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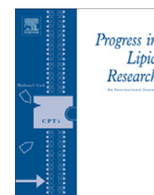

Review

Cannabinoids and omega-3/6 endocannabinoids as cell death and anticancer modulators

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ABSTRACT

Cannabinoids-endocannaboids are possible preventatives of common diseases including cancers. Cannabinoid receptors ($CB_{1/2}$, TRPV1) are central components of the system. Many disease-ameliorating effects of cannabinoids-endocannabinoids are receptor mediated, but many are not, indicating non-CBR signaling pathways. Cannabinoids-endocannabinoids are anti-inflammatory, anti-proliferative, anti-invasive, anti-metastatic and pro-apoptotic in most cancers, *in vitro* and *in vivo* in animals. They signal through p38, MAPK, JUN, PI3, AKT, ceramide, caspases, MMPs, PPARs, VEGF, NF-κB, p8, CHOP, TRB3 and pro-apoptotic oncogenes (p53,p21 waf1/cip1) to induce cell cycle arrest, autophagy, apoptosis and tumour inhibition. Paradoxically they are pro-proliferative and anti-apoptotic in some cancers. Differences in receptor expression and concentrations of cannabinoids in cancer and immune cells can elicit anti- or pro-cancer effects through different signal cascades (p38MAPK or PI3/AKT). Similarities between effects of cannabinoids-endocannabinoids, omega-3 LCPUFA and CLAs/CLnAs as anti-inflammatory, anti-angiogenic, anti-invasive anti-cancer agents indicate common signaling pathways. Evidence *in vivo* and *in vitro* shows EPA and DHA can form endocannabinoids that: (i) are ligands for $CB_{1/2}$ receptors and possibly TRPV-1, (ii) have non-receptor mediated bioactivity, (iii) induce cell cycle arrest, (iii) increase autophagy and apoptosis, and (iv) augment chemotherapeutic actions *in vitro*. They can also form bioactive, eicosanoid-like products that appear to be non-CBR ligands but have effects on PPARs and NF-kB transcription factors.

The use of cannabinoids in cancer treatment is currently limited to chemo- and radio-therapy-associated nausea and cancer-associated pain apart from one trial on brain tumours in patients. Further clinical studies are urgently required to determine the true potential of these intriguing, low toxicity compounds in cancer therapy. Particularly in view of their synergistic effects with chemotherapeutic agents similar to that observed for $n-3$ LCPUFA.

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Abbreviations: 2-AG, 2-arachidonoylglycerol; AEA, anadamide (arachidonoyl ethanolamide); Akt, protein kinase B; AMPK, adenosine monophosphate-activated protein kinase; ANG, angiopoetins; BAD, Bcl-2-associated death promoter; BAX, Bcl-2-associated X protein; BCl-2, B-cell lymphoma 2; CaMKKβ, calcium/calmodulin-dependent protein kinase kinase 2; CB, cannabinoid receptor; CBD, cannabidiol; cDNA, complementary DNA; CLA, conjugated linoleic acid; CLnA, conjugated linolenic acid; COX-2, cyclooxygenase 2; DHA, docosahexaenoic acid; DHEA, docosahexaenoyl ethanolamide; EPA, eicosapentaenoic acid; EPEA, eicosapentaenoyl ethanolamide; ERK, extracellular signal-regulated kinase; FAAH, fatty acid amide hydrolase; GPCR, G protein-coupled receptor; GPR55, G protein-coupled receptor 55; HETE, hydroxyeicosatetraenoic acid; HPETE, hydroperoxyeicosatetraenoic acid; ICAM, intercellular adhesion molecules; ID-1, DNA-binding protein inhibitor 1; JNK, c-Jun N-terminal kinase; LCPUFA, long chain poly unsaturated fatty acid; LOX, lipoxygenase; MAGL, monoacylglycerol lipase; MAPK, mitogen-activated protein kinase; MMPs, matrix metalloproteinases; mTORC1, mammalian target of rapamycin (mTOR) complex 1; NAAE, N-acylethanolamine acid amidase; NAE, N-acylethanolamine; NAPE, N-acylphosphatidyl ethanolamine; NAPE-PLD, N-acylphosphatidyl ethanolamine hydrolizing phospholipid D; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; PAI-1, plasminogen activator inhibitor 1; PGD2, prostaglandin D2; PGE2, prostaglandin E2; PI3K, phosphoinositide 3-kinase; PPAR, peroxisome proliferator-activated receptor; ROS, reactive oxygen species; RT-PCR, reverse transcription PCR; SiRNA, small interfering RNA; THC, tetrahydrocannabinol; TRB3, tribbles homolog 3; TRPV, transient receptor potential vanilloid receptor; VEGF, vascular endothelial growth factor.

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1. Introduction

1.1. Brief history and overview of the cannabinoid system

The medicinal and recreational properties of the plant *Cannabis sativa* Linnaeus, commonly referred to as hemp, hashish or marijuana, have been known and documented for centuries, particularly in Asia [1–3]. The therapeutic value of cannabis was first assessed scientifically by William O'Shaugnessy working in Calcutta in the early 19th century and publicised in the Western World [4]. Surprisingly, the extraction, isolation and structural identification of the most active component of the plant, trans-Δ^9-tetrahydrocannabinol (Δ^9-THC), was not reported until the publication by Gaoni & Mechoulam in1964 [5]. Since then approximately 88 unique terpenophenols with carbon side chains varying from C1 to C5 in length have been found in cannabis extracts [6,7]. They have been classified according to their structure. The antineoplastic effects of cannabinoids (e.g. THC) on cancer cells were recognised in the 1970s by Munson and colleagues [8,9]. (Examples for Δ^9-THC, Δ^8-THC, cannabinol, cannabidiol and cannabicyclol structures are shown in Fig. 1.)

These compounds are termed *phytocannabinoids*, due to their activation of the more recently identified classical cannabinoid receptors CB_1 and CB_2 and possibly TRPV-1 (transient receptor potential vanilloid 1). These receptors are recognized as vital components of the cannabinoid system through which the cannabinoids-endocannabinoids generally, but not exclusively, exert their effects although they were discovered only recently (see below).

Following the earlier determination of the structure of various phytocannabinoids and the discovery of the CB receptors in various

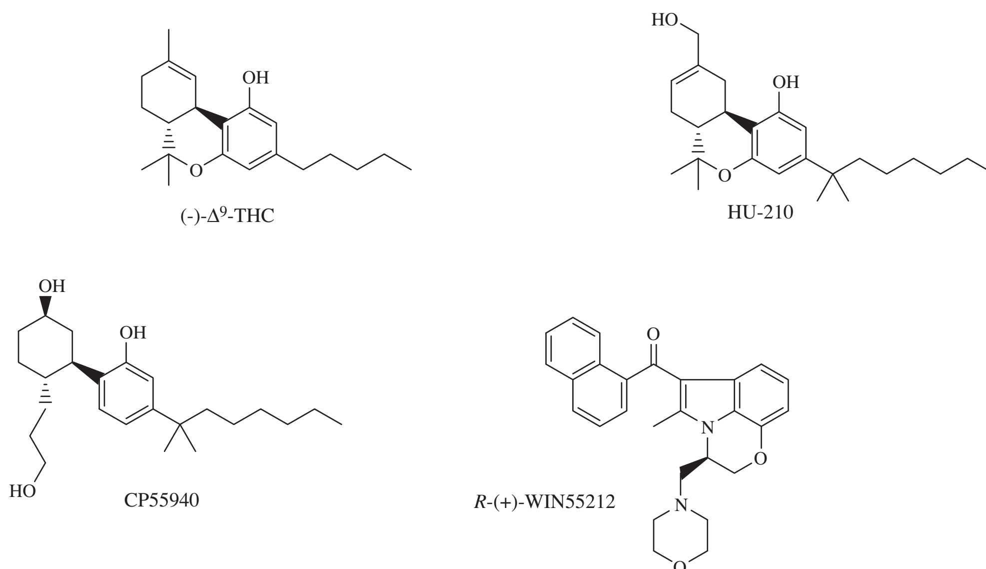

Fig. 1. Structure of classical and non-classical cannabinoids.

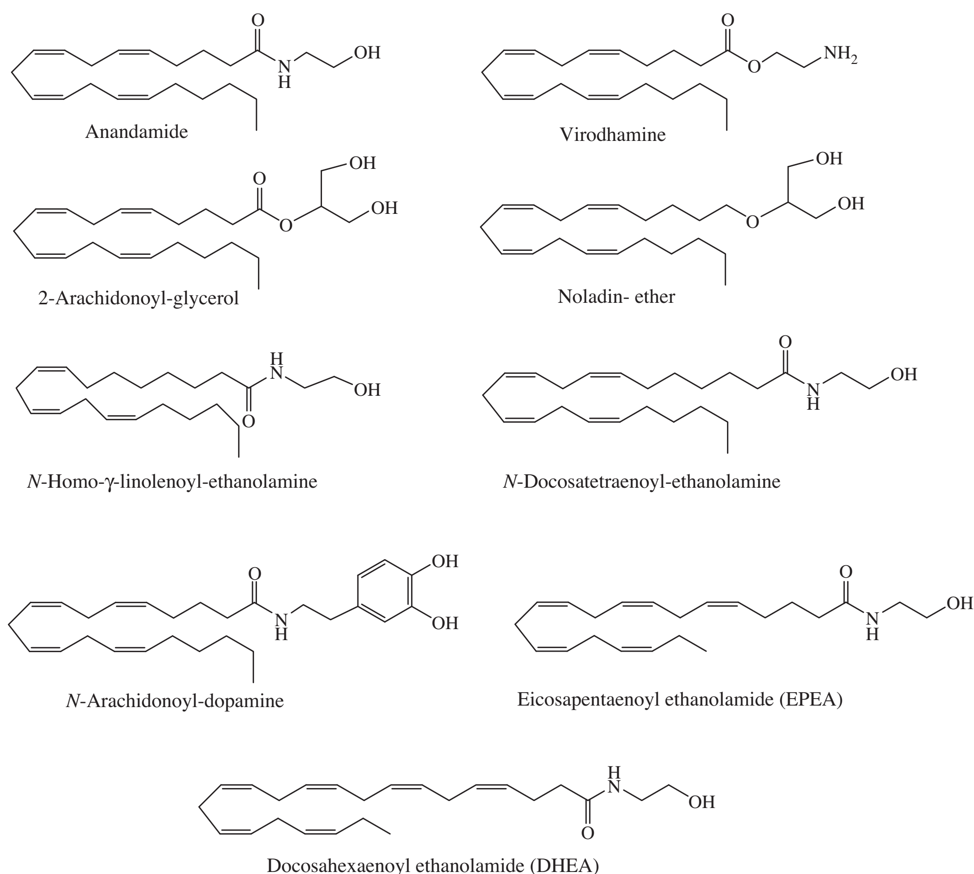

Fig. 2. Structure of endocannabinoids including $n-3$ and $n-6$ derivatives.

tissues, the quest for *synthetic analogs* of these plant cannabinoids that would hopefully exhibit greater potency grew apace and resulted in a number of interesting compounds being produced (for examples of structures for CP55940, WIN 55,212-2, JWH-133, HU-210, SR141716 (see Figs. 1–4) [1,2,10–18].

The discovery of two endogenously produced cannabinoids, now termed *endocannabinoids*, namely anandamide or arachidonoylethanolamide [AEA] and sn-2-arachidonoylglycerol [2-AG] [10,13,14] opened a new line of scientific enquiry. It explained, at least in part, the mode of action of cannabinoids in general and led to the identification of other endogenous saturated, monounsaturated and polyunsaturated fatty acid-derived N-acylethanolamides (NAEs) such as palmitoylethanolamide (PEA) and oleoylethanolamide (OEA). These compounds appear to have can-

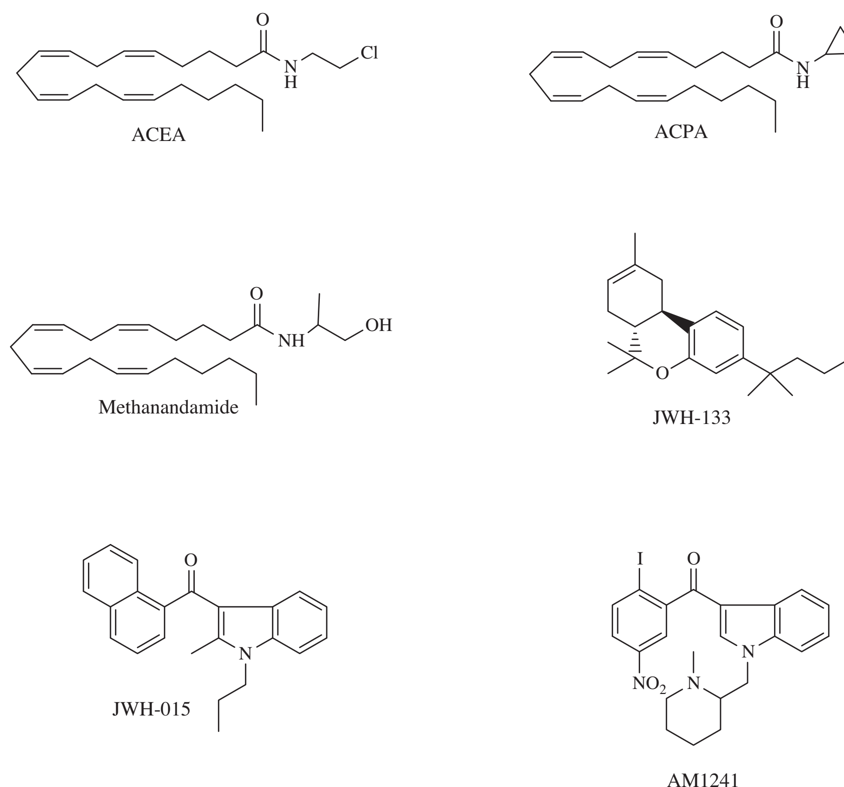

Fig. 3. Structure of some important synthetic selective CB_1 agonists.

nabinomimetic activity but do not bind the classical cannabinoid receptors mentioned above. It has been suggested that they may exert their cannabimimetic effects by acting as "entourage molecules" that prevent anandamide or other true cannabinoids being degraded by specific enzymes that regulate the concentrations of these compounds in tissues and are an integral component of the cannabinoid system. The two major degrading enzymes are fatty acylamide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) (see below); their inhibition increases the availability of the true endocannabinoids in cells [10,13,15–19]. It was also shown that cannabinoids-endocannabinoids can bind to other non-cannabinoid receptors like TRPA (transient receptor potential ankyrin), TRPM (transient receptor potential melastatin) and TRPV (transient receptor potential vanilloid) receptors and transcription factors like PPARs and NF-kB to exert their beneficial effects since a number of cannabinoid-endocannabinoid effects in cells and animal models are not attenuated by $CB_{1/2}$ receptor antagonists. Non-receptor mediated effects of cannabinoids-endocannabinoids have also been reported in various cells and tissues (see below and [10]). Clearly, such diverse modes of action indicate a complex, albeit intriguing, regulatory system.

The $n-3$ long-chain polyunsaturated fatty acids ($n-3$ LCPUFA, C-18 to C22), derived mainly from fish oils in the human diet, have long been regarded as having many significant health benefits. Epidemiological studies, animal studies *in vivo* and cell studies *in vitro* strongly suggest that their presence can attenuate/prevent the incidence of cardiovascular disease, many inflammatory disorders and also various aspects of the cancer process (antiangiogenic, antiadhesive, antiinvasive, pro-apoptotic, pro-cell cycle arrest). They are also capable of augmenting the efficacy of various chemotherapeutic agents [20–29]. Some of the reported health benefits of the $n-3$ LCPUFA have also been ascribed to conjugated linoleic and/or conjugated linolenic acids (CLAs/CLnAs, C18 PUFA with non-methylene interrupted double bonds in chain) *in vivo* and *in vitro* in various disease states, including cancer [24,25]. This is particularly true for their anti-inflammatory, anti-proliferative, anti-metatstaic, anti-angiogenic and pro-apototic effects. Interestingly, the effects of these fatty acids mirror many of the reported effects of cannabinoids on equally varied mammalian and human disorders and cancer types, both *in vivo* and *in vitro* (see below). The remarkable similarities between the reported health benefits/effects of $n-3$ LCPUFA, particularly EPA (20:5) and DHA (22:6), the CLAs and CLnAs and cannabinoids-endocannabinoids led to speculation that they could be due to: (i) their direct effects on the metabolic-signaling pathways resulting in the attenuation or prevention of various pathologies or (ii) to their subsequent conversion to the respective $n-3$, CLA or CLnA N-acylethanolamides (NAE) or (iii) to the further oxidative conversion of the N-acylethanolamides to the corresponding cyclooxygenase, lipoxygenase and/or cytochrome P_{450} derivatives [26] (see below).

