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Review

Endocannabinoid system modulation in cancer biology and therapy

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ABSTRACT

The discovery of the endocannabinoid system and the recognition of its potential impact in a plethora of pathological conditions, led to the development of therapeutic agents related to either the stimulation or antagonism of CB1 and CB2 cannabinoid receptors, the majority of which are actually tested in preclinical studies for the pharmacotherapy of several diseases. Endocannabinoid-related agents have been reported to affect multiple signaling pathways and biological processes involved in the development of cancer, displaying an interesting anti-proliferative, pro-apototic, anti-angiogenic and anti-metastatic activity both *in vitro* and *in vivo* in several models of cancer. Emerging evidence suggests that agonists of cannabinoid receptors, which share the useful property to discern between tumor cells and their non-transformed counterparts, could represent novel tumor-selective tools to treat cancer in addition to their already exploited use as palliative drugs to treat chemotherapy-induced nausea, pain and anorexia/weight loss in cancer patients. The aim of this review is to evidence and update the recent emerging knowledge about the role of the endocannabinoid system in cancer biology and the potentiality of its modulation in cancer therapy.

Contents

1. Introduction

During the past 15 years a remarkable amount of studies have been performed in order to understand the biological role of the endocannabinoid system and its regulatory functions in health and disease. Such studies have been prompted by the development of selective cannabinoid receptor antagonists and inhibitors of endocannabinoid metabolism and transport, as well as mice deficient in cannabinoid receptors or endocannabinoid-degrading enzyme FAAH, whereas synthesis inhibitors are not yet available. Since then, the endocannabinoid system has been implicated in a growing number of physiological functions, both in the central and peripheral nervous systems and in peripheral organs. More importantly, modulating the activity of the endocannabinoid system turned out to hold therapeutic promise in a wide range of disparate diseases and pathological conditions, ranging from mood and anxiety disorders, movement disorders such as Parkinson's and Huntington's disease, neuropathic pain, multiple sclerosis and spinal cord injury, to cancer, atherosclerosis, myocardial infarction, stroke, hypertension, glaucoma, obesity/metabolic syndrome and osteoporosis, to name just a few [1].

In particular, several components of the endocannabinoid system are interesting candidate targets or novel drugs for cancer treatment. The aim of this review is to evidence and update the

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recent emerging knowledge about the role of the endocannabinoid system in cancer biology and the potentiality of its modulation in cancer therapy.

2. Unraveling the potentiality of the endocannabinoid system as a target in cancer

The endocannabinoid system is highly conserved among species and the endocannabinoids are ubiquitously synthesized molecules, with an emerging modulating activity on proteins and nuclear factors that regulate cell proliferation, differentiation and survival. This suggests that the endocannabinoid signaling system could be involved in the control of fundamental processes of cell homeostasis and eventually in neoplastic transformation [2,3].

Numerous pharmacological studies have proposed that (endo)cannabinoids might directly inhibit tumor growth *in vitro* and in animal models such as xenograft tumors (induced by subcutaneous injection of tumor cells), chemically or genetically induced tumors in mice. The anti-tumor property is shared among natural and synthetic cannabinoids, including Δ^9-THC and cannabidiol, endocannabinoids analogs, and endocannabinoid-transport or -degradation inhibitors (e.g. VDM-11 and AA-5-HT) that have been shown to inhibit tumor growth and progression of several types of cancers including glioma, glioblastoma multiforme, breast, prostate and thyroid cancer, colon carcinoma, leukemia, lymphoid tumors and others. The proposed mechanisms at the basis of such efficacy are complex and may involve cytotoxic or cytostatic effects, apoptosis induction, anti-metastatic effect accompanied by inhibition of neo-angiogenesis and tumor cell migration [3]. Moreover the effect, depending on the type of (endo)cannabinoid and the target tissue, is CB1, CB2 or transient receptor potential vanilloid type 1 (TRPV1) receptor-dependent or sometimes receptor-independent (e.g. lipid rafts, cyclooxygenase, PPARγ) (Fig. 1).

2.1. Effects of (endo)cannabinoids in cancer: a plethora of mechanisms of action

As mentioned above, (endo)cannabinoids inhibit the proliferation of various tumor cells, possibly through inhibition of proliferative and oncogenic pathways like: adenylyl cyclase and cAMP/protein kinase A pathway [4], cell cycle blockade with induction of the cyclin-dependent kinase inhibitor $p27^{kip1}$ [5], decrease in epidermal growth factor receptor (EGF-R) expression and/or attenuation of EGF-R tyrosine kinase activity [6,7], decrease in the activity and/or expression of nerve growth factor, prolactin or vascular endothelial growth factor tyrosine kinase receptors [5,8,9] (see also Table 1).

(Endo)cannabinoids modulate MAPK/ERK and PI3K survival pathways. For instance, anandamide was reported to inhibit breast cancer cell proliferation through down-regulation of prolactin receptor, *brca1* gene product and the high affinity neurotrophins receptor *trk* [4,9]. The anti-proliferative effect was proportional to the degree of hormone dependency of the cell lines and the mechanism of effect rely on the inhibition of phospho-kinase A (PKA) pathway. Several intraepithelial or invasive prostatic cancers showed increased expression of EGF-R, EGF and transforming growth factor α (TGFα). Mimeault et al. showed that a micromolar concentration of anandamide inhibited EGF-induced proliferation of DU145 and PC3 prostate cancer cells, as well as of androgen-stimulated LNCaP cells, *via* G1 arrest, and down-regulated EGF-R levels. Both phenomena were CB1-mediated [7]. Similar growth arrest and receptor modulation were also reported for prolactin- and nerve growth factor-stimulated DU145 [8–10].

