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Cannabinoids and Gliomas

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Abstract Cannabinoids, the active components of *Cannabis sativa* L., act in the body by mimicking endogenous substances—the endocannabinoids—that activate specific cell surface receptors. Cannabinoids exert various palliative effects in cancer patients. In addition, cannabinoids inhibit the growth of different types of tumor cells, including glioma cells, in laboratory animals. They do so by modulating key cell signaling pathways, mostly the endoplasmic reticulum stress response, thereby inducing antitumoral actions such as the apoptotic death of tumor cells and the inhibition of tumor angiogenesis. Of interest, cannabinoids seem to be selective antitumoral compounds, as they kill glioma cells, but not their non-transformed astroglial counterparts. On the basis of these preclinical findings, a pilot clinical study of Δ^9-tetrahydrocannabinol (THC) in patients with recurrent glioblastoma multiforme has been recently run. The good safety profile of THC, together with its possible growth-inhibiting action on tumor cells, justifies the setting up of future trials aimed at evaluating the potential antitumoral activity of cannabinoids.

Keywords Cannabinoid · Receptor · Glioma · Cancer · Apoptosis · Angiogenesis · Experimental therapeutics · Clinical trial

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Gliomas

Gliomas are defined as those tumors that display histological, immunohistochemical, and ultrastructural evidence of glial differentiation. The World Health Organization classifies gliomas according to their cellular features (i.e., resembling astrocytes, oligodendrocytes, or ependymal cells) and their grade of malignancy (from I to IV) [1]. Glioblastoma multiforme (GBM), or grade IV astrocytoma, is the most frequent class of malignant primary brain tumors and one of the most aggressive forms of cancer. As a consequence, survival after diagnosis is normally just 6–12 months [1, 2], which is due mainly to the high invasiveness and proliferation rate of GBM. In addition, GBM exhibits a high resistance to standard chemotherapy and radiotherapy. These malignant features may be related to the varying mutations frequently found in these tumors that impact different key pathways involved in the control of cell proliferation, survival, differentiation, and DNA repair [1–3].

Current standard therapeutic strategies for the treatment of GBM are only palliative and include surgical resection and focal radiotherapy. A large number of chemotherapeutic agents (e.g., alkylating agents such as temozolomide and nitrosureas such as carmustine) have also been tested, but no remarkable improvement on patient survival has been achieved as yet [2, 4]. Likewise, although dendritic cell- and peptide-based immunotherapy strategies appear promising as a safe approach to induce an antitumor immune response [5], no immunotherapy or gene therapy trial performed to date has been significantly successful. It is therefore essential to develop new therapeutic strategies for the management of GBM, which will most likely require a combination of therapies to obtain significant clinical results. In this paper, we summarize the current knowledge

on how a family of compounds, the cannabinoids, exerts anti-glioma actions in laboratory animals and how a potential cannabinoid-based therapy for GBM might be envisaged.

Cannabinoids and Their Receptors

The hemp plant *Cannabis sativa* L. produces approximately 70 unique compounds known as cannabinoids, of which, Δ^9-tetrahydrocannabinol (THC) is the most important owing to its high potency and abundance in cannabis [6, 7]. THC exerts a wide variety of biological effects by mimicking endogenous substances—the endocannabinoids, including anandamide and 2-arachidonoylglycerol—that bind to and activate specific cannabinoid receptors [7, 8]. So far, two types of cannabinoid-specific $G_{i/o}$ protein-coupled receptors, CB_1 and CB_2, have been cloned and characterized from mammalian tissues [9]. Many of the effects of cannabinoids rely on CB_1 receptor activation. This receptor is particularly abundant in discrete areas of the brain and peripheral nerve terminals where it mediates endocannabinoid-dependent neuromodulation [10], but is also expressed in many extra-neural sites. In contrast, CB_2 receptors were first described in cells and tissues of the immune system and have long been believed to be absent from the brain. Recent data, however, question this notion and support the existence of CB_2 receptors in the central nervous system, specifically in microglial cells, astrocytes, some neuron subpopulations, and glioma cells [11].

