Chapter 18

Cannabidiol and Cancer — An Overview of the Preclinical Data

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Additional information is available at the end of the chapter

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1. Introduction

In this chapter the state of art of the pre-clinical evidences of cannabidiol (CBD) anti-tumour effects on different kinds of cancer will be discussed. As you will see in the following paragraphs, CBD is a phytocannabinoid devoid of any psychotropic activity which exerts some of its effects through modulation of different components of the endocannabinoid system. For a more clear comprehension, a brief introduction on the endocannabinoid system itself is here provided.

2. The endocannabinoid system: A brief overview

The endocannabinoid system (eCB) is a signalling system that consist of the cannabinoid CB_1 and CB_2 receptors, their intrinsic lipid ligands, named endocannabinoids (eCBs), such as the N-arachidonoylethanolamide (anandamide, AEA) and the 2-arachidonoylglycerol (2AG), and the associated enzymatic machinery, comprising transporters, biosynthetic and degradative enzymes. It has been recently discovered and was thoroughly explored by scientists in the past 15 years.

The cannabinoid CB_1 and CB_2 receptors are G protein coupled receptors. CB_1 receptors are mainly present in the central nervous system (CNS) with low to moderate expression in periphery; on the contrary, CB_2 receptors are highly localized in the immune system with much lower and more restricted distribution in the CNS [1,2].

Soon after the characterization of the cannabinoid receptors came the discovery of their endogenous ligands. The two major known endogenous ligands are anandamide (AEA) and 2-arachidonoylglycerol (2AG) [3-6]. Both derive from arachidonic acid and are produced postsynaptically from phospholipid precursors through activity-dependent activation of specific phospholipase enzymes [7]. Later on, a number of other eCB ligands have been discovered including N-arachidonoyldopamine (NADA), N-arachidonoylglycerolether and O-arachidonoylethanolamine [8].

AEA and 2AG are characterized by different biosynthetic and metabolic pathways, showing distinct mechanisms of regulation. AEA can be synthesized through different pathways from the phospholipid precursors N-arachidonoyl-phosphatidylethanolamine, the most important of which is a direct conversion catalysed by an N-acyl-phosphatidylethanolamine selective phosphodiesterase. 2AG principal way of synthesis is through activation of phospholipase C and subsequent production of diacylglycerol, which is rapidly converted to 2AG by diacylglycerol lipase. After its re-uptake, AEA is hydrolysed by the enzyme fatty acid amide hydrolase (FAAH), producing arachidonic acid and ethanolamine, while 2AG is primarily metabolized by monoacylglycerol lipase (MAG lipase), leading to the formation of arachidonic acid and glycerol [9]. eCBs may bind not only to the well known CB_1 and CB_2 receptors, but also to other receptors. For instance, AEA may activate the potential vanilloid receptor type 1 (TRPV1) intracellularly [10]. Moreover, 'orphan' G protein coupled receptor, GPR55, has been recently proposed as putative cannabinoid receptor [11], as has been the peroxisome proliferator activated receptor, PPAR [12,13]. CB_1 and CB_2 receptors still remain the best known targets for AEA and 2AG, though interacting with different affinity: AEA has the highest affinity to both receptors, whereas 2AG has the highest efficacy on both receptors [14].

eCB synthesis can be induced by physiological or pathological stimuli and results in their immediate release, with subsequent activation of cannabinoid receptors. Therefore eCBs are synthesized and released "on demand" by post-synaptic cells, involving the cleavage of membrane phospholipid precursors. Each member of the endocannabinoid machinery tightly controls the synthesis, release and degradation of the eCBs, finely tuning the signalling system.

The eCB system has been found altered in several diseases, as multiple sclerosis and spinal cord injury, neuropathic pain, cancer, atherosclerosis, stroke, myocardial infarction, hypertension, glaucoma, obesity/metabolic syndrome and osteoporosis, thus paving the way for new therapeutic strategies aimed at restoring normal eCB system functionality [15].

Currently, the term 'cannabinoid' refers more than 100 terpenophenols derived from *Cannabis sativa* [16], as well as to synthetic compounds that directly or indirectly interact with cannabinoid receptors. The most psychoactive component of the plant *Cannabis sativa* is Δ^9-tetrahydrocannabinol (Δ^9-THC): its biological actions as well as the ones of synthetic cannabinoid compounds (synthetic compounds active on cannabinoid receptors) are primarily mediated by CB_1 and CB_2 receptors. Cannabinoids classification comprises phytocannabinoids (subclassified in different categories according to their chemical structures, such as Δ^9-THC, Δ^8-THC, cannabinol, CBD and cannabicyclol), synthetic compounds active on cannabinoid receptors (i.e., JWH133, WIN55212-2, SR141716) and endocannabinoids (i.e., AEA and 2AG) which are produced endogenously (Figure 1).

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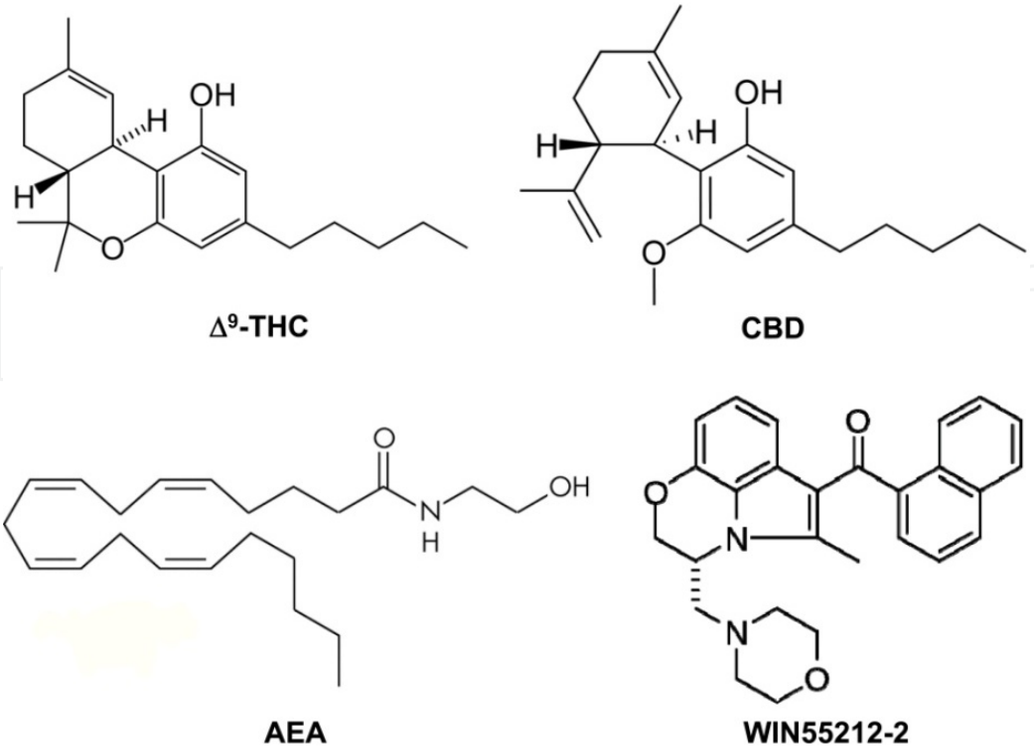