In a recent study the CB agonist WIN-55,212-2 treatment resulted in decreased LNCaP proliferation, androgen receptor expression, VEGF protein expression and secreted levels of PSA, a glycoprotein androgen receptor-regulated that is the most accepted marker of prostate cancer progression [11]. Antagonistic effect of (endo)cannabinoids on growth factor-induced proliferation has also been reported in glioma [12].

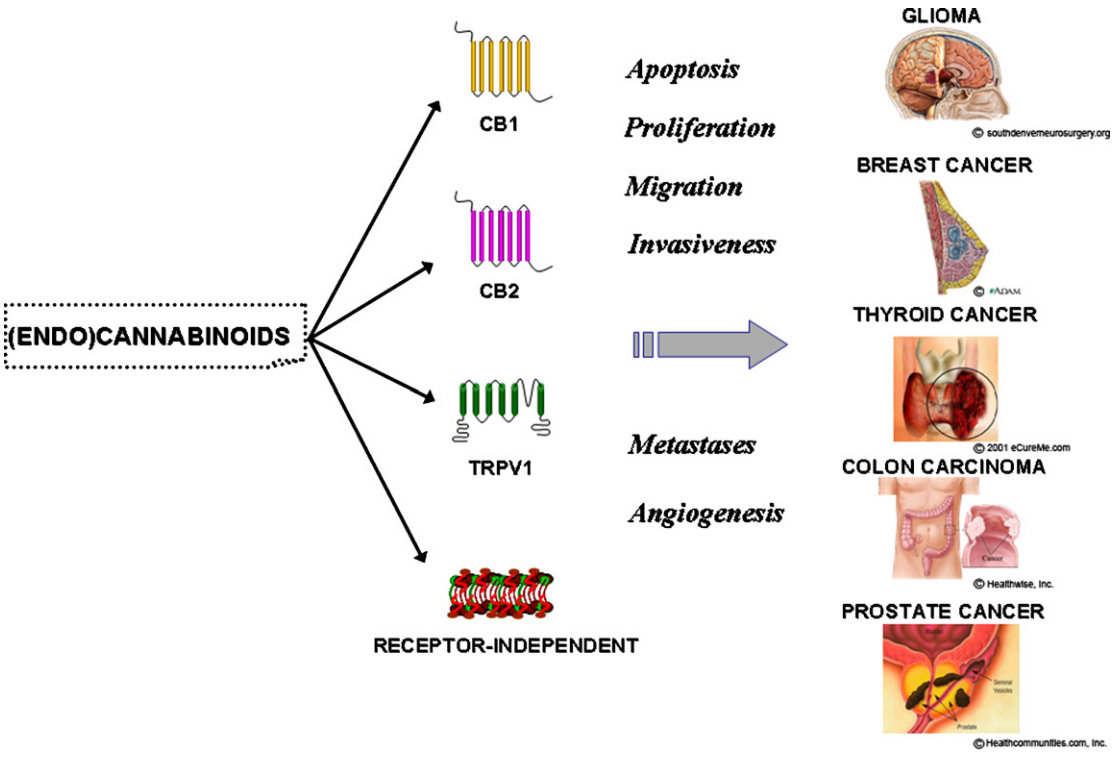

Fig. 1. Effects of (endo)cannabinoids in cancer.

Table 1
In vitro and *in vivo* effects of (endo)cannabinoids in cancer.