Extensive molecular and pharmacological studies have demonstrated that cannabinoids inhibit adenylyl cyclase through CB_1 and CB_2 receptors. The CB_1 receptor also modulates ion channels, inducing, for example, inhibition of N- and P/Q-type voltage-sensitive Ca^{2+} channels and activation of G-protein-activated inwardly rectifying K^+ channels [9]. Besides these well-established cannabinoid receptor-coupled signaling events, cannabinoid receptors also modulate several pathways that are more directly involved in the control of cell proliferation and survival, including extracellular signal-regulated kinase (ERK) [12], c-Jun N terminal kinase and p38 mitogen-activated protein kinase [13, 14], phosphatidylinositol 3-kinase (PI3K)/Akt [15], focal adhesion kinase [16], and the sphingomyelin cycle [17].

Antitumoral Activity of Cannabinoids

Cannabinoids have been known for several decades to exert palliative effects in cancer patients, and nowadays, capsules of THC (Marinol-TM) and its synthetic analogue nabilone (Cesamet-TM) are approved to treat nausea and emesis associated with cancer chemotherapy [18]. In addition, several clinical trials are testing other potential palliative properties of cannabinoids in oncology such as appetite stimulation and analgesia [19, 20]. Besides these palliative actions, cannabinoids have been proposed as potential antitumoral agents on the basis of experiments performed both in cultured cells and in animal models of cancer. These antiproliferative properties of cannabis compounds were first reported about 30 years ago when it was shown that THC inhibits lung adenocarcinoma cell growth in vitro and after oral administration in mice [21]. Although these observations were promising, further studies in this area were not performed until the late 1990s, mostly by Di Marzo, Bifulco and colleagues (reviewed in [22]) and Guzmán's group (reviewed in [19]). A number of plant-derived (for example, THC, and cannabidiol), synthetic (for example, WIN-55,212-2 and HU-210) and endogenous cannabinoids (for example, anandamide and 2-arachidonoylglycerol) are now known to exert antiproliferative actions on a wide spectrum of tumor cells in culture [19]. More importantly, cannabinoid administration to nude mice curbs the growth of various types of tumor xenografts, including lung carcinoma [21], glioma [23], thyroid epithelioma [24], lymphoma [25], skin carcinoma [26], pancreatic carcinoma [27], and melanoma [28]. The requirement of cannabinoid receptors for this antitumoral activity has been revealed by various biochemical and pharmacological approaches, in particular, by determining cannabinoid receptor expression in the tumors and by using selective cannabinoid receptor agonists and antagonists.

Antitumoral Activity of Cannabinoids in Gliomas

Most of our research on cannabinoid antitumoral action has focused on gliomas. Initial experiments in cultured glioma cells showed that incubation with cannabinoids induces cell death by an apoptotic mechanism [29]. Further studies with animal models showed that local administration of THC or WIN-55,212-2 reduced the size of tumors generated by intracranial inoculation of C6 glioma cells in rats, leading to complete eradication of gliomas and increased survival in one third of the treated rats [23]. Additional studies used tumor xenografts generated by subcutaneous injection of glioma cells in the flank of immune-deficient mice. Local administration of THC, WIN-55,212-2, or the selective CB_2 cannabinoid receptor agonist JWH-133 decreased the growth of tumors derived not only from the rat glioma C6 cell line but also from GBM cells obtained from tumor biopsies of patients [23, 30].

These and other studies also showed that activation of cannabinoid receptors on glioma cells modulates key signaling pathways involved in cell proliferation and survival.

Although the downstream events by which cannabinoids exert their antitumoral action in gliomas are not completely unraveled, there is substantial evidence for the involvement of at least two mechanisms: induction of apoptosis of tumor cells and inhibition of tumor angiogenesis (Fig. 1).

Induction of Apoptosis

Cannabinoids induce apoptosis of cultured glioma cells [23, 29]. Different studies have shown that this effect relies on the activation of cannabinoid receptors and the accumulation of the pro-apoptotic sphingolipid ceramide [23, 31, 32]. However, the molecular mechanisms involved in the triggering of the apoptotic signal by cannabinoids have started to be unraveled only very recently. By using a DNA array approach, we have identified a series of genes that are selectively up-regulated in cannabinoid-sensitive but not cannabinoid-resistant glioma cells upon THC treatment [33]. One of these genes was the stress-regulated protein p8 (also designated candidate of metastasis 1, Com-1) that belongs to the family of HMG-I/Y transcription factors and was originally described as a gene induced in acute pancreatitis [34]. Different experimental approaches confirmed that p8 up-regulation is essential for the pro-apoptotic and antitumoral action of cannabinoids in gliomas and pancreatic tumors [27, 33].