Figure 1. Chemical structures of Δ^9-tetrahydrocannabinol (Δ^9-THC), cannabidiol (CBD), anandamide (AEA) and WIN55212-2

3. Cannabinoids and cancer

Till now, most of cannabinoids applications in clinical practice of cancer patients comprised palliate wasting, emesis and pain that often accompany cancer. The discovery of a potential utility of these compounds for targeting and killing cancer cells led to a significant advancement in cannabinoid use in cancer treatment. In 1975 Munson et al. [17] demonstrated that the administration of Δ^9-THC, Δ^8-THC and cannabinol inhibited the growth of Lewis lung adenocarcinoma cells *in vitro* as well as *in vivo* after oral administration in mice. From then on, the interest in anti-carcinogenic properties of cannabinoids substantially decreased, until the discovery of the eCB system and the cloning of the specific cannabinoid receptors, CB_1 and CB_2. In the past decades, more and more evidences have contributed to assess and define the anti-tumourigenic effect of several cannabinoid compounds, including Δ^9-THC, cannabidiol (CBD), synthetic agonists, endocannabinoids and endocannabinoid transport or degradation inhibitors. These molecules have proved valuable against tumour cell proliferation and angiogenesis, and induce apoptosis in various cancer types, *i.e.* lung, glioma, thyroid, lymphoma, skin, pancreas, uterus, breast, prostate and colorectal carcinoma, both *in vitro* and *in vivo* [18-26]. Moreover, other mechanisms adopted by cannabinoids to counteract tumourigenesis are currently emerging and comprise interference with tumour neovascularization, cancer cell migration, adhesion, invasion and metastasisation [27].

Notwithstanding these promising anti-tumour effects, the clinical application of Δ^9-THC and other cannabinoid agonists is often limited by their unwanted psychoactive side effects. In line with this fact, in recent years increasing interest has been focused on non-psychoactive cannabinoid compounds with structural affinity for Δ^9-THC, such as cannabidiol (CBD). Interestingly, although its very low affinity for both CB_1 and CB_2 receptors, CBD has been recently reported to act with unexpectedly high potency *in vitro* as antagonist of CB_1 receptors in the mouse vas deferens [28] and brain [29] tissues, and to behave as inverse agonist at human CB_2 receptors [29]. Moreover, other putative CBD's molecular targets are TRPV, 5-HT_{1A}, GPR55 and PPARγ receptors (Figure 2).

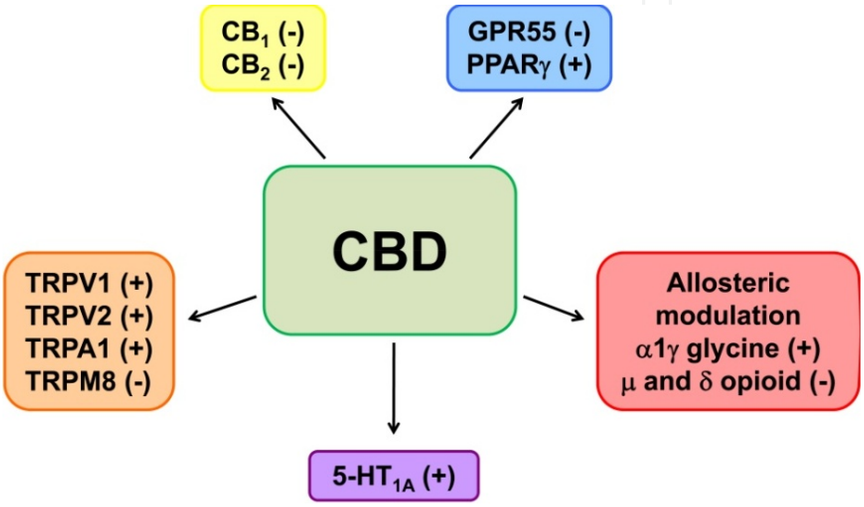

Figure 2. Some of the potential biological targets of CBD (with the permission of the authors [30])

Several studies reported the beneficial effects of CBD in the treatment of pain and spasticity and other CNS pathologies, as multiple sclerosis, Huntington disease, Parkinson etc. Moreover, many reports demonstrated its anti-tumour effects: it is characterized by a pro-apoptotic anti-metastatic activity and inhibits cancer cell migration, adhesion and invasion.

The aim of this chapter is to report the state of art on the efficacy of CBD in the modulation of the different steps of tumourigenesis in several types of cancer. The results here presented highlight the importance of future further exploration of CBD and/or of CBD analogues as alternative agents for tumour therapy, at least for breast cancer, glioma, lung tumour and leukaemia. The data available so far are summarized in Table 1 and are discussed in detail in the following paragraphs.

