Cannabimimetic fatty acid derivatives in cancer and inflammation

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Abstract

Evidence for the role of the cannabimimetic fatty acid derivatives (CFADs), i.e. anandamide (arachidonylethanolamide, AEA), 2-arachidonoylglycerol (2-AG) and palmitoylethanolamide (PEA), in the control of inflammation and of the proliferation of tumor cells is reviewed here. The biosynthesis of AEA, PEA, or 2-AG can be induced by stimulation with either \textsuperscript{2+} ionophores, lipopolysaccharide, or platelet activating factor in macrophages, and by ionomycin or antigen challenge in rat basophilic leukemia (RBL-2H3) cells (a widely used model for mast cells). These cells also inactivate CFADs through re-uptake and/or hydrolysis and/or esterification processes. AEA and PEA modulate cytokine and/or arachidonate release from macrophages in vitro, regulate serotonin secretion from RBL-2H3 cells, and are analgesic in some animal models of inflammatory pain. However, the involvement of endogenous CFADs and cannabinoid CB\textsubscript{1} and CB\textsubscript{2} receptors in these effects is still controversial. In human breast and prostate cancer cells, AEA and 2-AG, but not PEA, potently inhibit prolactin and/or nerve growth factor (NGF)-induced cell proliferation. Vanillyl-derivatives of anandamide, such as olvanil and arvanil, exhibit even higher anti-proliferative activity. These effects are due to suppression of the levels of the 100 kDa prolactin receptor or of the high affinity NGF receptors (\textit{trk}), are mediated by CB\textsubscript{1}-like cannabinoid receptors, and are enhanced by other CFADs. Inhibition of adenylyl cyclase and activation of mitogen-activated protein kinase underlie the anti-mitogenic actions of AEA. The possibility that CFADs act as local inhibitors of the proliferation of human breast cancer is discussed here. © 2000 Elsevier Science Inc. All rights reserved.

Keywords: Anandamide; 2-Arachidonoyl-glycerol; Palmitoylethanolamide; Tumor cell proliferation; Cancer; Cannabinoid; Receptor; Pain; Inflammation

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The several experimental and anedoctal reports of possible benefits originating from the use of the Indian hemp Cannabis sativa [1] or of the cannabinoids and their synthetic or natural derivatives [2], sparkled recently a debate on the possible medical use of marijuana [3,4]. However, still very little information is available on the possible therapeutic use of the endogenous counterparts of the cannabinoids, i.e. the cannabimimetic fatty acid derivatives (CFADs), anandamide (arachidonoyl-ethanolamide, AEA), 2-arachidonoylglycerol (2-AG), and palmitoylethanolamide (PEA) [5–8] (Fig. 1). Interestingly, of these compounds, only 2-AG seems to activate functionally both cannabinoid receptor subtypes described so far, the CB1 and CB2 receptors. In fact, although AEA seems to be more selective for the CB1 receptor, PEA does not bind to either subtype up to 1 μm concentrations, and the mechanism of action underlying its effects is still a matter of speculation [8]. Therefore, of these three compounds, only AEA and 2-AG can be defined as ‘endocannabinoids’, whereas, unless a new type of cannabinoid receptor for PEA is discovered, this compound can only be described as a CFAD. Because of the discovery of their cannabimimetic properties, these compounds have been assessed for a large number of possible pharmacological activities.

**Fig. 1. Chemical structures of some natural and synthetic cannabimimetic fatty acid derivatives (CFADs). The efficacy with which these compounds functionally activate the two cannabinoid receptor subtypes identified so far (CB1 and CB2) is also schematically described.**

1. Introduction

The several experimental and anedoctal reports of possible benefits originating from the use of the Indian hemp Cannabis sativa [1] or of the cannabinoids and their synthetic or natural derivatives [2], sparkled recently a debate on the possible medical use of marijuana [3,4]. However, still very little information is available on the possible therapeutic use of the endogenous counterparts of the cannabinoids, i.e. the cannabimimetic fatty acid derivatives (CFADs), anandamide (arachidonoyl-ethanolamide, AEA), 2-arachidonoylglycerol (2-AG), and palmitoylethanolamide (PEA) [5–8] (Fig. 1). Interestingly, of these compounds, only 2-AG seems to activate functionally both cannabinoid receptor subtypes described so far, the CB1 and CB2 receptors. In fact, although AEA seems to be more selective for the CB1 receptor, PEA does not bind to either subtype up to 1 μm concentrations, and the mechanism of action underlying its effects is still a matter of speculation [8]. Therefore, of these three compounds, only AEA and 2-AG can be defined as ‘endocannabinoids’, whereas, unless a new type of cannabinoid receptor for PEA is discovered, this compound can only be described as a CFAD. Because of the discovery of their cannabimimetic properties, these compounds have been assessed for a large number of possible pharmacological activities.
including neuroprotection [9,10], regulation of neurotransmission [11], anti-nociception [12], immune function modulation [13], vasodilation [14,15], and hormone regulation [16]. The potential use of cannabinoids and CFADs in neurological disorders also has been discussed [17]. The development of selective antagonists [18,19] for CB₁ and CB₂ receptors recently permitted to determine whether these proteins are involved in CFAD pharmacological actions. In this article, we shall focus on two of the most intriguing, as well as controversial, properties of CFADs, regarding the possible function of these metabolites as either anti-inflammatory agents or anti-mitotic mediators against cancer cell growth. If, on the one hand, the extent and mechanism of action of the anti-inflammatory activities of CFADs is still being debated, on the other hand, the possible therapeutic exploitation of the anti-cancer effects of AEA and 2-AG may be hindered by their immune-modulatory, and in some cases, immune-suppressant actions [13,20]. The experimental evidence that has led to suggest for these compounds a role as anti-inflammatory and anti-tumoral lipid mediators, as well as the potential for their application to the cure or prevention of inflammatory diseases and cancer, will be critically discussed here.