The suggestion in (ii) above does have some support from recent studies in animals *in vivo*, tissues *ex vivo* and different cell types *in vitro* [26–38]. Addition of the $n-3$ LCPUFA, eicosapentaenoic acid (EPA) and/or docosahexaenoic acid (DHA), to prostate cancer cells [38] and 3T3 adipocytes [31] resulted in increased production of the respective ethanolamide derivatives (EPEA and DHEA). Similarly, studies *in vivo* in animals and man have also shown the presence of EPEA and DHEA in tissues, including plasma. Increased intake of $n-3$ LCPUFA enhanced production of the corresponding $n-3$ ethanolamides in brain, liver, gut and plasma [31–37]. Furthermore, Meijerink et al. [34] recently reported that EPEA and DHEA inhibited lipopolysaccharide-induced nitric oxide production in a macrophage cell line and DHEA suppressed the production of inflammatory MCP-1 (monocyte chemotactic protein-1). Previous studies have shown $n-3$ ethanolamides can bind to CB_1 receptors [39–41] and we recently published evidence to show that both the EPEA and DHEA can bind to and activate both CB_1 and CB_2 receptors in prostate cancer cells [42]. The further oxi-

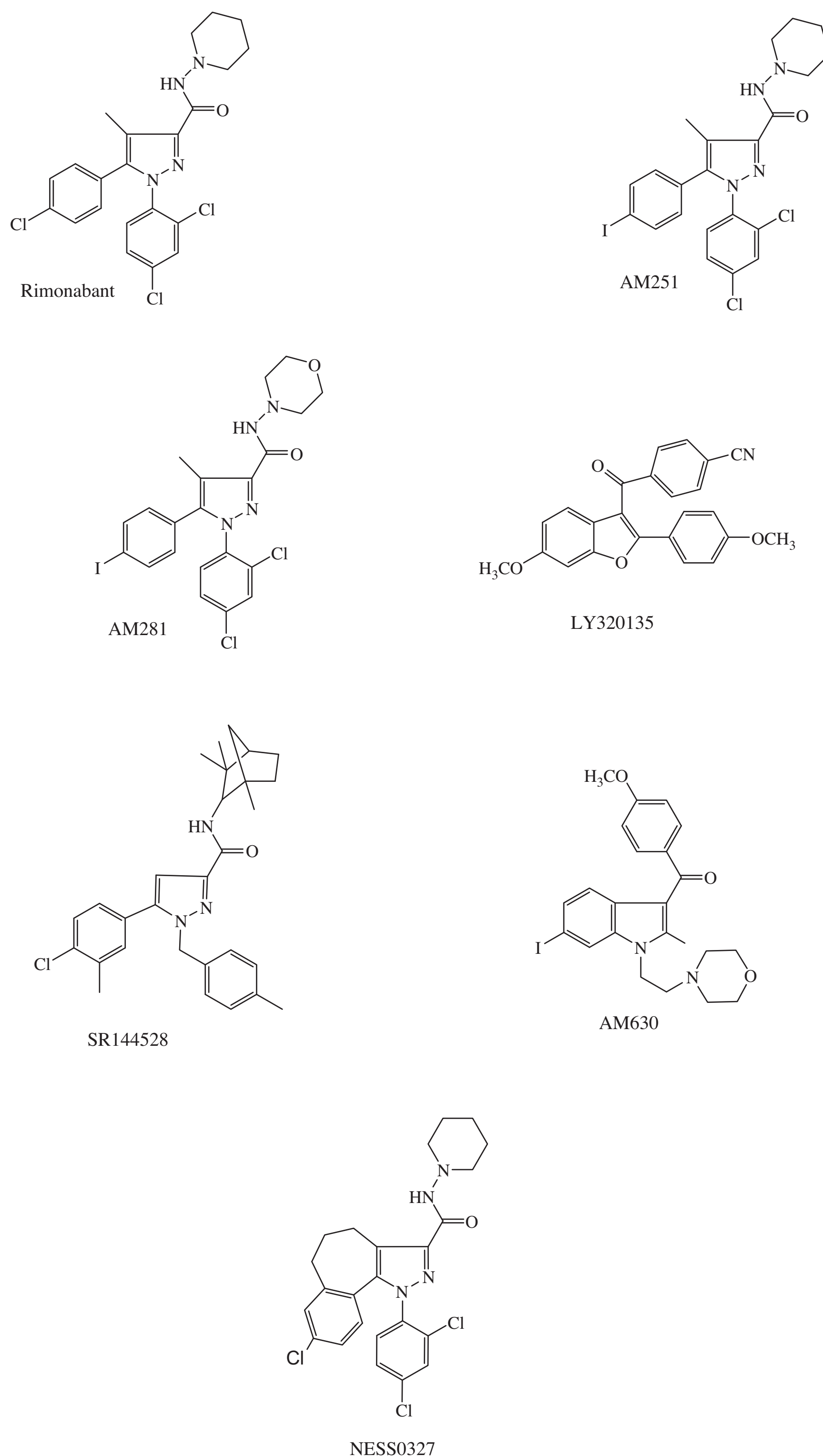

Fig. 4. Structure of $CB_{1/2}$ synthetic antagonists.

dative metabolism in cells and tissues of AEA and 2-AG to a plethora of potentially bioactive derivatives has also been reported. The physiological significance of these compounds has not been determined sufficiently to allow a definitive role to be ascribed to them (see below).

From the foregoing brief outline it is evident that the endocannabinoid system (ECS) *per se* consists of: (i) a number of receptors with varying specificities and differential tissue/cell distribution that are pivotal components of the system; (ii) the endogenously synthesised (endocannabinoid) ligands of these receptors with their differing specificities for these receptors; (iii) their non-receptor mediated effects; (iv) the relevant membrane transporters and (v) the enzymes responsible for the synthesis and degradation of these ligands. Some of the latter are also responsible for the syn-

thesis and degradation of a number of fatty acid-derived primary ethanolamines, acylethanolamides and 2-acylmonoacylglycerides. These can also influence cancer cell proliferation, metastasis and apoptosis through mechanisms that are not clearly understood at present and may not always involve binding to the classical $CB_{1/2}$ receptors (see below). Some of the oxidative catabolic enzymes such as cyclooxygenases, lipoxygenases and cytochrome-P_{450} are able to further oxidise endocannabinoids to their respective bioactive derivatives [11,26,43–61]. Some of these, particularly the prostaglandin derivatives, appear not to act as $CB_{1/2}$ receptor agonists or eicosanoid receptor agonists but may act through other receptors such as PPARs [61] (see below).

A more detailed, but by no means all encompassing, review of the cannabinoid system and its putative role in cancer is presented below.

2. Cannabinoid receptors

The two classical cannabinoid receptor subtypes were only identified and cloned in the early 1990s despite the fact that the effects of cannabis have been known for centuries. This discovery indicated the existence of endogenous ligands and resulted in the concept of a cannabinoid-endocannabinoid system in mammalian physiology. The cannabinoid receptor-1 (CB_1) is located mainly, but not exclusively, in the central nervous system [10–14,43] and the CB_2 receptor is expressed mainly, but not solely, peripherally in the immune system (see above and [7–14,43–46]). Both receptors are members of the G-protein coupled receptor super-family acting through inhibition of adenylcyclase and activation of ERK/MAPK, PI3K/AKT and/or p8, TRB3, mTORC1to regulate a multitude of complex cell signaling pathways that influence cell proliferation, autophagia, cell death (apoptosis) [10,43,47,48]. There is evidence for the existence of other cannabinoid receptors such as transient receptor potential vanilloid type 1 (TRPV1) and GRP55,5 (orphan) receptors as well as receptors (i.e. TRPA1, TRPV2–4, TRPM8) that appear to be activated to some extent by a variety of cannabinoids-endocannabinoids but are not regarded as classical cannabinoid receptors at present (see below). Non-receptor mediated, cannabimimetic effects of cannabinoids, endocannabinoids and non-cannabinoid acylethanolamines on receptors like the transcription factors PPARs and NF-κB can also occur and significantly influence cell proliferation and apoptosis in a variety of tissues [10,11,49–51]. The importance of the interactions between various receptor and non-receptor mediated effects of cannabinoids-endocannabinoids; the relative expression and differential distribution of the classical $CB_{1/2}$ receptors in cancers and normal cells/tissues and the differences in coupling to major cell signaling pathways, will be discussed in detail below.

Cannabinoid compounds that exert their actions through binding and activating the various CB receptors have been simply classified into three main categories as mentioned above. Of these, the *endocannabinoids* that are released on demand in response to various physiological and pathological stimuli, are the natural, endogenous ligands of the CB receptors. However, externally administered phytocannabinoids and their synthetic analogs are also effective ligands for these receptors and they can activate a variety of cell signaling pathways, some of which may confer health benefits in mammalian systems, including in man. Paradoxically, the opposite effects have also been reported for certain cancers, with some of these compounds promoting cell proliferation and procancer effects under certain conditions [5–7].

Clearly, the role of the cannabinoid-endocannabinoid system in the regulation of anticancer or pro-cancer mechanisms is a complex one. The reader is referred to a number of excellent recent reviews on this topic that address the issues, often from somewhat different perspectives [7–11,43,45,46,55,57,62–66]. The objective of this review is to provide a general overview and update of current cannabinoid-endocannabinoid research and to highlight certain intriguing aspects of recent advances in the field that may have potential therapeutic benefits in cancer treatment and care. The intriguing similarities between the anticancer effects of endocannabinoids, particularly the omega-6 arachidonoyl derivative anandamide and 2-AG and the effects of omega-3 LCPUFA, CLAs/CLnAs and their respective ethanolamide derivatives will be discussed in relation to the possible beneficial effects they can exert. The question whether these effects are due to parent fatty acids *per se* or due to their respective endocannabinoid derivatives or even to their further oxidation to bioactive eicosanoid-like derivatives will also be considered.

2.1. CB_1 and CB_2 receptors

The CB_1 and CB_2 receptors are currently regarded as the only true cannabinoid receptors although the vanilloid receptor TPRV-1 has been mooted as a potential CB_3 receptor; other TRP receptors are also activated by certain cannabinoids (see below). Agonists of these receptors can also bind and activate other ion channels and proteins and elicit signal transduction in cells/tissues (see below and Pertwee et al. (2010) for detailed review). In 1990 Matsuda et al. [67] cloned an orphan G protein-coupled receptor from a cDNA library of rat brain cortex and found that it mediated the characteristic pharmacological effects of Δ^9-THC, the major psychoactive component of cannabis. Thus, the first true cannabinoid receptor, now termed CB_1, was identified in neurological tissue. This was followed by the cloning of both human and rat cDNA analogs that encoded proteins that had different amino acid chain lengths between the species; i.e. 327 and 473 amino acids respectively for man and rat, but they had a 97–99% sequence homology with one another [68]. A non-neurological G protein coupled CB_2 receptor was then identified and cloned from the DNA of human promyelocytic leukemic HL60 cells This gene encoded a much shorter protein, only 360 amino acids long, that had only 40% homology with the CB_1 receptor. Ligand-activation of both receptors was shown to inhibit adenylate cyclase activity and to activate mitogen activated protein kinase (MAPK) by signal transduction through $G_{i/o}$ proteins [68,69]. The CB_1 receptor was also shown to transduce signals through G_s protein coupling [70–72]. Apart from the major orthosteric binding site for cannabinoids, the CB_1 receptor also possesses allosteric binding sites that allow various secondary ligands to augment or inhibit/attenuate the activation of this receptor by the main, direct agonists; an important point that further adds to the complexity of the system [73].

As mentioned above, the possible existence of a novel cannabinoid "CB_3" receptor has been mooted because of certain agonistic/antagonistic effects of some $CB_{1/2}$ receptor ligands on channels or non-$CB_{1/2}$ receptors, although their potencies differ from those observed with classical $CB_{1/2}$ receptors. Pertwee et al. [10], in their extensive and detailed review of the subject, proposed that any such novel receptor should meet a number of the five criteria they listed (see Pertwee et al. [10] for criteria). They stated that one of the transient receptor potential (TRP) superfamily of cation channels, of which more than 50 members have been characterized, namely the transient receptor potential vanilloid-1 (TRPV-1) did meet three of the five criteria, at least in part. An important piece of evidence in support of TRPV-1 as a putative CB_3 receptor is that it is activated by endogenously released anandamide when the latter's degradation is prevented by inhibition of the fatty acid amide hydrolase (FAAH) enzyme (see below). The authors concluded that further research was needed to determine categorically if TRPV-1 should be regarded as a novel ionotropic cannabinoid CB_3 receptor or perhaps a dual TRPV-1/CB_3 channel. Similarly, other TRP ion

channels are activated to some extent by a variety of cannabinoids but it is not certain that they can be classified as cannabinoid receptors at present since some are also activated by various other ligands (see below).

2.1.1. Occurrence and distribution in normal and cancer tissues

As mentioned above, the CB_1 receptors occur mainly, but not exclusively, in neurological tissues at the terminal ends of central and peripheral neurons where their main role appears to be the inhibition of various excitatory and inhibitory neurotransmitters [10,16,17,68]. The extent of expression and sites of distribution of CB_1 receptors throughout the central nervous system suggests that their ligand-specific activation can modulate cognitive, memory and motor functions as well as analgesia [10]. Recent evidence shows that CB_1 receptors can also be expressed in peripheral, non-neuronal cells like immune cells and various cancer cells and tissues [10,16,17,68].

CB_1 expression was detected by immunohistochemistry, RT-PCR, immunofluorescence and/or Western blot methods in 14–28% of human breast cancer tumour tissue [74,75]. No correlation was observed between CB_1 and the expression of the ErbB2 tyrosine kinase receptor in tissues that expressed both receptors [74]. CB_1 receptor expression was also detected in various breast and prostate cancer cell lines [42,75–83]. High CB_1 receptor expression in prostate cancer tissue was associated with high severity of the disease and poor prognosis [78]. Similarly, CB_2 receptor immuno-reactivity was detected in 72% of human breast tumour tissue and in 91% of ErbB2-positive tumour tissue. These observations indicate a possible link between CB_2 and ErbB2 expression but not CB_1 and ErbB2 expression [74]. Another study observed that CB_2 immuno-reactivity was only present in 35% of human breast tumour tissue [75]. The reason for these differences in % expression are not clear and the latter study did not specify the ErbB2 status of the tumours examined. CB_2 receptor expression, like that of CB_1, was also detected in various breast and prostate cancer cell lines using similar methods to those described for CB_1 [42,75–84].

Clearly, further research is required in order to delineate any definitive correlation between the extent of expression of CB receptors in tumours and cancer severity, progression and outcome.

2.2. TRP receptors

The transient receptor potential (TRP) superfamily of cation channels consists of more than 50 members in nature with 28 members in mammalian systems [10,85]. They are involved in the signal transduction of a wide range of stimuli, including the effects elicited by endogenous lipids [86]. This makes them interesting targets for the putative health benefits of a variety of lipid derivatives including various phospholipids and acylethanolamides [10]. Interestingly, it has been shown that mutations in some of these TRP's may be linked to diseases common in man. Their expression is often increased in various pathologies, including cancer [10,85]. Six types of TRP channels from three subfamilies have been suggested as candidates for binding exogenous phytocannabinoids and *in situ* synthesised endocannabinoids; namely TRPV1, TRPV2, TRPV3, TRPV4, TRPM8 and TRPA1 [10]. Of these, the vanilloid receptor-1 (TRPV-1), first cloned as a receptor for the vanilloid capsaicin, has been mooted as the most likely candidate to fulfill the criteria for its classification as an "ionotropic cannabinoid receptor" [87]. This receptor is predominantly, but not exclusively, expressed in sensory neurons [88–94]. It is also expressed in non-neuronal cells including epithelial, endothelial and smooth muscle cells as well as in lymphocytes, hepatocytes and pancreatic cells [10,93]. Markedly increased expression of TRPV-1 was also found in human prostate carcinoma and squamous cell carcinomas of the tongue compared to normal tissue but this did not correlate directly with the malignancy score of the tumour [83,91]. However, TRPV-1 expression is susceptible to modulation by inflammatory and pathological processes and can be activated by a variety of exogenous and endogenous cannabinoids, particularly the arachidonic acid-derived anandamide. Evidence *in vivo* in support of this role for TRPV-1 was obtained using TRPV-1 knockout mice and by the observations that various phytocannabinoids and some synthetic cannabinoids that do not activate $CB_{1/2}$ receptors exhibit significant potency toward this receptor, albeit usually at a lower potency than toward $CB_{1/2}$ receptors [7,10]. It is conceivable that the non-CB receptor effects of the $n-3$ ethanolamides observed in prostate cancer cells are attributable to TRPV1 activation [42].

TRPV2 is activated by several phytocannabinoids. These include cannabidiol (CBD), THC and cannabinol, with CBD showing greatest potency in a cell-based calcium mobilisation assay. CBD also elicited a concentration-dependent release of calcitonin gene-related peptide (CGRP) from cultuted rat neurones that was independent of CB and TRPV1 receptor activation [92]. The plant cannabinoids THC, CBD and various synthetic analogs were shown to affect the activity and gene expression of TRPV1–4 ion channels (activation and desentisation) in mouse gastrointestinal tracts with different degrees of potency [93,94].

The expression of $CB_{1/2}$ and TRPV1–4 receptors were differentially regulated by dietary DHA in a dose-dependent manner in rats [95]. CB_1 and TRPV1 were upregulated, as assessed by RT-PCR and Western blot analysis, whilst TRPV2 was down-regulated; CB_2, TRPV3 and TRPV4 were not affected in hippocampal neurons. Correlations with spatial memory tests led the authors to postulate that certain sub-types of endocannabinoid/endovannilloid receptors could be involved in enhanced spatial memory induced by DHA supplementation [95]. It is conceivable that the reported benefits of $n-3$ LCPUFA supplementation, particularly DHA, to mood changes, depression and ADHD in man [96] could be due to activation of the cannabinoid receptors in the brain by the endocannabinoid derivatives, but this requires experimental verification.

Transient receptor potential melastatin-8 (TRPM8) and transient receptor potential ankyrin-1 (TRPA-1) are two subtypes of transient receptor potential channels different from TRPV receptors. Both are involved in thermosensation and are activated by cold temperatures as well as natural compounds such as menthol and icilin (i.e. TRPM8) and irritants such as mustard oil, acrolein, isothiocyanates (i.e. TRPA1) [10]. Various plant-derived cannabinoids and the endocannabinoid annandamide, but not 2-AG, have been shown to affect these receptors by either acivating (i.e. TRPA1) or inhibiting activation (i.e. TRPM8) [10,93,97–99]. Phytocannabinoids, cannabis extracts and endocannabinoids can efficiently antagonise the stimulatory effects of menthol and icilin on intracellular Ca^{++} elevation in HEK293 cells transfected with TRPM8 and in rat dorsal root ganglion (DRG) neurons in a cannabinoid receptor-independent manner. CBD, THC, CBD acid, THC acid, cannabichromene (CBC) and cannabigerl (CBG) all induced TRPA1-mediated Ca^{++} elevation in the cells with efficacies comparable to mustard seed isothiocyanates [93,97–99]. The differential effects on TRPA1 and TRPM8 are interesting in that the latter is regarded as a survival channel and is overexpressed in prostate cancer cells; its deactivation, as reported with cannabinoids, would be expected to elicit proapoptotic effects in these cells as shown by De Petrocellis et al. [93].

Clearly, various cannabinoids can influence the expression and activity of these channels but whether this defines them as classical CB receptors remains to be verified [10]. Furthermore, it is not clear at present how, and to what extent, the various $n-3$ endocannabinoids or putative CLA/CLnA cannabinoid derivatives can bind and activate these channels. This leaves a rich seam for future research.

2.3. G protein-coupled receptor 55 (GPR55)

Suggestions that the GPR55 receptor, or orphan receptor, can be activated by different types of cannabinoids is less compelling and inconclusive and requires further research to establish its role in the cannabinoid/endocannabinoid system [10]. Activation by various phytocannabinoids (e.g. Δ^9-THC) and endocannabinoids (e.g. anandamide, 2-AG) (see below) has frequently been reported in the literature. The present data, however, is conflicting in that both positive and negative effects were found in relation to GTPγS binding, calcium mobilization, ERK1/2 phosphorylation, β-arrestin recruitment and GPR55 internalisation [10]. The reason for such inconsistencies in ligand effectiveness is not clear at present. They tend to preclude GPR55 being designated a true cannabinoid receptor at this juncture. Future observations could, however, result in this designation being revised.