The acute increase of p8 levels after cannabinoid treatment triggers a cascade of events that involves the up-regulation of the activating transcription factor 4 (ATF-4) and the C/EBP-homologous protein (CHOP, also called DDIT3 and GADD153). These two transcription factors cooperate in the induction of the tribbles homologue 3 (TRB3, also called TRIB3), a pseudokinase that has been implicated in the induction of apoptosis of tumor cells and neurons [35]. In line with this observation, selective knockdown of ATF-4 and TRB3 prevented cannabinoid-induced apoptosis, indicating that this signaling route also operates in glioma cells after treatment with cannabinoids [33] (Fig. 2). ATF-4, CHOP, and TRB3 (together with other genes selectively induced upon THC treatment of glioma cells) [33] participate in the endoplasmic reticulum (ER) stress response. A series of ER alterations such as calcium depletion, protein misfolding, and impairment of protein trafficking to the Golgi triggers this response, which involves attenuation of protein synthesis and selective transcription and translation of a series of genes, mainly involved in favoring correct protein folding [36]. When these ER alterations cannot be repaired by the ER stress response, the damaged cells undergo apoptosis. Several stimuli, including ischemia [37], viral infection [38, 39], and drugs such as tunicamycin [35] or cisplatin [40], induce apoptosis through this pathway. Of interest, cannabinoid-induced ceramide accumulation and ER stress induction seem to be closely linked. Thus, inhibition of ceramide synthesis de novo prevents THC-induced p8, ATF-4, CHOP, and TRB3 up-regulation [33] as well as ER dilation (authors' unpublished observations), indicating that ceramide accumulation is an early event in cannabinoid-triggered ER stress and apoptosis in glioma cells.

Unlike this pro-apoptotic action of cannabinoids on transformed cells, treatment of primary cultured astrocytes with these compounds does not trigger ceramide accumu-

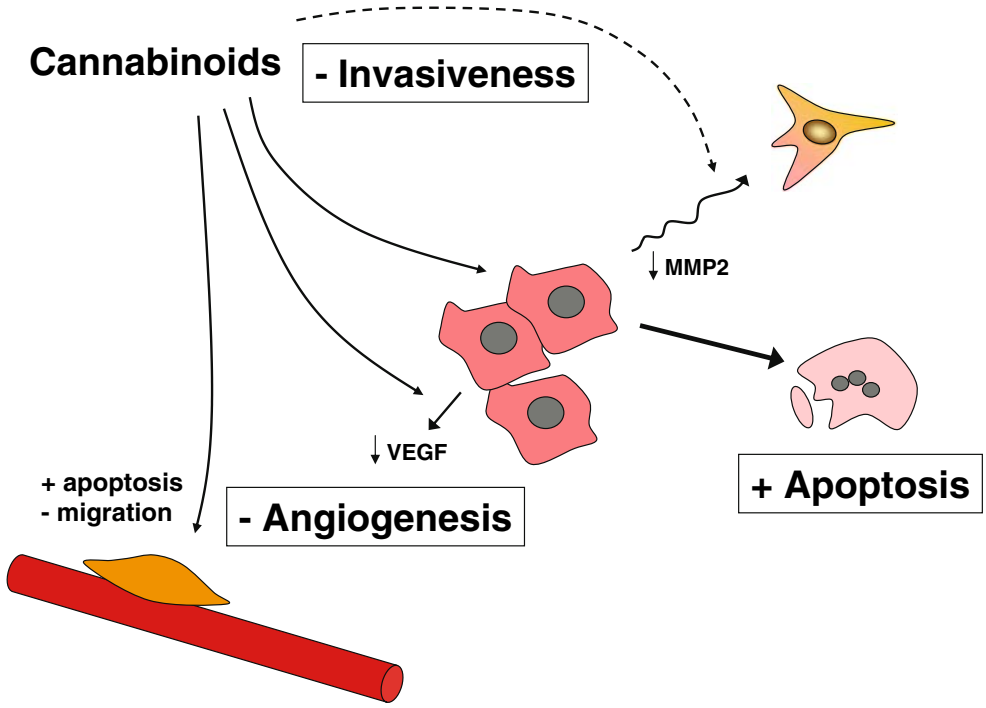

Fig. 1 Antitumoral effect of cannabinoids in gliomas. Cannabinoid administration to mice decreases the growth of gliomas by several mechanisms, including at least: (1) reduction of tumor angiogenesis, (2) induction of tumor cell apoptosis, and perhaps (3) inhibition of tumor cell migration and invasiveness