4. CBD and breast cancer

In 2006 Ligresti et al. [31] first demonstrated that CBD potently and selectively inhibits the proliferation of human breast cancer cells. This compound was able to strongly inhibit the growth of different breast tumour cell lines (MCF7, MDA-MB-231) *in vitro*, with an IC_{50} of

Cancer	*In vitro effect*	Receptor involvement	ROS production	Molecular cell signalling	Autophagy	Apoptosis	*In vivo effect*	Reference
Breast	↓ proliferation	CB_2; TRPV1	↑	NC	NC	+	↓ xenografts growth ↓ lung metastases	[26]
	↓ viability	non-CB_1; non-CB_2; non-TRPV1	↑	↓ pAkt; ↑ cytochrome C Bid translocation	+	+	NC	[29]
	↓ invasion	NC	↑	↓ Id-1; ↑ pERK	NC	NC	↓ tumour growth ↓ size and number of metastases	[27,28]
Glioma	↓ proliferation	non-CB_1; partial-CB_2; non-TRPV1	↑	↓ pERK; ↓ pAkt; ↓ HIF-1α ↑ cytochrome C caspase activation	NC	+	↓ tumour growth	[33,34,37,38]
	↓ proliferation and invasiveness	NC	NC	NC	NC	NC	NC	[35]
	↓ migration	non-CB_1; non-CB_2; non-TRPV1	NC	Ptx insensitive	NC	NC	NC	[39]
	↓ invasiveness	NC	NC	↓ Id-1	NC	NC	NC	[46]
Leukaemia	↓ viability	NC	NC	caspase-3 activation	NC	+	NC	[40]
	↓ viability	CB_2	↑	↓ p-p38 caspase activation; ↓ Bid ↑ cytochrome C	NC	+	↓ tumour burden ↑ tumour cell apoptosis	[41]
Lung	↓ invasion	CB_1; CB_2; TRPV1	NC	↑ p-p38; ↑ p-ERK; ↑ TIMP-1 ↓ PAI-1	NC	NC	↓ lung metastases ↓ PAI-1 in xenografts	[49,50]
Thyroid	cytostatic effect	NC	NC	NC	NC	+	NC	[26]
Thymoma	↓ viability	NC	↑	NC	NC	+	NC	[53]
Colon	↓ proliferation	CB_1; TRPV1; PPARγ	NC	↓ Akt; ↑ 2AG ↑ caspase-3; ↑ COX-2	NC	+	↓ ACF, polyps and tumours	[55]

NC=not checked ↑ increase; ↓ decrease

Table 1. Effects of CBD on different types of cancer

about 6 μM. Worth to note, its effect on non cancer cells resulted far less potent. Further on, by the use of xenografts obtained by *s.c.* injection into athymic mice of human MDA-MB-231 breast carcinoma, the authors demonstrated that CBD and CBD-rich extracts (containing approximately 70% CBD together with lesser amounts of other cannabinoids) not only inhibited tumour growth *in vivo*, but also reduced lung metastases deriving from intrapaw injection of MDA-MB-231 cells. The possible cellular and molecular mechanisms underlying these effects may include induction of a combination of mechanisms by CBD, involving direct TRPV1 activation and/or CB_2 indirect activation (via FAAH), as well as induction of oxidative stress.

Subsequent work form McAllister and coworkers [32] demonstrated that CBD not only affected breast cancer cell proliferation, but it also interfered with invasion and metastasis, two other crucial final and fatal steps of breast cancer cell progression. Analysis on three different groups of cannabinoid compounds (phytocannabinoids with affinity for CB_1 and CB_2 receptors; phytocannabinoids with no appreciable affinity for CB_1 and CB_2 receptors; synthetic compounds with affinity for CB_1 and CB_2 receptors) revealed that CBD was shown to be one of the most effective inhibitors of human breast cancer cell proliferation, being as potent as Δ^9-THC and CP55940 in inhibiting MDA-MB-231 and MDA-MB-436 cell growth respectively, and resulting as the most potent inhibitor of the MDA-MB-231 cell migration. By the use of Boyden chamber the authors also investigated the effect of several cannabinoids on the ability of MDA-MB-231-the most aggressive breast cancer cell line-to migrate and invade a reconstituted basement membrane: CBD demonstrated once more to be the most potent inhibitor of cell invasion. Further investigation concerned the identification of the cellular mechanism underlying CBD effect on cell growth and invasion. CBD was predicted to act through the regulation of key genes involved in the control of cell proliferation and invasion. In particular, attention was focused on Id-1 protein, an inhibitor of basic helix-loop helix transcription factors, which overexpression in breast cancer cells stimulates proliferation, migration and invasion. In fact, CBD exerted inhibition of Id-1 expression at the same concentrations already proved to be effective against proliferation and invasion (0.1, 1 and 1.5 μM). It is worth noting that CBD has no effect on invasiveness in cells that ectopically expressed Id-1. CBD therefore revealed a non toxic exogenous agent able to significantly decrease Id-1 expression in breast cancer cells and, at the same time, effective at reducing tumour aggressiveness.

Later work from the same group elucidated the cellular pathways involved in the down-regulation of Id-1 expression by CBD and leading to inhibition of human breast cancer proliferation and invasiveness [33]. First of all, they demonstrated that CBD-induced up-regulation of the extracellular signal-regulated kinase phosphorylation (p-ERK) was responsible for inhibition of Id-1 expression and subsequent human breast cancer cell proliferation and invasion. Indeed, the ERK inhibitor, U0126, partially reverted the ability of CBD to inhibit proliferation and invasion and attenuated its effect on Id-1 expression. Besides ERK up-regulation, also the production of reactive oxygen species (ROS) seems to play a role in CBD inhibitory effect on Id-1. In fact, CBD ability to inhibit proliferation, invasion and Id-1 expression was reversed by the use of tocopherol, a ROS scavenger. In addition to Id-1, CBD also

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modulates the pro-differentiation factor Id-2, inducing its up-regulation. Consistent with the work from Ligresti [31], CBD also demonstrated *in vivo* efficacy: it reduced primary tumour mass as well as the size and number of metastatic foci in two models of metastasis.