2.4. Peroxisome proliferator activated receptors (PPARs)

PPARs are ligand-activated transcription factors of the nuclear receptor family that, when activated, alter the transcription of a number of target genes encoding proteins/enzymes involved in lipid turnover and metabolism [10,12,100–104]. The major agonists of the three PPAR isoforms (PPARα, PPARβ, and PPARγ) tend to be endogenous monounsaturated and long chain polyunsaturated fatty acids (LCPUFA-C16- and >C18) and their various oxidative derivatives. These include those derived from oxidative metabolism through the cyclooxygenase, lipoxygenase (i.e. eicosanoids) and cytochrome P_{450} pathways. A number of endocannabinoids (e.g. anandamide, 2-AG), phytocannabinoids (e.g. Δ^9-THC) and synthetic cannabinoids (e.g. WIN 55,212-2, ajulemic acid), that are also fatty acid derivatives, were shown to be PPAR agonists but their activation potency was generally much lower than that observed with their classical cannabinoid receptors [10]. Furthermore, fatty acid ethanolamides that are not agonists for $CB_{1/2}$ receptors (e.g. oleoyl- and palmitoyl ethanolamide) can significantly activate PPARα. Of interest, for the objectives of this review, are the observations that oxidative derivatives of endocannabinoids, including 2-AG derivatives, of the cyclooxygenase/lipoxygenase/P_{450} pathways are also potent activators of PPARs [10]. It is conceivable, but not proven yet, that the omega-3 ethanolamides, similar to their omega-6 counterparts, are also activators of PPARs.

Jhaveri et al. [50] observed that endocannabinoids can activate PPAR receptors *in vivo* in animals. They used an animal model of inflammation, namely the carrageenan inflamed hind paw. Inhibition of fatty acid amide hydrolase (FAAH) (see below) and cyclooxygenase-2 (COX-2) by URB597 and nimesulide respectively increased levels of AEA, PEA and 2-AG and attenuated hyperalgesia and hind paw oedema. Both AEA and PEA are ligands for PPARα and the authors observed that antagonism of this PPAR, but not of PPARγ, blocked the inhibitory effects of both nimesulide and URB597 on hyperalgesia. These observations strongly suggest that both FAAH and COX-2 play a role in endocannabinoid metabolism in this model *in vivo*, possibly by increasing the availability of the endocannabinoids or/and their COX-2 derivatives and thereby the activation of the PPARα receptor. Clearly, endocannabinoids are endogenous agonists of PPARs, particularly PPARα, but also PPAR-γ [10,17,50]. A number of oxidative derivatives of LOX and P_{450} enzyme activities are also PPAR agonists [105] (see below).

2.5. Other putative cannabinoid receptors

Pertwee et al. [10] described in detail the arguments for and against other G-protein-coupled receptors, ligand-gated ion channels, muscarinic and nicotinic acetylcholine receptors and glycine receptors being considered as putative cannabinoid receptors just because certain cannabinoids have been shown to activate and elicit signal transduction through these receptors. The lysophospholipid receptors $S1P_{1-5}$ and LPA_{1-3} are GPCRs that are most closely related to $CB_{1/2}$ receptors but have evolved in a different branch of the GPCR superfamily. As mentioned above, at present CB_1 and CB_2 are regarded as the only *bona fide* cannabinoid receptors although the TRPV1 receptor may well come to be considered as a CB_3 receptor in the future (see above and [10]). Other members of the TRP family of ion channels have been discussed above.

2.6. Expression of cannabinoid receptors in cancer

The classical $CB_{1/2}$ receptors and the TRPV1 receptor appear to have an important role to play in the various anticancer effects of their ligands *in vitro* and *in vivo* when acting individually or in concert. Although the level of their expression appears to differ both between different types of cancers (cancer cells) and between cancers and normal tissues (cells), this does not always hold true. In some cancers, no differences in expression of these receptors between cancer and benign or normal tissues/cells have been reported (see above and below). It is not clear at present how the differential expression and regulation of these receptors correlates with the severity of the cancer or the potency of any anticancer effects of the cannabinoids. Furthermore, little is known regarding the specific and apparent complex interactions and potencies between different cannabinoid ligands, their differential availability in various cancers and the relative expression and availability of the three main receptor types. It has been suggested that increased expression of these receptors in tumour compared to normal tissue, when it occurs, is a protective mechanism to prevent normal cells/tissues succumbing to the pro-aptotic and antiproliferative effects of the cannabinoid agonists [43,55].

2.6.1. CB receptors

In some tumours, such as lymphoma [106], myeloid leukemia [107], liver carcinoma [108], prostate cancer cells [83], astrocytomas [109] and pancreatic cancer [110], increased expression of both CB_1 and CB_2 receptors has indeed been observed compared to normal tissue/cells. However, in other tumours such as astroglial [111], pituitary adenomas [112], non-melanoma skin cancer [113], human colorectal cancer [114,115] and Kaposi's sarcoma [116] the expression levels of both receptors, although generally increased, did not differ significantly between normal and cancer tissue/cells [43]. This suggests that the protective hypothesis mentioned above does not pertain to the normal tissues of such cancers/tumours. Furthermore, in other types of tumour, the expression of individual CB_1 or CB_2 receptors was elevated independently of one another. For example, a significant increase in CB_2 receptor expression alone was observed in endometrial carcinoma samples compared with normal tissue [115]. This was also observed in primary breast cancer tissue compared with normal breast tissue; also in receptor negative compared with receptor positive breast tumours [75] and in over 90% of ErbB2 positive compared with negative breast tumours [74]. The enhanced expression of the CB_2 receptor was observed to correlate positively with the histological grade of breast cancer [80], astrocytoma grades [117], and in adult and pediatric brain tumours [118]. However, this is not the case for all cancers and no correlation between disease severity and CB_2 expression was found in pancreatic cancer patients [43,57,119] (for a more detailed discussion of receptor expression in cancer see [43,7]).

An over-expression of the CB_1 receptor alone has been detected in human gastric cancer cells (HGC-27) and in rhabdomyosarcoma biopsy samples [120]. Furthermore, high expression of CB_1, but not CB_2, in pancreatic cancer in combination with low FAAH and MGL, was associated with a shorter survival time [119]. Similarly, an increased CB_1 immuno-reactivity in prostate cancer, greater or equal

to the median value of 2 on a simple scoring system from 0 (absent = 0) to 3 (=intense), was found to be associated with increased severity of disease and a poor prognosis [43,57,82]. Unfortunately, the prognostic value of enhanced CB receptor expression in prostate cancer is difficult to assess because expression was found to be highest in patients with benign prostatic hyperplasia compared to control and cancer patients [43,57,83].

Interestingly, over-expression of both CB receptors correlated with an improved prognosis in a cohort of 64 hepatocellular carcinoma patients [121]. Clearly, the prognostic value of overexpression of the CB receptors in different forms of cancer is not universal and depends on cancer type/tissue as well as ligand concentration and relative receptor expression since this can determine the link to different signaling cascades. Cudaback et al. [51] developed astrocytoma subclones that stably expressed defined but different levels of CB_1 or CB_2 (high, median and low) and determined the extent to which the cannabinoid agonist CP55940, a potent agonist for both CB receptors, could regulate different kinase cascades and induce apoptosis in these cells [51]. The authors concluded that cannabinoids only induced apotosis in cells expressing low levels of either of the receptors and that this effect was coupled to the ERK1/2 kinase cascade. In cells expressing high levels of the CB receptors, cannabinoids at low concentrations elicited coupling to the pro-survival AKT kinase signal pathway and did not induce apoptosis until present at high concentrations when all subclones were similarly affected. These effects with high cannabinoid levels were found to be independent of the level of receptor expression, i.e. the compounds bypassed the receptors, but still elicited their effects through coupling to the pro-apoptotic ERK1/2 kinase cascade. In other words, the same agonist engaging either of the CB receptors, but at different concentrations, or not binding to either of the receptors at high concentrations, can differentially couple to distinct kinases and elicit opposing outcomes (apoptosis or survival). This was suggested to be due to differences between the membrane receptors and the amino acid sequences of their intracellular loops and/or differences in positioning of the ligand within the binding pocket of the CB_1 and CB_2 receptors, leading to differential G protein coupling and subsequent activation of the specific kinase signal pathways (ERK1/2 or AKT) [51,122]. Cudaback et al. [51] concluded that this differential effect on G protein coupling and kinase signal activation would preclude the use of cannabinoids in low submicromolar concentrations, but not high concentrations, as pro-apoptotic therapeutic agents in brain tumours unless the concomitant inhibition of the pro-survival AKT pathway was ensured. As mentioned above, direct injection of high concentrations of cannabinoids into transplanted C6 astrocytomas in rat brain, eradicated significant proportions of the malignant cells without affecting healthy brain tissue [123]. Direct injection of chemotherapeutic agents into brain tumour mass is a routine procedure for neurosurgeons [124]. This suggested to the authors that such administration of high cannabinoid concentrations should not be overlooked as a possible therapy for brain tumours [123]. Indeed, a clinical trial on patients with recurrent glioblastome multiforme where THC was injected directly into the tumours showed promise as a treatment modality and highlighted the lack of toxicity of the THC [124]. Conceivably, other solid tumours in different tissues could also be treated in this way.

It is also not clear at present if such differential, concentration-dependent activation of CB receptors occurs in cancer cells other than astrocytomas. It is, however, a plausible suggestion and highlights the requirement to clearly characterise CB receptors in individual cancers/tumours before contemplating cannabinoid therapy.

A recent report showed that CB_1 receptor expression in human colorectal cancer, but not normal tissue, had been silenced by epigenetic hypermethylation of its promoter region [125] (see below). Whether this occurs in other cancers and whether the increased expression observed with increasing severity of certain cancers can be ascribed to increased hypomethylation that unmasks the promoter silencing is not known at present. Similarly, whether cannabinoids-endocannabinoids can alter the methylation state is unclear at present but is worthy of consideration since such epigenetic effects, although heritable, can also be modified by dietary factors [126] (see below).

2.6.2. TRP receptors

Most studies on receptor expression and their correlation with cancer severity and prognosis have focused on the CB receptors. However, the over-expression of the TRPV1 receptor has also been implicated as a factor in the regulation of human prostate carcinoma [83] and squamous cell carcinoma of the tongue although the latter showed no correlation with the degree of malignancy [91]. However, a down-regulation of TRPV1 (mRNA, protein) in urothelial bladder cancer (UC) specimens/cell lines compared to normal tissue/cells did correlate with tumour progression and was considered to be a possible negative prognostic marker for bladder cancer [127–129]. Activation of TRPV1 in UC cells with capsaicin, the vanilloid agonist, induced cell cycle arrest in G_0/G_1 phase and increased apoptosis [128]. Similar evidence for a negative correlation between TRPV1 expression (mRNA, protein) and disease score was obtained with glioma cells where capsaicin also induced apoptosis through an increased calcium influx and p38 activation but not ERK/MAPK activation; these effects were prevented by the TRPV1 antagonist capsazepine [130]. It is interesting to note, in the context of the similarities between $n-3$ LCPUFA and endocannabinoid effects on various ion channels, that the $n-3$ fatty acids, particularly DHA, have been reported to directly activate TRPV1 in a phosphorylation dependent manner and they displace the binding of the ultrapotent TRPV1 ligand resinoferratoxin [131].

TRPV2 is another vanilloid receptor that has been associated with carcinogenesis and is also activated by certain cannabinoids (e.g. cannabidiol, Δ^9-THC) although it is not, at present, regarded as a true cannabinoid receptor according to the criteria set out by Pertwee et al. [10]. This receptor was also significantly expressed (mRNA, protein) in benign astrocyte tissue but decreased progressively in glioma tissue with increasing tumour histology grading. Furthermore, transfection of TRPV2 into glioma cells resulted in reduced cell viability and increased Fas-induced apoptosis [132]. Clearly, activation of TRPV2, like TRPV1, also exerts a negative control on glioma cell survival and proliferation. Such activation could be induced by the cannabinoid-endocannabinoid agonists mentioned above and/or other cannabinoid agonists not mentioned. Expression of TRPV2 (mRNA, protein) also correlated with the tumour grade/stage in bladder cancer (UC) tissue/cells with a progressive decline in the short splice-variant of the receptor (s-TRPV2) up to a complete loss in the highest tumour grade tissue/cell lines [133]. This contrasts markedly with the observation that TRPV2 expression was enhanced in higher-grade compared to lower-grade UC cell lines and that increasing concentrations of cannabidiol, a cannabinoid agonist of TRPV2, increased intracellular calcium levels and decreased the cell viability [134]. The above findings do agree with the expression profile of TRPV1 in bladder cancer mentioned above [128,130]. The reasons for the reported discrepancies in the expression profiles of the TRPV1 and TRPV2 receptors in cancer cells/tissues are not clear at present but illustrate the complexity of studying the changes in expression of the multiplicity of receptors in cells/tissues of different cancer types.

TRPV6 channel-receptors, although not known to be cannabinoid receptors, have also been reported to be highly expressed in advanced prostate cancer and to correlate positively, and signifi-

cantly, with high Gleeson grading (>7) whilst being undetected in healthy and benign prostate tissue [135]. The authors [135] observed that TRPV6 was directly involved in the control of LNCaP cell lines by increasing proliferation, cell survival and resistance to apoptosis. These observations were supported by the work of Zhao et al. [136] who used siRNA silencing of TRPV6 to assess the effects on cell proliferation, cell cycle phase and apoptosis. The siRNA inhibited the transcription of the receptor, inhibited the proliferation of the LNCaP cells, arrested their cell cycle in the Go and G1 phase and induced apoptosis.

TRPV6 upregulation has also been demonstrated in other malignancies including breast, thyroid, colon and ovary [137]. It is apparent from these observations that the TRPV receptors are both pro- and anti-carcinogenic depending on the type of receptor and type and grading of the cancer. It is not known at present if the regulation of the TRP receptors involves changes in DNA methylation (epigenetics).

The role of TRPM8 and TRPA1 in cancer was briefly referred to above. TRPM8 expression is important for the survival of androgen receptor-dependent prostate cancer cells. Various cannabinoids can activate TRPA1 and inhibit or desensitise the TRPM8 responsiveness in cells overexpressing the latter to varying extents thereby eliciting apoptotic effects in the cells [93,98,99].

Clearly, cancers are extremely heterogeneous in their and this heterogeneity appears to be mirrored in the diversity of the expression levels of the principal receptors involved in the cannabinoid system, even within the category of a single cancer [53]. This suggests that any strategy for developing cannabinoids or other agonists/antagonists as treatment modules for a specific cancer would need to characterize the expression of the individual CB and TRPV receptors in the specific cancer type. Furthermore, in order to be a viable proposition as a therapeutic modality it would be necessary to understand the interactions or cross-talk between the different receptors in a specific cancer; a complex and difficult task but not an impossible one.

3. Cannabinoid receptor agonists and antagonists

A number of excellent reviews on the $CB_{1/2}$ receptor agonists and antagonists have been published, including the enzymic pathways of their synthesis and degradation in mammalian tissues and cells; the reader is referred to these reviews for a more comprehensive description of these important compounds and their actions [53,65,15,138–141].

3.1. $CB_{1/2}$ receptor ligands

The compounds that have been reported to bind to cannabinoid receptors fall into four main groups: (1) Agonists that target both receptors with similar potency, (2) CB_1- and CB_2-selective agonists, (3) CB_1 and CB_2 selective antagonists/inverse agonists, and (4) neutral antagonists.

3.1.1. $CB_{1/2}$ receptor agonists

Turning first to the CB_1/CB_2 cannabinoid agonists, these fall essentially into four chemical classes. The first of these, the "*classical cannabinoids*", includes the main psychoactive constituent of cannabis, Δ^9-tetrahydrocannabinol, Δ^9-THC), and a synthetic compound, (−)-11-hydroxy-Δ^8-THC-dimethylheptyl (HU-210). HU-210 displays high potency and efficacy as a CB_1 and CB_2 receptor agonist [142,143]. Δ^9-THC displays much lower CB_1 and CB_2 affinity and efficacy than HU-210. The second chemical class, *non-classical cannabinoids*, includes the compound CP 55940, which is one of the most widely used pharmacological tools in cannabinoid research [144] CP55940 has a bicyclic structure and behaves as a CB_1 and CB_2 receptor full agonist [145]. The third chemical class consists of "*aminoalkylindoles*", of which WIN 55,212-2 is an important member (Fig. 1). It also displays high potency and efficacy as a CB_1 and CB_2 receptor agonist [146,147]. In contrast, its (−)-(*S*)-enantiomer is inactive at cannabinoid receptors both *in vitro* and *in vivo* [148]. Finally, the fourth class of cannabinoid CB_1/CB_2 receptor agonists is made up of the "*endocannabinoids*". Their structure is markedly different from both classical and non-classical cannabinoids (Fig. 2). The most intensively studied compounds within this class are the two endocannabinoids, anandamide (AEA) and 2-arachidonoylglycerol (2-AG) (see below for more details). Certain endogenously synthesised *N*-acyl-ethanolamides derived from fatty acids that are not eicosanoid precursors, e.g. OEA, PEA, as well as some that are, do not act as ligands for CB receptors; they may elicit "chaperone" effects thereby enhancing the binding of true ligands (see below).

The selective CB_1 cannabinoid agonists include the synthetic AEA analogs, R-(+)-methanandamide-arachidonyl-2′-chloroethylamide (ACEA), and arachidonylcyclopropylamide (ACPA), and a single 2-AG analog, noladin ether (see Pertwee, 2010 [10]). Compounds able to activate CB_2 receptors selectively are: the classical cannabinoid, JWH-133, the non-classical cannabinoid HU-308, and the aminoalkylindoles, JWH-015and AM1241 (Fig. 3) [10].

3.1.2. $CB_{1/2}$ receptor antagonists

Among the compounds that are able to block cannabinoid CB_1 receptors selectively, are the diarylpyrazole, rimonabant, and its structural analogs, AM251 and AM281. Another such compound is LY320135, the structure of which resembles that of rimonabant [17]. AM630 (6-iodopravodoline), the diarylpyrazole, SR144528, JTE-907 [149] and the triaryl bis-sulphones, Sch.225336, Sch.356036 and Sch.414319, are all compounds that can block the cannabinoid CB_2 receptors [10]. All these CB_1 and CB_2 receptor antagonists can also behave as inverse agonists, compounds that can produce a decrease in the spontaneous coupling of CB_1 or CB_2 receptors to their G protein effector mechanism [150]. They therefore differ from "neutral antagonists", which are compounds that are able to displace an agonist from its orthosteric-binding pocket without inducing signs of inverse agonism. Such compounds are thought to include: (a) certain structural analogs of rimonabant, for example AM6527, AM4113, VCHSR and NESS 0327 (Fig. 4); (b) the CBD-analog, O-2654 and (c) Δ^8- and Δ^9-tetrahydrocannabivarin [15].

3.2. Cannabis-derived ligands

Among the compounds that have been identified in *C. sativa* L. so far, at least 85 are terpenophenolic compounds, so called "phytocannabinoids" [6,151]. The most investigated phytocannabinoids are cannabidiol (CBD), Δ^9-THC and cannabinol. Δ^9-THC is considered to be the main psychotropic constituent of *cannabis* whereas CBD lacks psychotropic activity but does possess anti-inflammatory and anti-psychotic properties. The structure and the stereochemistry of Δ^9-THC and CBD was elucidated in the 1960s [152–156], whereas the structure of cannabinol was determined earlier by Jacob and Todd [157] and independently by Adams et al. [158]. Several other phytocannabinoids were also identified at about the same time, for example cannabigerol [5], cannabichromene [159], cannabicyclol [160,161] and Δ^9-tetrahydrocannabivarin (propyl-Δ^9-THC) [162].