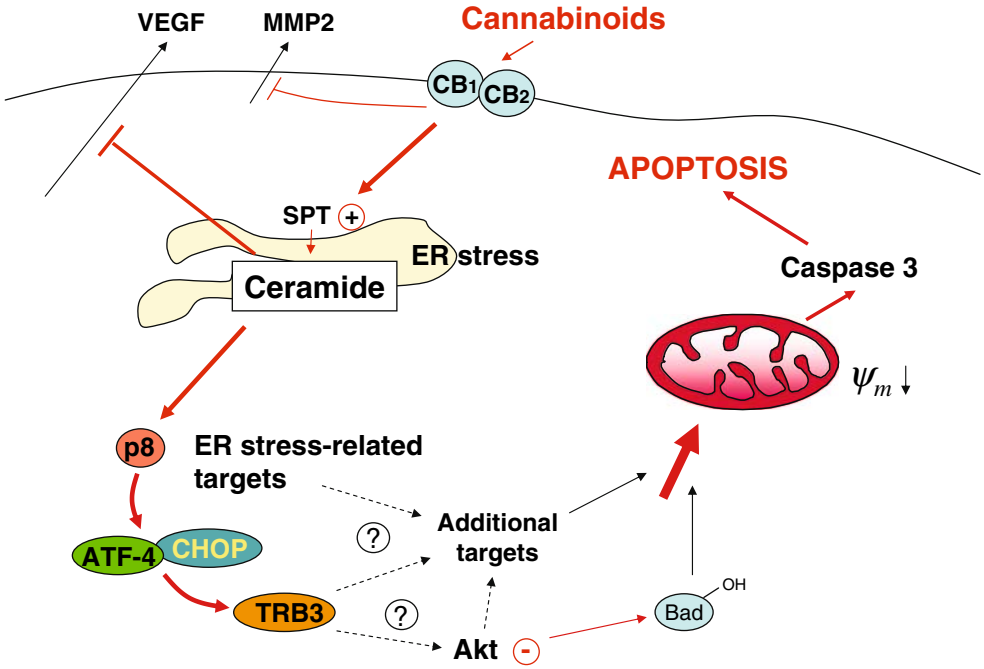

Fig. 2 Mechanism of cannabinoid pro-apoptotic action in glioma cells. Cannabinoid treatment induces apoptosis of glioma cells via ceramide accumulation and activation of an ER stress-related pathway. The stress-regulated protein p8 plays a key role in this effect by controlling the expression of ATF-4, CHOP and TRB3. This cascade of events triggers the activation of the mitochondrial intrinsic apoptotic pathway through mechanisms that have not been unraveled as yet. Cannabinoids also decrease the expression of various tumor-progression molecules such as VEGF and MMP2

lation [41] or induction of the aforementioned ER stress-related genes [33] (Fig. 3). Furthermore, cannabinoids promote the survival of astrocytes [42], oligodendrocytes [43], and neurons [44] in different models of injury, suggesting that the antiproliferative effect of cannabinoids is selective for brain-tumor cells, the viability of normal brain cells being unaffected or even favored by cannabinoid challenge.

The processes downstream of ER stress activation involved in the execution of cannabinoid-induced apoptosis of glioma cells are only partially understood. Decreased mitochondrial membrane potential and caspase 3 activation are observed in cannabinoid-treated glioma cells [33, 45], suggesting that execution of apoptosis occurs via activation of the mitochondrial intrinsic pathway (Fig. 2), a mechanism that is involved in the induction of apoptosis by cannabinoids in other types of tumor cells [46, 47]. Cannabinoid treatment induces loss of mitochondrial membrane potential in $p8^{+/+}$, but not p8-deficient mouse embryonic fibroblasts, suggesting that the p8-regulated