2. CFADs and inflammation

The anti-inflammatory properties of Cannabis preparations, which are for example particularly useful for the alleviation of asthma, have been known for millennia [1]. Stemming from this knowledge, recent studies led to the development of the potent anti-inflammatory cannabinoid acids (for a recent review see [21] and the article by Burstein et al. in this issue), compounds that, unlike marijuana’s major psychoactive component, Δ⁹-tetrahydrocannabinol (THC), are devoid of psychotropic activity and are very weak ligands of either CB₁ or CB₂ receptors. These studies, together with observations of little anti-inflammatory activity with oral THC [22], seem to suggest that the anti-inflammatory properties of cannabinoids are not necessarily mediated by cannabinoid receptors. Recent investigations, reviewed in the next four chapters, addressed the possibility that endogenous metabolites such as the CFADs could also behave as anti-inflammatory agents and serve as templates for the development of new drugs.

2.1. Biosynthesis and inactivation of CFADs in immune cells

The first clue that CFADs could act as endogenous modulators of the immune and inflammatory response came from the finding that blood cells involved in these pathophysiological situations synthesize these compounds on treatment with various stimuli, and inactivate them through several mechanisms. Thus, rat basophilic leukemia (RBL-2H3) cells, a widely employed model for the study of mast cells, was shown to respond to ionomycin stimulation and immunological challenge—two stimuli leading to cell degranulation and serotonin release—by producing AEA and PEA together with other fatty acid ethanolamides [23]. These cells inactivate the two compounds by distinct saturable and temperature-sensitive uptake processes followed by the hydrolysis to ethanolamine and the corresponding fatty acids [23] catalyzed by fatty acid amide hydrolase (FAAH) (see Ref. [24] and the article

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by Ueda and Yamamoto in this issue). The AEA membrane transporter in RBL-2H3 cells was shown later to be inhibited by oleylethanolamide, phloretin and, particularly, fatty acid vanillyl-amides such as olvanil and arvanil (Fig. 1) [25]. Intact RBL-2H3 cells were also shown to inactivate 2-AG through a re-uptake mechanism—that did not seem to be temperature-sensitive and was immediately followed by enzymatic hydrolysis to glycerol and arachidonic acid (AA), as well as by direct esterification into membrane phospholipids [26]. The major enzyme involved in 2-AG hydrolysis in these cells seemed to be FAAH, although the presence of other enzymes serving this purpose could not be ruled out. Mouse J774 macrophages were also shown to produce AEA and PEA [23,27] together with 2-AG [28] on stimulation with ionomycin. More physiologically relevant stimuli, such as lipopolysaccharide (LPS) and platelet activating factor (PAF), were found to induce AEA formation in mouse RAW 264.7 macrophages [29]. LPS was also shown to induce AEA and 2-AG biosynthesis in rat circulating macrophages [28,30] and 2-AG formation in rat platelets [31]. Interestingly, mouse peritoneal macrophages stimulated with A23187 did not exhibit an enhanced formation of AEA and PEA [99]. However, in this recent study, unlike previous ones [23,27–31], no FAAH inhibitor was introduced in the incubation medium to prevent the degradation of CFADs formed de novo. In fact, macrophages rapidly inactivate AEA, PEA [23], and 2-AG [28], the latter compound being degraded following passive diffusion into cells and subsequent re-esterification into (phospho)glycerides and/or hydrolysis to glycerol and AA. Interestingly, enzymatic 2-AG hydrolysis by macrophages (and platelets) was down-regulated by LPS [28]. U937 monocytes [32], as well as human platelets [33], express FAAH as well as an AEA membrane transporter that can be activated by NO-donors, thus raising the possibility that AEA levels in these cells may be under the control of mediators that induce NO release. Finally, although the metabolism of CFADs in lymphocytes has not been investigated yet, AEA was poorly hydrolyzed by human neutrophils, where instead oxidation by lipoxygenases seems to be the major metabolic pathway [34]. However, one of the major products of these reactions, $12(S)$-hydroxy-$5Z,8Z,10Z,14Z$-eicosatetraenoyl-ethanolamide, was still very active as a cannabinoid receptor ligand and did not seem to be recognized by FAAH, thus suggesting that, in some cases, lipoxygenase-mediated oxidation of AEA may be used to prolong the half-life of this substance in vivo rather than inactivating it.