Tumor type	(Endo)cannabinoid	Mechanism	*In vitro* effect	*In vivo* effect	Ref.
Breast cancer					
	AEA (2–10 μM) 2-AG (2–10 μM) HU210 (≥4 μM)	CB1	Inhibition of the mitogen-induced stimulation of the G0/G1-S phase in human breast cancer cell lines (MCF7, T47D, EFM-19)		[8]
	AEA (≥2 μM) 2-AG, HU210 (≥1 μM)	CB1	Inhibition of NGF-induced proliferation Inhibition adenylyl cyclase; down-regulation PRLr TRK		[4,9]
	AEA (10 μM)	CB1	S phase arrest; induction Chk1 intra-S phase checkpoint in MDA-MB-231 cells		[13]
	AEA (10 μM and 0.5 mg/kg/dose)	CB1	Inhibition of adhesion and migration (MDA-MB-231 cells)	Reduction of number and dimension of metastatic nodes in TSA-E1 mice breast xenograft tumor	[39]
	THC (≤5 μM)	Immunosuppression		Increased tumor growth and metastasis; *in vivo*, decreased anti-tumor immune response in mouse mammary carcinoma (4T1)	[50]
	THC (≥12 μM)	CB2	G2/M phase transition blockade through Cdc2 and apoptosis induction (MCF7; T47D; MDA-MB-231; MDA-MB-468). Activation of JunD		[14,51]
	Cannabidiol (8–12 μM)	CB2; TRPV1	Inhibition of proliferation; apoptosis induction (MCF-7; MDA-MB-231)		[46]
	Rimonabant (0.1 μM and 0.7 mg/kg/dose)	CB1; lipid rafts	Inhibition of proliferation; G1 arrest (MCF-7; MDA-MB-231; T47D)	Growth inhibition of breast xenografts tumors	[52]
	THC (≤1 μM)	Non-CB1; non-CB2	Stimulation of proliferation in absence of CB receptors expression (MCF7)		[53]
Prostate cancer					
	AEA, R-(+)-MET (≥2 μM)	CB1, CB2	Inhibition of mitogen-induced proliferation, G1 arrest of androgen-independent prostate cancer cells (PC3, DU145)		[7,9]
	THC (1 μM)	Non-CB	Apoptosis		[54]
	AEA, R-(+)-MET (≥2 μM)	CB1	Inhibition of mitogen-induced proliferation, G1 arrest of androgen-dependent prostate cancer cells (LNCaP)		[7]
	WIN-55,212-2 (≥2.5 μM)	CB1, CB2	Dose- and time-dependent induction of apoptosis; decreased expression of AR and PSA (LNCaP)		[11]
	R-(+)-MET (0.1–0.2 μM)	CB1, CB2	Increased proliferation and AR expression (LNCaP)		[55]
	CAY10401 (0.1–10 μM)	FAAH	Decreased cell migration by FAAH inhibition (PC3)		[44]
Glioma and brain cancers					
	THC (1 μM)	CB1, CB2	Apoptosis *via* ceramide *de novo* synthesis (rat glioma cell line C6)	*In vivo*, regression of C6-derived glioma	[17,21,56]
	JWH133, WIN-55,212-2 (0.1 μM)	CB2	Apoptosis *via* ceramide *de novo* synthesis (C6)		
	WIN-55,212-2 (15 μM)	n.d.	Apoptosis *via* activation of caspase cascade (C6)		
	JWH-133 (50 μg/die)	CB2		Inhibited growth of astrocitoma tumors induced in deficient mice	[56]
	THC (1 μM) WIN-55,212-2	CB1	Decreased proliferation and increased cell death (human glioblastoma multiforme cell line GBM)		[57]
	R-(+)-MET (1–10 μM)	COX-2	Apoptosis induction (human neuroglioma cells)		[23]
	THC (1.5 μM) JW133 (50 μg/d)	Non-CB	Cell invasion inhibition through MMP-2 down-regulation (C6)	Glioma growth and MMP-2 inhibition	[45]
Thyroid cancer					
	Met-F-AEA (0.5 mg/kg/d)	CB1		*In vivo*, inhibited growth of thyroid xenografts tumors induced in nude mice	[58]
				Inhibited the development of lung metastases	[5]
	Met-F-AEA (0.5 mg/kg/d) VDM-11, AA-5-HT (5 mg/kg/d) Rimonabant (0.7 mg/kg/dose)	CB1 FAAH, AMT CB1		Inhibited growth of thyroid tumor xenografts induced in athymic mice	[59]

Table 1 (*Continued*)

Tumor type	(Endo)cannabinoid	Mechanism	*In vitro* effect	*In vivo* effect	Ref.
Hematological cancers					
	AEA (10 μM)	TRPV1	Apoptosis induction *via* TRPV1 (lymphoma U937 cells)		[60]
	THC (10 μM), HU210 (5 μM)	CB2	Apoptosis induction *via* CB2 (lymphoma cell lines)		[61]
	AEA, WIN-55,212-2 Rimonabant (1–10 μM)	CB1, CB2 CB1	Growth inhibition; apoptosis induction (mantle cell lymphoma cell lines)		[62,26]
	THC (1–5 μM); JWH-133	CB2	Apoptosis *via* CB2 (human leukaemia cells)		[63]
	THC (14–25 μM) CBD (6–20 μM)	CB Non-CB	Growth inhibition; apoptosis induction (C6 glioma cells)		[46]
	R(+)methAEA (10 μM) (5 mg/kg)	CB1, CB2	Cell death induction (mantle cell lymphoma, chronic lymphatic leukemia)	Reduction of tumor size and mitotic index in mantle cell lymphoma xenografts	[64]
Gastro-intestinal cancers					
Colon cancer	AEA (25 μM)	COX-2	Cell death *via* COX-2 (colorectal carcinoma)		[65]
	N-arachidonoylser (5 mg/kg) HU210 (0.1 mg/kg)	FAAH Non-CB		Reduced precancerous lesions in the mouse colon	[66]
	HU210 (3 μM) Anandamide (23 μM)	Non-CB	Inhibition of colon cancer cells viability. Synergism with 5-fluorouracil (Caco-2 cells)		[67]
	Arachinodyl-2′-chloroethylamide CB13 (100 nM) (2.5 mg/kg/d)	CB1 CB2	Apoptosis induction and increased ceramide levels in colon cancer cells (DLD-1 and HT29 cells)	Reduced growth of a xenograft colon cancer in mouse	[31]
		CB1		Loss of CB1 accelerates intestinal tumor growth	[68]
Pancreatic cancer	THC (2 μM and 15 mg/kg/die)	CB2, ceramide	Apoptosis induction through ceramide in pancreatic tumor cells (Panc1; MiaPaCa2)	Inhibited growth of xenografts and intrapancreatic tumors	[19]
Hepatocarcinoma	WIN 55,212-2 (5–10 μM)	PPAR-γ	Apoptosis induction through PPARγ		[32]
Cholangio carcinoma	AEA (10 μM)	Non-CB, lipid rafts	Inhibition of proliferation and apoptosis induction through ceramide accumulation		[69]
Other cancers					
Skin cancer	JWH-133, WIN-55,212-2 (1.58 μg)	CB1, CB2		Inhibited growth of mouse skin carcinomas induced in nude mice	[19]
Lung carcinoma	THC (100 mg/kg)	n.d.		Growth inhibition	[70]
	THC (0.1–0.3 μM)	EGF-R	Increased proliferation (lung cancer cells NCI-H292)		[71]
Cervical carcinoma	R(+)methAEA	COX-2	Apoptosis induction through a COX-2 and PPARγ-dependent pathway		[72]