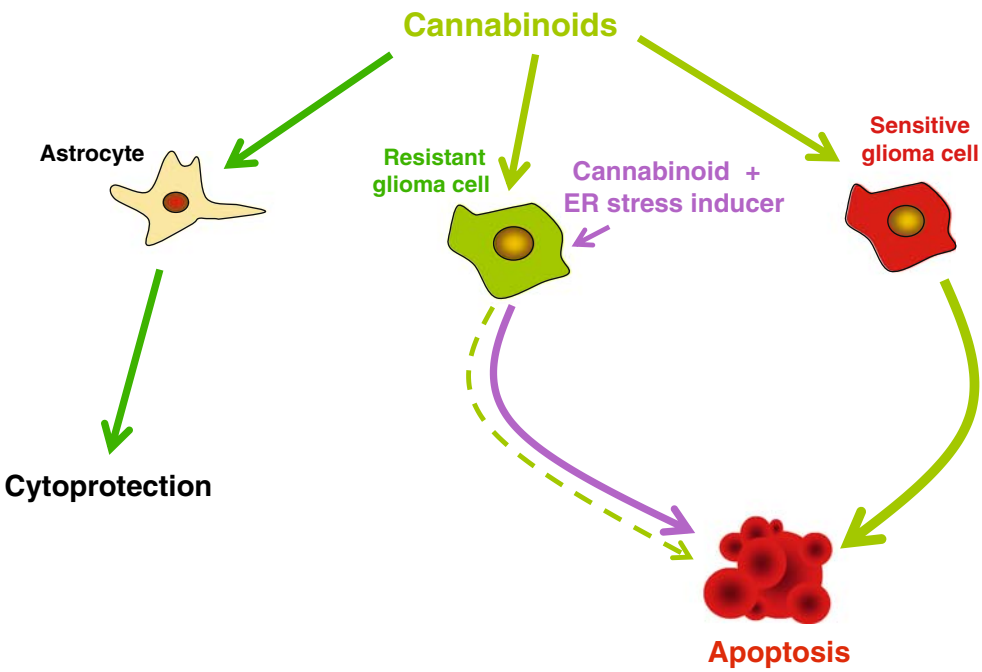

Fig. 3 Synergy of cannabinoids and endoplasmic reticulum stress inducers. Cannabinoid-induced activation of the ER stress pro-apoptotic pathway is blunted in cannabinoid-resistant glioma cells. This resistance can be overcome by co-treatment with cannabinoids and ER stress-inducing drugs. Cannabinoids do not activate the ER stress pathway in astrocytes; on the contrary, they protect these cells from different pro-apoptotic stimuli

pathway described above is required for the activation of the mitochondrial pro-apoptotic pathway. On the other hand, cannabinoids inhibit Akt in glioma cells, an effect that is prevented by pharmacological blockade of ceramide synthesis de novo [32]. In addition, cannabinoids lead to decreased phosphorylation of the BH3-only protein Bad [45], an Akt and extracellular signal-regulated protein kinase (ERK) cascade target which phosphorylation inhibits apoptosis via the intrinsic pathway. These observations suggest that regulation of Akt could be involved in the connection between the ceramide/p8-regulated pathway and the activation of the mitochondrial pro-apoptotic route (Fig. 2). Modulation of ERK, as well as of the other mitogen-activated protein kinases, could also participate in the induction of apoptosis by cannabinoids in gliomas [23]. Intriguingly, both inhibition (e.g., [45]) and activation (e.g., [23]) of ERK have been proposed to participate in this effect. Further research is therefore necessary to clarify the involvement of this signaling cascade in cannabinoid-induced apoptosis.

Inhibition of Tumor Angiogenesis

To grow beyond minimal size, tumors must generate a new vascular supply (angiogenesis) for purposes of cell nutrition, gas exchange, and waste disposal, and therefore, blocking the angiogenic process constitutes one of the most promising antitumoral approaches currently available. Immunohistochemical analyses in mouse models of glioma [48], skin carcinoma [26], and melanoma [28] have shown that cannabinoid administration turns the vascular hyperplasia characteristic of actively growing tumors to a pattern of blood vessels characterized by small, differentiated, and impermeable capillaries. This is associated with a reduced expression of vascular endothelial growth factor (VEGF) and other proangiogenic cytokines such as angiopoietin-2 and placental growth factor [26, 48, 49], as well as of type 1 [49] and type 2 [50] VEGF receptors, in cannabinoid-treated tumors. Pharmacological inhibition of ceramide synthesis de novo abrogates the antitumoral and antiangiogenic effect of cannabinoids in vivo and decreases VEGF production by glioma cells in vitro and by gliomas in vivo [50], indicating that ceramide plays a general role in cannabinoid antitumoral action.

