A Glance Over the Cannabinoid Machinery to Design New Anti-Angiogenic Compounds

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Abstract: Aim of the present review is to summarize the different evidences regarding the ability of cannabinoids to control new vessels formation, and in this way, to suggest new possible molecular targets for the development of drugs which may be helpful in the management of different pathological condition associated to angiogenesis.

Key Words: Angiogenesis, cannabinoids, *Cannabis*, CB receptors, cancer, inflammation, palmitoylthanolamide.

ANGIOGENESIS

Angiogenesis, the development of new capillaries form pre-existing vessels, is an essential process during all life stages. Starting from fetal development where the growth of a vascular system is one of the earliest event in organogenesis, angiogenesis also occurs in adulthood, during wound healing and restoration of blood flow to injured tissues [1].

New blood vessel formation by angiogenesis involves the degradation of extra-cellular matrix combined with sprouting and migration of endothelial cells from preexisting capillaries [2]. Blood vessels consist of two main cell types: endothelial cells and mural cells [3]. Angiogenesis requires the coordinate activation of endothelial cells, which migrate and proliferate in response to growth factors to form functional vessels [4].

A very sensitive interplay of growth and inhibitor factors finely regulate angiogenesis, whereas their imbalance can lead to the ignition and progression of new vessel formation [1]. Vascular endothelial growth factor (VEGF) is a potent angiogenic peptide with biologic effects including regulation of hematopoietic stem cell development, extracellular matrix remodeling, and inflammatory cytokine formation. VEGF is both a vascular growth factor and a vascular permeability factor [5]. In fact, VEGF directly induces migration and mitosis of endothelial cells [6] and indirectly vasodilatation through the release of nitric oxide (NO) [7] by eNOS. Other important pro-angiogenic mediators are represented by angiogenin, angiopoietin-1/Tie2, TNF-α, IGF family, IL-8 and NO, that represent the right arm of the so called "angiogenic balance" [1].

Accordingly to this theory, the normally quiescent vasculature can be activated to sprout new capillaries by a change in the relative balance of angiogenesis' inducers and inhibitors. In fact, angiogenesis is a process tightly regulated and influenced by the positive factors mentioned above, as well as by negative regulatory factors, as angiostatin and endostatin. These factors are proteolytic fragments of larger proteins that act as negative regulators of angiogenesis [1] through the binding to specific integrins and stimulating many kinases including PKC, ERK1/2, Akt and FAK. Furthermore, it has been demonstrated that many exogenous substances, including the natural products fumagillin, borreledin, and withaferin, are able to inhibit angiogenesis [8].

Beside the angiogenic balance so far mentioned, another important stimulus, allowing the new vessel development, comes from the serine and cysteine protease families. These proteins known as matrix metalloproteinase (MMP) are required to degrade the extracellular matrix (ECM) and let endothelial cells to migrate as new vascular 'sprout' [9]. In addition, protease activity contributes to the release of positive and negative angiogenic factors from the ECM and cell surface. The pro-angiogenic milieu seems to derive from a pro-inflammatory/ immune response initiated by different cell type, including T helper cells and mast cells [10].

During the last decade the mast cells have been considered the *deus ex machina* for new vessel formation. Mast cells are strategically localized in tight contact with blood vessels where they release vasoactive compound, such as VEGF, TNF-α, together with important ECM degrading enzymes, as MMPs and chymase [11]. Chymases belongs to a family of serine protease with chymotrypsin-like activity, fairly selectively expressed by mast cells [12]. Not only chymases contribute directly to matrix destruction through the cleavage of proteins (e.g. fibronectin and collagen), but they also cooperate indirectly to activate MMPs. Therefore, mast cells, unlike other cells, represent a self-dependent angiogenic inducer: they secrete and activate MMPs, immediately leading to matrix degradation, and contemporaneously they release endothelial cell activating factors, thus they initiate endothelial "tube" formation and progression [13].

ROLE OF ANGIOGENESIS AND ITS CONTROL

Angiogenesis contributes to the development and progression of a variety of physio-pathological conditions [14].

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Physiologically, angiogenesis occurs during embryogenesis, development of follicles, corpus luteum formation, embryonic implantation and wound healing [15].