(Endo)cannabinoids have been shown to interfere with the regulation of cell cycle, affecting several components of the cell cycle machinery. Indeed, anandamide arrests the proliferation of human breast cancer cells MDA-MB-231 in the S phase of the cell cycle as a consequence of the specific loss in Cdk2 activity, up-regulation of p21waf and a reduced formation of the active complex cyclin E/Cdk2 kinase [13]. Anandamide activates a cell cycle checkpoint, through Chk1 activation and Cdc25A proteolysis, thereby preventing Cdk2 activation by dephosphorylation on critical inhibitory residues (Thr14/Tyr15), which arrests cells in S phase. Also THC inhibits breast cancer cell proliferation by blocking the progression of cell cycle in G2/M phase *via* the down-regulation of Cdc2. In this case, the effects were mediated by CB2 receptors, however CB2-selective antagonists significantly but not totally prevented such effects, pointing to the existence of CB receptor-independent mechanism [14]. Treatment of human prostate cancer LNCaP cells with WIN-55,212-2 caused an arrest of the cells in the G0/G1 phase of the cell cycle, sustained by the activation of ERK1/2, induction of p27/KIP1 and inhibition of cyclin D. G0/G1 arrest up-regulated the Bax/BCl-2 ratio and activated caspases, resulting consequently in an induction of apoptosis. Moreover, WIN-55,212-2 treatment of the cells resulted in a dose-dependent decrease in protein expression of cyclin D1, cyclin D2 and cyclin E, as well as cdk2, cdk4 and cdk6, pRb and its molecular partner, the transcriptional factor E2F. WIN-55,212-2 caused a dose-dependent decrease in the protein expression of DP-1 and DP-2 that form heterodimeric complexes with E2F essential for its activity [15]. THC administration elicited G0/G1 cell cycle blockade in glioblastoma multiforme cells through suppression of E2F1 and Cyclin A and up-regulation of the cell cycle inhibitor p16(INK4A) [16].

As regard to the pro-apoptotic effect of (endo)cannabinoids in tumor cells, collected results give a complex scenario with different mechanisms of action: increased synthesis of the pro-apoptotic sphingolipid ceramide [17,18], ceramide-dependent up-regulation of the stress protein p8 and several downstream stress-related genes expressed in the endoplasmic reticulum (ATF-4, CHOP, and TRB3) [19], prolonged activation of the Raf1/extracellular signal-regulated kinase cascade [18], inhibition of Akt [20,21], c-Jun NH2-terminal kinase and p38 mitogen-activated protein kinase [17,22–24]. The anti-tumoral action of cannabinoids on glioma may be exerted either *via* the CB1 or the CB2 receptor. THC induced apoptosis of C6 glioma cells by a pathway involving CB1 receptor, sustained generation of the pro-apoptotic lipid ceramide and

prolonged activation of Raf1/MEK/ERK cascade [17]. A role for BCL-2 family members, such as Bad, has also been hypothesized [21]. Pro-apoptotic effect may rely also on a CB1 receptor-independent stimulation of sphingomyelin breakdown [25]. In lymphoma and leukemia cell lines, CB agonists such as THC and WIN-55,212-2, induced apoptosis CB-dependent through ceramide accumulation and final caspase activation through p38MAPK signaling pathway [26], down-regulation of RAF1/MAPK pathway and translocation of BAD to mitochondria [27]. A common event was the depolarization of mitochondria with cytochrome *c* release [26–28]. Recently it has been reported that CB agonists are mitocondrial inhibitors, since decrease oxygen consumption and mitochondrial membrane potential, increase mitochondrial hydrogen peroxide production, thus inducing apoptosis [29]. In colon carcinoma cells THC treatment resulted in CB1-mediated inhibition of both RAS-MAPK/ERK and PI3K-AKT survival signaling cascades, two key cell survival pathways frequently deregulated in colorectal tumors. The inhibition of ERK and AKT activity by THC was accompanied by the activation of the pro-apoptotic BCL-2 family member BAD [30]. Interestingly CB agonists induced colon carcinoma cell death through TNFα-stimulated ceramide synthesis. Therefore, TNFα could act as a link between cannabinoid receptor activation and ceramide production [31]. In hepatocarcinoma HepG2 cells WIN-55,212-2 induced a clear apoptotic effect accompanied by up-regulation of the death-signaling factors Bax, Bcl-X(S), t-Bid, down-regulation of the survival factors survivin, phospho-AKT, Hsp72 and Bcl-2, JNK/p38 MAPK pathway activation and mitochondrial depolarization. In HepG2 cells, WIN-55,212-2 markedly increased the level of the transcription factor PPARgamma in a dose- and time-dependent manner [32].

CB2 agonist JWH133 and mixed CB1/CB2 agonist WIN-55,212-2 induced higher rate of apoptosis in anaplastic thyroid carcinoma cells ARO/IL-12 (transfected with IL-12 gene) than in ARO cells [33]. Furthermore, the mechanism through which anandamide induces apoptosis in cells expressing both functional cannabinoid and vanilloid receptors is still controversial and might depend on the experimental conditions used. It is important to remark that prolonged anandamide incubation times (5–6 days) in DU145 and PC3 prostate cancer cells were able to induce massive apoptosis. This effect was mediated by CB1/2 *via* cellular ceramide accumulation, and was absent in LNCaP cells [7].