2.2. Effect of CFADs on inflammatory mediator release from tissues and cells

A few studies have addressed the possibility that CFADs may act on immune cells and somatic neurons to release inflammatory or pro-inflammatory mediators such as serotonin, cytokines, AA, NO, and calcitonin-gene related peptide (CGRP), although the involvement of cannabinoid receptors in these effects has not always been assessed. PEA was shown to inhibit the antigen-induced secretion of serotonin from both rat mast cells and RBL-2H3 cells [35]. Under the same conditions potent synthetic cannabinoids were not more active than PEA, whereas AEA was inactive and inhibited the cannabinoid-induced down-regulatory effect. The authors also provided evidence for the presence of CB$_2$, but not CB$_1$, cannabinoid receptors in RBL-2H3 cells. However, subsequent studies (reviewed in [36]) showed that PEA is unable to bind with a high affinity to either of the two cannabinoid receptor subtypes,
thus raising the possibility that the effect of PEA in mast cells and basophils was due to the interaction with proteins different from these two receptors.

Independent experiments from Burstein’s and our laboratories, based on early reports of the activation of phospholipase A\textsubscript{2} enzymes by cannabinoids (reviewed in [37]), showed that AEA, but not PEA, can induce AA release from mouse macrophage cell lines [38,39]. The effect was observed with low-medium range mm concentrations of the compound, and was blocked by either pertussis-toxin [38] or the selective CB\textsubscript{2} receptor antagonist SR144528 [39]. We have found a similar effect with AEA in RBL-2H3 cells (Table 1). Also in this case the effect could not be observed with PEA and was blocked by pertussis-toxin pretreatment of cells. In both J774 macrophages and RBL-2H3 cells, the effect of AEA on AA release was attenuated by phospholipase A\textsubscript{2} inhibitors (Table 1). It should be mentioned, however, that other authors have observed the release of AA with AEA also in cells that do not express CB\textsubscript{1} or CB\textsubscript{2} cannabinoid receptors [40,41]. Whatever its mechanism of action, AEA-induced AA release may lead to the formation of eicosanoids with opposing actions in the immune and inflammatory response, such as prostaglandin E\textsubscript{2} from macrophages and leukotrienes from mast cells.

AA release from monocytes stimulated with AEA (30 \textmu m) was also observed by Berdyshev et al. [42] who reported an inhibitory effect of this compound as well as of PEA on FMLP-stimulated interleukin-6 (IL-6) and -8 and (in the case of AEA only) tumor necrosis factor (TNF)-\alpha (TNF-\alpha) production. The effect was bi-phasic, being observed only at 3–30 nM doses and then disappearing at higher (0.3–3 \textmu m) doses, where an inhibitory effect on cytokine soluble receptors was observed instead. The participation of cannabinoid receptors in these effects was not investigated. At the level of the CNS, however, AEA was found to

<table>
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<tr>
<th>Substrate</th>
<th>J774 Macrophages</th>
<th>RBL-2H3 Basophils</th>
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<tr>
<td>None</td>
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<td>100</td>
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<tr>
<td>AEA 6 \textmu m</td>
<td>101 \pm 3</td>
<td>115 \pm 10</td>
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<td>304 \pm 35</td>
<td>212 \pm 10</td>
</tr>
<tr>
<td>PEA 25 \mu m</td>
<td>116 \pm 13</td>
<td>N.D.</td>
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<tr>
<td>50 \mu m</td>
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<td>109 \pm 13</td>
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<td>100 \mu m</td>
<td>117 \pm 27</td>
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<td>115 \pm 5*</td>
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<td>25 \mu m + PTX</td>
<td>148 \pm 41*</td>
<td>115 \pm 4*</td>
</tr>
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<td>50 \mu m + PTX</td>
<td>160 \pm 30*</td>
<td>154 \pm 4*</td>
</tr>
<tr>
<td>AEA 25 \mu m + AACF\textsubscript{3}</td>
<td>122 \pm 22*</td>
<td>127 \pm 4*</td>
</tr>
<tr>
<td>AEA 25 \mu m + DEDA</td>
<td>134 \pm 25*</td>
<td>N.D.</td>
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The effect was measured in cells prelabelled for 16 h with [\textsuperscript{3}H]AA (0.1 \mu Ci/ml) and then washed and stimulated for 15 min with the drugs (or combination of drugs) shown. Radioactivity released in the supernatant was then measured by liquid scintillation counting (the complete method is described in Ref. [38]). PTX, pertussis toxin (200 ng/ml, 6 h pre-treatment of cells). AACF\textsubscript{3}, arachidonoyl trifluoromethylketone, 15 \mu m. DEDA, 7,7\'-di-methyl-eicosadienoic acid, 20 \mu m. *\textit{P} < 0.05 (Student’s \textit{t}-test) compared to the corresponding sample obtained at the same concentration of AEA or PEA. N.D., not determined.
enhance the release of the anti-inflammatory IL-6 from astrocytes infected with Theiler’s murine encephalomyelitis virus. The effect was blocked by the CB$_1$ antagonist SR141716A, and suggested a possible neuroprotective role of cannabinoid receptors in neurological disorders such as multiple sclerosis [10]. More recently, the potent cannabinoid agonist CP-55,940, but not AEA and THC, was shown to induce IL-8 and β-chemokine monocyte chemotactic protein-1 gene expression in unstimulated HL60 cells [98]. The effect was due to activation of CB$_2$ receptors, which do not seem to be efficiently activated by either THC or AEA [8].

