinoids in cerebrospinal fluid of dogs with epileptic seizure disorder.* BMC veterinary research, 2013. **9**(1): p. 262.
24. Lu, H.-C. and K. Mackie, *An introduction to the endogenous cannabinoid system.* Biological psychiatry, 2016. **79**(7): p. 516-525.
25. Blankman, J.L., G.M. Simon, and B.F. Cravatt, *A comprehensive profile of brain enzymes that hydrolyze the endocannabinoid 2-arachidonoylglycerol.* Chemistry & biology, 2007. **14**(12): p. 1347-1356.
26. Marrs, W.R., et al., *The serine hydrolase ABHD6 controls the accumulation and efficacy of 2-AG at cannabinoid receptors.* Nature neuroscience, 2010. **13**(8): p. 951-957.
27. Bisogno, T., et al., *Molecular targets for cannabidiol and its synthetic analogues: effect on vanilloid VR1 receptors and on the cellular uptake and enzymatic hydrolysis of anandamide.* British journal of pharmacology, 2001. **134**(4): p. 845-852.
28. Parsons, L.H. and Y.L. Hurd, *Endocannabinoid signalling in reward and addiction.* Nature Reviews Neuroscience, 2015. **16**(10): p. 579-594.
29. Di Marzo, V., N. Stella, and A. Zimmer, *Endocannabinoid signalling and the deteriorating brain.* Nature Reviews Neuroscience, 2015. **16**(1): p. 30-42.
30. Kano, M., et al., *Endocannabinoid-mediated control of synaptic transmission.* Physiological reviews, 2009. **89**(1): p. 309-380.
31. Pacher, P., S. Bátkai, and G. Kunos, *The endocannabinoid system as an emerging target of pharmacotherapy.* Pharmacological reviews, 2006. **58**(3): p. 389-462.
32. Maccarrone, M., et al., *Endocannabinoid signaling at the periphery: 50 years after THC.* Trends in pharmacological sciences, 2015. **36**(5): p. 277-296.
33. Di Marzo, V., *'Endocannabinoids' and other fatty acid derivatives with cannabimimetic properties: biochemistry and possible physiopathological relevance.* Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism, 1998. **1392**(2-3): p. 153-175.
34. Romero, J., et al., *The endogenous cannabinoid receptor ligand, anandamide, inhibits the motor behavior: role of nigrostriatal dopaminergic neurons.* Life sciences, 1995. **56**(23-24): p. 2033-2040.
35. Romero, J., et al., *Involvement of GABA (B) receptors in the motor inhibition produced by agonists of brain cannabinoid receptors.* Behavioural pharmacology, 1996. **7**(3): p. 299-302.
36. Castellano, C., et al., *The effects of anandamide on memory consolidation in mice involve both D1 and D2 dopamine receptors.* Behavioural pharmacology, 1997. **8**(8): p. 707-712.
37. Terranova, J.-P., et al., *Improvement of memory in rodents by the selective CB1 cannabinoid receptor antagonist, SR 141716.* Psychopharmacology, 1996. **126**(2): p. 165-172.

38. Freund, T.F., I. Katona, and D. Piomelli, *Role of endogenous cannabinoids in synaptic signaling.* Physiological reviews, 2003.
39. FemándeZ-Ruiz, J.J., et al., *Time course of the effects of different cannabimimetics on prolactin and gonadotrophin secretion: evidence for the presence of CB1 receptors in hypothalamic structures and their involvement in the effects of cannabimimetics.* Biochemical pharmacology, 1997. **53**(12): p. 1919-1927.
40. Wenger, T., et al., *Arachidonyl ethanolamide (anandamide) activates the parvocellular part of hypothalamic paraventricular nucleus.* Biochemical and biophysical research communications, 1997. **237**(3): p. 724-728.
41. Gorzalka, B.B., M.N. Hill, and C.J. Hillard, *Regulation of endocannabinoid signaling by stress: implications for stress-related affective disorders.* Neuroscience & Biobehavioral Reviews, 2008. **32**(6): p. 1152-1160.
42. Steiner, M.A. and C.T. Wotjak, *Role of the endocannabinoid system in regulation of the hypothalamic-pituitary-adrenocortical axis.* Progress in brain research, 2008. **170**: p. 397-432.
43. Hill, M.N., et al., *Regional alterations in the endocannabinoid system in an animal model of depression: effects of concurrent antidepressant treatment.* Journal of neurochemistry, 2008. **106**(6): p. 2322-2336.
44. Reznikov, L.R., L.P. Reagan, and J.R. Fadel, *Activation of phenotypically distinct neuronal subpopulations in the anterior subdivision of the rat basolateral amygdala following acute and repeated stress.* Journal of Comparative Neurology, 2008. **508**(3): p. 458-472.
45. Domenici, M.R., et al., *Cannabinoid receptor type 1 located on presynaptic terminals of principal neurons in the forebrain controls glutamatergic synaptic transmission.* Journal of Neuroscience, 2006. **26**(21): p. 5794-5799.
46. Zhu, P.J. and D.M. Lovinger, *Retrograde endocannabinoid signaling in a postsynaptic neuron/synaptic bouton preparation from basolateral amygdala.* Journal of Neuroscience, 2005. **25**(26): p. 6199-6207.
47. Woodhams, S.G., et al., *The role of the endocannabinoid system in pain.* Pain control, 2015: p. 119-143.
48. Calignano, A., et al., *Control of pain initiation by endogenous cannabinoids.* Nature, 1998. **394**(6690): p. 277-281.
49. Alkaitis, M.S., et al., *Evidence for a role of endocannabinoids, astrocytes and p38 phosphorylation in the resolution of postoperative pain.* PloS one, 2010. **5**(5): p. e10891.