The most recent contribute to the elucidation of the cellular mechanism elicited by CBD to induce cell death in breast cancer cells comes from an excellent paper of Shrivastava et al. [34]. According to the experiments that they performed on both estrogen receptor positive and estrogen receptor negative breast cancer cells, CBD (0.1-10 μM for 24 h) induced a concentration-dependent cell death. Worth to note, the effective concentrations of CBD in tumour cells have little effect on MCF-10A cells, a line of non tumourigenic, mammary cells. This effect resulted to be independent of CB_1, CB_2 and TRPV1 receptor activation. Cell morphology assessed through electron microscopy revealed to be consistent with the coexistence of autophagy and apoptosis. These events were promoted by the induction of endoplasmic reticulum (ER) stress and the inhibition of Akt/mTOR/4EBP1 signalling. Also ROS production induced by CBD seems to play a role, since ROS inhibition through tocopherol blocked the induction of apoptosis and autophagy. Further investigation of the cellular mechanisms involved in CBD-induced programmed cell death (PCD) demonstrated that this compound reduced mitochondrial membrane potential, triggered the translocation of the Beclin2 Interacting Protein (Bid) to the mitochondria and the release of cytochrome C to the cytosol and, ultimately, the activation of the intrinsic apoptotic pathway. The relationship between CBD-induced apoptosis and autophagic cell death was also explored through blockade of each form of the PCD with specific caspase and autophagy inhibitors. In the first case, caspase inhibition reduced CBD pro-apoptotic effect and corresponded to lower levels of protein markers in breast cancer cells. On the other hand, the inhibition of autophagy enhanced the level of CBD-induced apoptosis and determined an increase in protein marker expression. As suggested by these data, blockade of CBD-induced autophagy likely results in a compensatory increase of apoptosis as an alternative means of PCD. CBD-induced PCD thus depends on a precise balance between apoptosis and autophagy which could be mediated by Beclin1. Though its mechanism of action is not clearly elucidated, Beclin1 is considered a key signalling molecule in the autophagic process. It has been recently suggested that Beclin1 is cleaved by caspases and that such cleaved form is incapable of inducing autophagy [31,32]. CBD treatment (5 to 10 μM) resulted in the cleavage of Beclin1. This is consistent with induction of caspases activity and suggests that Beclin1 may likely have a role in CBD-mediated cell death. The consequent cleavage product translocates to the mitochondria, where it induces apoptosis by enhancing the release of cytochrome C [35,36]. The cleavage and consequent translocation of cleaved Beclin1 to the mitochondria induced by CBD treatment may thus be crucial among the mechanisms elicited by CBD to modulate the balance between autophagy and apoptosis in breast cancer cells.

As a whole this work highlights the presence of a complex mutual regulation of autophagy and mitochondria-mediated apoptosis in CBD-induced breast cancer cell death. The very promising data here reported support the idea that this non toxic compound could likely

represent either a new therapeutic opportunity for breast cancer treatment or the starting point for a second generation compound to be tested in clinic.

In Figure 3 it is depicted a schematic representation of the signalling pathways associated with CBD effect in breast cancer cell proliferation and invasion.

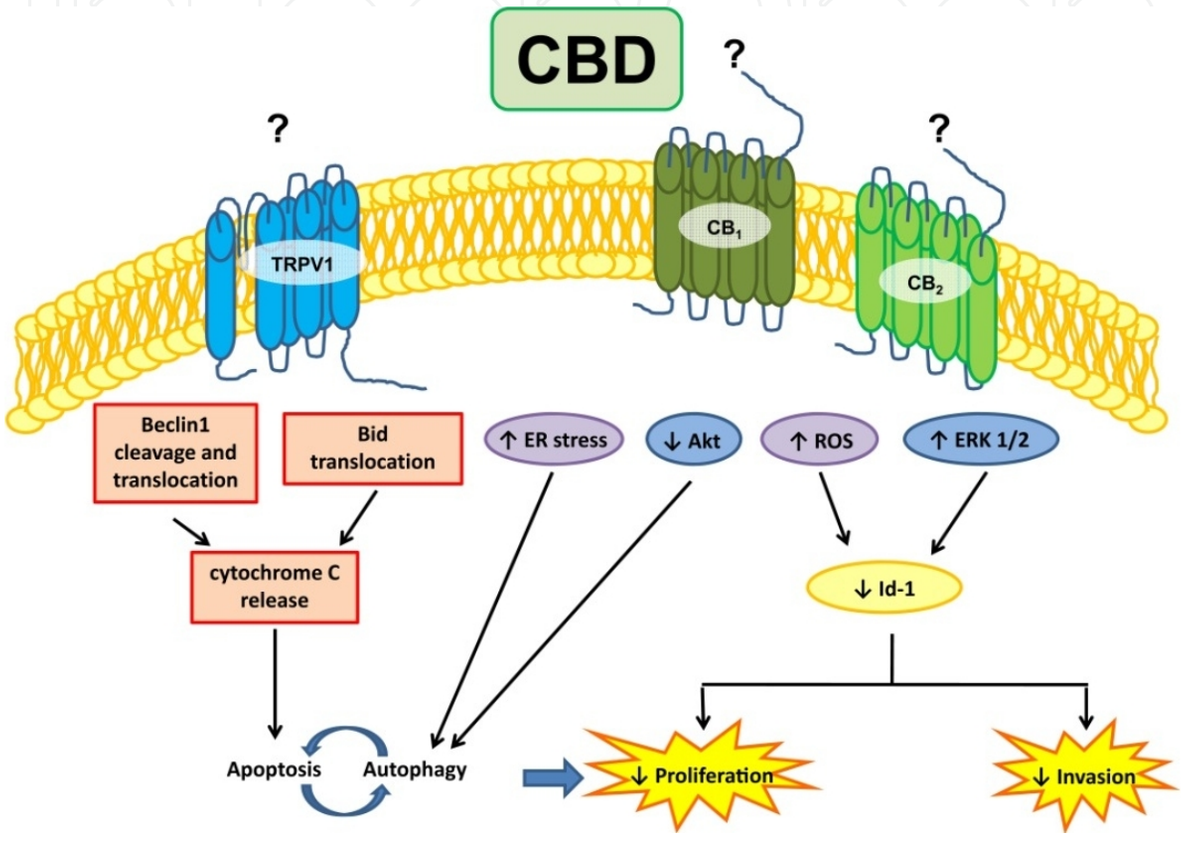

Figure 3. Schematic representation of the signalling pathways associated with CBD effects on breast cancer (with the permission of the authors [30])

5. CBD and glioma

Moreover, CBD also exhibited anti-tumoural properties in gliomas, a class of brain tumours of glial origin characterized by a high morphological and genetic heterogeneity accounting for over 30% of all primary brain tumours. Gliomas are characterized by a high proliferative rate, aggressive invasiveness and insensitivity to radio-and chemotherapy, and are considered among the most rapidly growing and devastating solid tumours.