2.2. (Endo)cannabinoids as emerging suppressors of tumor progression: inhibition of metastasis formation and angiogenesis

In addition to their direct anti-proliferative and pro-apoptotic effects on tumor growth *in vitro* and *in vivo*, cannabinoid-related drugs have been reported to affect tumor progression through the inhibition of key events which are cell migration, invasion, metastasis formation and tumor neo-angiogenesis (see also Table 1). These hallmarks of solid tumors are directly related to the degree of malignancy, disease outcome and mortality.

As proposed in 1971 by Folkman, tumor growth and metastasis formation are angiogenesis-dependent processes and hence, blocking angiogenesis is turned out to be a fundamental therapeutic strategy to arrest tumor growth [34]. The recent studies on the endocannabinoid system have provided strong evidence for a key role of the endocannabinoids in the control of cell-signaling pathways involved in cancer cell growth, invasion and metastasis processes, in a way dependent on CB receptor activation. In particular, it has been suggested that the anti-tumor effect of cannabinoid-related drugs could be partially ascribed to the inhibition of tumor neo-angiogenesis *in vivo*, both indirectly through an inhibition of the angiogenic factors produced by tumor cells and directly through an action on endothelium. Indeed, endocannabinoids inhibited angiogenesis in thyroid tumors *via* down-regulation of the pro-angiogenic growth factor VEGF and its receptor Flt-1 expression, interfered with endothelial cell migration also inducing endothelial cell apoptosis, thereby counteracting thyroid, skin, glioma and melanoma cancer growth *in vivo* [5,6,35,36]. In addition, cannabinoid treatment of gliomas, inhibited the expression of VEGF, angiopoietin-2, matrix metalloproteinase-2 (MMP-2) and hypoxia-inducible factor 1-α (HIF-1α). Intra-tumoral administration of THC to two patients affected by glioblastoma multiforme, was reported to decrease both VEGF and VEGFR-2 activation in the tumors [37]. We recently reported direct evidence for an anti-angiogenic activity of the endocannabinoid anandamide, *in vitro* and *in vivo*, supporting its reported anti-tumor efficacy and providing also new evidence for a role of the endocannabinoid system in the angiogenic process [38].

In both angiogenesis and metastasis formation processes a key role is played by cell migration and invasion through the extracellular matrix components. The anti-angiogenic effect of anandamide was indeed due also to the inhibition of bFGF-induced chemotaxis, capillary-like tube formation and morphogenesis and MMP-2 degrading activity [38]. The anti-tumor effects of (endo)cannabinoids have been related to a direct regulation of tumor cell migration in different types of cancers. Anandamide inhibited both adhesion and migration of the highly invasive metastatic breast cancer cell lines MDA-MB-231 and TSA-E1, on type IV collagen, the major component of the basement membrane [39] and of colon carcinoma cells SW480 through the activation of CB1 receptors [40]. Inhibition of migration and invasion by anandamide and THC was reported also in lung cancer cells and human cervical cancer cells [41,42]. In androgen-independent prostate cancer cell lines PC3 and DU145, endogenous 2-AG and CB1 agonists reduced invasion through the CB1-dependent inhibition of adenylyl cyclase, decreasing phospho-kinase A activity [43]. Also FAAH pharmacological inhibition or siRNA knockdown decreased cell invasion [44]. The signaling pathways involved in invasion inhibition are numerous: inhibition of FAK/Src signaling [39], of endogenous tissue inhibitor of metalloproteinases TIMP-1 [41], of metalloproteinase MMP-2 [45,38]. The inhibitory properties exerted *in vitro* by several CB1 agonists have been widely confirmed in animal models of lung metastases formation where an inhibition of number and dimension of metastatic nodes has been observed [39,46,42]. Since endocannabinoids, through the CB2 receptor, physiologically modulate the recruitment of monocytes to inflammatory sites and the production of chemokines [47,48], it would be interesting to study these aspects in the context of cancer pathogenesis.

As reported for cell proliferation, the variable effects on cell migration that in some cell types seems to be stimulated by (endo)cannabinoids – e.g. induced migration in human embryonic kidney 293 cells [49] – seem to be dependent on both cellular differentiation degree, receptor levels and specific activation of different receptors.

2.3. An open question: potential increased cancer risk by (endo)cannabinoids

In the light of the available literature, potential tumor-promoting effects of (endo)cannabinoid-related drugs have to be taken into account. Hart et al. [71] reported pro-proliferative effects of cannabinoids in different cancer cell lines at submicromolar doses, very low if compared to both the anti-proliferative and pro-apoptotic doses widely reported (in the micromolar range) and to the concentration achieved *in vivo* for most anti-cancer drugs during a chemotherapy protocol. However, in the same paper the authors reported that at micromolar doses cannabinoids induced tumor cell apoptosis, highlighting a likely bimodal action of cannabinoid agonists on cancer cell growth, with low con-

centrations being pro-proliferative and high concentrations being anti-proliferative. On the contrary it has been reported that submicromolar doses of anandamide and free arachidonic acid stimulated hematopoietic and lymphoid cell growth *via* a CB receptor-independent MAPK activation potentiating growth factor-induced proliferation [102]. Noteworthy, growth promoting effects have not been reported in hematological cancers, where indeed anandamide at micromolar doses inhibited cell growth also inducing apoptosis [60,62,64].