3.3. Endocannabinoid ligands

As mentioned above, the first endocannabinoid to be discovered was anandamide, or AEA [162,163]. This is arachidonoyl ethanolamide, the ethanolamine derivative of arachidonic acid, and it be-

haves as both a partial CB_1 and CB_2 receptor agonist and a TRPV1 agonist [162–166]. A second endocannabinoid, 2-arachidonoyl-glycerol (2-AG), is an ester of arachidonic acid and glycerol. This was first isolated from canine gut [13] and from the brain [167]. Later, virodhamine, an endogenous molecule with the same molecular weight as AEA, was discovered. In this molecule, arachidonic acid and ethanolamine are joined by an ester linkage and not by an amide linkage as in AEA [168]. This compound has been reported to behave as a CB_2 receptor agonist and a CB_1 receptor antagonist/inverse agonist. Other long-chain PUFA derivatives include *N*-dihomo-γ-linolenoylethanolamide and *N*-docosa-tetraenoyl-ethanolamine, which are also thought to be endocannabinoids [169]. Other compounds with endocannabinoid characteristics include oleamide, *N*-oleoyl dopamine, 2-arachidonoylglycerylether (noladin ether) [150,169,170] and also the omega-3 ethanolamides, docosahexaenoyl ethanolamide (DHEA) and eicosapentaenoyl ethanolamide (EPEA) [34,42]. Interestingly, the latter two compounds, derived from the *n*−3 LCPUFA of fish oil, activate both CB receptors and have recently been shown, for the first time, to display a greater anti-proliferative potency than that of their parent *n*−3 fatty acids, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), in LNCaP and PC3 prostate cancer cells [42]. Receptor antagonist studies also suggested that some DHEA and EPEA effects were $CB_{1/2}$ receptor-independent [42]. Indeed, our group hypothesised that *n*−3 LCPUFA may exert their known anticancer effects, at least in part, after conversion to their respective endocannabinoids. However, *n*−3 LCPUFA at physiological concentrations (1–10 μM) have also been reported to interact directly with the TRPV1 receptor, with DHA acting as an effective agonist, whereas EPA and LNA are effective inhibitors [131]. Whether this is a direct effect or due to endocannabinoid conversion is unclear at present.

It has previously been well documented that the *n*−3 LCPUFA from fish oil, as well as certain conjugated forms of linoleic acid and linolenic acid, induced similar anticancer effects to those observed for their ethanolamide derivatives and the phyto- and synthetic cannabinoids as well as the *n*−6-endocannabinoids. They were also able to augment the chemotherapeutic potency of docetaxel (a taxotere derived from the pacific yew tree commonly used in cancer therapy) in prostate cancer cells [21,23,25,30,171,172] a similar anti-proliferative, anti-cancer effect in prostate cancer cells was recently observed with the ethanolamide derivatives of the *n*−3 LCPUFA EPEA and DHEA [38].

Although 2-AG is an endocannabinoid the effects of the 2-MAGs derived from the omega-3 and conjugated fatty acids on the CB or TRPV1 receptors and on cancer cell autophagy/apoptosis have not been reported to our knowledge.

3.3.1. Biosynthesis of the endocannabinoids

Endocannabinoids are not stored in cells like classical neurotransmitters but are rapidly formed from membrane phospholipids ‘on demand’. To date, the biosynthetic pathways of two endocannabinoids, AEA and 2-AG, have been most investigated (Fig. 5a and b).

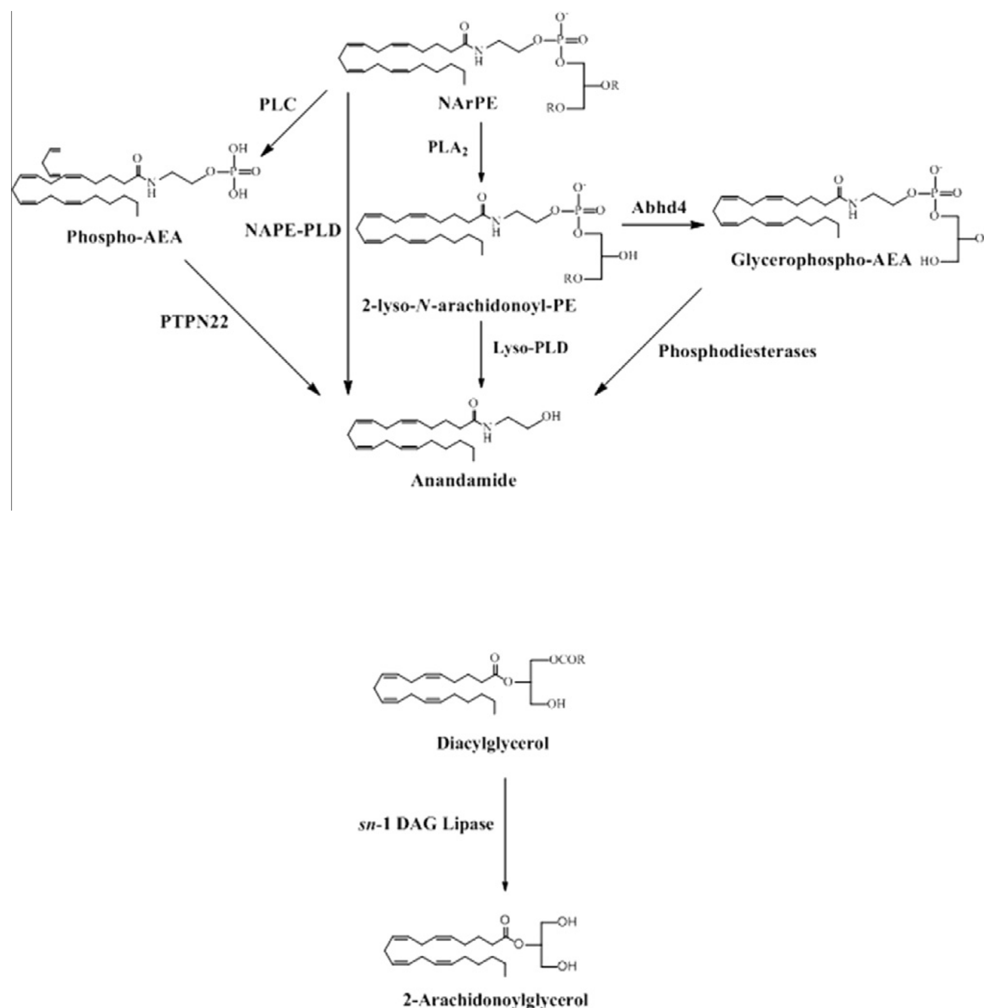

Fig. 5. Pathways for the synthesis of endocannabinoids (a) ethanolamide derivatives; (b) 2-acylglycerol derivatives.

3.3.1.1. Anandamide (AEA) and n−3 homologues. The endocannabinoid, AEA, belongs to the large family of *N*-acylethanolamines (NAEs) [173–175]. Its main biosynthetic pathway consists of a two-step process (Fig. 5a). In the first step, *N*-acyl-phosphatidylethanolamine (NAPE) is formed from phosphatidyl-ethanolamine (PE) by a calcium-dependent *N*-acyltransferase (Ca-NAT). This NAPE is subsequently broken down to form NAE in a process that is catalysed by NAPE-hydrolyzing phospholipase D (NAPE-PLD) [174,176–178].

The enzyme NAPE-PLD is a member of the metallo-β-lactamase family, a large superfamily comprising a variety of hydrolases [179,180]. However, there is no sequence homology between NAPE-PLD and well-known PLDs. NAPE-PLD is widely distributed in various rat [173,181], mouse [182] and bovine tissues [183], the highest activity having been detected in rat heart [173,181]. NAPE-PLD mRNA has also been detected in human prostate epithelial cells and prostate cancer cells (PC-3, DU-145 and LNCaP) [184]. There is also a second biosynthetic pathway in which AEA is formed from *N*-acyl-lysophosphatidyl-ethanolamine by a lysophospholipase D-like enzyme (lysoPLD) [185]. *N*-Acyl-lysophosphatidyl-ethanolamine is in turn produced from NAPE by the enzyme PLA2 (Fig. 5a).

In 2006, Simon and Cravatt [186] identified an additional enzyme, Abhd4, that is involved in the biosynthesis of AEA. This enzyme belongs to the α/β-hydrolase family, the members of which are characterized by the presence of a α/β-hydrolase fold [187]. It can act on either NAPE or lyso-NAPE to generate glycerol-phospho-arachidonoyl-ethanolamide (GpAEA) that in turn is converted to AEA in the presence of a phosphodiesterase. In the same year, Liu et al. [188] obtained evidence for an alternative pathway in which NAPE is hydrolyzed to phospho-anandamide (pAEA) by phospholipase C (PLC), and pAEA is dephosphorylated by PTPN22 (protein tyrosine phosphatase non-receptor type 22) to AEA.

Finally, it was shown that AEA could also be enzymatically generated from arachidonic acid and ethanolamine when both these compounds are present at very high concentrations [189–192]. This condensation reaction has been attributed to the enzyme, FAAH, which can catalyze not only the hydrolysis of anandamide to arachidonic acid and ethanolamine, but also the reverse reaction in which anandamide is synthesized from these two compounds, albeit only when they are present at rather high, possibly non-physiological, concentrations [193].

Although in the past, endocannabinoid research has been largely focused on the AA derivatives, i.e. anandamide and 2-AG, some evidence for the presence of the *n*−3 LCPUFA homologues in mammalian tissues has existed for a number of years. Bisogno et al. [28] identified DHEA (docosahexaenoylethanolamide) and 2-DHG (docosahexaenoylglycerol) in bovine retina and showed their synthesis from DHA in retina membranes in 1999. Of interest are the observations that the levels of anandamide and other *N*-acylethanolamines in human plasma and various animal tissues are closely correlated with the dietary intakes and availability of the corresponding free fatty acids. These findings indicated that these compounds are formed in animals including man and that availability of precursor substrate in membrane phospholipids *in vivo* is an important determinant of formation [24–38,194–196]. Animal studies also showed the presence of ethanolamides of *n*−6 and *n*−3 LCPUFA in various tissues *ex vivo* (see below). The specific synthetic pathways for the *n*−3 LCPUFA derivatives EPEA and DHEA, or their 2-AG derivatives, have not been elucidated to date but it is reasonable to assume that the same general pathways described above (Fig. 5a and b) for other fatty acids are involved. In support of this suggestion, Artman et al. [33] observed that feeding arachidonic acid (AA) to rats increased jejunum, but not liver, levels of anandamide and 2-AG. Feeding a fish oil diet (*n*−3) decreased liver levels of *N*-acylethanolamines and their precursor fatty acids but increased the corresponding EPA and DHA levels and that of their *N*-acylethanolamide derivatives; the *n*−3 2-monoglyceride derivatives were not determined. The AA and FO diets had no effect on any lipids, precursor or derivative, in the brain. This suggested that *n*−6 and *n*−3 LCPUFA undergo similar conversions in rats but they are dependent on precursor fatty acids and the extent of conversion appears to differ between tissues. DHEA, but not EPEA, has also been detected in human plasma. This probably reflects the levels of their precursors in human plasma since the levels of plasma anandamide and other *N*-acylethanolamines in women correlated with the levels of the corresponding free fatty acids [194]. This would explain the decreased anandamide and/or 2-AG found in some experiments when *n*−3 fatty acids or phospholipids (e.g. krill oil) are administered in rodent diets [31,35,196]. Furthermore, a lifelong deficiency in *n*−3 LCPUFA in mice markedly reduced endocannabinoid-mediated neuronal functions due to an uncoupling of the presynaptic CB_1 receptors from their effector $G_{i/o}$ proteins resulting in impaired emotional behavior. This indicated the importance of *n*−3 LCPUFA in the functioning of the endocannabinoid system in the brain [196].

Recent observations showed that increased availability of EPA and DHA *in vitro* in prostate cancer [38] and adipose 3T3-L1 cells [31] also resulted in enhanced concentrations of their respective *N*-acylethanolamides (EPEA and DHEA) in these cells. This further supported the suggestion that the *n*−3 LCPUFA can be formed by the same, or similar, pathways as other *N*-acylethanolamides. These observations indicated the possibility of manipulating (increasing) the levels of these compounds in cells/tissues *in vivo* by dietary means as well as by the pharmacological use of antagonists/inhibitors of receptors, transporters and degradation enzymes to achieve potential anticancer benefits.

3.3.1.2. 2-Arachidonoylglycerol (2-AG) and n−3 homologues. AEA and 2-AG are biosynthesised through different pathways in tissues and cells, despite their structural and functional similarities, [197]. 2-AG is one of the major monoacylglycerols present in animal tissue. The main biosynthetic pathway consists of hydrolysis by PLC of inositol phospholipids containing arachidonic acid at the sn-2 position and further hydrolysis by diacylglycerol lipase (DAGL) of the arachidonic acid-containing diacylglycerol (DAG) to give 2-AG (Fig. 5b) [197,198].

In 2003, human DAGL was cloned and further characterized and was shown to exist as two closely related genes designated α and β [198,199]. Both isoforms display selectivity by hydrolyzing the sn-1 position of DAG, rather than its sn-2 position, and exhibit optimal activity at pH 7. Both enzymes are stimulated by glutathione and Ca^{2+} and inhibited by Serine/Cystine-hydrolase inhibitors such as *p*-hydroxy-mercuri-benzoate and $HgCl_2$ [199]. Pharmacological studies have revealed that DAGLα activity is required for axonal growth and guidance in the developing brain [199,200]. Moreover, in adult brain, DAGLα has been found to play an essential role in the regulation of retrograde synaptic plasticity and neurogenesis [201]. It is also possible that 2-AG may be produced by the sequential hydrolysis of PI, first by PLA1, and then by lyso PI-specific PLC [19].

Although Bisogno et al. [28] showed the presence of 2-DHG, the homologue of 2-AG derived from DHA, in bovine retina, the synthesis of this *n*−3 derived 2-monoacylglycerol has not been investigated in detail in mammalian tissues/cells. Again, it is presumed that the EPA and DHA derivatives of 2-AG are synthesised by the same pathways as the AA derivative. Although this has not been clearly determined to date it is actively being investigated in our group [38]. In the literature, the main focus of the effects of omega-3 LCPUFA supplementation *in vivo* on endocannabinoid formation related largely to the decrease in the omega-6 derivatives,

due to the increased levels/availability of the competing omega-3 fatty acids from the diet [35,26,195]. Increased levels of the $n-3$ endocannabinoids probably elicit effects that are not just due to a reduced level of the corresponding $n-6$ derivatives. This requires further investigation in order to understand the relative importance of the two families of endocannabinoids.

Clearly, the level and type of endocannabinoid produced *in vivo* and *in vitro* can be altered by precursor availability and highlights the use of dietary modification, particularly dietary omega-3 LCPUFA, as a means of achieving such changes; possibly in conjunction with an inhibitor of FAAH to decrease degradation and increase availability (see below).

3.3.2. Degradation of endocannabinoids

After targeting their receptors, the endocannabinoids, AEA and 2-AG, are inactivated by a two-step process. The first process is one of "facilitated diffusion" that results in endocannabinoid transport from the extracellular to the intracellular space. Whether this cellular uptake depends on the presence of an "endocannabinoid membrane transporter" is currently a subject for debate as no such transporter has yet been cloned. Such a transporter mechanism could explain, at least in part, some of the non-receptor effects of endocannabinoids. After the uptake, the endocannabinoids tend to be metabolized by either hydrolysis or oxidation reactions.

3.3.2.1. FAAH. The main enzyme involved in AEA hydrolysis is FAAH (FAAH-1) (fatty acid amide hydrolase). This enzyme, which was first cloned by Cravatt and co-workers [202] and is an integral membrane protein widely distributed in various tissues of rat [202–204], mouse [205,206], and human [207,208], is able to hydrolyse not only AEA but also other bioactive fatty acid amides such as oleamide [207,209], and *N*-acyl-taurines [210]. Its optimal pH lies within the range 8.5–10. A second isoform of FAAH, FAAH-2, has recently been identified [208]. FAAH-2 is more effective at metabolizing oleamide than AEA or other NAEs. FAAH-1 and FAAH-2 are located in the cytosolic and luminal sides of intracellular membranes, respectively.

It is probable that the $n-3$ ethanolamides, now known to be present in mammalian tissues and cells [28,31–37,42], will also be hydrolysed by the FAAH enzymes *in situ*. This is supported by the observations of Brown et al. [42], in prostate cancer cells, where treatment with FAAH inhibitors resulted in increased levels of ethanolamides; these findings require verification in other cells/tissues.

3.3.2.2. NAAA. In addition to the 2 FAAH isozymes, another enzyme involved in the hydrolysis of AEA is NAAA (*N*-acylethanolamine acid amidase) [211,212] which is present in the cellular lysosomes or the Golgi apparatus in cells, in contrast to the ER distribution of FAAH. It must be emphasized that the substrate specificity for NAAA is ca. eightfold greater for palmitoylethanolamide (PEA) than for anandamide, with the enzyme showing only very low activity toward the latter [19]. Whether this enzyme contributes significantly to anadamide catabolism under physiological conditions is unclear at present. NAAA is an *N*-glycosylated protein similar to other lysosomal hydrolases, including acid ceramidase [19,213,214]. Its optimal pH is 4.5–5 [19,184,215,216]. Millimolar concentrations of DTT as well as non-ionic detergents such as Triton X-100 and Nonidet P-40 are required to promote its full activity [211,215,216]. NAAA is highly expressed in a number of blood cell lines and particularly in macrophages in various rodent tissues. In humans, NAAA mRNA was expressed most abundantly in prostate followed by leukocytes, liver, spleen, kidney and pancreas [19]. Prostate cancer cell lines like PC3, LNCaP and DU-145 also express high levels of NAAA [19]. The physiological role of NAAA is currently unclear. Its contribution to NAE levels in the brain appears to be minor compared to FAAH and it has been suggested that it is mainly involved in NAE degradation in peripheral tissues and macrophages. It may also have a role in removing the NAEs in degenerating tissues that normally accumulate these compounds [19,15].

3.3.2.3. MAG lipase, ABHD6 and ABHD12. 2-AG is also metabolized by FAAH, although to a lesser extent than AEA [217,218]. However, the major 2-AG metabolizing enzyme is MAG lipase, an enzyme that is responsible for about 85% of the 2-AG hydrolyzing activity of mouse brain [217]. 2-AG is also catalysed by two integral membrane proteins, α/β-hydrolase domain containing protei $n-6$ (ABHD6) and -12 (ABHD12). As with the $n-6$ endocannabinoids, it is presumed, but not known definitively, that MAG–lipase also hydrolyses the $n-3$ derivatives to a similar extent.

3.3.2.4. COX, LOX and P_{450} enzymes. These oxidative enzymes can be perceived as endocannabinoid degrading enzymes since they can reduce the levels of these compounds in cells/tissues. However, these enzymes catalyse the production of a host of bioactive lipid derivatives that can elicit numerous physiological responses and can influence carcinogenesis and tumour progression. They are also capable of forming eicosanoid-like derivatives from precursor endocannabinoids, some of which have biological activity (see below).