In a seminal paper Jacobsson et al. [37] first demonstrated that CBD displayed a serum-dependent effect on C6 murine glioma cell proliferation. Massi et al. [38] afterwards reported

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in 2004 an effective inhibition of U87-MG and U373 human glioma cell proliferation *in vitro* following CBD treatment, associated with the induction of apoptosis. Exposure of both cell lines to CBD for 24 h induced a concentration-and time-dependent inhibition of the mitochondrial oxidative metabolism, with an IC_{50} value of 25 μM. The authors then evaluated the effect of intratumoural *in vivo* treatment with CBD, in tumour xenografts generated by subcutaneous injection of glioma cells in the flank region of immune-deficient mice. The treatment caused a significant reduction (60% mean) of the tumour growth over a 23-day period of observation [38]. Data from flow-cytometric analysis and ssDNA detection assay indicated that this reduction was correlated to CBD ability to induce a PCD. Of note, subsequent investigation demonstrated that CBD as no effect on the viability of non transformed primary glial cells [39].

Analysis of the cellular mechanisms responsible for CBD anti-proliferative effect demonstrated the lack of significant stimulation of cannabinoid and vanilloid receptors [38]. The sole CB_2 receptor antagonist SR144528 only weakly and transiently reverted CBD effect, a partial antagonism being present only over a 24 h-period and restricted to just one glioma cell lineage. Further data from the same work demonstrated for the first time that CBD anti-tumour effect depend on its ability to induce an oxidative stress state in the tumour cells: indeed this molecule induced an elevated and early production of ROS, a corresponding depletion of intracellular glutathione and an increase of activity of the GSH-associated enzymes. Consistent with these evidences, the anti-proliferative effect of the drug was reversed by the anti-oxidant, tocopherol. It is worth noting that CBD did not affect ROS production in non transformed primary glial cells [39]. Subsequent investigation of the cellular events implicated in glioma cell death demonstrated that CBD induces a time-dependent release of cytochrome C and activation of caspase-8, caspase-9 and caspase-3: both the intrinsic and the extrinsic pathways of apoptosis result therefore involved in CBD effect [39].

Further support to the efficacy of CBD in inhibiting the growth of different glioblastoma cell lines (SF126, U251 and U87-MG) comes from a work from Marcu et al. [40]. In their paper the authors demonstrated not only that CBD appears more potent that Δ^9-THC, but also that combination treatment of Δ^9-THC with CBD determined an enhancement of Δ^9-THC inhibitory effect on glioblastoma cell growth, but not on invasiveness [40]. Accordingly, Torres et al. [41] recently confirmed that association of CBD with Δ^9-THC treatment greatly reduced the viability of several human glioma cells and determined an enhancement of both autophagy and apoptosis, with corresponding triggering of caspase-3 activation. Exposure of xenografts generated from U87-MG cells in nude mice to submaximal concentrations of CBD in combination with Δ^9-THC inhibited tumour growth at a higher extent than the treatment with the individual compounds: these data suggest a potential use of the combinatory therapy as a strategy to allow reduction of the amount of the psychoactive Δ^9-THC in potential cancer treatment.

The cellular mechanism involved in the synergistic action of the combined therapy were investigated by Marcu et al. [40]: double treatment with CBD and Δ^9-THC *in vitro* induced cell cycle arrest, stimulation of ROS production and sustained activation of caspases-3,-7 and-9, with consequent induction of apoptosis, together with specific modulation of the extracellular signal-regulated kinase, ERK. Exposure to each individual compound was unable to induce

these specific effects, suggesting that the signal transduction pathways affected by the combination treatment were unique. Recent results form Parolaro's group [42] apparently disagree with Marcu's data, as in their hands CBD *per se* strongly down-regulates ERK, as well as another signalling molecule playing a crucial role in tumour cell proliferation, such as PI3K/Akt, both in U87-MG cells and in cannabinoid-resistant T98G human glioma cell lines. Moreover, CBD also profoundly down-regulated the hypoxia-inducible transcription factor HIF-1α, one of the most critical stimuli for cell survival, motility and tumour angiogenesis. These evidences suggest that HIF-1α, together with ERK and Akt, represent three of the multiple molecular targets through which the CBD exerts its anti-neoplastic activity [42].

Talking about the mechanisms elicited by CBD to prevent glioma growth, further *ex vivo* investigations, performed on glioma tumour tissues excised from nude mice treated *in vivo* with CBD, showed its ability to modulate the lipoxygenases (LOX) pathway and the endocannabinoid system [43]. Biochemical analysis indicated a significant decrease of 5-LOX activity and content and of its end-product leukotriene B4, as well as a marked stimulation of the fatty acid amide hydrolase (FAAH), and a decrease of AEA content. In contrast, cyclooxygenase (COX)-2 activity and content were not affected by CBD.

CBD ability to reduce tumour growth is not only restricted to cell proliferation, but several data also support its effects on glioma cell migration and invasiveness. As a matter of fact, CBD inhibited U87-MG glioma cells migration in a concentration-dependent way, at concentrations lower than those required to inhibit cell proliferation [44]. Treatment with the selective cannabinoid receptor antagonists SR141716 (CB_1), SR144528 (CB_2) or by pretreatment with pertussis toxin were not able to antagonize CBD anti-migratory effect, thus indicating no involvement of classical cannabinoid receptors and/or $G_{i/o}$ protein coupled receptors. Cell invasiveness, evaluated through a reconstituted basement membrane in a Boyden chamber test, was reduced after CBD treatment in U251 glioma lineage [40].

Worth to note, an activation of TRPV2 receptor by CBD have recently [45] demonstrated to inhibit human glioma cell proliferation and overcome carmustine resistance of glioma cells. This mechanisms seems to involve increase in Ca^{2+}influx and consequent drug uptake, synergizing with cytotoxic agents to induce apoptosis of glioma cells. Interestingly this effect was limited to tumour cells, whereas it is absent in normal human astrocytes.

At last, Soroceanu and coworkers [46] recently investigated the role of the transcriptional regulator Id-1 in modulating the invasiveness of glioma cells. As previously reported for breast cancer, Id-1 expression levels positively correlate with glioma cell invasiveness in culture and with histopathological grades in patient biopsies. The authors showed that CBD significantly down-regulates Id-1 gene expression and associated glioma cell invasiveness and self-renewal, and glioma progression *in vivo*, thus suggesting that Id-1 may be an additional target for CBD.

As a whole, CBD seems to counteract glioma cell proliferation and invasion through multiple mechanisms. Most of them are summarized in Figure 4.