Modulation of NO synthesis is likely to play an important role in the possible anti-inflammatory activity of AEA, as shown by recent studies carried out in both mammalian and invertebrate immunocytes [43,44]. AEA induced immune cell rounding through activation of cannabinoid receptors and stimulation of constitutive NO synthase [43]. The latter effect is probably mediated by cannabinoid-receptor induction of intracellular Ca$^{2+}$ transients [44], and may result in turn in the indirect inhibition of adenylyl cyclase, thereby causing, for example, disruption of nuclear factor kappa B action and inhibition of inducible NO synthase [45].

Finally, AEA was recently shown to variedly affect the release of the potent vasodilatory peptide CGRP from sensory fibers, possibly depending on the co-localization in these neurons of CB$_1$ and capsaicin (i.e. vanilloid) receptors. It was found that very low doses of AEA inhibited capsaicin-induced CGRP release from rat hindpaw skin through a CB$_1$-mediated mechanism, thus leading to inhibition of capsaicin-evoked plasma extravasation into the hindpaw and to an anti-hyperalgesic action [46]. Subsequent studies showed, however, that CB$_1$ receptors, by being mostly localized on neurons intrinsic to the spinal cord, are not expressed in all capsaicin-sensitive sensory neurons [47]. Accordingly, AEA, but not PEA or 2-AG, was recently found to induce CGRP release from peri-vascular sensory neurons through a CB$_1$ receptor-independent mechanism involving the direct activation of vanilloid receptors [48]. Thus, it is possible that AEA exerts anti-inflammatory or pro-inflammatory responses by either decreasing or increasing CGRP levels, respectively at low or at high nM concentrations, depending on whether or not CB$_1$ and vanilloid receptors are co-localized on sensory neurons.

2.3. Anti-inflammatory actions of CFADs in animal models

The first reports that PEA could be a potent anti-inflammatory mediator dates back to the late 1950’s and early 1960’s (see [49] for review). Since then, the possible therapeutic use of this compound as an anti-inflammatory agent has been pointed out by Mazzari et al. [50], who found that, when administered orally to rats, PEA causes a unique spectrum of actions, including reduction of: 1) substance P-induced mast cell degranulation and extravasation; 2) passive cutaneous anaphylaxis-induced extravasation; 3) carrageenan-induced edema and hyperalgesia; 4) formalin-induced edema, and 5) dextran-induced edema. More recently, a potent and CB$_1$-mediated effect against carrageenan-induced thermal hyperalgesia and edema in rats was described also for AEA [46]. Indirect evidence for the involvement of endogenous cannabinoids in the down-modulation of mechanical allodynia produced by injections of complete Freund’s adjuvant (CFA) in rat hindpaw was also gained by using the
selective CB₁ antagonist SR141716A, which cause enhanced mechanical sensitivity ‘contra-
lateral’ to the inflammation site [51]. However, in another similar study, the effect of THC,
but not anandamide, was blocked by SR141716A, which, when administered alone, failed to
alter paw pressure thresholds in either CFA-treated or control rats [52]. Two previous studies
had shown that peripheral administration of either AEA or PEA could produce strong
anti-hyperalgesic effects in the rodent formalin paradigm of inflammatory pain [53,54]. In
one case, the two compounds were found to act in synergy, and their effects were blocked
by selective CB₁ and CB₂ receptor antagonists, respectively, which were also shown to
induce hyperalgesia when administered alone [54]. However, recent work showed no
consistent hyperalgesic or pro-inflammatory effect by either SR141716A or the CB₂ antag-
onist SR144528 in the rat or mouse formalin test [59,96] or in the mouse AA-induced ear
thickness test [97], respectively. Thus, although there seems to be a general consensus for a
possible analgesic action of exogenous CFADs against somatic inflammatory pain, the
possible existence of an endogenous tone controlling this pathological condition via canna-
binoid receptors is still being debated.

Along with somatic pain, also visceral inflammatory pain can be reduced by AEA and
PEA, as shown by Jaggar et al. [53,55]. Unlike AEA, however, PEA did not prevent the
viscero-visceral hyperreflexia associated with nerve growth factor (NGF)-induced inflam-
mination of the rat urinary bladder, thus suggesting that this compound becomes effective as
an analgesic only when an inflammatory state is established [55].