3.3.2.4.1. COX-2 enzyme. Many cancer cells overexpress COX-2, but not the constitutive COX-1, whilst cannabinoids and endocannabinoids can further induce expression [46,219–221]. Both AEA and 2-AG, and perhaps the omega-3 ethanolamide and 2-monoacylglycerol derivatives, can also be degraded by oxidation mechanisms involving catalysis by cyclooxygenase-2 (COX-2), [31,49,56,59,60,219,222–226] (Fig. 6). This would result in the reduction of the tissue endocannabinoid levels and the increased production of prostaglandin ethanolamides (PGEA) from AEA [60,224,227] and of glyceryl prostaglandins (PG-G) from 2-AG [26,58,59,228]. Yu et al. [227] showed that COX-2 could oxygenate AEA but not COX-1, indicating a substrate specificity for the two isoforms. The reason for this appeared to be a fourfold higher K_m for AEA compared to AA. Consequently, the maximal rate of AEA oxygenation was between 18% and 27% of that found for AA. The PGE_2, PGD_2, PGI_2, PGF_2-α and TXA_2 derivatives of AEA and 2-AG have been detected in cellular and subcellular systems [26,58,59,228]. These eicosanoid ethanolamides do not appear to act as ligands for the $CB_{1/2}$ receptors or of any of the EP1–4 eicosanoid receptors but they have been shown to act through other receptors such as PPARs and NF-kB [26,61,229]. They can also inhibit adenylcyclase activity and consequently the eicosanoid production in immune cells (see below). This is related to the reported anti-inflammatory properties of endocannabinoids [62,230]. Although a variety of AEA eicosanoid-like products have been detected in cells/tissues it must be remembered that their production levels are much lower than the corresponding AA derivatives [26]. Whether they have any physiological significance is not clear and could depend on the localized concentrations in cells/tissues. By contrast, COX-2 can oxidise 2-AG to a similar extent as AA whilst COX-1 utilises this substrate poorly [26,228]. The products of the reaction were a number of glyceryl esters of PGE_2 and their relative quantities were not significantly different from those derived from AA. It is conceivable that $n-3$ monoglycerides will be oxidised in a similar manner to 2-AG but this requires verification. These compounds do not activate the classical CB receptors but they are capable of eliciting a plethora of signaling events through other receptors, most of which have not been determined yet. Delineating the possible role of such compounds in cancer metastasis, autophagy and apoptosis would be an interesting objective.

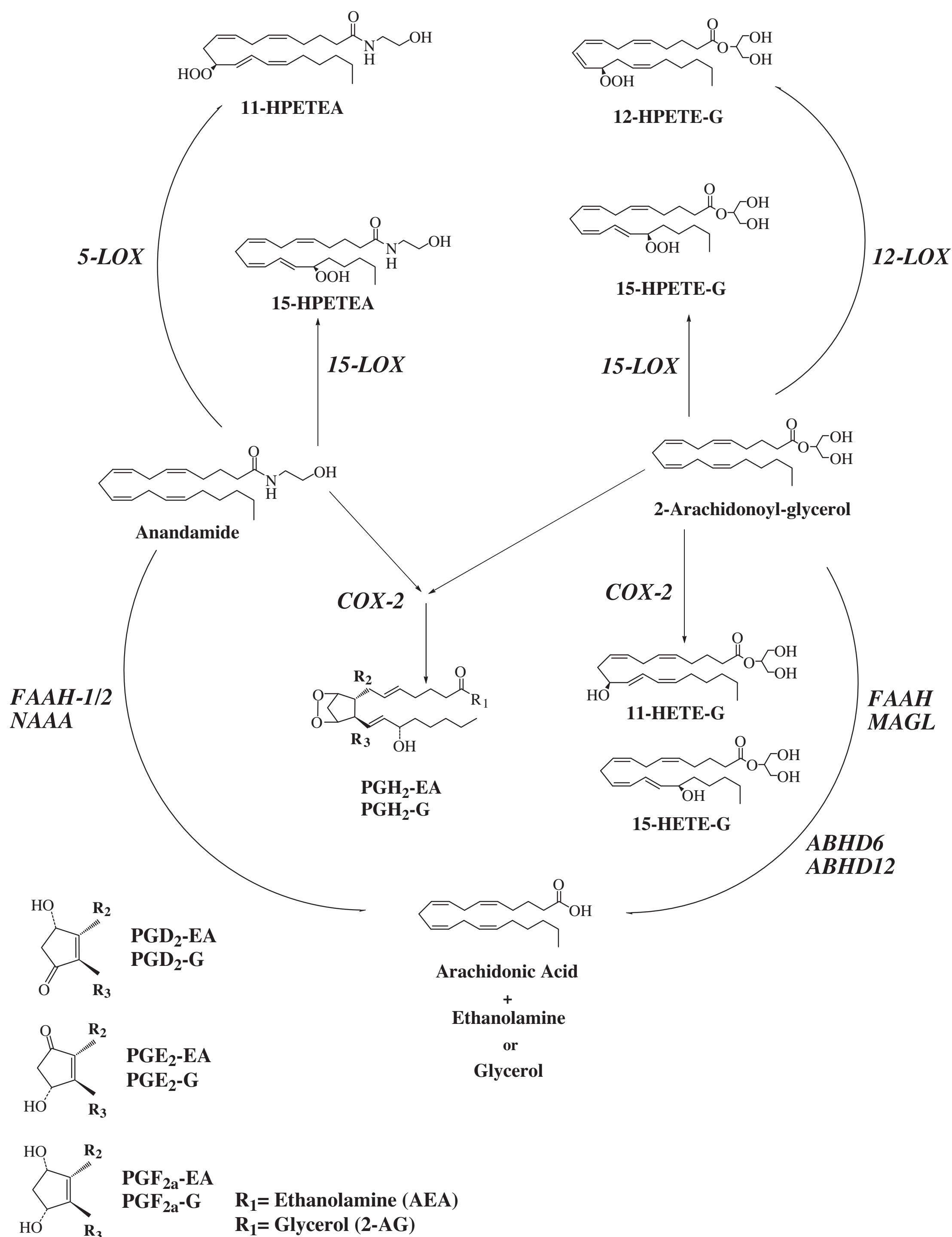

Fig. 6. Oxidative metabolism of endocannabinoids through the COX and LOX pathways. LOX, lypoxygenase; COX, cyclooxygenase; FAAH, fatty acid amide hydrolase; MAGL, monoacylglycerol lipase; NAAA, *N*-acylethanolamine-hydrolyzing acid amidase; ABHD, α/β-hydrolase domain.

3.3.2.4.2. LOX enzymes. $n-6$-, and presumably $n-3$-LCPUFA, are also metabolized by the lipoxygenase enzymes (5-, 12- and 15-LOX) in mammalian systems. Arachidonic acid metabolism by these enzymes, resulting in various leukotriene products, has been implicated in cancer development [231]. The role of the $n-3$ homologues would be expected to have the opposite effects since the parent $n-3$ LCPUFA elicit anticancer effects whilst parent $n-6$ LCPUFAs have pro-cancer effects [232]. As with the COX-2 enzyme, the LOX enzymes are also able to metabolise the endocannabinoid AEA and 2-AG resulting in the formation of the respective hydroxyeicosatetraenoic acid (HETE) and hydroperoxyeicosatetraenoic acid (HPETE) derivatives [26,52,59,225] (Fig. 6). Further studies with different LOXs from various tissues/cells showed that AEA oxidation was roughly comparable to that of AA, except that human platelet 12-LOX was only slightly active and porcine 5-LOX was inactive toward AEA [26,233,234]. Clearly, different tissues can exhibit different substrate specificities or findings are due to differences in methodology. These derivatives, in contrast to the COX derivatives, are reported to be involved in endogenous cannabinoid signaling and consequently add yet an-

other dimension to the complexity of endocannabinoid effects in cancer. Intriguingly, the non-psychoactive CBD appears to exert its antitumour effects through attenuation of 5-LOX expression in U-87 glioma cells since a selective 5-LOX inhibitor (MK-886) significantly enhanced the antimitotic effect of CBD whereas COX-2 inhibitors were ineffective [26,233–235].

The putative formation of eicosanoid derivatives of the $n-3$ ethanolamides and $n-3$ 2-AGs is an intriguing concept. These compounds could exhibit anti-inflammatory effects similar to their EPA and DHA parent fatty acids and their COX and LOX derivatives, the resolvins and protectins [236,237]. The formation of prostaglandin ethanolamides and prostaglandin glyceryl esters from the $n-3$ LCPUFA, EPA and DHA, has not been reported to our knowledge. Similarly, it is not known, to our knowledge, if the CLAs and CLnAs are metabolised to their respective ethanolamide and monoglyceride derivatives, let alone if these compounds are then further oxidised to a variety of eicosanoid-like derivatives. The significant effect of the parent fatty acids on cancer and CVD, as well as on inflammation *in vitro* and *in vivo* in animals (see above), warrants further investigation of their oxidative metabolism. This area of endocannabinoid research has, unfortunately, not been a focus of significant research to date.

3.3.2.4.3. P_{450} enzymes. Numerous mammalian cytochrome P_{450} enzymes exist and are involved in the oxidative metabolism of xenobiotics and they display a wide range of substrate specificities [26]. They also oxidise $n-6$ and $n-3$ fatty acids. Unsurprisingly, anandamide has also been shown to undergo oxidation by several human cytochrome P_{450} isoenzymes, including CYP3A4, CYP4F2, CYP4X1 and the polymorphic CYP2D6, resulting in a number of structurally diverse epoxy derivatives such as 20-hydroxyepoxyeicosatrienoic acid ethanolamide (20-HEET-EA) and 20-hydroxyepoxyeicosatetraenoic acid ethanolamide (20-HETE-EA) [105]. The 5,6 epoxyeicosatrienoic acid ethanolamide (5,6 EET-EA) was also a potent, functional agonist of the CB_2 receptor [105]. By contrast, little evidence exists for the oxidation of 2-AG by any P_{450} enzymes from different tissue preparations [26,238,239]. Although $n-3$ LCPUFA undergo P_{450} metabolism to the HETE and HPETE derivatives it is not known at present if the $n-3$ endocannabinoids also form their respective P_{450} derivatives, although it is highly likely.

Clearly, the metabolism of the endocannabinoids by the different oxidative pathways mentioned above and the possible functional potencies of the various derivatives suggests a potentially important area of research in the cannabinoid/endocannabinoid field that may lead to important therapeutic agents in cancer.

4. Anticancer mechanisms of cannabinoids and endocabnnabinoids

The extent of endogenous synthesis and catabolism of the various ligands within cells and tissues and the extent of the differential expression of the various receptors (e.g. $CB_{1/2}$; TRPV1) appears to be important in determining the signaling cascades through the various kinases. This signaling results in either pro-autophagic, pro-apoptotic, anti-proliferative or anti-apoptotic, pro-proliferative effects that have been observed in some cancer cells/tissues [51] (see below). The observations that malignant cells and tissues can increase their endocannabinoid and NAE concentrations, including anandamide, and upregulate their $CB_{1/2}$ receptor levels compared to non-malignant cells/tissues may be important in understanding their role in carcinogenesis (see below). Since inhibition of the NAE hydrolyzing enzymes results in increased availability of the NAEs, including anandamide, these enzymes are regarded as novel targets for the development of therapeutic drugs for a variety of diseases including anti-cancer drugs. Inhibition of the NAE hydrolyzing enzymes could also lead to greater metabolism of the endocannabinoids through other pathways, including the oxidative pathways mentioned above, with an increase in the levels and numbers of bioactive, oxidative derivatives.

The signaling mechanisms underlying the reported anti-cancer effects of the cannabinoids are as complex and varied as the expression levels of their receptors and the activity of their synthesis and degradation pathways (see above). They are generally involved in anticancer effects at all the major stages in carcinogenesis. This includes: (i) inhibition of the initiation and growth of tumours, due to inhibition of cell proliferation through cell cycle arrest and increased apoptotis; (ii) inhibition of cancer cell vascular adhesiveness, invasiveness and metastasis, which prevents tumour spread; (iii) inhibition of angiogenesis, which prevents oxygen and nutrient supply to the tumour. Similar anticancer effects at these stages of cancer development have been reported for both $n-3$ LCPUFA and CLAs/CLNAs [24,25] (see below).

4.1. Inhibition of cell proliferation

The anticancer properties of cannabinoids have been recognised for some time since Δ^9-THC was shown to inhibit lung adenocarcinoma cell growth *in vitro* and *in vivo*, [8–11,43,45,46,55,57,62–64]. Since then, several studies have shown that cannabinoids, including endocannabinoids, exhibit anticancer effects in various cancer cells and tissue types (see below). The primary mechanisms believed to account for these anticancer effects are induction/activation of autophagy, apoptosis, cell cycle arrest and activation of cell signaling pathways, such as the p38 MAPK, ERK pathways and the ceramide, p8, CHOP, mTORC1 pathway, often in various combinations depending on the receptor/agonist availability (see above). The available evidence suggests that these effects can occur either through CB receptor-dependent or independent mechanisms. This is indicative of, as yet unidentified, cannabinoid/endocannabinoid receptors or possible non-receptor mediated effects of these compounds. Several non-apoptotic mechanisms of inhibition have also been described (see below).

4.1.1. Activation of autophagy

Autophagy is a highly conserved process whereby cellular components are enclosed in double-membrane vesicles termed autophagosomes and transferred to lysosomes for degradation and recycling of products. This process has a pivotal role in cellular homeostasis but it can also be a form of programmed cell death akin to apoptosis (type I programmed cell death) and is termed "type II programmed cell death". Paradoxically, because it can also play a cytoprotective role in situations of nutrient deprivation, the process appears to play an important role in the opposing mechanisms, namely promotion of tumour progression and cancer cell survival. Reasons for this dual action of autophagy are not clear at present [47]. Recent observations [47,48,240] indicated that the plant cannabinoid (Δ9-THC) and a CB_2 receptor agonist (JWH-015) can induce autophagy and cancer cell death in various tumour cells including glioma, astrocytoma, pancreatic and hepatic cancer cells; they do not affect this process in non-transformed cells. Pharmacological or genetically elicited inhibition of autophagy prevented cannabinoid-induced cell death and apoptosis; blocking apoptosis alone prevented cell death but not the autophagy induced by these compounds. These findings suggested to the authors that induction of autophagy is part of the mechanism by which cannabinoids promote apoptotic death in cancer cells/tumours. Studies *in vivo*, in animals, showed that cannabinoid treatment reduced growth of tumour xenografts, derived from human astrocytoma cells and mouse embryonic fibroblasts, by activating autophagy and apoptosis. This did not occur in autophagy-defi-

cient tumours generated by subcutaneous injection of Arg5$^{-/-}$ MEFs. These findings indicated the relevance of cannabinoid-induced autophagy *in vivo* in animal models. Preliminary data from the analysis of samples from two glioblastoma multiforme patients indicated that THC administration could also induce autophagy and cell death in human tumours [47,48]. The authors proposed two different pathways for cannabinoid-induced cell death through autophagy in cancer cells, both of which required the binding to and activation of the CB_2 receptor and induction of endoplasmic reticular (ER) stress signaling [47,48] (Fig. 7). One pathway involved the upregulation of the pseudokinase Tribble homologue 3 (TRB3) by ceramide and the stress regulated protein p8 (candidate of metastasis-1, Com-1) and subsequent inhibition of serine-threonine kinase Akt/mammalian target of rapamycin C-1(Akt/mTORC-1). The second pathway involved activation of adenosine monophosphate activated kinase (AMPK) via CaMKKβ. Both pathways lead to firstly autophagy, which occurs upstream of apoptosis, and then to apoptotic cell death (THC—ER-stress—autophagy—tumour cell death).

Cannabidiol (CBD), the major non-psychotic component of cannabis, was also shown to induce cell death in MDA-MB-231 breast cancer cells but independently of cannabinoid and vallinoid receptor activation [241]. The authors reported the coexistence of both autophagy and apoptosis in CBD treated cells, along with an increased ER stress and subsequent inhibition of AKT and mTORC signaling, as shown by decreased phosphorylated mTORC, 4EBP1 and cyclin D1. In these studies CBD reduced mitochondrial membrane potential, increased cytochrome c release to the cytosol and increased apoptosis. This appeared to depend on increased generation of reactive oxygen species (ROS) because when ROS was inhibited, the induction of autophagy and apoptosis was blocked. CBD also triggered oxidative stress and caspase activation in human glioma cells [242]. Ligresti et al. [81] were the first to show that CBD elicited proapototic effects in cancer cells by production of ROS. It would appear that a common effector in apoptotis is the prior production of ROS, e.g. ceramide induces ER stress and ROS formation followed by autophagy and apoptosis (see above).

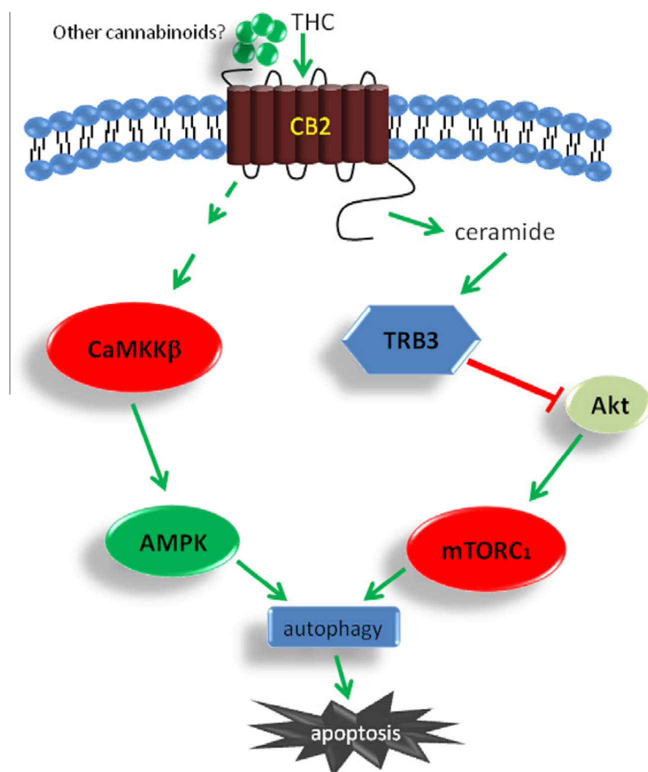

Fig. 7. Activation of cannabinoid receptor 2 may lead to autophagy and apoptosis by upregulating TRB3, and thereby inhibiting Akt/mTORC1 pathway, or may also initiate autophagy by activating AMPK. (THC = tetrahydrocannabinol, CB2 = cannabinoid receptor 2, CaMKKβ = calcium/calmodulin-dependent protein kinase kinase 2, TRB3 = Tribbles homolog 3, Akt = protein kinase B, AMPK = adenosine monophosphate-activated protein kinase, mTORC1 = mammalian target of rapamycin (mTOR) complex 1).