50. Richardson, J.D., L. Aanonsen, and K.M. Hargreaves, *SR 141716A, a cannabinoid receptor antagonist, produces hyperalgesia in untreated mice.* European journal of pharmacology, 1997. **319**(2-3): p. R3-R4.
51. Sierra, S., N. Luquin, and J. Navarro-Otano, *The endocannabinoid system in cardiovascular function: novel insights and clinical implications.* Clinical Autonomic Research, 2018. **28**(1): p. 35-52.
52. Hillard, C.J., *Endocannabinoids and vascular function.* Journal of Pharmacology and Experimental Therapeutics, 2000. **294**(1): p. 27-32.
53. Pratt, P.F., et al., *N-arachidonylethanolamide relaxation of bovine coronary artery is not mediated by CB1 cannabinoid receptor.* American Journal of Physiology-Heart and Circulatory Physiology, 1998. **274**(1): p. H375-H381.

54. Facci, L., et al., *Mast cells express a peripheral cannabinoid receptor with differential sensitivity to anandamide and palmitoylethanolamide.* Proceedings of the National Academy of Sciences, 1995. **92**(8): p. 3376-3380.
55. Mazzari, S., et al., *N-(2-hydroxyethyl) hexadecanamide is orally active in reducing edema formation and inflammatory hyperalgesia by down-modulating mast cell activation.* European journal of pharmacology, 1996. **300**(3): p. 227-236.
56. Bisogno, T., et al., *Biosynthesis, uptake, and degradation of anandamide and palmitoylethanolamide in leukocytes.* Journal of Biological Chemistry, 1997. **272**(6): p. 3315-3323.
57. Wagner, J.A., et al., *Activation of peripheral CB 1 cannabinoid receptors in haemorrhagic shock.* Nature, 1997. **390**(6659): p. 518-521.
58. Köse, S., et al., *Human bone marrow mesenchymal stem cells secrete endocannabinoids that stimulate in vitro hematopoietic stem cell migration effectively comparable to beta-adrenergic stimulation.* Experimental hematology, 2018. **57**: p. 30-41. e1.
59. Almogi-Hazan, O. and R. Or, *Cannabis, the Endocannabinoid System and Immunity—the Journey from the Bedside to the Bench and Back.* International Journal of Molecular Sciences, 2020. **21**(12): p. 4448.
60. Berdyshev, E.V., et al., *Influence of fatty acid ethanolamides and Δ9-tetrahydrocannabinol on cytokine and arachidonate release by mononuclear cells.* European journal of pharmacology, 1997. **330**(2-3): p. 231-240.
61. Cabral, G.A. and L. Griffin-Thomas, *Emerging role of the cannabinoid receptor CB2 in immune regulation: therapeutic prospects for neuroinflammation.* Expert reviews in molecular medicine, 2009. **11**.
62. Larose, M.-C., et al., *Mechanisms of human eosinophil migration induced by the combination of IL-5 and the endocannabinoid 2-arachidonoyl-glycerol.* Journal of allergy and clinical immunology, 2014. **133**(5): p. 1480-1482. e3.
63. Turcotte, C., et al., *The CB 2 receptor and its role as a regulator of inflammation.* Cellular and Molecular Life Sciences, 2016. **73**(23): p. 4449-4470.
64. Szabady, R.L., et al., *Intestinal P-glycoprotein exports endocannabinoids to prevent inflammation and maintain homeostasis.* The Journal of clinical investigation, 2018. **128**(9): p. 4044-4056.
65. Ortega-Gutiérrez, S., E. Molina-Holgado, and C. Guaza, *Effect of anandamide uptake inhibition in the production of nitric oxide and in the release of cytokines in astrocyte cultures.* Glia, 2005. **52**(2): p. 163-168.
66. Chiurchiù, V., et al., *Distinct modulation of human myeloid and plasmacytoid dendritic cells by anandamide in multiple sclerosis.* Annals of neurology, 2013. **73**(5): p. 626-636.
67. Eljaschewitsch, E., et al., *The endocannabinoid anandamide protects neurons during CNS inflammation by induction of MKP-1 in microglial cells.* Neuron, 2006. **49**(1): p. 67-79.
68. Correa, F., et al., *Anandamide enhances IL-10 production in activated microglia by targeting CB2 receptors: Roles of ERK1/2, JNK, and NF-κB.* Glia, 2010. **58**(2): p. 135-147.
69. Correa, F., et al., *The endocannabinoid anandamide downregulates IL-23 and IL-12 subunits in a viral model of multiple sclerosis: evidence for a cross-talk between IL-12p70/IL-23 axis and IL-10 in microglial cells.* Brain, behavior, and immunity, 2011. **25**(4): p. 736-749.

70. Malek, N., et al., *Anandamide, acting via CB2 receptors, alleviates LPS-induced neuroinflammation in rat primary microglial cultures.* Neural plasticity, 2015. **2015**.
71. Kurihara, R., et al., *Effects of peripheral cannabinoid receptor ligands on motility and polarization in neutrophil-like HL60 cells and human neutrophils.* Journal of Biological Chemistry, 2006. **281**(18): p. 12908-12918.
72. Eisenstein, T.K., et al., *Anandamide and Δ9-tetrahydrocannabinol directly inhibit cells of the immune system via CB2 receptors.* Journal of neuroimmunology, 2007. **189**(1-2): p. 17-22.
73. Cencioni, M.T., et al., *Anandamide suppresses proliferation and cytokine release from primary human T-lymphocytes mainly via CB 2 receptors.* PloS one, 2010. **5**(1): p. e8688.