Finally, two recent investigations have addressed the potential anti-inflammatory activity
of CFADs in airway hyper-reactivity, which was suggested by the well known broncho-
dilatory and anti-asthma properties of Cannabis preparations [1]. In one study it was found
that anandamide did not affect dynamic compliance, total pulmonary resistance, tidal vol-
ume, or breathing frequency in guinea pigs treated with an aerosol of A23187, although it did
reduce significantly airway epithelial injury and pulmonary leukocytosis without preventing
peribronchiolar granulocytic accumulation [56]. In the other study, it was reported that both
AEA and PEA could weakly inhibit TNF-α levels in bronchoalveolar lavage fluid of
LPS-treated mice, and that AEA could also inhibit neutrophil recruitment [57]. These data
suggest that CFADs have moderate anti-inflammatory activity also in the airways, although
they seem to lack the ability to directly relax the airway smooth muscle.

2.4. Levels of CFADs in inflamed tissues

Thus far, three studies have attempted to correlate CFAD levels in tissues with the onset
of inflammatory states. In one case, Kondo et al. [58] examined the levels of several
acylethanolamides, including PEA and AEA, as well as of their phospholipid biosynthetic
precursors, the N-acyl-phosphatidylethanolamines, in rat testes treated with cadmium chlo-
ride, a chemical known to induce inflammation and degeneration of this tissue. The authors
found a striking increase of the amounts of all acylethanolamides and, correspondingly, of
their precursors, in cadmium-treated versus vehicle-treated testes. Interestingly, however,
PEA levels seemed to be stimulated to a higher extent (39-fold) than AEA levels (fivefold).
In another set of studies we have analyzed, by using gas chromatography-mass spectrometry,
the levels of CFADs in rat hindpaw skin after injection of 1, 2.5 or 5% formalin, as well as
in the NGF- or turpentine-treated rat urinary bladder. We found no statistically significant variation in the levels of AEA, PEA or 2-AG, nor in FAAH-like activity, in these tissues (Table 2), thus suggesting that endogenous CFADs may not be released in these animal models of inflammation (Ref. [59] and Di Marzo and Rice, unpublished observations).

### 3. CFADs and cancer

There have been in the past, and very recently too, numerous reports on the anti-cancer activity of THC and its analogs. Several studies on the anti-mitotic and anti-neoplastic activity of THC were published in the early 1970’s, when large amounts of synthetic cannabinoids became available. THC was found to retard dose-dependently the growth of Lewis lung adenocarcinoma and to inhibit Friend leukemia virus-induced splenomegaly in vivo [60], probably by reducing the rate of DNA synthesis in tumor cells [61–63]. Antimitotic effects for THC were found against L1210 and K-562 leukemia cells [64,65], whereas later studies showed that this cannabinoid could also delay the cell differentiation of human leukemia cells in vitro [66]. In almost all these studies, non-psychotropic cannabinoids such as cannabidiol were also tested and found to be active, albeit less potent than THC. In one study in particular [64], where several cannabinoids were tested, the rank of potency found for these compounds did not correspond to their relative activity as ligands of cannabinoid receptors, determined in later binding studies. Indeed, recent experiments showed that a non-psychoactive cannabinoid, cannabigerol, is an inhibitor of human oral epithelioid cell and NIH 3T3 fibroblast growth in vitro [67], and that THC can induce apoptosis in C6 glioma and PC-3 prostate cells through cannabinoid-receptor independent mechanisms [69]. On the other hand, THC was shown to disrupt the cytoskeletal organization of differentiated rather than undifferentiated PC-12 cells [70], an effect more recently observed also for the synthetic cannabinoid HU-210, but not for its non-psychoactive enantiomer HU-211, and due to inhibition of tubulin and actin expression [71]. Thus,
cannabinoids seem to inhibit cancer cell growth through several mechanisms, some of which (e.g. the induction of apoptosis) are not necessarily mediated by CB₁ receptors, whereas some others (e.g. the disruption of the cytoskeleton or the inhibition of DNA synthesis) may be due, at least in part, to activation of cannabinoid receptors. Unfortunately, despite the fact that sensitive techniques for the rapid measurement of both CB₁ and CB₂ receptor expression in tissues (e.g. the reverse-transcriptase polymerase chain reaction (PCR) [RT-PCR] for the quantitation of RNA transcripts and Western immunoblotting for the determination of the proteins) are now available, no study has examined yet whether cannabinoid receptors are over-expressed in some tumor as compared to healthy tissues or cells. Furthermore, although CFADs are produced and metabolized by numerous tumor cells (for example see Refs. [72–75]), and polyunsaturated fatty acids are known to inhibit cancer cell growth (see [76] for review), so far only a few studies have aimed at assessing whether these compounds exert a possible anti-tumor activity. These studies will be reviewed in the next chapters.