However, during this process an imbalance may contribute to numerous malignant, inflammatory, ischemic, infectious and immune disorders. In cancer as well as in diabetic retinopathy and rheumatoid arthritis, the excessive angiogenesis feeds diseased tissue and destroys normal one. Conversely, insufficient angiogenesis occurs in particular conditions such as coronary heart disease, stroke and delayed wound healing, where inadequate blood vessel growth leads to poor circulation and tissue death.

Therefore, it is substantial to develop new angiogenesis modulator [14]. On one hand, it will be useful to stimulate new vessel formation during neurodegenerative disorders, whereas an increased blood supply could minimize neuronal damage in the brain, or during heart attack where new coronary vessels could help repairing a damaged heart.

On the other hand, there is a growing evidence that anti-angiogenic drugs will improve future therapies of diseases like cancer, rheumatoid arthritis and ocular neo-vascularization [16]. Agents able to block angiogenesis during endometriosis could prevent the implantation of uterine tissue outside the uterus, while during retinal disease angiogenesis' inhibitors could help to reduce abnormal blood vessels formation in the eye [17].

Nowadays, there are new evidences that an alternative promising anti-angiogenic approach could come from natural and synthetic Marijuana derivates, which represent the cannabimimetic "clan".

CANNABIMIMETIC "CLAN"

Cannabis Sativa L contains a total of 483 identified chemical entities belonging to different chemical classes. Amongst them, cannabinoids (CBs) are the most distinctive class of compounds, that exist only in this plant [18]. Most CBs contain 21 carbon atoms, but there are some variations in the length of the C-3 side chain attached to the aromatic ring. Classical CBs are ABC tricyclic terpenic compounds bearing a benzopyran moiety and they are insoluble in water but soluble in lipids, alcohols, and other non-polar organic solvents [19]. Most of CB effects in mammalian tissues are mediated by the activation of two specific receptors, named CB receptor and numbered chronologically on the base of their discovery by a subscript (CB_1 and CB_2). CBreceptors have seven-trans-membrane-structure coupled to pertussis toxin-sensitive Gi/o proteins able to inhibit adenylyl cyclase activity, to activate the mitogen-activated protein kinase and immediately to initiate early gene signaling pathway(s) [20, 21]. In addition, CB_1 receptors are coupled through Gi/o proteins to various types of potassium and calcium channels. The CB_1 receptors have been cloned from rat, mouse, and human tissues and they exhibit from 97% to 99% amino acid sequence identity across species. CB_1 receptor mRNA and protein are found primarily in brain and neuronal tissue [22]. The CB_2 receptor exhibits only 48% homology with the CB_1. The CBs receptors cloning lead to the discovery of their endogenous ligands, known as endocannabinoids (endoCB) [23]. The first to be discovered was the ethanolamide of arachidonic acid, known as anandamide (AEA). AEA. was shown to bind the CB_1 receptor with modest affinity (Ki=61 nM), to have low affinity with the CB_2 receptor (Ki=1930 nM) [24], and to behave as partial agonist in biochemical and pharmacological tests characterizing CB activity.

A second important endocannabinoid, 2-arachidonoylglycerol (2-AG), binds weakly to both CB_1 (Ki=472 nM) and CB_2 (Ki=1400 nM) receptors [25]. 2-AG was isolated from intestinal [25] and brain tissues [26] and it is present in the brain at concentrations approximately 170-fold higher than AEA. In extending the endoCB research, palmitoylthanolamide (PEA), a C16:0 fatty acid derivate, where the carboxylate function is amidated by the primary amine of ethanolamine, was isolated in mammalian brain, liver and skeletal muscle. The ability of PEA to activate CBs receptors is still under debate; in fact while PEA does not interact with CB_1 at physiologically relevant concentration (Ki=23.8μM), it has been suggested that PEA acts, at least in particular cell types, as an endogenous ligands for CB_2 receptors (Ki=139.μM) [27].

