Chapter 11
Cannabinoid Signaling in Glioma Cells

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Abstract Cannabinoids are a group of structurally heterogeneous but pharmacologically related compounds, including plant-derived cannabinoids, synthetic substances and endogenous cannabinoids, such as anandamide and 2-arachidonoylglycerol. Cannabinoids elicit a wide range of central and peripheral effects mostly mediated through cannabinoid receptors. There are two types of specific $G_{i/o}$-protein-coupled receptors cloned so far, called CB1 and CB2, although an existence of additional cannabinoid-binding receptors has been suggested. CB1 and CB2 differ in their predicted amino acid sequence, tissue distribution, physiological role and signaling mechanisms. Significant alterations of a balance in the cannabinoid system between the levels of endogenous ligands and their receptors occur during malignant transformation in various types of cancer, including gliomas. Cannabinoids exert anti-proliferative action in tumor cells. Induction of cell death by cannabinoid treatment relies on the generation of a pro-apoptotic sphingolipid ceramide and disruption of signaling pathways crucial for regulation of cellular proliferation, differentiation or apoptosis. Increased ceramide levels lead also to ER-stress and autophagy in drug-treated glioblastoma cells.

Keywords Cannabinoids • Apoptosis • Autophagy • ER-stress • Gliomas

Abbreviations

AEA	Anandamide, arachidonoylethanolamide
2-AG	2-Arachidonoylglycerol
Akt	Protein kinase B/Akt
ATF4	Activating transcription factor 4
cAMP	Cylic adenosine monophosphate
CB1	Cannabinoid receptor type 1
CB2	Cannabinoid receptor type 2
CHOP	The C/EBP-homologous protein

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DAG	Diacylglycerol
eIF2α	Eukaryotic translation initiation factor 2α
ER	Endoplasmic reticulum
ERK1/2	Extracellular signal-regulated kinase 1/2
FAAH	Fatty acid amide hydrolase
IP_3	Inositol 1,4,5-trisphosphate
LPS	Lipopolysacharide
MAPK	Mitogen-activated protein kinase
MEK	MAP kinase-ERK kinase
MGL	Monoacylglycerol lipase
mTORC1	Mammalian target of rapamycin, complex 1
NAPE	N-Arachidonylphosphatidylethanolamide
PI3K	Phosphatidylinositol 3-kinase
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
Δ^9-THC	(−)-*trans*-Δ^9-Tetrahydrocannabinol
TRB3	Pseudo-kinase tribbles homologue 3
TRPV1	Transient receptor potential cation channel subfamily V member 1, capsaicin or vanilloid receptor
WIN55,212-2	Synthetic cannabinoid

11.1 Introduction

Preparations from *Cannabis sativa*, the hemp plant, have been used for centuries for both medicinal and recreational purposes (Howlett et al. 2002; Mackie 2006). Isolation of the active components of the plant, called cannabinoids, in 1960s, as much as subsequent cloning of cannabinoid receptors, discovery of their endogenous ligands and development of synthetic cannabinoids contributed to an intensive burst of cannabinoid research. Along with our expanding comprehension of mechanisms of cannabinoids action, targeting cannabinoid signaling for therapeutic purposes has inevitably emerged as an interesting area of scientific and clinical investigations.

One of the most extensively studied applications of cannabinoids is their potential use as anti-cancer agents. Anti-proliferative effects of cannabinoids have been reported in various cultured cancer cells, including neural, breast, prostate, skin, thyroid cancer cells and leukemia cells. Several studies demonstrated anti-tumor activity of cannabinoids in animal models (Guzman 2003). Since the first publication by Sanchez and co-workers in 1998, providing evidence that cannabinoids are effective in inducing glioma cell death, a growing interest of several groups, including ours, has been focused on understanding of molecular mechanisms of cannabinoid signaling in glioma cells and therapeutic potential of cannabinoids in glial tumors (Sanchez et al. 1998).

Signaling pathways and intracellular processes underlying cannabinoid action on glioma cells are reviewed here. Due to the number of studies carried out in the recent years on mechanisms of anti-proliferative effects of cannabinoids, these mechanisms are described in details. A brief overview of the endocannabinoid system, canonical signal transduction pathways coupled to the activation of cannabinoid receptors and current scientific data relevant to the use of cannabinoids in treatments of glioblastomas are also included.