Other reported effects of cannabinoids might be related to the inhibition of tumor angiogenesis and invasiveness by these compounds (Fig. 1). Thus, activation of cannabinoid receptors on vascular endothelial cells in culture inhibits cell migration and survival [48]. Endothelial cell apoptosis was also potently triggered by cannabinoid quinonoid derivatives, although this action seems to be cannabinoid receptor-independent [51]. In addition, cannabinoid administration to glioma-bearing mice decreases the activity and expression of matrix metalloproteinase-2 (MMP2), a proteolytic enzyme that allows tissue breakdown and remodeling during angiogenesis and metastasis [48]. In line with this notion, cannabinoid intraperitoneal injection reduces the number of metastatic nodes derived from melanoma [28], lung [49], and breast [52] cancer cells injected into the paws of mice.

Other Potential Targets of Cannabinoid Action

The identification of the cell(s) from which gliomas originate is still a matter of debate. Although neoplastic transformation of differentiated glial cells was, for many years, the most accepted hypothesis to explain the origin of gliomas, recent findings support the existence of a stem cell-derived origin for different types of cancers such as gliomas, hematopoietic, breast, and prostate tumors [53]. In particular, glioma-derived stem-like cells, which may represent the consequence of transformation of the normal neural stem cell compartment, seem to be crucial for the malignancy of gliomas [54]. We have recently shown that glioma stem-like cells derived from GBM biopsies and glioma cell lines express CB_1 and CB_2 cannabinoid receptors, the activation of which promotes cell differentiation and inhibits gliomagenesis [55]. Interestingly, gene array experiments indicated that cannabinoid receptor activation on glioma stem-like cells down-regulates epidermal growth factor (EGF) and fibroblast growth factor receptors, in line with the suggestion that cannabinoids mediate at least part of their apoptotic actions on skin and prostate cancer cells by attenuating EGF receptor expression [26, 56] and/or tyrosine kinase activity [26]. In addition, the antiproliferative action of cannabinoids in breast, prostate, and thyroid cancer cells may involve a decrease in the activity and/or expression of prolactin [57], nerve growth factor [58], and type 1 VEGF receptors [49]. Furthermore, cannabinoids inhibit type 2 VEGF receptor activation in glioma cells [50]. Taken together, these results indicate that attenuation of signaling through tyrosine kinase receptors may constitute a common mechanism of cannabinoid growth-inhibiting action.

Cannabinoids as Potential Therapeutic Agents for the Treatment of Gliomas

On the basis of the aforementioned preclinical findings, we have recently conducted a pilot phase I clinical trial in which nine patients with actively growing recurrent GBM were administered THC intratumorally [59]. The patients had previously failed standard therapy (surgery and radiotherapy) and had clear evidence of tumor progression. The primary endpoint of the study was to determine the

safety of intracranial THC administration. THC action on length of survival and various tumor cell parameters was also evaluated. A dose escalation regime for THC administration was assessed. The initial dose of THC delivered to the patients was 20–40 μg at day 1, increasing progressively for 2–5 days up to 80–180 μg/day. The median duration of THC administration was 15 days. Under these conditions, cannabinoid delivery was safe and could be achieved without significant psychoactive effects. Median survival of the cohort from the beginning of cannabinoid administration was 24 weeks (95% CI, 15–33). THC decreased tumor cell proliferation (as determined by Ki67 immunostaining; [59]) and increased tumor cell apoptosis (as determined by active-caspase 3 immunostaining; [33]) when administered to two patients.

The good safety profile observed for THC, together with its possible antiproliferative action on tumor cells, may set the basis for future trials aimed at evaluating the potential antitumoral activity of cannabinoids. These possible new trials could involve one or more of the following modifications:

(1) Patients with newly diagnosed tumors. Pilot placebo-controlled trials for recurrent glioblastoma multiforme with temozolomide, a DNA-damaging agent that constitutes the current benchmark for the management of malignant gliomas, showed a very slight impact on overall length of survival (median survival=24 weeks; 6-month survival=46–60%) [60]. Further trials in patients with newly diagnosed tumors revealed a clear improvement in the therapeutic efficacy of temozolomide through the development of various administration regimes [2, 4, 61]. It is therefore conceivable that better outcomes could also be obtained with cannabinoid-based therapies in newly diagnosed gliomas.