It is not known at present if other cannabinoids or endocannabinoids (including $n-3$ endocannabinoids) or their respective analogs and derivatives elicit similar autophagic responses in cancer cells. The precise mechanisms that determine the anti-cancer effects of these compounds in relation to the initiation of autophagy and/or apoptosis require further clarification.

The observation that the reported effects of cannabinoids are specific for transformed cells and do not affect normal cells warrant further investigation as a possible cancer treatment modality.

4.1.2. Induction of apoptosis

Anticancer agents generally tend to inhibit cancer growth by promoting tumour cell apoptosis or programmed cell death (Fig. 8). The precise mechanisms through which cannabinoids induce apoptosis are unclear at present. Several mechanisms have been suggested, possibly indicating differences in cell type, in receptor expression and/or the available concentrations of agonists resulting in activation of different signaling pathways.

Salazar et al. [47], and Vara et al. [48] proposed that autophagy preceded apoptosis and suggested that two different mechanisms for this existed, both acting through the CB_2 receptor (see above). Many apoptotic effects of cannabinoids are linked to the CB receptors and subsequent activation of the MAPK signaling pathways such as p38 MAPK, JNK and ERK1/2. The MAP kinases are a family of serine/threonine kinases involved in signaling pathways that control cell differentiation, growth and proliferation, cell death and the response to cellular stress [243]. These kinase pathways initially involve cell surface receptors, followed by cell signaling and transcription factor activation and regulation of DNA tran-

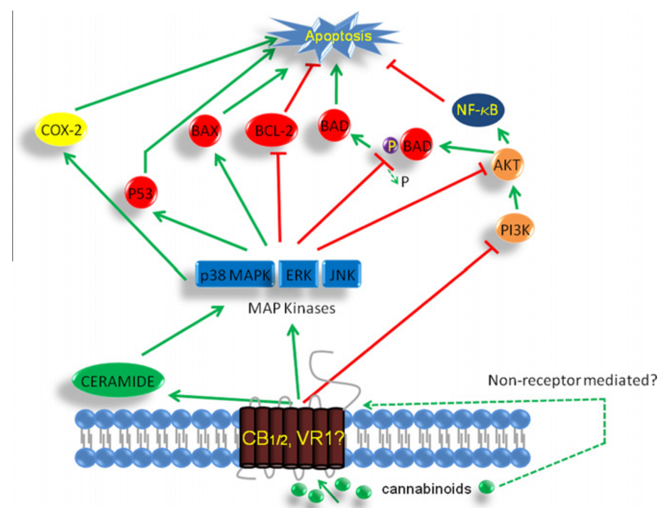

Fig. 8. Activation of cannabinoids receptors (or cannabinoids like receptors) is believed to activate map kinase family of proteins (MAPKs), which in turn can activate pro-apoptotic proteins, and inactivate anti-apoptotic proteins. Receptor activation can also inactivate the PI3K/Akt pathway, or COX-2 ($CB_{1/2}$ = cannabinoid receptor 1/2, VR1 = vanilloid receptor 1, MAPK = mitogen-activated protein kinase, ERK = extracellular signal-regulated kinase, JNK = c-Jun N-terminal kinase, COX-2 = cyclooxygenase 2, BAX* = Bcl-2–associated X protein, BCl-2** = B-cell lymphoma 2, BAD* = Bcl-2-associated death promoter, Akt = protein kinase B, PI3K = phosphoinositide 3-kinase, P = phosphorylation, NF-kB = nuclear factor kappa-light-chain-enhancer of activated B cells. * = pro-apoptotic, ** = anti-apoptotic).

scription that can trigger a number of downstream apoptotic responses. For example, activation of MAPK pathways have been described after treatment of prostate, liver, pancreatic cancer and leukemia cells [108,244–246] with Δ^9-THC, cannabidiol and WIN 55, 212-2. Furthermore, activation of these pathways appears to be both CB receptor dependent and non-dependent, as shown by the use of CB receptor antagonists in the studies of Giuliano et al. [108]. It is apparent, from different studies, that, although the activation of MAPK signaling pathways is a common factor in the anti-tumour effects of cannabinoids, the subsequent molecular cascades which result from this activation can be diverse and numerous. They are dependent on the type and extent of CB receptors expressed and the availability/concentration of agonists/antagonists [45].

Guiliano et al. [108] demonstrated that the activation of JNK/p38 MAPK pathway in liver cancer cells by WIN 55,212-2 was associated with the up-regulation of pro-apoptotic factors such as bax, bid and bcl-xs, and the corresponding down-regulation of anti-apoptotic, cell protective factors such as bcl-2 [108]. They also suggested that the induction of the PPARγ transcription factor played a key role in these effects. This PPAR transcription factor is activated by J series prostaglandins and the observation that anandamide can form prostaglandins, including the J series, suggested another mechanism for endocannabinoids to induce apoptosis [56] [see above]. Conversely, Jia et al. [245] demonstrated, in Δ^9-THC-treated leukemia cells, that inhibition, but not activation, of the ERK1/2 signaling pathway did not require p38 MAPK, or JNK. It did, however, lead to the phosphorylation and translocation of the pro-apoptotic protein, BAD, to the mitochondria, thereby activating apoptosis through caspase mediated mechanisms.

Cannabinoids are also believed to regulate other kinase signaling pathways, including the PI3K/Akt pathway. Phosphatidylinositol 3-kinases are a family of signal transducer enzymes that can activate Akt, a member of another serine/threonine kinase family. Akt is often found activated in many cancer types, and is regarded as having antiapoptotic and pro-survival functions [7,43,51,244,247]. It has direct antiapoptotic functions by phosphorylating BAD and thereby dissociating it from the BAX/BCL-2 complex and also indirect anti-apoptotic functions through activation of NF-κB, which is considered to have a pro-survival function [248]. This antiapoptotic role for cannabinoids is further supported by the finding that upregulation of TRB3 by THC and the CB_2 agonist JWH-015 results in downregulation of Akt prior to autophagy and apoptosis, as shown by Salazar et al. [47]. The effects of cannabinoids/endocannabinoids on this signaling pathway once again, appear to be conflicting because they can either activate or downregulate PI3K/Akt signaling pathways. In the brain, the neuroprotective properties of cannabinoids are suggested to be, in part, due to their ability to activate the PI3K/Akt cell survival pathway, similar to that shown for activation of the p38 MAPK pathway [249]. Ozaita et al. [250] showed that this activation, *in vivo*, in response to THC treatment in mice, was dependent on CB_1 receptors and resulted in Akt phosphorylation (activation and cell survival) in various parts of the brain.

In cancer cells, however, cannabinoids/endocannabinoids have been shown to inactivate Akt, resulting in increased apoptosis. Caffarel et al. [74] showed that both THC and JWH-133 inhibited tumour growth and induced apoptosis in ErbB2-positive breast cancer cells shown to express CB_2 receptors. They also showed that this increased apoptosis correlated with a decrease in Akt activation that was CB_2 dependent. Conversely, overexpression of activated Akt prevented the anti-tumour effects of these cannabinoids. Ethanolamide availability, receptor expression levels [51] and expression/activity levels of the kinases all seem to play a role in the anticancer effects of the cannabinoids.

In rat glioma C6 cells, WIN 55,212-2 treatment also reduced tumour growth and induced apoptosis, with a corresponding down-regulation of both the Akt and ERK1/2 signaling pathways [251]. The increase in apoptosis was shown to be a result of decreased phosphorylation of BAD, allowing it to exert its proapoptotic functions. Unfortunately, this study did not determine the specific role or expression levels of the CB receptors. This is pertinent since Cudaback et al. [51] observed that low concentrations of cannabinoids induced apoptosis only in astrocytoma cells expressing low levels of CB receptors that coupled to the ERK1/2 signal pathway. Cells expressing high levels of CB receptors were coupled to the pro-survival AKT pathway. The authors observed induction of apoptosis with high concentrations of cannabinoids, which agreed with previous findings [123,251–253]. They observed that this induction was independent of CB receptor-activation and AKT signaling but involved ERK1/2 signaling. It would appear that the effectiveness of the cannabinoids/endocannabinoids in inducing apoptosis is dependent on a number of factors. Not least the concentration of agonists and the level of expression of the receptors but also the ability to bypass these receptors and still activate apoptotic signals. This highlights a very complex and fascinating regulatory system worthy of more detailed investigation.

Another key modulator of apoptosis is the p53 protein. Activation of p53 by phosphorylation can lead to apoptosis, as a result of activation of multiple apoptotic pathways. In cortical neuron cells, p53 is necessary for Δ^9-THC to induce apoptosis, as shown by abrogating the effects of Δ^9-THC by using a p53 inhibitor and p53 siRNA [254]. Other studies in neuronal cells also showed that anandamide could increase p53 expression/activity through a CB_1 mediated pathway involving MAPKs [255].

In cancer cells, the story is less certain. In leukemic cell lines, Δ^9-THC could induce apoptosis and cell cycle arrest, but this was shown to be independent of CB_1 (and also of CB_2) [256]. Brown et al. [42] also showed pro-apoptotic effects in both p53 mutant (inactive) and wildtype prostate cancer cell lines when treated with endocannabinoids, suggesting p53 function is not essential for the apoptotic effects of the endocannabinoids in these cells. However, p53 was induced in prostate cancer cells after treatment with WIN 55,212-2, the CB receptor agonist [244]. It is interesting, in the context of p53 involvement in apoptosis, that hypomethylation, or silencing, of the p53 gene is associated with the development of lung cancer in male smokers [257], and that activation of p53 occurs when the DNA methyltransferase is inhibited, resulting in reduced epigenetic methylation [258]. It is possible that the induction of p53 by WIN 55,212-2 in prostate cancer cells was due to its increased phosphorylation (see below) or enhanced methylation of the gene encoding p53 or both. It is also likely, that due to the multiple pathways in which cannabinoids can exert their effects, that activation of p53 is one way to induce apoptosis, but is not an essential or main mechanism. As with many of the mechanisms of increased apoptosis mentioned in this chapter, it is entirely possible that p38 MAPK activation is responsible, since p38 MAPK is known to phosphorylate and activate p53 [259].

It is unclear at present whether the cannabinoids and endocannabinoids are directly inducing apoptosis, or if this arises purely as a result of activation of MAPK pathways and/or other receptor dependent and non-receptor dependent pathways. For example it has been extensively documented that cannabinoids-endocannabinoids can elicit the production of ceramide from membrane phospholipids, through activation of a specific PLD, and that ceramide is a potent pro-apoptotic lipid that induces ER stress and activates TRB3 and autophagy [47,48,246,260,261].

4.1.3. Induction of cell cycle arrest

As well as directly inducing apoptosis, several studies have also shown that cannabinoids can inhibit cancer cell proliferation by

arresting the cell cycle; this often leads to apoptosis. In particular, in breast cancer cells, several studies have shown that Δ^9-THC and anandamide can inhibit the cell cycle. After treatment with Δ^9-THC, cells were arrested in the G2–M phase and this was associated with down-regulation of cdc-2, an important regulator of the cell cycle, and a subsequent increase in apoptosis [80]. Anandamide treatment of breast cancer cells, using a metabolically stable analog, also led to cell cycle arrest, but this time in the S phase, with no associated increase in apoptosis [262]. This was associated with changes in expression and activity of several cell cycle proteins, including activation of chk1, degradation of cdc25A and reduction in cdk2 activity.

More recently, the hitherto little studied class of endocannabinoids, derived from $n-3$ fatty acids, have also been shown to induce cell cycle arrest in prostate cancer cells [42,263]. This appeared to be cell line specific with significant G1 arrest in LNCaP cells, and G2 arrest in PC3 cells, and was more pronounced for docosahexaenoylethanolamide (DHEA) than eicosapentaenoylethanolamide (EPEA). This cell cycle arrest was also associated with apoptosis, although the specific mechanisms leading to apoptosis were not explored [42]. Continuing in the vein of comparing endocannabinoid effects with $n-3$ LCPUFA and CLA effects, it is interesting to note that $n-3$ LCPUFA [21,23,50] as well as CLAs [24] and CLnAs [25], can attenuate breast and prostate cancer cell proliferation by arresting their cell cycles.

4.1.4. Other anti-proliferative mechanisms

Other mechanisms of inhibiting cancer cell proliferation also exist, and these can be either receptor-dependent or independent. Many of these mechanisms often result in the apoptotic death of cells.

4.1.4.1. Oxidation by cyclooxygenase-2 (COX-2). COX-2 is an enzyme which catalyses the production of prostaglandins and is often overexpressed in cancer cells. Several studies have demonstrated the role of COX-2 in cannabinoid-endocannabinoid induced apoptosis. Indeed, a metabolically stable analog of anandamide (*R*-(+)-methanandamide) induces COX-2 expression which in turn induces apoptosis in cervical cancer cells [219,264]. This apoptotic effect was reduced when COX-2 was inhibited, which also involved the activation of PPARγ The role of COX-2 in apoptotic cell death has also been shown in colon cancer cells and skin carcinoma cells treated with anandamide or anandamide analogs. In a recent study it was demonstrated that *R*-(+)-methanandamide and anandamide could trigger apoptosis in human neuroglioma cells after inducing COX-2 activation to increase prostaglandin E_2. Inhibition of COX-2, but not COX-1, by silencing with siRNA, prevented the apoptotic induction and the proapoptotic action of the anandamide analog was mimicked by PGE_2. Apoptosis and cell death were not affected by $CB_{1/2}$ receptor or TRPV1 receptor antagonists but were inhibited by COX-2 inhibitors [219]. Anandamide was also shown to induce COX-2-dependent cell death in apoptosis-resistant colon cancer cells [265]. These studies indicated that the COX-2 mediated apoptotic mechanism is CB receptor independent. Whether the COX-2 eicosanoid derivative was PGE_2 *per se* or the eicosanoid derivative of the anandamide was not clear (see below).

The endocannabinoids (AEA and 2-AG) *per se* are also substrates for the COX-2 enzyme and are metabolized to the corresponding prostaglandin derivatives (see above). These compounds are also substrates for the lipoxygenases (LOXs) and the P_{450} enzymes resulting in a plethora of their respective derivatives many of which have bioactivity in alternate pathways (see above). For example, anandamide can be converted to various ethanolamide PG derivatives, including a prostaglandin J_2 derivative which can potently activate PPARs [50,56,59,221–223,225,228,266] (see below).

4.1.4.2. Ceramide synthesis. The bioactive lipid ceramide is an important mediator in both extrinsic and intrinsic pathways of apoptosis [267]. Antiproliferative and apoptotic effects of anandamide in prostate cancer have been shown to involve the increased synthesis of ceramide via the de novo pathway [268], and more recently in mantle cell lymphoma, both *R*-(+)-methanandamide and WIN 55,212-2 induced cytotoxicity via upregulation of this ceramide pathway [269]. In this particular study, antagonists of CB_1 and CB_2 suggested, that this ceramide induction process was CB_1-mediated, and may also be partially mediated by CB_2. Carracedo et al. [110] demonstrated that treating pancreatic cancer cells with Δ^9-THC led to a CB_2-mediated, de novo synthesised, ceramide-dependent, up-regulation of the stress related protein, p8, and up-regulation of the endoplasmic reticulum associated, stress related genes ATF-4 and TRB3, resulting in apoptosis. Salazar et al. [47] also showed that ceramide synthesis was CB_2 dependent and involved ER stress and autophagy (see above).

4.1.4.3. Oxidative stress. Oxidative stress, leading to accumulation of reactive oxygen species (ROS), is a cellular stress factor that results in cellular damage, including DNA damage, which can increase the likelihood of cancer developing. Evidence for the role of cannabinoids in regulating the extent of oxidative stress is conflicting, sometimes increasing ROS-induced cell death and at other times protecting against it. It has been suggested that these marked opposing effects may be dependent on the available-concentration of cannabinoids (see above). The role of the CB receptors in the pro- or anti-oxidative effects is also unclear, and both CB receptor-dependent and -independent effects have been reported which may even differ between different cannabinoids [270].

Chen and Buck, [271] demonstrated that low doses of Δ^9-THC, cannabidiol and cannabinol, but not WIN 55,212-2, could prevent serum-deprived cell death through an antioxidant mechanism. This attenuation of oxidative cell death did not correlate with the affinity of the individual cannabinoids for the two CB receptors. Furthermore, it contrasts with the observations that cannabinoids actually elicit ROS formation as a mechanism for cell death in cancer cells (see above). Evidence for a CB receptor mediated mechanism was demonstrated when protecting kidney cells from cisplatin induced nephropathy [272]. In this study, a CB_2 receptor agonist was able to prevent cell death and attenuate accompanying oxidative stress after cisplatin treatment. Use of a CB_2 receptor knockout mouse further validated the role *in vivo* of CB_2 in this antioxidant response. CB_1 antagonists also decreased ROS production *in vitro* in endothelial cells and *in vivo* in mice, thereby improving endothelial function [273].

Conversely, several studies showed that cannabinoids could exert their anti-proliferative effects by inducing ROS-dependent cell death. Anandamide, for example, increased ROS production in cancer cells [242,274] and increased the susceptibility of hepatocytes to ROS damage [275]. Furthermore, Jacobssen and colleagues demonstrated that the anti-proliferative effects of anandamide and 2-AG on glioma cells could be totally inhibited by the antioxidant α-tocopherol [276].

Most, but not all, of the studies mentioned above involved non-malignant cells, and the specific anti-or pro-proliferative role of cannabinoids, due to modulation of the cellular ROS balance in cancer cells, remains to be elucidated.

4.2. Cannabinoid and endocannabinoid effects on cancer cell invasion and metastasis

Numerous publications have reported the effects of cannabinoids and endocannabinoids on cancer cell growth and proliferation, but in order for a tumour to disperse and spread (metastasise) to other tissues *in vivo*, it needs to produce invasive

cells that can be extravasated from the blood across membranes into other tissues/organs (Fig. 9). In order for the new malignant cells to grow into secondary tumours they need to develop new blood vessels (angiogenesis) in order to ensure an adequate supply of oxygen and nutrients for growth [277,278]. Angiogenesis involves the synthesis of specific angiogenic molecules including vascular endothelial growth factor (VEGF), angiopoietins 1 and 2 (Ang1 and Ang2), and matrix metalloproteases (MMPS). VEGF is one of the most potent and abundant pro-angiogenic molecules and is able to regulate most steps of angiogenesis that lead to increased capillary formation [279]. Angiopoietins are required for the formation of mature blood vessels [280] and MMPS are proteases that help to degrade proteins in vessel/tissue membranes, and thereby help to enhance the invasion and angiogenesis necessary for secondary tumours to form [281]. Adhesion molecules (such as ICAM-1 and VCAM-1) are also essential to allow extravasation of cells across the vascular wall [282]. There is obviously a large therapeutic potential for any natural or pharmaceutical compounds that can inhibit these adhesive, invasive metastatic processes of tumour cells whilst being relatively non-toxic for patients. Current evidence appears to suggest that cannabinoids/endocannabinoids could fulfill such criteria. However, such a suggestion may be an oversimplification since these compounds are also reported to enhance, rather than inhibit, the growth and metastasis of certain tumours under certain conditions. These contradictory observations may again be explained by the fact the pro-metastatic action of Δ^9-THC in cancer appears to depend on the level of expression of the cannabinoid receptors. For example, McKallip et al. [78] showed that Δ^9-THC could increase growth and metastasis of the mouse mammary carcinoma 4T1, which expressed little or no CB receptors *in vivo*. Conversely, in tumours that express CB receptors, such as A549 non-small cell lung cancer cells, Δ^9-THC inhibits tumour growth and migration *in vitro* and both growth and metastasis *in vivo*, when grown in immuno-compromised mice [283].