More appropriate is the concern about the immunosuppressive properties of cannabinoid agonists *in vivo* through the activation of CB2 receptor expressed by cells of the immune system and the consequent increased risk of tumor growth due to a repression of the natural anti-tumor immune response. A study demonstrated that exposure to THC led to increased tumor growth and metastasis of the mouse mammary carcinoma 4T1 which express low to undetectable levels of cannabinoid receptors, CB1 and CB2 [50]. These effects were due to the inhibition of the specific anti-tumor immune response *in vivo*. It seems very likely that relative levels of CB receptors expressed by tumor cells are important for the response to cannabinoid agonists. When these levels are high, both tumor and immune cells are targeted by cannabinoid agonists and the consequent effect is the inhibition of tumor growth. On the contrary, tumor cells expressing low or undetectable levels of CB receptors are resistant to the anti-proliferative effects and prevails immunosuppression through CB2 and hence enhanced tumor growth *in vivo*.

3. Cannabinoid receptors-independent effects: multiple players

In addition to the above reported effects on cancer, mediated by CB receptors, numerous signaling effects exerted by (endo)cannabinoids and, in particular, by anandamide and cannabidiol seem to be CB receptor-independent. It is well recognized that the transient receptor potential vanilloid type 1 is activated by various lipids including anandamide [73]. Indeed, anandamide induced neuroblastoma, lymphoma and uterine cervix carcinoma cell death through vanilloid receptors [60,74]. Furthermore, inhibition of cancer cell invasion through TIMP-1 by methanandamide is mediated by TRPV1 [41]. It has also been proposed that lipid rafts – membrane domains rich in sphingolipids and cholesterol – besides mediate anandamide effects through CB1 signaling [75,76], may directly be involved in receptor-independent apoptosis induced by anandamide. Indeed, in cholangiocarcinoma, anandamide anti-proliferative and pro-apoptotic action was facilitated by lipid rafts stabilization, ceramide accumulation and recruitment of FAS and FAS ligand into lipid rafts [69]. Another cellular protein that may be important in receptor-independent cell death endocannabinoid-induced is cyclooxygenase-2 (COX-2). COX-2 metabolizes arachidonic acid (AA) to prostaglandins (PGs) and elevated levels of both COX-2 and PGs have been measured in neoplastic tissues. COX-2 is also capable of metabolizing anandamide to PGE2-ethanolamide, PGF2-ethanolamide (PGF2-EA) and PGD2-ethanolamide (PGD2-EA) (PG-EA) [77,78]. Anandamide inhibited the growth, also inducing apoptosis, of colon carcinoma cell lines HT29 and HCA7/C29 (moderate and high COX-2 expressors, respectively) and in COX-2 transfected tumorigenic keratinocytes, but had little effect on the very low COX-2 expressing colon carcinoma cells SW480 and HaCaT keratinocytes [65,79]. Moreover, apoptosis induced by anandamide in neuroglioma cells was COX-2 mediated and not affected by antagonists of cannabinoid receptors (CB1, CB2) and vanilloid receptor 1 [23]. Some reports suggest that cannabinoid receptors could have a protective role against programmed cell death, as reported in human neuroblastoma and C6 cells, where anandamide induced apoptosis, *via* vanilloid receptors, increasing intracellular calcium concentration, activating COX, releasing cytochrome *c* and activating caspase 3 [60].

That is a different story for cannabidiol which does not have appreciable affinity for CB1 or CB2 receptors and lacks of psychotropic activities. Cannabidiol showed an interesting potential as anti-cancer drug. Indeed, it inhibited glioma and breast tumor growth *in vitro* and *in vivo* through induction of apoptosis, inhibition of cell migration and angiogenesis, all events CB and/or TRPV1 receptors-independent [80,81,46]. Interestingly, in breast cancer cells cannabidiol was able to inhibit the invasiveness through the inhibition, at the promoter level, of Id-1, an inhibitor of basic helix-loop-helix transcription factors strongly involved in tumor progression [82]. A quinone analog of cannabidiol, HU-331, was reported to exert a significant high efficacy against human cancer cell lines *in vitro* and against tumor grafts in nude mice. It is a highly specific inhibitor of topoisomerase II, compared with most known anti-cancer quinones [83]. Moreover, HU-331 inhibited angiogenesis by directly inducing apoptosis of vascular endothelial cells without changing the expression of pro- and anti-angiogenic cytokines and their receptors [84].

4. Some examples of combinatorial approaches *in vitro*

The potentiality of targeting the endocannabinoid system in cancer therapy is increasingly intriguing. Cannabinoid analogs could be used in selective regimens in combination with other chemotherapeutic drugs, in order to reduce doses, to avoid resistance and exert a more potent clinical impact. Some combinatorial approaches have been attempted *in vitro* and the results are encouraging. A synergistic interaction between 5-fluorouracil and HU210 has been reported in colorectal carcinoma cells. The effect was CB receptor-independent through the involvement of oxidative stress [67]. In leukemic cells a clear synergistic interaction has been reported between THC and the cytotoxic agents usually used in leukemia treatment. In these cells was also observed a sensitization by THC to the cytotoxic agents, eventually due to the down-regulation of phosphorylated ERK [85].

A considerable regression of thyroid tumors generated by inoculation of ARO/CB2 cells was observed in nude mice following local administration of the potent selective CB2 agonist JWH133. Interestingly, a significant increase in the induction of apoptosis by paclitaxel was reported in anaplastic thyroid carcinoma ARO cells transfected with CB2 transgene, indicating that tumor cells were sensitized to chemotherapy by CB2 receptor expression [33].