11.2 Cannabinoids and Their Receptors

Cannabinoids are a group of structurally heterogeneous but pharmacologically related compounds classified into three subtypes: plant-derived, synthetic and endogenous cannabinoids (Fig. 11.1). Plant-derived cannabinoids (phytocannabinoids) are uniquely found in the cannabis plant. Although the pharmacology of the majority of them is unknown, (−)-*trans*-Δ^9-tetrahydrocannabinol (Δ^9-THC) is recognized as the most potent out of approximately 70 identified phytocannabinoids. Various modifications of the chemical structure of natural cannabinoids led to generation of a still growing set of synthetic cannabinoids. Exogenous cannabinoids mimic the action of endogenous compounds, known as endocannabinoids, ubiquitously produced in both vertebrate and invertebrate tissues.

Two arachidonic acid derivatives, arachidonoylethanolamide (anandamide, AEA) and 2-arachidonoylglycerol (2-AG) are the best characterized endogenous cannabinoids, although some other amides and esters of long chain polyunsaturated fatty acids also exhibit cannabimimetic properties. Endocannabinoids are produced as rapidly inactivated lipid mediators and their levels are strictly controlled by a transporter system and hydrolyzing enzymes (Fig. 11.2). Biosynthesis of endocannabinoids is activated "on demand". AEA is produced from lipid membrane precursors upon the stimuli that increase intracellular concentrations of calcium. Increased calcium levels activate N-acyltransferase, the enzyme generating an AEA precursor N-arachidonylphosphatidylethanolamide (NAPE), which is then hydrolyzed by NAPE phospholipase D. Separate mechanisms have been described for 2-AG, which is most likely produced through the phospholipase C/diacylglycerol lipase pathway.

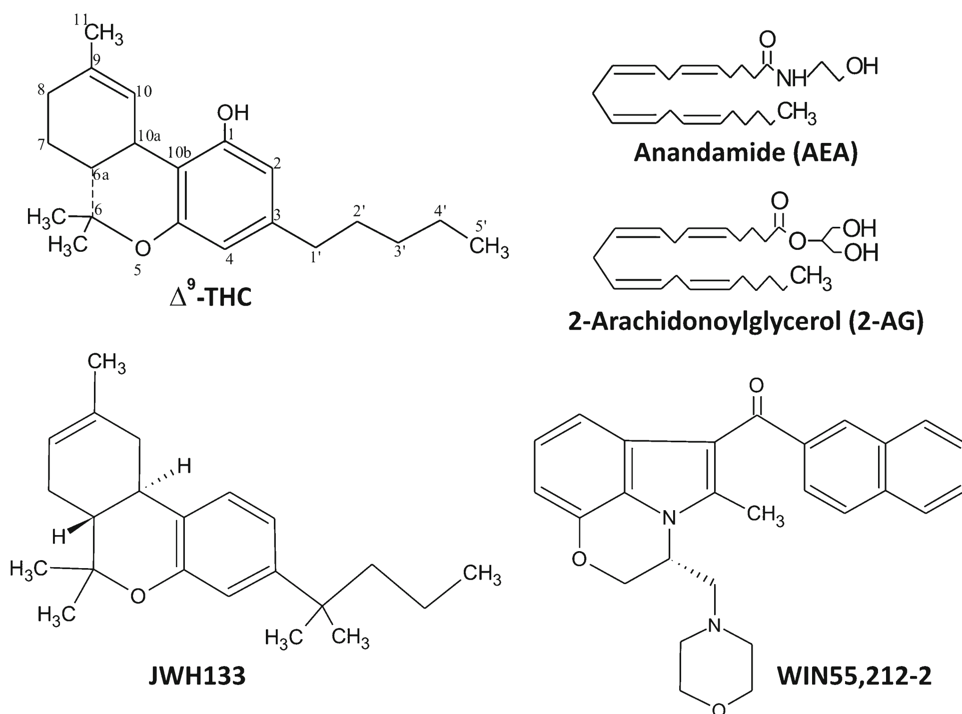

Fig. 11.1 Chemical structures of cannabinoids. Plant-derived Δ^9-THC ((−)-*trans*-Δ^9-tetrahydrocannabinol), endocannabinoids: anandamide (N-arachidonoylethanolamine) and 2-arachidonoylglycerol, synthetic cannabinoids JWH133, selective for non-psychoactive CB2 receptor ((6aR,10aR)-3-(1,1-Dimethylbutyl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6H-dibenzo[b,d] pyran), and WIN55,212-2, a CB1/CB2 receptor agonist ((R)-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl) pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone)