The common structural features, between the plant-derived CB agonist Δ9-tetrahydrocannabinol (THC) and the endoCB, are a polar head group and a hydrophobic chain with a terminal n-pentyl group [19]. The structure–activity relationship (SAR) studies of these compounds have recognized four pharmacophores: a phenolic hydroxyl (PH), a lipophylic side chain (SC), a northern aliphatic hydroxyl (NAH), and a southern aliphatic hydroxyl (SAH) [28]. It seems that the ''lipophilic side chain'' is the key pharmacophore that plays a crucial role in determining ligand affinity and selectivity towards CBreceptors as well as pharmacological potency [29]. Interpretation of SAR data for endoCBs is more complex since these fatty acid derivates are also agonists for non-CB_1, non-CB_2 receptors. Beside classical CB receptor, AEA also interacts with several non-CB receptors; among them the transient receptor potential vanilloid subtype 1 (TRPV1) channel, whom AEA binds an intracellular site [30]. Recently, AEA and 2-AG were reported to activate GPR55, an orphan G-protein-coupled receptor. The evidence of endoCBs' interaction with peroxisome-proliferator-activating receptors (PPARs) α and γ, although at high concentrations, has also been recently reviewed [31].

BIOSYNTHESIS AND DEGRADATION OF CANNABINOIDS AND ENDOCANNABINOIDS

CBs, like many other plant terpenoids, are biosynthesized *via* the deoxyxylulose phosphate pathway [32]. CBproduction starts with the combination of geranyl pyrophosphate and olivetolic acid to form cannabigerol (CBG). Moreover, CBG is independently converted to either cannabidiol (CBD) or cannabinol (CBC) by two separate enzymes. CBD is then enzymatically cyclized to THC.

Several pathways might exist for the endoCB synthesis. The biosynthesis of AEA involves the enzymatic transfer of an arachidonoyl group from the sn-1 position of phosphatidylcholine (PC) to the head group of a phosphatidylethanolamine (PE) by an N-acyltransferase (NAT) [33]. AEA is then released by an N-acylphosphatidylethanolamine-specific phospholipase D (NAPE-PLD) [34, 35] and possibly, to

some extent, *via* the corresponding N-arachidonoyl-lyso-phosphatidyl ethanolamine (NA-lyso-PE) [36]. Other fatty-acid ethanolamides, for example PEA or oleoylethanolamide, can also be formed through these pathways. 2-AG, is produced almost exclusively by the hydrolysis of diacylglycerols (DAGs) *via sn*1-selective DAG lipases (DAGLs) α and β. The major endoCBs, AEA and 2-AG, differ only in their head groups and they both derived from arachidonic acid, and each of them has the biologically important terminal n-pentyl tail.

After their cellular re-uptake, endoCBs are almost metabolized *via* fatty acid amide hydrolase (FAAH), an enzyme recognizing as substrates several esters, ethers and amides of fatty acid.

2AG is more specifically degraded *via* monoacylglycerol lipase (MAGL) or, at some extent, by other recently identified lipases, the αβ-hydrolases (ABH6) and 12 (ABH12), as well as FAAH.

Like 2-AG, a selective PEA metabolizing enzymes, referred as PEA-preferring acid amidase (PAA), has also been identified [35].

The cellular re-uptake mechanism of endoCB is not yet characterized and it is still controversial. However, this mechanism seems also to mediate the release of *de novo* synthesized endoCBs. Moreover, both AEA and 2-AG might become substrate for cyclooxygenase-2 (COX-2) and they give rise to the corresponding hydroperoxy derivatives. The AEA and 2-AG hydroperoxy derivates can then be converted to prostaglandin ethanolamides (prostamides) and prostaglandin glycerol esters, respectively, by various prostaglandin synthases [37].

ROLE OF ENDOCANNABINOID SYSTEM AND ITS CONTROL

EndoCBs, phytoCBs, and synthetic CBs affect several body functions and they have shown to be effective in a number of serious pathological conditions. Various diseases, such as anorexia, emesis, pain, inflammation, multiple sclerosis, neurodegenerative disorders (Parkinson's disease, Huntington's disease, Tourette's syndrome, Alzheimer's disease), epilepsy, glaucoma, osteoporosis, schizophrenia, cardiovascular disorders, cancer, obesity, and metabolic syndrome-related disorders, to name just a few, have the potential to be treated by CBs [38].

Since the multiple possible targets for endoCB system, at date, several pharmacological tools have been developed; behind the selective agonists, inverse agonists and antagonists for CB_1 and CB_2, also specific inhibitors of FAAH [39].