Since anandamide has been reported to activate a cell cycle checkpoint in breast cancer cells, through Chk1 induction and Cdc25A proteolysis, thus arresting cells in S phase [13], and such a mechanism of action has been already demonstrated for potent radiosensitizers like gemcitabine [86], combinatorial studies with radiotherapy could give interesting results.

Overall, the collected observations demonstrate for the first time that a combination approach with cannabinoid agonists and established cytotoxic agents may enhance cell death *in vitro* being useful in cancer therapy. It would be very interesting to perform combinatorial studies with commonly used chemotherapeutic drugs also in clinical trials.

5. Expression and function of endocannabinoids, endocannabinoid metabolizing enzymes and cannabinoid receptors in cancer

Endocannabinoid levels are finely modulated under physiological functions and pathological conditions. A transient increment appears to be an adaptive reaction to restore homeostasis when this is acutely and pathologically perturbed. However, in some chronic conditions, the alteration of the endocannabinoid system seems to

contribute to the progress and symptoms of the disease, such in the case of Parkinson's disease and Alzheimer disease [87].

In the matter of cancer, elevated levels of endocannabinoids (anandamide and 2-AG) have been reported in several types of tumors when compared with their normal counterparts, e.g. in glioblastoma, meningioma, pituitary adenoma, prostate and colon carcinoma and endometrial sarcoma [88–92] or in highly invasive human tumor cells [92]. As an example anandamide level, that in normal colon tissue is in the range of 75 nM, in colon carcinoma is two/three-fold elevated. The overall interpretation of these data could be that endocannabinoids act as endogenous tumor suppressors, mainly at the first stages of cancer development. However, contrasting data have been reported for gliomas [12], whereas endocannabinoid levels remained unchanged in pancreatic ductal carcinoma as compared to the normal pancreas [93].

Enzymes that synthesize and metabolize the endocannabinoids contribute to their effectiveness, limiting their local concentrations and actions. A correlation between endocannabinoid metabolizing enzymes (FAAH for anandamide and MAGL for 2-AG) and cancer has been investigated only in prostate adenocarcinomas, where an increase of FAAH protein expression compared to normal prostate tissue samples has been reported [44], and in pancreatic ductal adenocarcinomas where a correlation of high FAAH/MAGL levels and survival has been observed [93].

Predictably, also cannabinoid receptor levels seem to be a fundamental element for growth inhibitory effects of endocannabinoids. It has been reported that the expression of CB1 receptor was regulated in an opposite way in normal *vs* malignant cells. This pattern of expression seems to be a common mechanism of the general protection of normal cells from the pro-apoptotic and anti-proliferative effects of cannabinoid agonists [3]. THC induced apoptosis in several human cancer cell lines but showed less efficacy in non-transformed cell counterparts, that might be even protected from cell death [6,17,54,57]. Therefore, a relevant issue seems to be the evaluation of cannabinoid receptors expression in tumor *vs* normal tissues, in order to achieve a significant anti-tumor effect with cannabinoid agonists without immunosuppression, and also for the prognostic value that CB receptor levels could have alone or in association with other recognized prognostic markers. The issue on the mechanisms by which cannabinoid receptor expression is modulated has not been sufficiently investigated, however some suggestions proposed studying CB receptor expression in specific models, could give a general picture of the potential transcription factors involved. Indeed, THC induced a CB2 receptor-dependent transcription of the CB1 gene in T cells and T cell lymphoma lines mediated by IL4 release. Activation of the transcription factor STAT6 was required for such transactivation of the CB1 gene [103]. It has been recently reported that oral administration of specific *Lactobacillus* strains, induced CB2 receptor expression in colonic epithelial cells, through the NF-κB pathway, contributing in this murine model, to the modulation and restoration of the normal visceral pain perception [104]. CB1 receptor expression was induced in human colon cancer cells by 17-β-estradiol through a mechanism estrogen-receptor dependent [105]. Finally, chromatin immunoprecipitations studies have demonstrated that CB1 gene is a transcriptional target of PAX3/FKHR, a chimeric transcription factor found in alveolar rhabdomyosarcoma, where indeed CB1 receptor is highly over-expressed [106]. Further studies at the promoter level will clarify the transcriptional regulation of CB receptor expression. Moreover alternative spliced isoforms of CB1 (CB1a and CB1b) could reflect differences in its functionality in normal *vs* malignant tissues [107]. In addition to the transcriptional regulation, it is reasonable to speculate that the processes of biosynthesis, translocation across the ER membrane to the cell surface, and proteosomal degradation, could be a control point to regulate the expression, subcellular localization and function of CB receptors.