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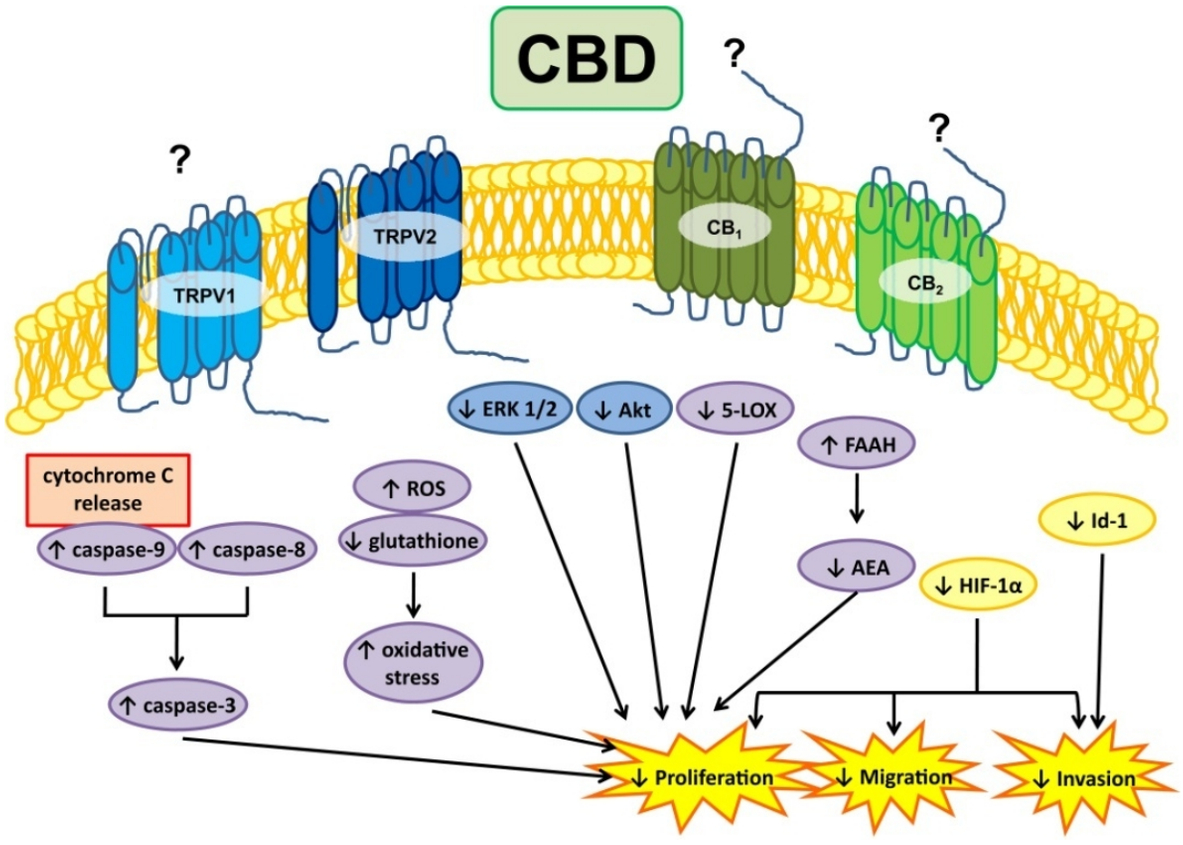

Figure 4. Schematic representation of the signalling pathways associated with CBD effects on glioma

6. CBD and leukaemia/lymphoma

Gallily et al. [47] first evidenced the possible role of CBD in the treatment of lymphoblastic diseases. They demonstrated that 24 h-treatment with CBD induced apoptosis in human acute myeloid leukaemia HL-60 cell line: the effect was dose-dependent and involved caspase-3 activation. On the contrary, CBD treatment had no effect on human monocytes from normal individuals.

By far the most important work in this field was from McKallip et al. [48]. They exposed the murine EL-4 lymphoma cell line and the human Jurkat and Molt-4 leukaemia cell lines to CBD (1-10 μM) and demonstrated that this compound is able to reduce the number of viable cells and to induce apoptosis, both *in vitro* and *in vivo*, in a CB_2 receptor dependent way. In particular, treatment with CBD *in vivo* led to a significant reduction in tumour burden and determined a higher level of apoptotic tumours in EL-4-bearing mice [48].

As far as the cellular mechanisms are concerned, CBD effect on Jurkat cells *in vitro* involved the activation of caspase-8, caspase-9, and caspase-3, caused the cleavage of poly(ADPribose) polymerase, and corresponded to a decrease in full-length Bid: these concerted events support

a possible cross-talk between the intrinsic and extrinsic apoptotic pathways. CBD exposure caused a loss of mitochondrial membrane potential and the subsequent release of cytochrome C, thus further confirming the crucial role of mitochondria in PCD regulation by CBD. Moreover, as previously observed in other tumour cells, CBD treatment corresponded to an increase of ROS production as well as in the expression of the NAD(P)H oxidases Nox4 and $p22^{phox}$. Treatment with the ROS scavengers or the NAD(P)H oxidase inhibitors prevented both CBD-mediated induction of apoptosis and ROS increase. Finally, CBD down-regulated phosphorylation and consequent activation of p38 mitogen-activated protein kinase, but did not affect p-ERK and p-JNK levels. Exposure to a CB_2-selective antagonist or to a ROS scavenger reverted this effect, thus suggesting that CBD may involve CB_2 receptors and regulation of Nox4 and $p22^{phox}$ expression in its action [48].

As a whole, CBD may represent a novel and highly selective candidate for leukaemia treatment. It is worth noting that previous evidences indicated that human leukaemias and lymphomas expressed significantly higher levels of CB_2 receptors compared to other tumour cell lines. For this reason the observation that CBD mediates apoptosis through CB_2 receptors is particularly relevant, suggesting the possibility that tumours of immune origin may be highly sensitive to the CB_2-mediated effects of CBD [49].

7. CBD and lung cancer

Lung cancer is characterized by a particularly aggressive biological nature and is, at present, poorly responsive to available therapy. For this reason, a series of targets and therapeutic strategies for its treatment are currently being investigated [50-53].