CANNABIMIMETIC DRUGS AND ANGIOGENESIS

The initial evidence of the antiangiogenic properties of CBs come from numerous study on their antitumoral activity. In fact, for the first time in 2003 Casanova *et al*. [40] discovered that CBs, beside the ability to induce tumour growth regression, were also able to prevent vessel sprouts in a model of skin tumour. The administration of a non selective CB_1/CB_2 agonist or a selective CB_2 agonist in a non-melanoma skin tumour, resulted in the development of smaller blood vessels, in line with the finding that blood vessel enlargement constitutes a prominent feature of skin tumour progression [40]. Moreover, in CB-treated carcinomas the expression of pro-angiogenic factors (VEGF, PIGF and Ang2) was depressed while anti-angiogenic factors remained unchanged [41]. The anti-angiogenenic property of CB-related drugs were then confirmed also in different tumour types as firstly in a model of gliomas in mice [41]. CB_2 receptor activation lead to a pattern of blood vessels characterized predominantly by very small and narrow capillaries compared to animals with gliomas that displayed micro vascular hyperplasia, in which proliferating blood vessels were lined by disorderly heaped up endothelial cells ultimately transformed into glomeruloid tufts [40].

The protective effects of CBs in tumor vascularization *in vivo* were also accompanied by changes in vascular functionality as vascular permeability. CBs inhibited glioma-associated angiogenesis not only by interfering with pro-angiogenic factor signalling (VEGF and Ang2) [41, 42] in the tumours but also by a direct agonism with CB receptors expressed on endothelial cells. The discovery of a functional CB_1 and CB_2 receptors on primary human endothelial cell-line [43], HUVEC, was followed by the assessment that the mixed CB_1/CB_2 agonist, WIN-55,212-2, directly induced vascular endothelial cell apoptosis and inhibited cell migration, both effects were reversed by the CB_1 and CB_2 selective antagonists, pointing to a real depression of cell locomotion (chemokinesis) rather than of cell chemotaxis [40]. CB receptor activation increased ERK activity in HUVEC, underlining the participation of the extracellular signal-regulated kinase (ERK) cascade in CB action.

An alternative pathway for the anti-angiogenic effects of CBs involves the blockage of p21*ras* activity, mediated through CB_1 receptor activation. The local administration of the stable AEA analogue and CB_1 receptor agonist Met-F-AEA in thyroids tumours, dramatically reduced the levels of VEGF and one of its receptors (Flt-1/VEGFR-1), thus indicating that CB treatment affected the VEGF signalling and, hence, tumour angiogenesis. These inhibitory effects of Met-F-AEA were attenuated by the selective CB_1 receptor antagonist, SR141716A, thus it suggests the involvement of CB_1 receptor activation in the anti-angiogenic effect of the compound [44].

However, the effect of CBs on angiogenesis are not mediated exclusively through CBs receptor activation; since HU-331, a cannabidiol derivate, whose precise pharmacodynamic profile remains to be established, probably acts through a non CB-receptor mechanism [45]. This fact is not surprising since vascular endothelial cells express various functional receptors apart from CB ones, including the tentative abnormal cannabidiol receptor [46], and the TRPV1 vanilloid receptor [47], controlling important cell functions such as migration [41], survival [42], and vascular tone [48]. In particular, HU-331 directly induces vascular endothelial cell apoptosis without affecting the expression of the most prominent cytokines and receptors involved in the control of angiogenesis; it blunts not only basal angiogenesis but also tumour angiogenesis. Nevertheless, it may not be ruled out that HU-331 interferes with intracellular pathways modulated by the pro/anti-angiogenic cytokines, as it has been shown previously for CBs receptor activation [45]. HU-331

affects the expression of a large number of genes involved in key vascular endothelial cell functions. They include the genes encoding for matrix metalloproteinase-1, the eicosanoid-generating enzyme COX-2, the bone growth/remodeling factor osteoprotegerin, the C-C chemokine monocyte chemotactic protein-1, the prothrombotic protein VWF, and the arachidonic acid-generating enzyme phospholipase A2 [45].