Until now there are only few studies investigating the association of CB receptors expression with tumor malignancy and disease outcome in cancer in general. Analyses of astrocytomas demonstrated that 70% of the tumors express CB1 and/or CB2 and the extent of CB2 expression was directly related with tumor malignancy [56]. In gliomas a higher expression of CB2 compared to CB1 was reported and was related to tumor grade. Importantly, also tumor-associated endothelial cells showed immunoreactivity for CB receptors similar to tumor cells [94]. Increased expression of CB1 and of both CB1 and CB2 has been reported respectively in mantle cell lymphoma [95] and in non-Hodgkin lymphoma of B cell type as compared to reactive lymph nodes [67]. In contrast, a greatly reduced expression of CB1, but not of CB2, was found in colon carcinoma compared with adjacent normal mucosa [68]. In breast cancer a correlation among CB2 expression, histological grade of the tumors and other markers of prognostic and predictive value, such as estrogen receptor, progesterone receptor, and ERBB2/HER-2 oncogene, has been observed [41]. In prostate cancer, Sarfaraz et al. previously reported that CB1 receptor expression by the human prostate cancer cell lines LNCaP (androgen-sensitive), DU145 and PC3 (androgen-independent) was higher than that seen in normal human prostate epithelial cells [11]. This was confirmed in prostate carcinoma specimens where expression of CB1 receptor and additionally of TRPV1 receptor was up-regulated and, for this latter, correlated with increasing tumor grades [96]. The level of CB1 in tumor tissue is associated with disease severity at diagnosis and outcome, even if the performed study suffers of some limitations and requires additional evaluations in metastatic tissues [97]. In pancreatic tumors high CB1 receptor expression was associated with a shorter survival time (median 6 months) than a low CB1 (median 16 months) [93]. In contrast, as regard to correlation with disease outcome, in hepatocellular carcinoma over-expression of CB1 and CB2 receptors correlated with improved prognosis [98]. These results suggest that the role of CB receptors expression in relation to prognosis and disease outcome is highly dependent on the specific cancer type. The topic of the regulation of CB receptors by factors naturally expressed in the tumor microenvironment is intriguing, in order to understand their role and biological relevance during carcinogenesis and tumor progression and to reinforce their potential prognostic value.

6. Clinical data: a long way to go

The potential application of cannabinoid agonists as anti-cancer agents is still at the preclinical level. Meanwhile cannabinoid-related drugs are emerging as valuable adjunctive agents for the management of multiple symptoms of cancer and of therapy-induced side effects. Indeed, available data support a broad spectrum of useful palliative properties, ranging from appetite stimulation, inhibition of nausea and emesis induced by chemo- or radiotherapy, pain relief, mood amelioration, and relief from insomnia [99]. Marinol, Cesamet and Sativex, three drugs based on Δ^9-THC, have been already approved by FDA for these indications [100].

Although the use of cannabinoid-derived drugs for medicinal purposes could be limited by the concerns on their psychotropic effects, they have shown a fair safety profile especially with respect to current chemotherapeutics, which all display toxic adverse effects.

However, despite an overall collected preclinical evidence on the therapeutic potential of cannabinoids and related drugs in several types of cancer, only a single pilot Phase I/II clinical trial, approved by the Spanish Ministry of Health in 2002, has been performed so far, and the results have been recently disclosed [101]. This study was aimed at evaluating the safety profile of THC administration and its anti-tumor efficacy in a cohort of nine terminal patients

affected by recurrent glioblastoma multiforme, an aggressive primary brain tumor with poor prognosis (6–12 months survival) and no available efficacious treatment. The main goal of this study was to assess the safety of intracranial administration of THC and to confirm the absence of significant psychotropic effects at the used regimen. Moreover the study reassured about the possibility that cannabinoids could have tumor-promoting effects, since THC administration did not induce tumor growth nor decreased patient survival. On the contrary, THC decreased tumor cell proliferation also inducing apoptosis, however it had only a slight impact on the overall median survival of the cohort (24 weeks). Although this pioneer study suffers of several limitations due to its design, the results are somehow encouraging and may be the point of departure of improved future trials. It will be interesting in the next future to perform other clinical studies aimed at evaluating the efficacy of cannabinoid agonists (not limited to THC) in cancer treatment in different types of tumors. To optimize the results such protocols should involve large cohorts of patients.

7. Conclusions

Acquired knowledge about the biological role of the endocannabinoid system and its regulatory functions in health and disease, have prompted to the development of therapeutic agents related to either the agonism or antagonism of CB1 and CB2 receptors, the majority of which are actually tested in preclinical studies for the pharmacotherapy of different pathologies. So far, some drugs targeting the endocannabinoid system have passed clinical trials and are now on the market as palliative agents to be used in chemotherapy-induced nausea and vomiting, pain relief and anorexia/weight loss in cancer patients. Noteworthy, endocannabinoid-related drugs show an interesting potential as anti-tumor drugs since the preclinical studies carried out, highlighted a good efficacy in several types of cancer. The mechanistic insights into the triggered cellular events, not at all explored, pointed out that (endo)cannabinoids simultaneously affect multiple signaling pathways and biological processes involved in the development of cancer showing anti-proliferative, pro-apototic, anti-angiogenetic and anti-metastatic activity both *in vitro* and *in vivo*. Importantly, these agents share the useful property to discern between tumor cells and their non-transformed counterparts, which are even protected from cell death, therefore displaying a tumor-selectivity that common cytotoxic agents do not have. The use of cannabinoid-derived medications as palliative drugs and the results from the unique clinical trial performed up to now in glioma patients with THC, reassured about the good-safety profile and the absence of significant psychotropic effects at the doses used. The potential adverse effects are endurable and fit well in the range of those induced by commonly used anti-tumor drugs. The development of CB2-selective agonists for the treatment of brain tumors, or CB1 agonists less hydrophobic or unable to pass the blood–brain barrier for peripheral tumors, will improve their efficacy reducing the risk of psychotic side effects. Finally the evaluation of cannabinoid receptors expression in tumors *vs* normal tissues and their association to well-known prognostic markers could give useful information about their prognostic value. It appears clear that the proposed anti-tumor efficacy of (endo)cannabinoid-related drugs alone or in combination with other chemotherapeutic drugs, intrinsically interesting but not completely investigated, needs a deeper knowledge at both pre-clinical and clinical level, in order to allow a safe translation into the clinical setting.

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