Ramer's research group is one of the most active interested in preclinical lung cancer research. They recently [54-56] evaluated CBD effects on A549 cells, a line of human lung carcinoma cells expressing both CB_1 and CB_2, as well as TRPV1 receptors, with particular focus on invasiveness. By the use of Matrigel invasion assay, they demonstrated that CBD induces an impaired invasion of A549 cells; this impairment was reversed by antagonists to both CB_1 and CB_2 receptors as well as to TRPV1. Moreover, they observed that invasion decrease determined by CBD treatment corresponded to an upregulation of tissue inhibitor of matrix metalloproteinases-1 (TIMP-1); on the other hand, knocking down of TIMP-1 expression through a siRNA approach was able to reverse the CBD-elicited decrease in tumour cell invasiveness. These evidences suggest that there may be a causal link between the TIMP-1 up-regulation and CBD anti-invasive action. CBD was also shown to induce phosphorylation of two mitogen-activated protein kinases, p38 and p42/44: these have been identified as upstream mechanisms leading to TIMP-1 induction and subsequent decrease of invasiveness. Interestingly, these events are all prevented by exposure to cannabinoids or TRPV1 receptor antagonists.

The authors also performed *in vivo* studies in thymic aplastic nude mice [54]: in such model, intravenous injection of CBD at a dose of 5 mg/kg in animals caused a significant inhibition of A549 lung metastasis, further supporting the possible therapeutic benefit of CBD adoption. Together with the strong inhibition of A549 cell invasion, CBD treatment induced a corre-

sponding down-regulation of the plasminogen activator inhibitor PAI-1 expression and secretion, another factor playing an important role in the regulation of cell spreading [55].

CBD-driven decrease in PAI-1 secretion and cell invasion were suppressed by exposure to CB_1 and CB_2 receptors as well as to TRPV1 receptor antagonists. Recombinant human PAI-1 led to a concentration-dependent up-regulation of invasiveness, while PAI-1 siRNA caused down-regulation, suggesting a crucial role of PAI-1 in A549 invasiveness. The same experiments were also performed in two other human lung cancer cell lines, H460 and H358, and key data were confirmed.

PAI-1 also revealed crucial *in vivo*: CBD treatment (5 mg/kg) of A549 xenografts of rats determined its significant down-regulation [55].

One of the most important aspects in these works resides in the dose-range at which CBD effectively inhibits invasion. In fact, treating human lung cancer cells for 72 h with CBD the authors observed a significant effect at a concentration as low as 0.01 μM, corresponding to a 33% inhibition when compared to control vehicle. At the same conditions [56] an equimolar concentration of Δ^9-THC caused a 68% inhibition of cell invasion. Nonetheless, it is worth noting that CBD anti-invasive effect occurred in a range of therapeutically relevant concentrations: as a matter of fact, as reported from a clinical study on healthy volunteers, CBD reached peak plasma concentrations between 0.01 μM and 0.05 μM following administration of SativexTM (1:1 ratio of Δ^9-THC and CBD) at the buccal dose of 10 mg or at the self-titrated doses during chronic therapy [57].

In their most recent work, Ramer et al. [58] demonstrated that CBD reduces cell viability in lung cancer cell lines (A549, H460) and primary cells from a patient with lung cancer, this effect being associated with apoptosis. The cellular mechanisms induced by CBD involve an initial up-regulation of COX-2 and PPAR-γ, both *in vitro*, in A549 and H460 cell lines, and *in vivo*, in A549-xenografted nude mice, and a subsequent nuclear translocation of PPAR- by COX-2-dependent prostaglandins.

As a whole, these evidences contribute to elucidate the mechanisms underlying CBD anti-invasive action on human lung cancer cells and strongly support its possible use as a therapeutic option for the treatment of highly invasive types of cancers.

8. CBD and endocrine tumours

Among the endocrine malignancies, the most common one is thyroid cancer. This kind of tumour is characterized by overactivation of Ras, that may depend on activating mutations in the ras gene or on overactivation of receptor tyrosine kinase receptors. According to these observations, new strategies for the treatment of thyroid cancer should reasonably point at Ras as crucial molecular target.

Ligresti et al. [31] first exposed KiMol cells – rat thyroid cells transformed with the v-K-ras oncogene – to CBD treatment: this phytocannabinoid caused anti-proliferative effects in

association with a cell cycle block at the G1/S phase transition, which was accompanied by a pro-apoptotic action.

Further studies from Lee et al. [59] demonstrated a marked CBD pro-apoptotic effect in both murine thymocytes and EL-4 thymoma cells: this effect was time-and concentration-related. In particular, according to time-course analyses, CBD-mediated apoptosis occurred earlier in EL-4 cells than in thymocytes. The cellular events triggered by CBD were similar in both T cells: ROS generation played a pivotal role, although the basal level of ROS in thymocytes was lower than that in EL-4 cells. Exposure to the glutathione precursor N-acetyl-L-cysteine (NAC) markedly attenuated CBD-driven induction of apoptosis, and reverted the decrease of cellular thiols levels.

In conclusion, unlike monocytes, T cells, both primary and immortalized, are all sensitive and similarly responding to CBD, as demonstrated by the fact that CBD induced oxidative stress in thymocytes, EL-4 cells and splenocytes [60]. ROS generation proved to play a central role in this response.

9. CBD and colon cancer

Colon cancer is one of the principal causes of morbidity and mortality in Western countries. Its aetiology seems to reside in a series of histopathological and molecular changes that transform normal colonic epithelial cells, give rise to Aberrant Crypt Foci (ACF) and polyps as intermediate steps, and at last develops a colorectal carcinoma.

First investigation about CBD effect on SW480 colon and LNCaP prostate carcinoma cells comes from Sreevalsan et al. [61]. They demonstrated that CBD inhibits cell growth in both cell lines. This anti-proliferative effect corresponds to an induction of apoptosis. Moreover, CBD induced mRNA expression of several dual specificity phosphatases and protein tyrosine phosphatases. This is particularly relevant given that the anti-tumoural effects of many cannabinoids include modulation of intracellular kinase. The pro-apoptotic activity of CBD was phosphatase-dependent in both cell lines, on the other hand, cannabinoid receptors mediated its effects only in prostate cancer cells, whereas their role in colon carcinoma is controversial.