74. Coopman, K., et al., *Temporal variation in CB2R levels following T lymphocyte activation: evidence that cannabinoids modulate CXCL12-induced chemotaxis.* International immunopharmacology, 2007. **7**(3): p. 360-371.
75. Joseph, J., et al., *Anandamide is an endogenous inhibitor for the migration of tumor cells and T lymphocytes.* Cancer Immunology, Immunotherapy, 2004. **53**(8): p. 723-728.
76. Rayman, N., et al., *Distinct expression profiles of the peripheral cannabinoid receptor in lymphoid tissues depending on receptor activation status.* The Journal of Immunology, 2004. **172**(4): p. 2111-2117.
77. Alberich Jordà, M., et al., *Hematopoietic cells expressing the peripheral cannabinoid receptor migrate in response to the endocannabinoid 2-arachidonoylglycerol.* Blood, The Journal of the American Society of Hematology, 2002. **99**(8): p. 2786-2793.
78. Tanikawa, T., K. Kurohane, and Y. Imai, *Induction of preferential chemotaxis of unstimulated B-lymphocytes by 2-arachidonoylglycerol in immunized mice.* Microbiology and immunology, 2007. **51**(10): p. 1013-1019.
79. Maestroni, G.J., *The endogenous cannabinoid 2-arachidonoyl glycerol as in vivo chemoattractant for dendritic cells and adjuvant for Th1 response to a soluble protein.* The FASEB journal, 2004. **18**(15): p. 1914-1916.
80. Oka, S., et al., *2-arachidonoylglycerol, an endogenous cannabinoid receptor ligand, induces the migration of EoL-1 human eosinophilic leukemia cells and human peripheral blood eosinophils.* Journal of leukocyte biology, 2004. **76**(5): p. 1002-1009.
81. Shiratsuchi, A., et al., *Involvement of cannabinoid receptor CB2 in dectin-1-mediated macrophage phagocytosis.* Immunology and cell biology, 2008. **86**(2): p. 179-184.
82. Kishimoto, S., et al., *2-arachidonoylglycerol induces the migration of HL-60 cells differentiated into macrophage-like cells and human peripheral blood monocytes through the cannabinoid CB2 receptor-dependent mechanism.* Journal of Biological Chemistry, 2003. **278**(27): p. 24469-24475.
83. Kishimoto, S., et al., *2-Arachidonoylglycerol, an endogenous cannabinoid receptor ligand, induces accelerated production of chemokines in HL-60 cells.* Journal of biochemistry, 2004. **135**(4): p. 517-524.
84. Walter, L., et al., *Nonpsychotropic cannabinoid receptors regulate microglial cell migration.* Journal of Neuroscience, 2003. **23**(4): p. 1398-1405.
85. Gokoh, M., et al., *2-Arachidonoylglycerol, an endogenous cannabinoid receptor ligand, induces rapid actin polymerization in HL-60 cells differentiated into macrophage-like cells.* Biochemical Journal, 2005. **386**(3): p. 583-589.
86. Kishimoto, S., et al., *Endogenous cannabinoid receptor ligand induces the migration of human natural killer cells.* Journal of biochemistry, 2005. **137**(2): p. 217-223.

87. Gasperi, V., et al., *2-Arachidonoylglycerol modulates human endothelial cell/leukocyte interactions by controlling selectin expression through CB1 and CB2 receptors.* The international journal of biochemistry & cell biology, 2014. **51**: p. 79-88.
88. Bari, M., et al., *The manifold actions of endocannabinoids on female and male reproductive events.* Front Biosci, 2011. **16**(498): p. e516.
89. Du Plessis, S.S., A. Agarwal, and A. Syriac, *Marijuana, phytocannabinoids, the endocannabinoid system, and male fertility.* Journal of assisted reproduction and genetics, 2015. **32**(11): p. 1575-1588.
90. O'Llenecia, S.W., A.C. Holloway, and S. Raha, *The role of the endocannabinoid system in female reproductive tissues.* Journal of ovarian research, 2019. **12**(1): p. 1-10.
91. Kolodny, R.C., et al., *Depression of plasma testosterone levels after chronic intensive marihuana use.* New England Journal of Medicine, 1974. **290**(16): p. 872-874.
92. Gammon, C.M., et al., *Regulation of gonadotropin-releasing hormone secretion by cannabinoids.* Endocrinology, 2005. **146**(10): p. 4491-4499.
93. Adams, I.B. and B.R. Martin, *Cannabis: pharmacology and toxicology in animals and humans.* Addiction, 1996. **91**(11): p. 1585-1614.
94. Russo, E.B., et al., *Phytochemical and genetic analyses of ancient cannabis from Central Asia.* Journal of experimental botany, 2008. **59**(15): p. 4171-4182.
95. Velasco, G., C. Sánchez, and M. Guzmán, *Towards the use of cannabinoids as antitumour agents.* Nature Reviews Cancer, 2012. **12**(6): p. 436-444.
96. Integrative, P., *Cannabis and Cannabinoids (PDQ®): Health Professional Version.* PDQ Cancer Information Summaries [Internet], 2002.
97. Nichols, M. *Medical cannabis*. in *International Symposia on Tropical and Temperate Horticulture-ISTTH2016 1205*. 2016.
98. Shevyrin, V. and Y.Y. Morzherin, *Cannabinoids: structures, effects, and classification.* Russian Chemical Bulletin, 2015. **64**(6): p. 1249-1266.
99. Montero-Oleas, N., et al., *Therapeutic use of cannabis and cannabinoids: an evidence mapping and appraisal of systematic reviews.* BMC complementary medicine and therapies, 2020. **20**(1): p. 1-15.