3.1. Natural and synthetic CFADs as inhibitors of breast and prostate cancer cell growth

When AEA was tested for its possible growth inhibitory action on several tumor cell lines, including mouse neuroblastoma and monocytoma, rat heart endothelioma, leukemia, and pheochromocytoma, and human mammary cancer cells, a potent anti-proliferative effect was only found in the latter cells [77]. All human breast cancer (HBCC) lines tested responded to a 3–5 day treatment with doses as little as 200 nM of the compound by proliferating less rapidly. EFM-19, T-47D and MCF-7 cells were more sensitive to AEA (IC₅₀ values between 0.5 and 2.5 μM), whereas BT-474 cells were less responsive (IC₅₀ = 6 μM) (Fig. 2). AEA was shown to inhibit the G1/S phase of the cell cycle and the incorporation of [³H]thymidine into DNA, and was not toxic to cells up to 100 μM, nor did induce apoptosis up to 10 μM. Although HBCCs rapidly degrade AEA, the effect of this compound was not due to AA produced from its hydrolysis, because inhibitors of FAAH enhanced AEA effect, and a metabolically stable AEA analogue, (R)-methanandamide (Met-AEA), and AA were more potent and less potent than AEA, respectively [77]. On the other hand AEA and other cannabimimetic agents, such as 2-AG and HU-210, exhibited the same rank of potency as inhibitors of EFM-19 cell proliferation and in binding assays performed using EFM-19 cell membranes and [³H]CP 55,940 as the high affinity cannabinoid ligand. Furthermore, the anti-proliferative effect of AEA was attenuated by the CB₁ antagonist SR141716A. These data suggested the presence of cannabinoid receptors in HBCC and their intermediacy in AEA-induced anti-mitotic effects. Indeed, in a subsequent study, MCF-7 and T-47D cells were found to express CB₁-like and, to a lesser extent, CB₂-like receptors and to contain specific binding sites for [³H]SR141716A that could be displaced by AEA at concentrations similar to those necessary to exert an anti-proliferative action [78]. Furthermore, this action was not influenced by co-incubation of cells with the CB₂-selective antagonist SR144528, and was not mimicked by a selective agonist of CB₂ receptors. Finally, it was noted that both the CB₁ transcript in RT-PCR analyses and the CB₁-immunoreactive band in Western immunoblot decreased in MCF-7 cells that had undergone several sub-culturing passages, and that the amount of [³H]SR141716A specific binding as well as the responsiveness of
these cells to AEA decreased correspondingly [78]. These data strongly suggest that the anti-proliferative action of AEA on HBCCs is mediated by CB$_1$-like receptors.

Breast cancer cells in culture are known to produce endogenous prolactin and to use it as an autocrine growth factor. Apart from the selectivity of AEA anti-cancer effect for HBCCs versus prolactin-insensitive cells, it was also noted that the responsiveness of different mammary cancer cell lines to a monoclonal antibody against prolactin and to AEA were directly related (Fig. 3). Furthermore, the anti-proliferative effects of sub-maximal doses of prolactin antibody and AEA were not additive, suggesting that the latter compound may act on the action rather than on the levels of prolactin. Accordingly, AEA inhibited the proliferative action of exogenous prolactin on EFM-19 cells at concentrations lower than those required to inhibit basal proliferation [77]. The hypothesis that AEA anti proliferative effects were due to interference with prolactin mitogenic action was confirmed by using the prostate cancer cell line DU-145, which does not produce endogenous prolactin and yet it responds to the exogenous hormone by proliferating more rapidly. It was found that a 3-day treatment with sub-μmolar concentrations of AEA, HU-210, 2-AG, Met-AEA, but not with a selective CB$_2$ agonist, causes inhibition of the prolactin-induced proliferation of DU-145 cells without significantly affecting the basal proliferation of these cells [78]. Again, the effect of AEA was blocked by a CB$_1$, but not a CB$_2$, receptor antagonist, in agreement with the finding of high levels of CB$_1$ receptor mRNA and protein, as well as of specific binding for [$^3$H]SR141716A, in DU-145 cells. This finding suggests that AEA and other compounds capable of functionally activating CB$_1$ receptors can, in principle, inhibit the proliferation of any prolactin-sensitive tumor cell that also expresses these receptors.

Fig. 2. Effect of anandamide on the basal proliferation rate of four different human breast cancer cell lines. Cells were treated with increasing concentrations of anandamide for 4–5 days (the compound being added at each change of medium), after which they were tripsinized and counted by a hemocytometer. See Ref. [77] for further experimental details.
In the attempt of increasing the cytostatic activity of natural CFADs we have tested a series of synthetic compounds, which were designed to satisfy two requirements: 1) higher stability toward hydrolysis, and 2) capability of interacting with other targets involved in the inhibition of cell proliferation. First, we tested non-hydrolyzable AEA analogs that lack the amide or ester bond typical of AEA and 2-AG but are still capable of binding to cannabinoid receptors. One of these compounds, named HU-313, was a potent anti-proliferative agent with all HBCC lines tested as well as with prolactin-stimulated DU-145 cells (with IC$_{50}$ values around 350 nM [Di Marzo, Melck and Mechoulam, unpublished data]) (Table 3). In the second case, we started from the observation that capsaicin is also a potent inhibitor of mammary cancer cell proliferation, although its mechanism of action is still not completely understood [79]. We synthesized a series of long chain $N$-acyl-vanillyl-amides (Fig. 1). Some

![Graph](image_url)

Fig. 3. Correlation between the sensitivities of different human breast cancer cell lines to anandamide and a monoclonal antibody against prolactin. On the $x$ axis the IC$_{50}$ values ($\mu$m) for anandamide induced inhibition of proliferation of MCF-7, T-47D, EFM-19, and BT-474 cells, respectively, are reported. On the $y$ axis, the percent inhibition of cell proliferation caused, under the same conditions, by incubation with 15 $\mu$g/ml of a monoclonal antibody against prolactin is reported. See Ref. [77] for further experimental details.