Izzo and coworkers recently [62] investigated the effect of CBD in a preclinical animal model of colon cancer based on azoxymethane (AOM) administration in mice. AOM causes in exposed cells occurrence of ACF (preneoplastic lesions), polyps, and tumour formation, as well as up-regulation of p-Akt, iNOS and COX-2 and the down-regulation of caspase-3. The authors demonstrated that CBD possesses effective chemopreventive properties: it reduced ACF, polyps and tumours and counteracted AOM-induced p-Akt and caspase-3 changes. Moreover, *in vitro* studies performed in colorectal carcinoma cell lines evidenced that CBD protected DNA from oxidative damage, increased endocannabinoid levels and reduced cell proliferation in a CB_1-, TRPV1-and PPAR-γ-antagonists sensitive manner.

Based on these promising data and on its safety records, it is likely to consider CBD a worthy candidate for clinical consideration in colon cancer prevention.

10. CBD and angiogenesis

More and more evidences indicate that a new promising therapeutic target for cancer therapy is represented by angiogenesis, the formation of new blood vessels from the pre-existing ones. Moreover, tumour angiogenesis is target of several cannabinoids, which not only down-regulate the production of pro-angiogenic factors in cancer cells, but also directly modulate endothelial cell growth [27]. It is hence surprising the lack of any data on CBD ability to influence angiogenesis.

As Solinas et al. recently demonstrated [63], CBD is a potent inhibitor of HUVE cell proliferation, migration and invasion, acting through the induction of endothelial cell cytostasis, but without triggering apoptosis. Interestingly, CBD affected endothelial cell differentiation into tubular capillaries and blocked the outgrowth of capillary-like structures from HUVEC spheroids *in vitro*. Moreover, CBD anti-angiogenic effect was also evident *in vivo* in a matrigel sponge model. The cellular mechanisms elicited seem to involve down-modulation of several molecules associated with angiogenesis, including MMP2 and MMP9, uPA, Endothelin-1, PDGF-AA and CXCL16.

These preliminary data are very promising and demonstrate for the first time that, besides its well known pro-apoptotic anti-proliferative and anti-invasive actions, CBD may also exert anti-angiogenic effects, thus further strengthening its potential application in cancer therapy.

11. Conclusion

Much interest has been addressed to CBD since it is a plant-derived cannabinoid devoid of psychoactive properties. Many evidences support its pro-apoptotic and anti-proliferative actions in different types of tumours, as well as its anti-migrative, anti-invasive, anti-metastatic and perhaps anti-angiogenic properties. This wide range of anti-tumour effects indicate that CBD may likely be a potent inhibitor of both cancer growth and spreading.

In this regard, it is worth noting that, at least *in vitro*, this compound seems to exert its anti-cancer effect selectively on cancer cells, without affecting normal cell viability. Moreover, CBD revealed extremely efficient in inhibiting tumour growth: its versatility can be ascribed to the modulation of multiple cellular pathways that control tumourigenesis and consequent altering of different intracellular signalling, depending on the cancer type considered.

In particular, among all, increase in ROS production appears to be one of the most common and crucial mechanisms induced by CBD to exert its effects in all of the considered cancer cell lines. Less obvious is the role of cannabinoid and vanilloid receptors in mediating CBD action. In some cases (lung, leukaemia, colon) exposure of the cells to specific antagonists clearly

demonstrated the essential contribution of these receptors. In other kinds of cancer (breast and glioma) their relevance appears only marginal or absent.

Of note, CBD demonstrated to efficiently inhibit cancer development not only *in vitro*, but also in several *in vivo* tests, determining reduction of tumour growth and, in some cases, of metastatization.

Nonetheless, the opportunity to introduce CBD in clinical practice for cancer treatment needs some consideration. A good starting point comes from high tolerability and from the lack of toxic effects. Indeed oral administration of CBD 700 mg/day for 6 weeks did not show any overt toxicity in humans [64] suggesting its possible exploitation for prolonged treatment. On the other hand, CBD oral absorption is slow and unpredictable, making it difficult to realize the optimal route of administration. Previous trials indicated that 6 weeks of oral CBD treatment 10 mg/kg/day induced a mean plasma level of CBD between 6 and 11 ng/ml (about 0.0036 μM) [65] and that this value did not differ significantly over the 6 weeks of administration. Of note, this same range of concentration was effective in inhibiting lung cancer cell invasion [55,56]: for instance in this case, the oral route could be the appropriate choice. Moreover, according to experimental data showing that combined treatment with CBD and Δ^9-THC could be more effective in reducing cancer cell proliferation, the co-administration may represent a better choice for cancer therapy. In line with this, oromucosal treatment with SativexTM 10 mg (a formulation consisting of 1:1 ratio of Δ^9-THC and CBD and recently approved for multiple sclerosis) provoked CBD plasma level of approximately 0.01 μM and up to 0.05 μM in humans, a concentration range effective in reducing lung cell invasion *in vitro*. As reported, the use of different associations of phytocannabinoids in a variable proportion might lead to a better outcome without pharmacokinetic interaction [66]. Another favourable aspect of oromucosal administration is its possible application in presence of nausea and vomiting. In view to overcome the several limitations to the systemic administration of cannabinoids in part derived from their high lipophilicity, a recent work from Hernán Pérez de la Ossa et al. [67] investigated the effects of CBD-and Δ^9THC-loaded poly-ε-caprolactone microparticles as an alternative delivery system for long-term cannabinoid administration in a murine xenograft model of glioma. Local administration of Δ^9-THC-, CBD-or a mixture (1:1 w:w) of Δ9THC- and CBD-loaded microparticles every 5 days to mice bearing glioma xenografts reduced tumour growth with the same efficacy than a daily local administration of the equivalent amount of those cannabinoids in solution. Moreover, treatment with cannabinoid-loaded microparticles enhanced apoptosis and decreased cell proliferation and angiogenesis in these tumours.

Moreover, it would be worth evaluating the possibility to use CBD (or Sativex) in combination with classical chemotherapeutic agents: this would enable to check for the presence of a synergistic effect that might potentially allow clinical chemotherapeutic dose reduction, and consequently reduce toxicity while maintaining efficacy.

Bearing in mind that CBD is already currently used in patients with multiple sclerosis, and in light of the findings here summarized, it can be concluded that CBD might be a good candidate for a future clinical approach and/or for the synthesis of a second generation compound, as suggested by McAllister's group. In view of these opportunities and based on the data here

presented, further research should be addressed to continue exploration of CBD and/or of CBD analogues as alternative agents for cancer therapy.
Nomenclature

The drug/molecular target nomenclature conforms to the BJP's Guide to Receptors and Channels [68].

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