100. Belendiuk, K.A., L.L. Baldini, and M.O. Bonn-Miller, *Narrative review of the safety and efficacy of marijuana for the treatment of commonly state-approved medical and psychiatric disorders.* Addiction Science & Clinical Practice, 2015. **10**(1): p. 1-10.
101. Grotenhermen, F. and K. Müller-Vahl, *The therapeutic potential of cannabis and cannabinoids.* Deutsches Ärzteblatt International, 2012. **109**(29-30): p. 495.
102. Guzman, M., *Cannabinoids: potential anticancer agents.* Nature reviews cancer, 2003. **3**(10): p. 745-755.
103. Sarfaraz, S., et al., *Cannabinoids for cancer treatment: progress and promise.* Cancer research, 2008. **68**(2): p. 339-342.
104. Katona, I. and T.F. Freund, *Endocannabinoid signaling as a synaptic circuit breaker in neurological disease.* Nature medicine, 2008. **14**(9): p. 923.
105. Pertwee, R.G., *Emerging strategies for exploiting cannabinoid receptor agonists as medicines.* British journal of pharmacology, 2009. **156**(3): p. 397-411.
106. Michalski, C.W., et al., *Cannabinoids in pancreatic cancer: correlation with survival and pain.* International journal of cancer, 2008. **122**(4): p. 742-750.
107. Wu, X., et al., *Alteration of endocannabinoid system in human gliomas.* Journal of neurochemistry, 2012. **120**(5): p. 842-849.

108. Ramer, R. and B. Hinz, *Cannabinoids as anticancer drugs*, in *Advances in Pharmacology*. 2017, Elsevier. p. 397-436.
109. Nithipatikom, K., et al., *2-Arachidonoylglycerol: a novel inhibitor of androgen-independent prostate cancer cell invasion.* Cancer research, 2004. **64**(24): p. 8826-8830.
110. Petersen, G., et al., *Endocannabinoid metabolism in human glioblastomas and meningiomas compared to human non-tumour brain tissue.* Journal of neurochemistry, 2005. **93**(2): p. 299-309.
111. Maccarrone, M., et al., *Gas chromatography–mass spectrometry analysis of endogenous cannabinoids in healthy and tumoral human brain and human cells in culture.* Journal of neurochemistry, 2001. **76**(2): p. 594-601.
112. Sailler, S., et al., *Regulation of circulating endocannabinoids associated with cancer and metastases in mice and humans.* Oncoscience, 2014. **1**(4): p. 272.
113. Gjerstorff, M.F., et al., *Identification of genes with altered expression in medullary breast cancer vs. ductal breast cancer and normal breast epithelia.* International journal of oncology, 2006. **28**(6): p. 1327-1335.
114. Endsley, M.P., et al., *Expression and function of fatty acid amide hydrolase in prostate cancer.* International journal of cancer, 2008. **123**(6): p. 1318-1326.
115. Thors, L., et al., *Fatty acid amide hydrolase in prostate cancer: association with disease severity and outcome, CB1 receptor expression and regulation by IL-4.* PloS one, 2010. **5**(8).
116. Wang, D., et al., *Loss of cannabinoid receptor 1 accelerates intestinal tumor growth.* Cancer research, 2008. **68**(15): p. 6468-6476.
117. Nomura, D.K., et al., *Monoacylglycerol lipase regulates a fatty acid network that promotes cancer pathogenesis.* Cell, 2010. **140**(1): p. 49-61.
118. Munson, A., et al., *Antineoplastic activity of cannabinoids.* Journal of the National Cancer Institute, 1975. **55**(3): p. 597-602.
119. De Petrocellis, L., et al., *The endogenous cannabinoid anandamide inhibits human breast cancer cell proliferation.* Proceedings of the National Academy of Sciences, 1998. **95**(14): p. 8375-8380.
120. Carracedo, A., et al., *The stress-regulated protein p8 mediates cannabinoid-induced apoptosis of tumor cells.* Cancer cell, 2006. **9**(4): p. 301-312.
121. Oesch, S., et al., *Cannabinoid receptor 1 is a potential drug target for treatment of translocation-positive rhabdomyosarcoma.* Molecular cancer therapeutics, 2009. **8**(7): p. 1838-1845.
122. Caffarel, M.M., et al., *Cannabinoids reduce ErbB2-driven breast cancer progression through Akt inhibition.* Molecular cancer, 2010. **9**(1): p. 196.
123. Jacobsson, S.O., T. Wallin, and C.J. Fowler, *Inhibition of rat C6 glioma cell proliferation by endogenous and synthetic cannabinoids. Relative involvement of cannabinoid and vanilloid receptors.* Journal of Pharmacology and Experimental Therapeutics, 2001. **299**(3): p. 951-959.
124. Hinz, B., et al., *Up-regulation of cyclooxygenase-2 expression is involved in R (+)-methanandamide-induced apoptotic death of human neuroglioma cells.* Molecular pharmacology, 2004. **66**(6): p. 1643-1651.
125. Ramer, R., et al., *Ceramide is involved in r (+)-methanandamide-induced cyclooxygenase-2 expression in human neuroglioma cells.* Molecular pharmacology, 2003. **64**(5): p. 1189-1198.

126. Ramer, R. and B. Hinz, *Antitumorigenic targets of cannabinoids–current status and implications.* Expert opinion on therapeutic targets, 2016. **20**(10): p. 1219-1235.
127. Casanova, M.L., et al., *Inhibition of skin tumor growth and angiogenesis in vivo by activation of cannabinoid receptors.* The Journal of clinical investigation, 2003. **111**(1): p. 43-50.
128. Picardi, P., et al., *Anandamide inhibits breast tumor-induced angiogenesis.* Translational Medicine@ UniSa, 2014. **10**: p. 8.