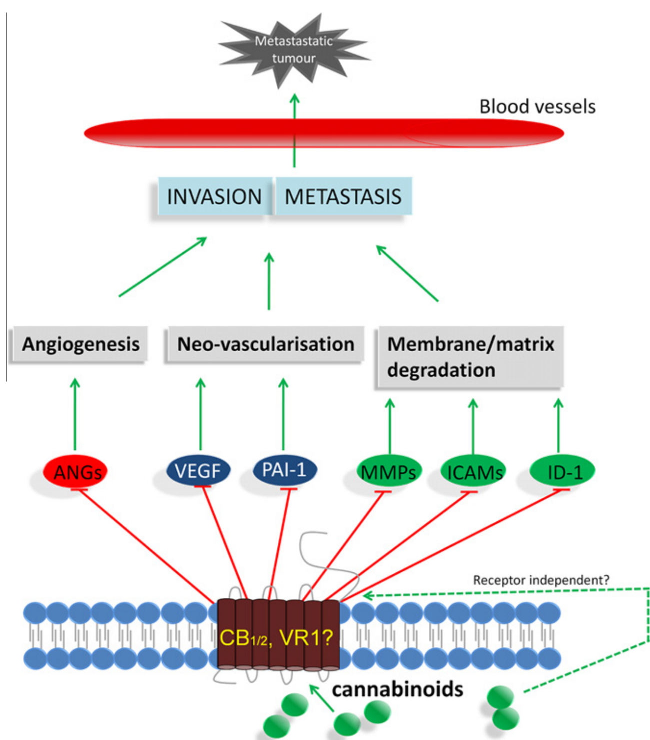

Fig. 9. Activation of cannabinoids receptors are known to inhibit many processes that lead to cancer cell invasion and metastasis, including pathways that regulate angiogenesis, tumour vascularisation, and tumour cells ability to degrade matrix membranes ($CB_{1/2}$ = cannabinoid receptor 1/2, VR1 = vanilloid receptor 1, ANGs = angiopoetins, VEGF = vascular endothelial growth factor, PAI-1 = plasminogen activator inhibitor 1, MMPs = matrix metalloproteinases, ICAMs = intercellular adhesion molecules, ID-1 = DNA-binding protein inhibitor 1).

A number of studies have investigated the role of synthetic cannabinoids, in particular WIN 55,212-2 (a CB_1 and CB_2 agonist) and JWH-133, a CB_2-selective agonist, on cancer metastasis. Various tumour types/cells have been used to determine the effects of these synthetic cannabinoids on angiogenesis, invasion and metastasis. In gastric cancer cells, treatment with WIN 55,212–2 inhibited cell growth and cell invasion by down-regulating the pro-invasive factors VEGF and MMP2. JWH-133 also resulted in reduced tumour growth and lung metastasis in a metastatic model system using both breast tumours and cell lines, *in vitro* and *in vivo* [75]. In gliomas, JWH-133 inhibited angiogenesis *in vivo* by decreasing pro-angiogenic factors including VEGF, Ang2 and MMP2 in the tumours and by inhibiting survival and migration of the vascular cells themselves [278]. Decreased vascularisation, with altered blood vessel morphology and reduced VEGF, have also been observed after treatment with WIN 55,212–2, JWH-133 and Ang2 of melanoma cell xenografts in mice [284]. In most of these studies, antagonists of CB_1 and CB_2 revealed that these processes were mediated through CB receptors.

Cannabinoids may further reduce the invasive potential of cancer cells by inhibiting their adhesion to the vascular endothelium. The adhesion molecules (ICAM-1 and VCAM-1), are a prerequisit for extravasation of circulating cells from blood vessels. They have been shown to be downregulated at the mRNA level after treatment with WIN 55,212-2 in astrocytes [285]. Interestingly, $n-3$ LCPUFA and CLAs/CLnAs also attenuate adhesion molecule expression on endothelial cells [24,286].

The phytocannabinoid, cannabidiol has been shown to inhibit breast cancer cell proliferation and invasion *in vitro* by reducing Id-1 expression as a result of modulating ERK pathway signaling and reactive oxygen species [287,288]. Inhibiting Id-1 has previously been shown to reduce tumour cell proliferation and invasiveness *in vitro* and *in vivo* [289]. In lung cancer cells, cannabidiol has also been shown to significantly inhibit the invasive potential of A549 lung cancer cells *in vitro* [290]. This action was suppressed using CB_1, CB_2 and TRPV1 antagonists, indicating a role for cannabinoid-receptor pathways and an interrelationship between the different receptors. This inhibition of invasive potential was also associated with a significant reduction in the expression of plasminogen activator inhibitor-1 PAI-1 and upregulation of an inhibitor of MMP1 [291]. Interestingly, a previous study in gliomal cells also demonstrated a reduction in invasion after cannabidiol treatment, but this did not involve cannabinoid receptors [292]. Perhaps the latter effect was mediated through TRPV1 receptors or was receptor independent and may represent different mechanisms that are activated in different cancer cell/tumour types.

The classical endocannabinoids have also been shown to inhibit angiogenesis and metastasis. In particular, a metabolically stable anandamide analog, 2-methyl-2′-F-anadamide (Met-F-AEA), was shown to inhibit the adhesion and migration of aggressive MDA-MB-231 breast cancer cells through a collagen IV matrix and to reduce the number of metastatic nodes *in vivo*, in a mouse model of metastasis [293]. A similar model was used to demonstrate that Met-F-AEA also reduced metastatic nodes and produced a significant inhibitory effect on VEGF production and VEGF receptor 1 [294]. The inhibitory effects of Met-F-AEA, in both studies, were reversed by antagonising CB_1, CB_2 antagonists were unfortunately not investigated. A similar role for $n-3$ endocannabinoids in inhibiting cancer invasion and metatstasis has, to our knowledge, not been elucidated to date but is currently under investigation in our laboratory.

The role of 2-AG in invasion and metastasis is somewhat more complicated. Endogenous 2-AG has been shown to inhibit invasion of prostate cancer cells. In particular by preventing its hydrolysis (or using a non-hydrolysable analog), and thereby increasing its endogenous levels, 2-AG can inhibit hormone independent prostate cancer cells through a CB_1 receptor dependent pathway [113]. However, adding exogenous 2-AG has the opposite effect and increases cell invasion [295]. This is thought to be due to increasing levels of AA, as a result of rapid hydrolysis of 2-AG by FAAH/MAGL, and further oxidative metabolism of AA to 12-hydroxyeicosatetraenoic acid, which is a promoter of PC3 cell invasion [296]. The 2-MG derivatives of $n-3$ LCPUFA should not elicit an increase in cell invasiveness since their hydrolysis would result in $n-3$ LCPUFA that have anti-adhesive and anti-invasive properties (see above).

The ability of different cannabinoids to inhibit angiogenesis and cell migration and metastasis, not only in tumour cells, but in some cases also in vascular endothelial plaque cells, suggests a possible therapeutic role, not only for inhibiting tumour and atherosclerotic plaque growth but also for preventing the spread and metastasis of a variety of malignant tumours. Taken together with their relatively safe toxicity profile, the potential for cannabinoids/endocannabinoids to halt the progression and spread of tumours warrants further study. However, some of these studies indicate that, although the endocannabinoids *per se* may inhibit invasion of cancer cells, the complexities of the pathways involved in their synthesis and metabolism need to be carefully considered and levels of respective cannabinoids/endocannabinoids, their metabolizing enzymes and their receptor expression may all be important in determining the final outcome of cannabinoid treatment. Being able to increase endogenous levels of endocannabinoids in cells either by directly increasing their synthesis, or by preventing their degradative metabolism, through dietary or pharmacological means, could be an important area for future research.

4.3. Cannabinoid and endocannabinoid-induced gene regulation

4.3.1. Epigenetic regulation

It has been suggested that tumorigenesis and cell invasiveness are driven by the hypomethylation of promoter regions of genes involved in tumour progression. The invasiveness of prostate cancer (PC3) cells was decreased *in vitro* by increasing the methylation state (silencing) of key cancer promoting genes [297]. Cannabinoid/endocannabinoids may influence cancer development and growth of tumours by altering the epigenetic regulation (methylation) of important cancer promoting genes. Epigenetics allows for the reversible regulation (silencing) of gene expression. This could prevent the initiation of cancer cells and/or their progression, invasiveness and metastasis. The ability to modulate epigenetic regulation by the production of endogenous cannabinoids, whose levels can be modulated by diet or by administration of plant-derived and synthetic cannabinoids, could be an important anti-cancer strategy for improving current therapeutic regimens.

Oxidative stress, often an accompanying or inducing factor in cancer, and $n-3$ LCPUFA, particularly EPA and DHA, are able to epigenetically regulate transcription of specific genes involved in lipid metabolism and cell regulation in tissues/cells, including cancer cells [298,299]. Feeding animals certain fatty acids results in mRNA changes in lipogenic enzymes within hours, and these changes remain as long as the fatty acids remain in the diet; they rapidly return to normal when the fatty acids are removed [300,301]. LCPUFA can also cause changes in gene expression by interacting directly with nuclear transcription factors when gene expression can be regulated in a matter of minutes [302]. This is apparently due to fatty acid sensitive response elements in the promoter regions of these genes that regulate their transcription [303,304]. It is, therefore, conceivable that endocannabinoids derived from LCPUFA, both $n-3$ and $n-6$, may exert similar effects on gene expression. Anandamide has been shown to regulate keratinocyte differentiation by inducing DNA hypermethylation that resulted in the downregulation or silencing of various genes. More importantly, it was also shown to increase methyltransferase activity *per se* [305]. These effects were mediated by the CB_1 receptor. The ability to alter DNA methyltransferase activity suggests that cannabinoids may be able to regulate the expression of many genes both positively and negatively, and consequently various metabolic pathways, by altering the methylation machinery in cells. The silencing of the CB_1 receptor by hypermethylation of the encoding gene, as mentioned above, appears to be another possible mechanism by which cannabinoids can regulate cancer [125]. It would be interesting to ascertain if decreased expression of p-glycoprotein (an efflux pump responsible for multidrug resistance when overexpressed in cancer cells) can be elicited by cannabinoid treatment of resistant cells [306]. Expression of p-glycoprotein was regulated by epigenetic methylation of its encoding gene DNA. The importance of the level of $CB_{1/2}$ receptor expression in modulating the apoptotic response of cancer cells/tissues to cannabinoids/endocannabinoid stimulation has been mentioned above [51]. The CB_1 receptor was silenced in human colorectal cancer tissue and in cancer cells, but not in normal tissue or cells [125]. This silencing was due to hypermethylation of the CpG dinucleotides in the promoter region of the Cnr1 gene encoding the CB_1 receptor. This highlighted a possible key role of epigenetic hypermethylation in the regulation of the cannabinoid/endocannabinoid system in colorectal and possibly other cancers [125]. Loss or inhibition of CB_1 in $Apc^{Min/+}$ mice accelerated intestinal tumour growth whilst activation or hypomethylation of the receptor attenuated this growth by decreasing cdc2 and inducing apoptosis by the down-regulation of the anti-apoptotic protein survivin; this was mediated by the cAMP-dependent PKA pathway [125]. PKA can regulate cdc2 expression/activity through the action of two cdc2 kinase regulators in oocytes [307]. A decrease in cdc2 and survivin protein was also observed in breast cancer cells treated with Δ^9-THC again suggesting that PKA pathway regulates survivin expression [80].

The epigenetic methylation status of genes may be regulable by diet as observed for the MnSOD gene in human buccal cells [126]. However, it is not known at present if $n-3$ LCPUFA or their endocannabinoid derivatives are able to modify the methylation state of various genes involved in tumour promotion or inhibition. The reduction in the methylation status of the CB_1 recptor mentioned above would suggest that cannabinoids may be able to exert anticancer effects by this process. This is an exciting area for future cannabinoid research in relation to cancer and other disease states. Clearly, the ability of cannabinoids/endocannabinoids to alter multiple molecular pathways and processes involved in cancer, either by CB receptor dependent or independent mechanisms or by directly affecting gene expression, suggests that their potential use as cancer therapeutic agents warrants further research.

5. Cannabinoids-endocannabinoids and immune functions in cancer

A number of studies have shown that the CB_2 receptor is largely, but not exclusively, expressed in cells and tissues associated with the immune system, including tumours of immune origin such as leukemia and lymphoma cell lines [11,45,62,308,309]. Furthermore, the major effects of cannabinoids/endocannabinoids on im-

mune-cell function appear to be through activation of the CB_2 rather than the CB_1 receptor.

5.1. CB_2 receptors and immune function

Some studies have suggested that cannabinoid/endocannabinoid activation of the CB_2 receptor in immune cells, resulting in their apoptosis, can lead to attenuation of certain immune cell functions, particularly attenuation of inflammatory cytokine formation [43,78]. This impairment in immune function raised concerns about the possibility of these compounds eliciting pro-cancer effects because of a decrease in immune surveillance of cancer cells and the enhanced pro-proliferative and pro-cancer mechanisms.

Inhibiting pro-inflammatory cytokines and eicosanoids is generally regarded as beneficial in the prevention of disease states with an autoimmune-inflammatory component like MS, rheumatoid arthritis and diabetes (see below). Furthermore, as mentioned above, the CB_2 receptor is also expressed to varying extents in cancer cell lines and tissues. The phyto-, synthetic- and endocannabinoids, Δ^9-THC, HU-210 and anandamide respectively, and the synthetic CB_2-selective agonist JWH-015 all suppressed proliferation and induced apoptosis in cancer cell lines [308,309]. Similar effects were observed in primary acute lymphoblastic leukemia cells [308] and primary human T-lymphocytes [309]. Δ^9-THC also inhibited the growth of xenografted lymphoma cells in mice and increased the survival-time of the animals [308]. This led the authors to suggest that targeting CB_2 receptors in tumours of immune-cell origin could be a viable therapeutic approach for these cancers. However, this suggestion does require further research because of the adverse pro-proliferative and tumour-promoting effects that have been reported for low, sub-micromolar doses of various cannabinoids/endocannabinoids in different cancer cell lines [45,51,309,310]. Interestingly, high concentrations of cannabinoids in the micromolar range, as opposed to low, sub-micromolar concentrations, are widely reported to induce anti-proliferation and pro-apotosis in these cell lines [45,51,309–311]. This is indicative of a bimodal response of the cell lines to the cannabinoids/endocannabinoids [45,51] and could reflect the expression level of the $CB_{1/2}$ receptors as has been suggested for astrocytomas [51]. Determining expression level of $CB_{1/2}$ receptors and the possible availability of various cannabinoids/endocannabinoids for every cancer type to ensure anti- as opposed to pro-cancer effects would be an extremely difficult and time consuming way of identifying practical therapeutic application of these compounds. The simple concept of using high concentrations of cannabinoids/endocannabinoids in cancer therapy and bypassing the $CB_{1/2}$ receptors and selectively killing malignant cells without harming normal cells, as suggested by Guzman et al. [124], could be a viable treatment option (see below under clinical aspects). Whether such high concentrations of agonist involve the TRPV1 receptor or other receptors or are completely non-receptor mediated, which is possible, is not clear at present.

5.1.1. Anandamide and immune function

One of the first fatty acid amides to be studied with respect to its potential to modulate immunity was arachidonyl ethanolamide (anandamide), primarily because it was an arachidonic acid metabolite. Arachidonic acid metabolism, particularly its conversion to eicosanoids, has long been associated with enhanced immune/inflammatory responses and increased pro-cancer effects, particularly growth and metastasis [30]. The possibility that another, rather novel, metabolite of arachidonic acid, i.e. anandamide, could be produced as a result of immune-cell activation and that it could modulate immune cell functions was intriguing. It is also conceivable that the effects of arachidonic acid on immune cells could be due, at least in part, to its prior conversion to the ethanolamide derivative, as suggested by our underlying hypothesis for the effects of $n-3$ ethanolamides in cancer and cell functions (see below). One of the first studies to address this question found that anandamide did not have any significant effect on T-cell proliferation when they were stimulated using either anti-CD3 antibody, a polyclonal stimulus, or directly by circumventing the plasma receptors by using the DAG analog, phorbol-12-myristate-13-acetate (PMA) and the calcium ionophore, ionomycin [312]. The authors also found that anandamide did not affect antibody-forming B-cells. This suggested that anandamide was unable to affect adaptive immune responses i.e. T or B cell activity. However, a later study contrasted with these findings and showed that anandamide could inhibit plaque-forming antibody-producing colonies of B-cells in the picomolar to nanomolar range [313]; indicating another example of the differences in effects [314] elicited by cannabinoids depending on differences in their concentration. The lower concentrations of endocannabinoids are more likely to be physiologically relevant, although higher concentrations may be of therapeutic value. The above authors also demonstrated that this suppressive action of anandamide was reversed by cannabinoid CB_2 receptor antagonists, but not by CB_1 antagonists. This clearly indicated that the potent suppression of antibody production by anandamide was through activation of the CB_2 receptor. It is not certain at present whether this effect of anandamide is a protective or pathogenic response. The suppression of colony formation/antibody production by anandamide would appear to be an undesirable side effect of cannabinoids, however, it could also be a regulatory response of endogenously produced anandamide i.e. its normal function may be to switch-off the processes involved in antibody production as a negative feedback mechanism. The effects of anandamide on other adaptive immune functions have been poorly studied, however, some studies suggest that there is little effect of anandamide on T-cell functions. T-cell activation is critically important in immune responses to viruses and also for anti-tumour immunity. The study of Lissoni et al. [314] focused on the actions of anandamide on various T-cell functions. They showed that anandamide did not alter either basal or IL-2-stimulated T-cell proliferation, nor did it affect differentiation into regulatory T-cells (Treg) or the production of the inhibitory cytokine IL-10, a major marker of Treg activation. This study also assessed the effects of anandamide on target cell cytotoxicity of T-cells, an indicator of the anti-tumour killing capability of T-cells. Human blood-derived T-cells were assessed for their ability to kill target K562 cells as determined by the release of Cr51, a reliable and unambiguous indicator of cell killing. Anandamide at concentrations of either 30 nM or 3 μM (spanning 5-orders of magnitude) did not alter IL-2-enhanced cell killing [314]. This is an important observation with respect to the role of anandamide in cancer therapy because it appears that it does not have any deleterious effects on anticancer immune activity. However, the findings of Cencioni et al. [309] contrast with the above insofar that AEA was observed to suppress the proliferation of activated, primary, human peripheral T-lymphocytes as well as the release of the cytokines IL-2, TNF-α and IFN-γ. Their findings did agree with Lissoni et al. [314] in so far that AEA was not cytotoxic to the T-cell. Cencioni et al. [309] further showed that the immuno-suppression elicited by AEA was largely dependent on CB_2 receptor activation. This was because the effect could be mimicked by the selective CB_2 receptor agonist JWH-015 and blocked by the CB_2-selective antagonist SR144528, whereas selective CB_1 agonists and antagonists were without effect. The authors also reported immuno-suppression, but not cytotoxity, by AEA of the newly identified cytokine IL-17, derived from the unique T-cell subset Th-17. This cytokine is responsible for host defense against extracellular pathogens and is involved in the increased inflammation in autoimmune dis-

orders like multiple sclerosis (MS), arthritis and diabetes [315]. Most immunosuppressive therapies are cytotoxic to T-cells and increase the likelihood of infection in patients. Consequently, a natural compound like anandamide, that suppresses the pro-inflammatory element of the Th-17 cells but preserves cell viability and therefore the protective immune responses, could be an invaluable, novel therapy to prevent autoimmune disorders in patients [309].