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<table>
<thead>
<tr>
<th>Substance</th>
<th>MCF-7</th>
<th>T-47D</th>
</tr>
</thead>
<tbody>
<tr>
<td>HU-313</td>
<td>$0.41 \pm 0.10$</td>
<td>$0.30 \pm 0.09$</td>
</tr>
<tr>
<td>Olvanil</td>
<td>$1.60 \pm 0.18$</td>
<td>$0.75 \pm 0.06$</td>
</tr>
<tr>
<td>Arvanil</td>
<td>$0.40 \pm 0.15$</td>
<td>$0.35 \pm 0.03$</td>
</tr>
</tbody>
</table>

For further details on the cell proliferation assays see Refs. [77,78].
of these compounds were shown to be potent inhibitors of AEA re-uptake, as well as ligands for CB\textsubscript{1}, but not CB\textsubscript{2}, receptors, and to be capable of also activating vanilloid receptors [25]. We found that one of these compounds, arvanil, was among the most potent inhibitors of basal HBCC and prolactin-induced DU-145 cell proliferation developed to date (Table 3) [25,80].

Finally, we also tested other natural CFADs that do not bind to cannabinoid receptors with high affinity, i.e. PEA and the putative sleep-inducing factor, oleamide (cis-9-octadecenoamide, OA, Fig. 1, [81]). The latter compound is also a substrate for FAAH [81,82] and exhibits a spectrum of biological activities that may be due, in part, to the enhancement of endogenous cannabinoid levels [36,83]. We found that PEA did not inhibit HBCC proliferation up to 10 \( \mu \text{m} \), whereas OA did, although at concentrations approximately fivefold higher than those required to AEA to exhibit the same effect [75]. Interestingly, the effect of 10 \( \mu \text{m} \) OA was blocked by SR141716A, suggesting that the action of this CFAD was mediated by endogenous cannabinoids. Both OA at an inactive dose and PEA (1–10 \( \mu \text{m} \)) significantly enhanced the anti-proliferative effect of AEA (Ref. [75] and [Melck, De Petrocellis, and Di Marzo, unpublished data]). This last finding may have important physiological relevance, because OA and PEA are produced by EFM-19 cells in amounts higher than AEA [75], and may significantly improve the anti-mitogenic action of this compound by behaving as ‘entourage’ compounds [84].

3.2. CFADs as possible inhibitors of growth factor receptor expression

Evidence discussed in the previous section pointed to prolactin action as a likely target for AEA and 2-AG anti-proliferative action on HBCCs. Therefore, we investigated the effects of 3–5 day treatment of these cells with AEA on prolactin receptor expression. We found a strong down-regulation by AEA of the levels of the high molecular weight form of this protein, the effect being reversed by SR141716A [77]. Interestingly, AEA also potently reduced the levels of this receptor in DU-145 cells [78], again in a SR141716A-sensitive fashion, thus providing strong evidence that, in prolactin-responsive cells that express CB\textsubscript{1} receptors, activation of these receptors by CFADs may potentially lead to the inhibition of proliferation through interference with prolactin action.

We also wanted to assess if CFADs were able to inhibit the mitogenic action of other growth factors. Although it was known that CB\textsubscript{1} receptor agonists do not modulate estrogen receptor activation [85], reports in the literature pointed to NGF as a possible facilitating factor for the establishment and growth of breast and prostate tumors (for example see Refs. [86,87]). Therefore, we studied the effect of AEA and 2-AG on HBCC proliferation induced by this neurotrophin. We found that the two compounds, as well as Met-AEA, arvanil and HU-210, but not a CB\textsubscript{2}-selective agonist, potently inhibited NGF-induced proliferation of MCF-7 cells [78]. The effect of AEA was blocked by SR141716A but not by the CB\textsubscript{2}-selective antagonist, and was enhanced by PEA, which alone exerted no significant effect (Ref. [78], and [Melck, De Petrocellis, and Di Marzo, unpublished data]). The same CB\textsubscript{1} agonists potently down-regulated the expression of the high affinity NGF receptors, the \( \text{trk} \) proteins, which are highly expressed in MCF-7 cells [86] and, unlike the low affinity p75 NGF receptor, have been implicated in NGF tumor-promoting action [88]. SR141716A
counteracted, and PEA enhanced, also this effect of AEA, thus strongly suggesting that the inhibition of NGF-induced MCF-7 proliferation caused by CB₁ receptor activation was due to suppression of trk levels (Ref. [78], and [Melck, De Petrocellis, and Di Marzo, unpublished data]).