129. Kogan, N.M., et al., *A cannabinoid anticancer quinone, HU-331, is more potent and less cardiotoxic than doxorubicin: a comparative in vivo study.* Journal of Pharmacology and Experimental Therapeutics, 2007. **322**(2): p. 646-653.
130. Ramer, R., et al., *Decrease of plasminogen activator inhibitor-1 may contribute to the anti-invasive action of cannabidiol on human lung cancer cells.* Pharmaceutical research, 2010. **27**(10): p. 2162-2174.
131. McAllister, S.D., et al., *Pathways mediating the effects of cannabidiol on the reduction of breast cancer cell proliferation, invasion, and metastasis.* Breast cancer research and treatment, 2011. **129**(1): p. 37-47.
132. McAllister, S.D., et al., *Cannabidiol as a novel inhibitor of Id-1 gene expression in aggressive breast cancer cells.* Molecular cancer therapeutics, 2007. **6**(11): p. 2921-2927.
133. Soroceanu, L., et al., *Id-1 is a key transcriptional regulator of glioblastoma aggressiveness and a novel therapeutic target.* Cancer research, 2013. **73**(5): p. 1559-1569.
134. Blázquez, C., et al., *Cannabinoids inhibit glioma cell invasion by down-regulating matrix metalloproteinase-2 expression.* Cancer research, 2008. **68**(6): p. 1945-1952.
135. Guzmán, M., I. Galve-Roperh, and C. Sánchez, *Ceramide: a new second messenger of cannabinoid action.* Trends in pharmacological sciences, 2001. **22**(1): p. 19-22.
136. del Pulgar, T.G., et al., *Cannabinoids protect astrocytes from ceramide-induced apoptosis through the phosphatidylinositol 3-kinase/protein kinase B pathway.* Journal of Biological Chemistry, 2002. **277**(39): p. 36527-36533.
137. Bifulco, M., et al., *Control by the endogenous cannabinoid system of ras oncogene-dependent tumor growth.* The FASEB Journal, 2001. **15**(14): p. 2745-2747.
138. Schwarz, H., F.J. Blanco, and M. Lotz, *Anadamide, an endogenous cannabinoid receptor agonist inhibits lymphocyte proliferation and induces apoptosis.* Journal of neuroimmunology, 1994. **55**(1): p. 107-115.
139. Valk, P., et al., *Anandamide, a natural ligand for the peripheral cannabinoid receptor is a novel synergistic growth factor for hematopoietic cells.* Blood, The Journal of the American Society of Hematology, 1997. **90**(4): p. 1448-1457.
140. Freundt-Revilla, J., et al., *Spatial distribution of cannabinoid receptor type 1 (CB1) in normal canine central and peripheral nervous system.* PLoS One, 2017. **12**(7): p. e0181064.
141. Pirone, A., et al., *Parvalbumin expression in the claustrum of the adult dog. An immunohistochemical and topographical study with comparative notes on the structure of the nucleus.* Journal of chemical neuroanatomy, 2015. **64**: p. 33-42.
142. Thomas, A., et al., *Cannabidiol displays unexpectedly high potency as an antagonist of CB1 and CB2 receptor agonists in vitro.* British journal of pharmacology, 2007. **150**(5): p. 613-623.

143. Salazar, M., et al., *Cannabinoid action induces autophagy-mediated cell death through stimulation of ER stress in human glioma cells.* The Journal of clinical investigation, 2009. **119**(5): p. 1359-1372.
144. Carracedo, A., et al., *Cannabinoids induce apoptosis of pancreatic tumor cells via endoplasmic reticulum stress–related genes.* Cancer research, 2006. **66**(13): p. 6748-6755.
145. Vara, D., et al., *Anti-tumoral action of cannabinoids on hepatocellular carcinoma: role of AMPK-dependent activation of autophagy.* Cell Death & Differentiation, 2011. **18**(7): p. 1099-1111.
146. Preet, A., R. Ganju, and J. Groopman, *Δ 9-Tetrahydrocannabinol inhibits epithelial growth factor-induced lung cancer cell migration in vitro as well as its growth and metastasis in vivo.* Oncogene, 2008. **27**(3): p. 339-346.
147. Bifulco, M., et al., *Endocannabinoids in endocrine and related tumours.* Endocrine-related cancer, 2008. **15**(2): p. 391.
148. Molina, J.R., A.A. Adjei, and J.R. Jett, *Advances in chemotherapy of non-small cell lung cancer.* Chest, 2006. **130**(4): p. 1211-1219.
149. McKallip, R.J., et al., *Targeting CB2 cannabinoid receptors as a novel therapy to treat malignant lymphoblastic disease.* Blood, The Journal of the American Society of Hematology, 2002. **100**(2): p. 627-634.
150. Jia, W., et al., *Δ9-tetrahydrocannabinol-induced apoptosis in Jurkat leukemia T cells is regulated by translocation of Bad to mitochondria.* Molecular Cancer Research, 2006. **4**(8): p. 549-562.
151. Gustafsson, K., et al., *Cannabinoid receptor-mediated apoptosis induced by R (+)-methanandamide and Win55, 212-2 is associated with ceramide accumulation and p38 activation in mantle cell lymphoma.* Molecular pharmacology, 2006. **70**(5): p. 1612-1620.
152. Gustafsson, K., et al., *Expression of cannabinoid receptors type 1 and type 2 in non-Hodgkin lymphoma: Growth inhibition by receptor activation.* International journal of cancer, 2008. **123**(5): p. 1025-1033.
153. Blázquez, C., et al., *Cannabinoid receptors as novel targets for the treatment of melanoma.* The FASEB journal, 2006. **20**(14): p. 2633-2635.