(2) THC in combination with temozolomide. GBM—particularly when relapse occurs—is an extremely lethal disease. The success of potential treatments is usually hampered by factors such as the rapid growth, remarkable heterogeneity, high degree of infiltration, and extreme resistance to chemotherapy displayed by these tumors. It is therefore conceivable that combined therapies could provide better results than single-agent therapies. For example, by synergizing via complementary signaling pathways, THC plus temozolomide might exert a more potent clinical impact than either THC or temozolomide alone.

(3) Non-invasive administration route. Although intratumoral delivery may allow a high local concentration of the drug in situ, in the case of large tumors such as actively growing recurrent GBM, the local perfusion through a catheter placed at one point of the tumor constitutes an obvious limitation of the technique. In addition, a non-invasive, less traumatic route would be more desirable for clinical practice. Alternative or complementary options for THC administration would include oral capsules and oro-mucosal sprays.

(4) Other cannabinoid receptors ligands. Although the use of cannabinoids in medicine may be limited by their well-known psychotropic effects, it is generally believed that cannabinoids display a good drug safety profile and that their potential adverse effects are within the range of those accepted for other medications, especially in cancer treatment [19, 20]. In line with this idea, THC delivery in the aforementioned clinical study was safe and could be achieved without overt psychoactive effects. As the possible antitumoral action of nabilone has never been evaluated preclinically, THC remains as the only cannabinoid receptor agonist currently available for cancer clinical trials. Nonetheless, most likely, THC is not the most appropriate cannabinoid agonist for future antitumoral strategies owing to its high hydrophobicity, relatively weak agonistic potency, and ability to elicit CB_1 receptor-mediated psychoactivity. The current synthetic cannabinoid agonists that have been reported to exert antitumoral actions in animal models and that could theoretically circumvent, at least in part, the pharmacokinetic and pharmacodynamic limitations of THC (e.g., WIN-55,212-2, a more potent and less hydrophobic CB_1/CB_2-mixed agonist [23], and JWH-133, a more potent CB_2-selective agonist [30]) have not been developed yet for their use in the clinic. However, because some of these agonists are currently of interest for drug development in other areas, notably pain control, they may become available for clinical trials in the not too distant future.

(5) Other types of tumors. As mentioned above, we and others have shown that THC and synthetic cannabinoids, besides their anti-glioma activity, inhibit the growth of different types of tumor xenografts in mice (see above). Trials on these and other types of tumors might also be run to test the antitumoral activity of cannabinoids in these malignant diseases.

Future Perspectives

One of the most striking features of gliomas is their high resistance to conventional chemotherapy. In addition, these tumors exhibit a great heterogeneity due to the frequent presence within the same tumor of various cell subpopulations with different morphologic and even genetic characteristics. Moreover, these different cells show different sensitivity to chemotherapy or radiotherapy. Thus, it is widely believed that strategies aimed at reducing the

mortality caused by these tumors should consist of targeted therapies capable of providing the most efficacious treatment for each individual cell subpopulation, tumor, and patient. This new therapeutic approach would require not only the utilization of new cocktails of chemotherapeutic drugs but, more importantly, the identification of the markers associated with the resistance of tumor cells to these new therapies. The significant antiproliferative action of cannabinoids in animal models of gliomas, together with their low toxicity compared with other chemotherapeutic agents, might make these compounds promising new ingredients of antitumoral cocktails for the management of GBM. Studies performed in our laboratory suggest that resistance of glioma cells to cannabinoid treatment correlates with the ability of these cells to block the activation of the ER stress pathway (authors' unpublished observations). In addition, we have observed that agents that induce ER stress interacts synergistically with cannabinoids [33]. Likewise, overexpression of p8 or TRB3 sensitizes resistant glioma cells to a further treatment with cannabinoids [33]. These observations suggest that activation of the aforementioned ER stress pathway should be investigated as a potential strategy to enhance the response of gliomas to chemotherapy. Another factor that should be taken into account is the expression level of cannabinoid receptors in the cells of the tumor to be treated. Research to be performed during the next few years should help to clarify which are the optimal conditions of cannabinoid utilization by identifying the factors that confer resistance to cannabinoid treatment as well as the most efficient approaches for enhancing their antitumoral activity alone or in combination with other therapies.

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