In summary, it seems that some CFADs can act as selective inhibitors of cancer cell proliferation through a hormone/growth factor–dependent mechanism that may have little to do with the anti-tumor activities previously described for THC and other cannabinoids. To fully appreciate the patho-physiological significance of these findings it will be of interest to assess whether the effects of AEA and 2-AG on prolactin receptors and trk proteins can be extended to other growth factors and other tumor cells or normal tissues.

What are the intracellular events that, once triggered by CB₁ receptor activation, lead to suppression of the levels of prolactin and NGF receptors? We found that forskolin—an activator of adenylyl cyclase—and PD 098059—an inhibitor of the mitogen-activated protein kinase (MAPK) pathway—block AEA inhibition of human breast cancer cell proliferation (when this was induced by either prolactin or NGF) and AEA-induced down-regulation of prolactin and NGF trk receptors. On the other hand, RpCAMPs, an inhibitor of the cAMP-selective protein kinase A, mimicked these two effects of AEA [89]. These data, together with the observation that AEA inhibits adenylyl cyclase and activates MAPK in MCF-7 cells [89], strongly suggest that the down-regulation of prolactin receptors and trk proteins is due, at least in part, to CB₁-mediated inhibition of adenylyl cyclase and activation of MAPK (Fig. 4), two effects reported also for other cannabinoid receptor agonists and other cells (see [90] for review). Indeed, modulation of either the cAMP/protein kinase A pathway or the MAPK signaling cascade have been described in the past to underlie the inhibition of the expression of numerous genes, including trk [91].

4. Conclusive remarks

The data described in this article clearly point to CFADs as possible templates for the development of novel and potent anti-inflammatory and anti-tumor drugs. However, whether these compounds behave as endogenous modulators of hyper-reactivity and cancer cell proliferation under patho-physiological conditions still remains to be determined. Experimental evidence available in the literature strongly suggests that, on the one hand, CFADs are produced (and subsequently inactivated) by cells and tissues challenged with inflammatory stimuli, and, on the other hand, some of the exogenous compounds significantly down-regulate the inflammatory response. However, the possibility of endogenous CFADs being produced to counteract, for example, the edema and pain associated with inflammation is still being debated. It is possible that these metabolites are produced by mast cells, basophils, and macrophages and act on these cells as well as on vascular endothelial and smooth muscle cells to modulate inflammatory humoral and cellular responses. The use of cannabinoid receptor antagonists devoid of reverse agonist activity, or of selective inhibitors of CFAD degradation (see [92] for review) will help clarifying the role of endogenous CFADs in this context, and of CB₁ and CB₂ receptors in CFAD and, particularly, PEA anti-inflammatory actions. In fact, although PEA analgesic action in the formalin-treated
mouse hindpaw was blocked by a CB$_2$ receptor antagonist [54], this metabolite does not significantly activate either of the two known cannabinoid receptor subtypes [36]. The possibility that PEA behaves as an ‘enhancer’ of endogenous AEA action may explain these contradictory data and is currently being investigated in our laboratory. Finally, the involvement of vanilloid receptors in the possible pro- or (after receptor desensitization) anti-inflammatory action of anandamide also needs to be examined in detail.

Although intriguing, the hypothesis of a physiological role for CFADs as local suppressors of the growth of some growth factor-dependent tumors is still speculative. If it is true that these compounds inhibit the prolactin- and NGF-induced proliferation of HBCCs by suppressing the expression of receptors for these two growth factors, little is known on the possible up-regulation of the biosynthesis of CFADs and of cannabinoid receptors in tumors versus healthy tissues. Indeed, even the role in the onset and growth of tumors of prolactin and NGF, the two targets identified so far for CFAD anti-proliferative actions in vitro, needs to be corroborated by further data. It is worthwhile noting, however, that compounds that prolong AEA half-life in HBCCs also behave as weak inhibitors of cell proliferation when administered alone, thus suggesting that endogenous cannabinoids may control tumor cell growth [75,89]. The recently reported presence of 2-AG in human milk [93] may also be relevant to the anti-mitogenic properties of this compound. In fact, all the HBCC lines tested so far were originally established from tumors deriving from the milk duct epithelium. It is possible that 2-AG present in milk contributes preventing the growth of HBCC foci in vivo.
As to the therapeutic applications of CFAD derivatives as anti-cancer agents, this possibility will have to await the results of appropriate in vivo studies, and should be analyzed in view of reports showing that THC, by exerting an immune-depressant action, may facilitate rather than inhibit cancer cell growth [94,95]. However, very recent findings seem to suggest that this adverse effect of THC is mediated by CB2 receptors [94], thus leaving open the possibility that CB1 receptor selective agonists may be used as anti-tumor agents against growth-factor-induced cancer cell proliferation.

In conclusion, still much work needs to be conducted to investigate the possible function of endogenous CFADs as local suppressors of inflammation and cancer cell proliferation. The data reviewed in this article, however, support the case for the development of new anti-inflammatory and anti-cancer drugs from these natural fatty acid derivatives.

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References