154. Figueiredo, A.S., et al., *Suppression of vascular endothelial growth factor expression by cannabinoids in a canine osteosarcoma cell line.* Veterinary Medicine: Research and Reports, 2013. **4**: p. 31-34.
155. Mon, M.J., et al., *Influence of Δ9-tetrahydrocannabinol on cell proliferation and macromolecular biosynthesis in human cells.* Biochemical pharmacology, 1978. **27**(13): p. 1759-1765.
156. Blevins, R. and D. Smith, *Effects of delta-9-tetrahydrocannabinol on cultured HeLa cell growth and development.* Growth, 1980. **44**(2): p. 133-138.
157. Olea-Herrero, N., et al., *Inhibition of human tumour prostate PC-3 cell growth by cannabinoids R (+)-Methanandamide and JWH-015: involvement of CB 2.* British journal of cancer, 2009. **101**(6): p. 940-950.
158. De Petrocellis, L. and V. Di Marzo, *An introduction to the endocannabinoid system: from the early to the latest concepts.* Best practice & research Clinical endocrinology & metabolism, 2009. **23**(1): p. 1-15.

159. Basu, S., A. Ray, and B.N. Dittel, *Cannabinoid receptor 2 is critical for the homing and retention of marginal zone B lineage cells and for efficient T-independent immune responses.* The Journal of Immunology, 2011. **187**(11): p. 5720-5732.
160. Iannotti, F.A., V. Di Marzo, and S. Petrosino, *Endocannabinoids and endocannabinoid-related mediators: targets, metabolism and role in neurological disorders.* Progress in lipid research, 2016. **62**: p. 107-128.
161. Zygmunt, P.M., et al., *Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide.* Nature, 1999. **400**(6743): p. 452-457.
162. Ramer, R., R. Schwarz, and B. Hinz, *Modulation of the endocannabinoid system as a potential anticancer strategy.* Frontiers in pharmacology, 2019. **10**: p. 430.
163. Wasik, A.M., B. Christensson, and B. Sander. *The role of cannabinoid receptors and the endocannabinoid system in mantle cell lymphoma and other non-Hodgkin lymphomas*. in *Seminars in cancer biology*. 2011. Elsevier.
164. Pisanti, S., et al., *The endocannabinoid signaling system in cancer.* Trends in pharmacological sciences, 2013. **34**(5): p. 273-282.
165. Wasik, A.M., et al., *Perturbations of the endocannabinoid system in mantle cell lymphoma: correlations to clinical and pathological features.* Oncoscience, 2014. **1**(8): p. 550.
166. Flygare, J., et al., *Cannabinoid receptor ligands mediate growth inhibition and cell death in mantle cell lymphoma.* FEBS letters, 2005. **579**(30): p. 6885-6889.
167. Pondugula, S.R., et al., *Predictable hematological markers associated with cognitive decline in valid rodent models of cognitive impairment.* Toxicol Mech Methods, 2020. **30**(6): p. 454-461.
168. Majrashi, M., et al., *Dopaminergic neurotoxic effects of 3-TFMPP derivatives.* Life sciences, 2018. **209**: p. 357-369.
169. Hong, S., et al., *Agmatine protects cultured retinal ganglion cells from tumor necrosis factor-alpha-induced apoptosis.* Life sciences, 2009. **84**(1-2): p. 28-32.
170. Dhanasekaran, M., B. Tharakan, and B.V. Manyam, *Antiparkinson drug–Mucuna pruriens shows antioxidant and metal chelating activity.* Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives, 2008. **22**(1): p. 6-11.
171. Giustarini, D., et al., *Nitrite and nitrate measurement by Griess reagent in human plasma: evaluation of interferences and standardization.* Methods in enzymology, 2008. **440**: p. 361-380.
172. Almaghrabi, M., *Investigate the dopaminergic neurotoxicity profile of designer drugs (Piperazine derivatives).* 2018.
173. Ramsay, R.R., et al., *Energy-driven uptake of N-methyl-4-phenylpyridine by brain mitochondria mediates the neurotoxicity of MPTP.* Life sciences, 1986. **39**(7): p. 581-588.
174. Ahuja, M., et al., *Immunological alteration & toxic molecular inductions leading to cognitive impairment & neurotoxicity in transgenic mouse model of Alzheimer's disease.* Life Sci, 2017. **177**: p. 49-59.
175. Bhattacharya, D., et al., *Assessment of the cerebellar neurotoxic effects of nicotine in prenatal alcohol exposure in rats.* Life sciences, 2018. **194**: p. 177-184.
176. Al Saqr, A., et al., *Elucidating the anti-melanoma effect and mechanisms of Hispolon.* Life sciences, 2020. **256**: p. 117702.

177. Copeland, R.A., et al., *Mechanism of selective inhibition of the inducible isoform of prostaglandin G/H synthase.* Proceedings of the National Academy of Sciences, 1994. **91**(23): p. 11202-11206.
178. Usha, R., et al., *Region-specific attenuation of a trypsin-like protease in substantia nigra following dopaminergic neurotoxicity by 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine.* Brain research, 2000. **882**(1-2): p. 191-195.
179. Nong, L., et al., *CB1 and CB2 receptor mRNA expression in human peripheral blood mononuclear cells (PBMC) from various donor types*, in *Neuroimmune circuits, drugs of abuse, and infectious diseases*. 2002, Springer. p. 229-233.
180. Zhang, J., et al., *The potential relevance of the endocannabinoid, 2-arachidonoylglycerol, in diffuse large B-cell lymphoma.* Oncoscience, 2016. **3**(1): p. 31.
181. Sobolewski, C., et al., *The role of cyclooxygenase-2 in cell proliferation and cell death in human malignancies.* International journal of cell biology, 2010. **2010**.