The contrasting reports of the effects of anandamide on T-cells and lymphocyte function are difficult to reconcile but could relate to the type of cells used, their CB expression and the concentration of the compounds involved. The concerns relating to the pro- and anti-cancer effects of cannabinoids are in the minority and these compounds are generally recognized as being antiproliferative and anti-tumorigenic (see below).

It is well documented that the parent $n-3$ LCPUFA, like the CLAs and CLNAs, are able to attenuate inflammatory cytokine formation and adhesion molecule expression on endothelial cells [24,25,286,303].

5.1.1.1. Anandamide attenuation of TNF-α. Inflammation is intimately involved in the of the initiation and progression of many forms of cancer and attenuation/inhibition of inflammation by pharmacological or dietary supplements is regarded as being of positive benefit [316–318]. Anandamide has been reported to inhibit proliferation of several cancer cell types including breast cancer [319] and prostate cancer [268], indicating a direct beneficial anti-cancer effect. However, a potential drawback is that anandamide can also have a counteracting effect in that it also appears to be cytoprotective under certain conditions (see above), this could be detrimental to the killing of cancer cells. An alternative cell-controlling, anti-cancer mechanism is the production of TNF-α from immune cells which can bind to target cells and induce apoptosis. This particular action of TNF-α was suppressed by anandamide since the TNF-induced cytotoxicity in L929 cells was reduced by anandamide indicating an anti-apoptotic action [320]. Clearly, in an *in vivo* situation the effects of cannabinoids/endocannabinoids on non-immune cancers would depend on the matrix of cells present, including immune cells, and the outcome could be very different to that observed with a pure cancer cell line in culture since the attenuation of immune cell inflammatory responses could reduce cancer cell apoptosis.

The production of TNF-α is a primary inflammatory, innate immune response and is extensively regulated by both arachidonic acid and its oxygenated metabolites, especially prostanoids, including PGE_2. However, this axis has been very poorly studied for the effects of cannabinoids-endocannabinoids. There have been several studies that have demonstrated that anandamide can ameliorate the end-symptoms of inflammatory responses such as ulcerative colitis and gingivitis [321,322]. These indicate that anandamide is able to attenuate pro-inflammatory cytokine release and indeed it has been suggested that it is an endogenous modulator that limits colon inflammation [323]. In addition, macrophages have themselves been shown to produce anandamide [324] and this was upregulated by inflammatory stimuli such as lipopolysaccharide (LPS) [325]. Furthermore, anandamide can inhibit the NF-kB pathway, the main transcription factor pathway involved in the response to stress stimuli and expression of pro-inflammatory cytokines [326]. These observations clearly point to an important regulatory axis for cytokine production by anandamide. Similar effects of $n-3$ ethanolamides have not been reported to our knowledge but they would be expected to attenuate proinflammatory cytokine production in a manner akin to that of the parent fatty acids (see above).

5.1.1.2. Anandamide attenuation of neutrophil migration. Anandamide can also modulate the actions of neutrophils, a critical primary antibacterial defense mechanism, by downregulating their migratory activity [327]. This particular action appears to occur in a manner that does not involve CB_1 or CB_2 receptors. Although neutrophil migration can be inhibited by anandamide in the nanomolar range it does not appear to affect the respiratory burst activity of human blood-derived neutrophils [328]. This suggests that anandamide may immobilize neutrophils at particular key sites whilst allowing their killing capabilities to remain intact.

5.2. Anandamide oxidative metabolism in immune cells

With the discovery that arachidonic acid could undergo conversion to arachidonoyl ethanolamide, it was suggested that this metabolite could also be a substrate for COX-2 and be converted to a prostaglandin-amide (see above). Indeed, it is also possible that the free carboxyl groups in the formed prostanoids could be amidated. The latter has not been addressed to any great extent, however, there is information regarding the formation of prostamides from anandamide. One of the first of these to be described was PGE_2-ethanolamide. It was shown that radiolabelled anandamide was converted by human COX-2, but not COX-1, to radio-labeled PGE_2-ethanolamide which was confirmed by mass spectrometry [227]. A later study confirmed that macrophages were able to produce not only PGE_2-ethanolamide but also PGD_2-ethanolamide [59]. It was shown that lipopolysaccharide and interferon-gamma stimulation of macrophages (a potent inflammatory stimulus) resulted in the production of PGD_2-ethanolamide when cells were pre-exposed to anandamide. This was ascertained by mass spectrometry and it was also shown that COX inhibitors prevented the formation of the PGD_2-ethanolamide confirming a COX-mediated cyclo-oxygenation of anandamide [59]. These observations strongly suggested that PGD_2-ethanolamide, in addition to PGE_2-ethanolamide, played an important role in controlling innate immune cell activity, particularly during the development of an inflammatory response. It would appear that the pharmacology of prostamides differs from that of the precursor anandamide, or other endocannabinoids, since they do not appear to act through CB receptors [329]. This was also shown by an earlier study where it was confirmed that PGE_2-ethanolamide could bind to all 4 EP receptors in a similar manner to PGE_2 but did not bind to the CB receptors [61]. This study compared both the binding and functional activity of PGE_2 and PGE_2-ethanolamide and found that PGE_2 had in the order of 1000-fold greater affinity for each of the 4 EP receptors compared to the ethanolamide derivative. Interestingly, the pK_i for PGE_2-ethanolamide was within a narrow range for all four receptors [61]. The smooth muscle activity in response to both prostanoids was also compared in different tissues with different EP receptors; PGE_2 was always more potent than PGE_2-ethanolamide. However, this difference in potency between the two derivatives was less than that observed for their binding activity to the ER receptors, the PGE_2 being only in the order of 10- to 100-fold greater than the ethanolamide derivative [61]. This raises the question of why there would be dual activity for each of the EP receptors elicited by both PGE_2 and PGE_2-ethanolamide. Similarly, the question of whether the actions of PGE_2 are mediated by PGE_2-ethanolamide in some cases has nor been answered, although the differences in receptor affinity between the PGE_2 and its ethanolamide would appear to preclude this likelihood. Presumably, the relative concentrations of the two derivatives would be a deciding factor. It has been suggested that many studies that determined PGE levels by ELISA immunoassay may have actually been measuring PGE-ethanolamide as it has been recently recognized that the antibodies used in these assays can also have cross-reactivity with PGE_2-ethanolamide indicating that the presence of PGE species

may have been mis-indentified and may have indicated the presence of PGE_2-ethanolamide [330].

Not only anandamide but also 2-AG was shown to be a substrate for COX-2 in immune cells [59] resulting in the formation of glycerol-prostaglandins (see Section 3 above). Anandamide is also a substrate for the lipoxygenase enzymes LOX-5, LOX 12, LOX15 in immune cells (see Section 3 above).

It seems reasonable to assume that EPEA and DHEA can also be converted to PGE_3-ethanolamides as well as other $n-3$ series prostamides in immune cells, as was observed for prostate cancer cells and 3T3-L1 adipocytes [31,42]. However, this has not been addressed to any significant extent. It may prove to be an extremely important regulatory axis considering the inhibitory effects of the omega-3 LCPUFA on inflammatory processes and especially on immune cell activity. PGs are the most potent regulators of all immune cell functions and the possible role of the eicosanoid ethanolamides could be an important focus for future research.

5.3. $n-3$-derived endocannabinoids and immune function

As mentioned previously, arachidonic acid, an $n-6$ LCPUFA, can be converted to the ethanolamide derivative, anandamide and that $n-3$ LCPUFA can be similarly converted to their respective derivatives. This was supported by observations that both EPA-ethanolamide (EPEA) and DHA-ethanolamide (DHEA) have been detected recently in various tissues in animals and man, especially animal neuronal tissue, but also in liver, gut, [32,33], (see above). Prostate cancer cells were also able to convert EPA and DHA to their respective ethanolamides [42], as were 3T3-L1 adipocytes [31]. The latter authors demonstrated that DHEA, but not EPEA, was present in human plasma [31]. These studies showed that the tissue levels of ethanolamides are elevated by the corresponding precursor fatty acid, either through dietary supplementation or addition to cell incubations. The concentration of anandamide in macrophages has also been shown to respond positively to inflammatory stimuli [324]. The presence in, and effects on, immune cells of the omega-3 LCPUFA derivatives, EPEA and DHEA, have to date been poorly studied. However, a recent study demonstrated that EPEA and DHEA both attenuate lipopolysaccharide-induced nitric oxide production in a macrophage cell line at concentrations up to 10 μM [34]. The effects of these compounds are elicited at comparatively high concentrations (i.e. up to 10 μM). Their concentrations in cells/tissues are unknown but probably only exist in the nM or even picoM range. It must be emphasized that we do not know the physiological concentrations of the $n-3$ endocannabinoids in cells where they are synthesized on demand at intracellular sites. Their concentration at these sites would not necessarily reflect the average concentration within the cell or tissue. Clearly, any addition of exogenous $n-3$ ethanolamides would need to be at greater concentrations than required at the site of action due to the binding capacity of the albumin in the incubation medium used in the studies and the activity of degradation enzymes in the cells (see above).

It was also shown that DHEA could suppress the production of the pro-inflammatory cytokine MCP-1 and that both effects occurred at the level of gene expression, i.e. the mRNA for both NOS and MCP-1 was suppressed [34]. These observations indicated that the *N*-acyl ethanolamine derivatives of $n-3$ LCPUFA, similar to the fatty acids *per se*, could contribute to the inhibition of inflammatory responses in immune cells. Control of the formation or degradation of these ethanolamides could play a critical role in the endogenous regulation of immune/inflammatory diseases (see above).

As mentioned previously, the $n-3$ LCPUFA, EPA and DHA, and the CLAs/CLnAs are able to inhibit immune-inflammatory cytokine formation in cells and tissues [24,25,303,316,317]. This again raises the question of the identity of the active agent(s) in this process: the parent fatty acids or the ethanolamide derivatives or both. The possibility of oxidative metabolites such as the eicosanoid derivatives being active agents cannot be precluded as this stage (see above).The observation that effects of anandamide could be prevented by COX inhibitors supports the latter suggestion [265].

6. Existing and potential therapeutic applications of cannabinoids and endocannabinoids in cancer

There is a wealth of data derived from cell culture studies *in vitro* and promising observations of anticancer therapeutic potential of cannabinoids in animal models with various cancer xenografts *in vivo* (see above). Cannabinoids inhibited growth and angiogenesis of gliomas in animal models [117,123,278,331]. Of importance is the fact that the antiproliferative, apoptotic effects of cannabinoids appear to be tumour-selective, affecting tumour cells but not normal brain cells such as astrocytes, oligodendrocytes and neurons; indeed the latter appeared to be protected by the cannabinoid treatment [14,123,332,333]. With such encouraging preclinical, animal data it is somewhat surprising that to date the only human, clinical findings relating to possible anti-tumour effects of cannabinoids is enshrined in one pilot study (see above) using nine terminally ill patients with recurrent glioblastoma multiforme, an aggressive primary brain tumour with very poor prognosis where normal survival was only ca. 6–12 months [124]. The study, similar to the rat study above [123,278,331] involved the direct administration of Δ^9-THC into the tumour. It was primarily undertaken in 2002 to ascertain the safety profile of this compound in this type of intracranial administration and to emphasise the lack of significant psychotropic effects. The authors concluded that the compound did not induce tumour growth or reduce patient survival. It did decrease tumour cell proliferation and increase apoptosis and did not present any deleterious safety issues. However, due to the small numbers of patients no definitive conclusions could be made regarding the overall efficacy of the compound and the mode of treatment.

Many tumours, like colorectal [334] and prostate tumours [268] express high levels of COX-2 that normally makes them resistant to induction of apoptosis. Selective COX-2 inhibitors were shown to suppress growth of human colon epithelial cells and to enhance the response to conventional chemotherapy agents [335] and consequently have been proposed as possible anticancer agents, alone or in combination with standard chemotherapy agents. Interestingly, AEA induced non-apoptotic cell death in these tumours, suggesting cannabinoids/endocannabinoids could be beneficial in treating such resistant cancers but without the deleterious side effects of COX-2 inhibitors. Such adjunct effects of cannabinoids warrant further preclinical and clinical studies. Similar adjunct augmenting effects on chemotherapy with $n-3$ LCPUFA and CLAs have been shown *in vitro* and in animals *in vivo* (see above) but similar effects with $n-3$ ethanolamdes have not been reported.

Although limited in perspective, the overall indication from accumulated data relating to cannabinoids and cancer strongly suggest that these compounds may have an important role to play as possible curative agents or adjunct chemotherapeutic agents in cancer, with the caveats mentioned above relating to possible anti-immune effects and cancer enhancement. Although the evidence for cannbinoids acting as potential anticancer agents is mainly derived from non-clinical studies in cells and animal models, these compounds have been used as palliative agents to optimize the management of a variety of cancer-associated symptoms and to attenuate a number of chemotherapy side effects [57,336]. Cannabinoids exhibit a broad spectrum of palliative properties when

used as adjuncts to standard cancer therapies including appetite stimulation that counters the anorexia of some forms of cancer; the inhibition of nausea and vomiting often associated with chemo- and radiotherapy; pain relief associated with cancer and cancer metastasis; amelioration of depression associated with the disease and importantly, the amelioration of insomnia [57,336,337].

Two synthetic derivatives of Δ^9-THC, Marinol and Cesamet are approved by the FDA in the USA for palliative, medicinal use in chemotherapy-induced nausea and vomiting in patients who failed to respond to conventional anti-emetic compounds. Cannabinoids also interact with dopaminergic, serotoninergic, monaminergic, noradrenergic and opioid receptors/systems that are involved in emesis. Evidence points to cannabinoids acting on CB_1 receptors in the dorsal-vagal complex of the brainstem that controls the vomiting reflex. Also, their presence in the GI tract may indicate a role in emesis [336]. Sativex, a combination drug containing both Δ^9-THC and CBD, has been licensed in Canada since 2005 as a pain-relief drug for cancer patients.

Cancer-induced anorexia/cachexia is an important risk factor in patients with certain forms of cancer that can reduce life expectancy considerably. Numerous animal studies have shown that cannabinoids can stimulate appetite and increase food intake [336,338]. A number of clinical studies support the orexigenic effect of THC and a synthetic cannabinoid, dronabinol, has been approved by the FDA in the USA for treating anorexia in AIDS patients. However, the clinical evidence for anti-cachexia effects of cannabinoids is still rather limited. A phase-II trial to assess the appetite stimulating effect of THC in patients with advanced cancer had a positive outcome [336,339]. Furthermore, megestrol, a standard orexigenic agent, was superior in reducing anorexia in advanced cancer patients to that of dronabinol treatment [336,340]. Disappointingly, the first phase-III trial in patients with cancer cachexia compared the effects of cannabinoids, a standard cannabis extract and a placebo on appetite stimulation, quality of life, mood and nausea and found no significant differences between the groups [341]. Clearly, further research into the clinically relevant aspects of cannabinoids in combating cancer cachexia is required. Of interest in this area is the well documented anti-cachechtic effects of $n-3$ LCPUFA in animals and man [342,343] suggesting that the application of these $n-3$ LCPUFA (see above) or a combination of plant cannabinoids and $n-3$ LCPUFA or $n-3$ ethanolamines might be of even greater benefit than the use of the plant extracts/derivatives alone. Our group previously supplemented terminally ill prostate cancer patients with $n-3$ LCPUFA from fish and observed an enhancement in well being and mood with an increase in weight gain and muscle strength and decrease in levels of prostate specific antigen (PSA) in the patients (McClinton, Heys, Wahle et al. unpublished observations). The interactions of $n-3$ LCPUFA and their cannabinoid derivatives are worthy of further study in relation to their individual or combined effects on cancer cachexia as well as on anorexia.

7. Conclusions

From the foregoing it can be ascertained that the cannabinoids-endocannabinoids exhibit great potential as anticancer agents in their own right or as adjunct therapies to standard chemo-, and possibly radio-therapy over and above their current clinical uses in amelioration of cancer pain, emesis and weight loss. The role of $n-3$ ethanolamides and $n-3$, 2-acylglycerols have not been studied in any detail and they could provide interesting developments as nutraceuticals considering some of the health benefits attributed to their parent LCPUFA, namely EPA and DHA, in ameliorating diseases including cancer. The possibility that endocannabinoid derivatives of the conjugated linoleic acids (CLAs) and conjugated linolenic acids (CLnAs) can also be formed has not been reported. Evidence exists that these fatty acids also elicit anticancer effects in cells and confer cardiovascular and cancer health benefits in animal models of disease (see above).

The concerns regarding the pro-cancer effects sometimes reported in the literature, possibly relating to differences in receptor expression and cannabinoid-endocannabinoid availability, also need to be addressed and defined.

Indications are that a number of the anticancer effects of cannabinoids-endocannabinoids could possibly be mediated by their secondary conversion to their respective eicosanoid or even epoxy derivatives and this warrants further investigation and clarification in the light of the differential effects of $n-6$ and $n-3$-derived eicosanoids in cancer and the role of the resolvins and protectins derived from LCPUFA by their COX/LOX metabolism. It is interesting that the availability of fatty acids can determine the level of endocannabinoid synthesis and this highlights the possibility of dietary supplementation as a possible adjunct therapy in cancer.

It is also intriguing that the $n-3$ LCPUFA, apparently in their natural form, and the CLAs/CLnAs are ascribed similar beneficial health effects to the cannabinoids-endocannabinoids and the sequence and type of metabolites formed and the cell signaling mechanisms underlying these effects need to be clearly delineated in order to understand the full potential of the cannabinoids-endocannabinoids as anticancer agents. The cannabinoids-endocannabinoids will continue to be a fascinating and potentially clinically translatable area of research for many years.

The overriding evidence suggests that these compounds, natural or synthetic, are friends rather than foes with regard to the battle against cancer.

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