All these results have encouraged testing CB in a pilot clinical study in patients with recurrent glioblastoma multiforme. The intracranial administration of THC reveals a fair safety profile, together with its possible antiproliferative action. Moreover, THC tended to decrease tumour vascularisation but only in two of nine enrolled patients [49], therefore even though the effect was not statistically significant; it sets the basis for future trials aimed to evaluate the potential anti-angiogenic activity of CBs.

The antiangiogenic profile of CBs is not limited exclusively to tumour-associated angiogenesis. In fact, recent publications have supported the role of CBs in the control of new vessel formation during chronic inflammation. In line with the effect on cancer, the activation of CB_1 and CB_2 receptors resulted in switching off the inflammatory scenario by the down regulation of angiogenic pathways. The effects of CBs are mediated by the control of NF-κB activation, one of the most important transcription factor that regulates the transcription of several pro-inflammatory and pro-angiogenic mediators including for instance TNF-α, VEGF, iNOS, COX-2, MMPs, and therefore resulting in new vessels formation's reduction [50].

Some recent studies have identified mast cells as a specific cellular target for CB anti-angiogenic effects, at least in granulomatous-type of chronic inflammation. Mast cells, in fact, express both functional CB receptors and their activation leads to the control of mast cell behaviour. Both CB_1 and CB_2 selective agonists reduce the transcription and expression of chymase, a serine protease with chymotrypsin-like activity, fairly selectively expressed by mast cells and strongly implicated in new vessel formation [10]. However, CBs receptor activation was able to reduce mast cells infiltration in inflamed tissues without affecting mast cell degranulation, which is the pivotal event in mast cell activation. Interestingly, other CB-related drugs resulted able to control not only mast cell infiltration, but also mast cell degranulation, and in this way, reducing the release of the plethora of pro-angiogenic factors from activated mast cells, they prevent new blood vessels formation. This is the case of Adelmidrol, a PEA analogue, whose physicochemical properties favour its usages in skin disorders, as λ-carrageenin-induced granuloma in rats. Ademidrol, almost completely abrogates the granuloma-associated angiogenesis by the modulation of mast cell degranulation at least, by the inhibition of the release of vasoactive VEGF and TNF-a and, in parallel, of several enzymes involved in the degradation of connective tissue, as MMP-9 and Chymase, which are necessary for the advancement of the new formed vessels [11].

DISCUSSION AND CONCLUSIONS

The aim of the present review was to summarize the recent findings on the emerging role played by cannabinomimetic compounds in the control of angiogenesis suggest new possible pharmacological approach. As previously cited, CBs strongly inhibit new vessel formation in different pathological conditions from inflammation to cancer.

Here for the first time, it is possible recognized at least three different cellular targets in CB anti-angiogenic action. At the beginning, the antiangiogenic properties of CB have been linked to their ability to inhibit the pro-angiogenic cascade in several tumour cells [40]. Soon after, it was discovered that CB directly affected endothelial cell migration and survival [42], thus affecting the new tube capillary formation. At the end, the third cellular target of CB effect was recognized in mast cells, at least in inflammatory skin disorders [10].

Almost all the CB effects seem to be related to the activation of the two CB receptor subtypes, showing the way to a future use of CB receptor agonists, in particular the selective CB_2 agonists which lack in undesired psychotropic effects. In fact, despite the emerging evidence regarding putative therapeutical activities of CBs, their effective introduction in the clinical use is still controversial and strongly limited by unavoidable central side effects exhibited by many of them.

In this scenario, new CB-related drugs with similar anti-angiogenic profile, as the quinone HU311 o PEA, which only weakly interact with CB receptors, may represent the most promising candidate for clinical utilization due to their remarkable lack of any cognitive and psychoactive actions, in addition to their excellent tolerability in humans [45]. The antiangiogenic efficacy showed by these compounds may be explained by the involvement of different functional receptors, apart from CB receptor, including the tentative abnormal cannabidiol receptor, the TRPV1 vanilloid receptor, orphan receptor (GPR55) and even uncloned receptor. In conclusion, it is conceivable to hypothesize the usage of cannabinomimetic compounds, including PEA and its congeners which, by the selective control of angiogenesis, may subsequently prevent several pathological condition accounting for the resolution of the related pathologies.

Cannabinomimetic compounds, as anti-angiogenic drugs, will improve future therapies of diseases like cancer, rheumatoid arthritis, chronic skin disorders and ocular neovascularisation.

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