182. Patsos, H.A., et al., *The endogenous cannabinoid, anandamide, induces COX-2-dependent cell death in apoptosis-resistant colon cancer cells.* International journal of oncology, 2010. **37**(1): p. 187-193.
183. Adinolfi, B., et al., *Anticancer activity of anandamide in human cutaneous melanoma cells.* European journal of pharmacology, 2013. **718**(1-3): p. 154-159.
184. Yamaji, K., et al., *Anandamide induces apoptosis in human endothelial cells: its regulation system and clinical implications.* Thrombosis and haemostasis, 2003. **89**(05): p. 875-884.
185. Athanasiou, A., et al., *Cannabinoid receptor agonists are mitochondrial inhibitors: a unified hypothesis of how cannabinoids modulate mitochondrial function and induce cell death.* Biochemical and biophysical research communications, 2007. **364**(1): p. 131-137.
186. Tucker, A. and M. Friedman, *Effects of cannabinoids on L1210 murine leukemia. 1. Inhibition of DNA synthesis.* Research communications in chemical pathology and pharmacology, 1977. **17**(4): p. 703-714.
187. Ito, D., A.M. Frantz, and J.F. Modiano, *Canine lymphoma as a comparative model for human non-Hodgkin lymphoma: recent progress and applications.* Veterinary immunology and immunopathology, 2014. **159**(3-4): p. 192-201.
188. Zandvliet, M., *Canine lymphoma: a review.* Veterinary Quarterly, 2016. **36**(2): p. 76-104.
189. Breen, M. and J.F. Modiano, *Evolutionarily conserved cytogenetic changes in hematological malignancies of dogs and humans–man and his best friend share more than companionship.* Chromosome Research, 2008. **16**(1): p. 145-154.
190. Castor, A., et al., *Distinct patterns of hematopoietic stem cell involvement in acute lymphoblastic leukemia.* Nature medicine, 2005. **11**(6): p. 630-637.
191. Challa, A., et al., *Population depletion activates autonomous CD154-dependent survival in biopsylike Burkitt lymphoma cells.* Blood, The Journal of the American Society of Hematology, 2002. **99**(9): p. 3411-3418.
192. Sánchez, C., et al., *Inhibition of glioma growth in vivo by selective activation of the CB2 cannabinoid receptor.* Cancer research, 2001. **61**(15): p. 5784-5789.
193. Bifulco, M., et al., *Control by the endogenous cannabinoid system of ras oncogene-dependent tumor growth.* The FASEB Journal, 2001. **15**(14): p. 1-17.
194. Velasco, G., et al., *Hypothesis: cannabinoid therapy for the treatment of gliomas?* Neuropharmacology, 2004. **47**(3): p. 315-323.

195. Sarker, K.P., et al., *Anandamide induces apoptosis of PC-12 cells: involvement of superoxide and caspase-3.* FEBS letters, 2000. **472**(1): p. 39-44.
196. Maccarrone, M., et al., *Anandamide induces apoptosis in human cells via vanilloid receptors: evidence for a protective role of cannabinoid receptors.* Journal of Biological Chemistry, 2000. **275**(41): p. 31938-31945.
197. Blasco-Benito, S., et al., *Appraising the "entourage effect": Antitumor action of a pure cannabinoid versus a botanical drug preparation in preclinical models of breast cancer.* Biochemical pharmacology, 2018. **157**: p. 285-293.
198. Milian, L., et al., *Cannabinoid receptor expression in non-small cell lung cancer. Effectiveness of tetrahydrocannabinol and cannabidiol inhibiting cell proliferation and epithelial-mesenchymal transition in vitro.* PLoS One, 2020. **15**(2): p. e0228909.
199. Sarfaraz, S., et al., *Cannabinoid receptor as a novel target for the treatment of prostate cancer.* Cancer research, 2005. **65**(5): p. 1635-1641.
200. Caffarel, M., et al., *& Sanchez, C.(2010). Cannabinoids reduce ErB2-driven breast cancer progression through Akt inhibition.* Molecular Cancer. **9**: p. 196.
201. Guindon, J. and A.G. Hohmann, *The endocannabinoid system and cancer: therapeutic implication.* British journal of pharmacology, 2011. **163**(7): p. 1447-1463.
202. Pertwee, R.G., et al., *International Union of Basic and Clinical Pharmacology. LXXIX. Cannabinoid receptors and their ligands: beyond CB1 and CB2.* Pharmacological reviews, 2010. **62**(4): p. 588-631.
203. Pertwee, R.G., *Ligands that target cannabinoid receptors in the brain: from THC to anandamide and beyond.* Addiction biology, 2008. **13**(2): p. 147-159.
204. Zheng, M., et al., *Evaluation of differential cytotoxic effects of the oil spill dispersant Corexit 9500.* Life sciences, 2014. **95**(2): p. 108-117.
205. Ohkawa, H., N. Ohishi, and K. Yagi, *Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction.* Analytical biochemistry, 1979. **95**(2): p. 351-358.
206. Anand, U., et al., *CBD Effects on TRPV1 Signaling Pathways in Cultured DRG Neurons.* Journal of Pain Research, 2020. **13**: p. 2269.
207. Derocq, J.-M., et al., *Cannabinoids enhance human B-cell growth at low nanomolar concentrations.* FEBS letters, 1995. **369**(2-3): p. 177-182.
208. Carayon, P., et al., *Modulation and functional involvement of CB2 peripheral cannabinoid receptors during B-cell differentiation.* Blood, The Journal of the American Society of Hematology, 1998. **92**(10): p. 3605-3615.
209. Massi, P., et al., *The non-psychoactive cannabidiol triggers caspase activation and oxidative stress in human glioma cells.* Cellular and Molecular Life Sciences CMLS, 2006. **63**(17): p. 2057-2066.
210. McKallip, R.J., et al., *Cannabidiol-induced apoptosis in human leukemia cells: a novel role of cannabidiol in the regulation of p22phox and Nox4 expression.* Molecular Pharmacology, 2006. **70**(3): p. 897-908.
211. Marcu, J.P., et al., *Cannabidiol enhances the inhibitory effects of Δ9-tetrahydrocannabinol on human glioblastoma cell proliferation and survival.* Molecular cancer therapeutics, 2010. **9**(1): p. 180-189.
212. Rimmerman, N., et al., *Direct modulation of the outer mitochondrial membrane channel, voltage-dependent anion channel 1 (VDAC1) by cannabidiol: a novel mechanism for cannabinoid-induced cell death.* Cell death & disease, 2013. **4**(12): p. e949-e949.

213. Singer, E., et al., *Reactive oxygen species-mediated therapeutic response and resistance in glioblastoma.* Cell death & disease, 2015. **6**(1): p. e1601-e1601.
214. McAllister, S.D., L. Soroceanu, and P.-Y. Desprez, *The antitumor activity of plant-derived non-psychoactive cannabinoids.* Journal of neuroimmune pharmacology, 2015. **10**(2): p. 255-267.
215. Meng, J., et al., *Combination therapy using co-encapsulated resveratrol and paclitaxel in liposomes for drug resistance reversal in breast cancer cells in vivo.* Scientific reports, 2016. **6**(1): p. 1-11.
216. Galustian, C. and A.G. Dalgleish, *Article Commentary: The Power of the Web in Cancer Drug Discovery and Clinical Trial Design: Research without a Laboratory?* Cancer informatics, 2010. **9**: p. CIN. S3191.
217. Lehne, G., *P-glycoprotein as a drug target in the treatment of multidrug resistant cancer.* Current drug targets, 2000. **1**(1): p. 85-99.
218. Lavi, O., M.M. Gottesman, and D. Levy, *The dynamics of drug resistance: a mathematical perspective.* Drug Resistance Updates, 2012. **15**(1-2): p. 90-97.
219. Hu, C.-M.J. and L. Zhang, *Nanoparticle-based combination therapy toward overcoming drug resistance in cancer.* Biochemical pharmacology, 2012. **83**(8): p. 1104-1111.
220. Hiss, D.C., G.A. Gabriels, and P.I. Folb, *Combination of tunicamycin with anticancer drugs synergistically enhances their toxicity in multidrug-resistant human ovarian cystadenocarcinoma cells.* Cancer cell international, 2007. **7**(1): p. 1-14.
221. Iwamoto, T., *Clinical application of drug delivery systems in cancer chemotherapy: review of the efficacy and side effects of approved drugs.* Biological and Pharmaceutical Bulletin, 2013. **36**(5): p. 715-718.
222. Kummar, S., et al., *Utilizing targeted cancer therapeutic agents in combination: novel approaches and urgent requirements.* Nature reviews Drug discovery, 2010. **9**(11): p. 843-856.
223. Hosoya, K., et al., *Comparison of COAP and UW-19 protocols for dogs with multicentric lymphoma.* Journal of Veterinary Internal Medicine, 2007. **21**(6): p. 1355-1363.
224. Harrison, J.S., et al., *Leukemia and lymphoma society.* 2012.
225. Impellizeri, J.A., et al., *The role of rituximab in the treatment of canine lymphoma: an ex vivo evaluation.* The Veterinary Journal, 2006. **171**(3): p. 556-558.
226. Majchrzak, A., et al., *In vitro cytotoxicity of ranpirnase (onconase) in combination with components of R-CHOP regimen against diffuse large B cell lymphoma (DLBCL) cell line.* Advances in Hygiene & Experimental Medicine/Postepy Higieny i Medycyny Doswiadczalnej, 2013. **67**.
227. Hinz, B. and R. Ramer, *Anti-tumour actions of cannabinoids.* British journal of pharmacology, 2019. **176**(10): p. 1384-1394.
228. Ramer, R. and B. Hinz, *Cannabinoids as anticancer drugs.* Advances in Pharmacology, 2017. **80**: p. 397-436.
229. Ramsey, F. and D. Schafer, *The statistical sleuth: a course in methods of data analysis*. 2012: Cengage Learning.
230. Rodriguez, R.N., *Sas.* Wiley Interdisciplinary Reviews: Computational Statistics, 2011. **3**(1): p. 1-11.
231. Chou, T.-C., *Drug combination studies and their synergy quantification using the Chou-Talalay method.* Cancer research, 2010. **70**(2): p. 440-446.

232. Chou, T.-C. and P. Talalay, *Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors.* Advances in enzyme regulation, 1984. **22**: p. 27-55.
233. Huang, R.-y., et al., *Isobologram analysis: a comprehensive review of methodology and current research.* Frontiers in pharmacology, 2019. **10**: p. 1222.
234. Gustafsson, S.B., et al., *Cannabinoid receptor-independent cytotoxic effects of cannabinoids in human colorectal carcinoma cells: synergism with 5-fluorouracil.* Cancer chemotherapy and pharmacology, 2009. **63**(4): p. 691-701.
235. Andradas, C., et al., *Assessment of cannabidiol and Δ9-Tetrahydrocannabiol in mouse models of Medulloblastoma and Ependymoma.* Cancers, 2021. **13**(2): p. 330.
236. Strong, T., et al., *Synergistic Effect of Cannabidiol with Conventional Chemotherapy Treatment.* Blood, 2018. **132**: p. 5382.