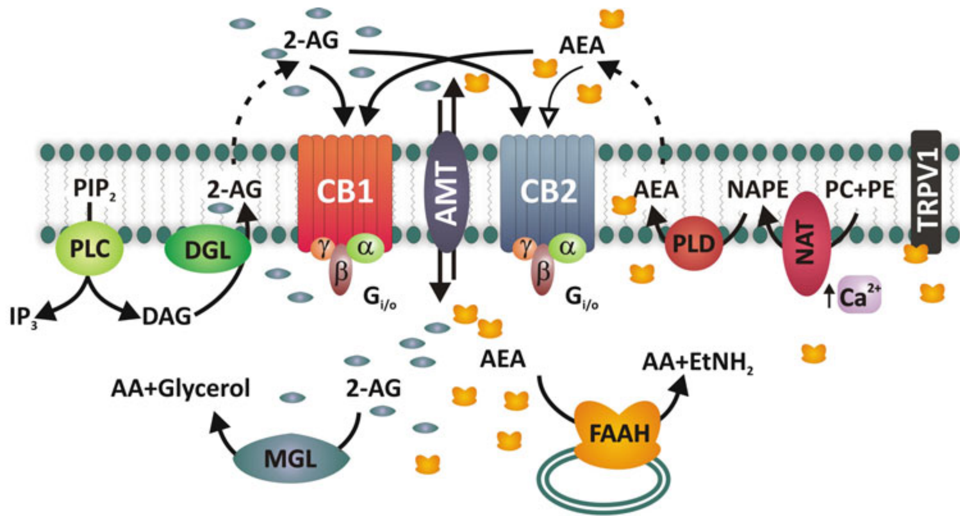

Fig. 11.2 The endocannabinoid system. Two arachidonic acid derivatives, anandamide (AEA) and 2-arachidonoyl glycerol (2-AG), are produced by cleavage of lipid precursors in response to increased calcium levels or receptor stimulation. AEA is generated from *N*-arachidonoylphosphatidylethanolamine (NAPE). NAPE originates from transfer of arachidonic acid from the *sn*-1 position of 1,2-*sn*-diarachidonoylphosphatidylcholine (PC) to phosphatidylethanolamine (PE), catalyzed by a Ca^{2+}-dependent *N*-acyltransferase (NAT). NAPE is cleaved by phospholipase D (PLD) to release AEA and phosphatidic acid. 2-AG is synthesized in two steps via generation of 1-acyl-2-arachidonoylglycerol (diacylglycerol, or DAG) form phospholipids (such as phosphatidylinositol-4,5-bisphosphate PIP_2) by phospholipase C (PLC) and subsequent hydrolysis of DAG by a diacylglycerol lipase (DGL). AEA and 2-AG are rapidly removed from the extracellular space possibly through a common purported high-affinity transporter (AMT), a carrier possibly working in both inward and outward directions. Once taken up by cells, AEA is a substrate for the fatty acid amide hydrolase (FAAH), which breaks the amide bond and releases arachidonic acid (AA) and ethanolamine ($EtNH_2$). 2-AG is primarily degraded by a specific monoacylglycerol lipase (MGL). Both AEA and 2-AG bind to and activate CB1 and CB2 receptors; however, AEA is a weaker agonist than 2-AG at CB1 and is only a partial agonist at CB2. The transient receptor potential cation channel subfamily V member 1 (TRPV1) is another key molecular target of AEA, but importantly not of 2-AG. The binding site of TRPV1 receptors for AEA is on an intracellular domain. All elements of the ECS are located in the plasma membrane except from FAAH, which is bound to intracellular membranes, and MGL, which is cytosolic

Endocannabinoid production and release by glial cells may underlie the neuroprotective properties of cannabinoids in experimental models, however its physiological significance is still unknown.

Although endocannabinoids, lipophilic in nature, can freely cross cell membranes, evidence suggests the existence of mechanisms facilitating endocannabinoid internalization. Endocannabinoids are transported into cells by a purported high-affinity membrane transporter to undergo enzymatic hydrolysis. Two degradative enzymes for endocannabinoids have been described so far: the fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MGL), responsible mainly for AEA and 2-AG degradation, respectively (Fig. 11.2). FAAH and MGL exhibit a wide distribution in the rat and human central nervous system, but are mostly confined to neurons. FAAH is primarily a postsynaptic enzyme, whereas MGL localizes to presynaptic terminals. There are conflicting data on the expression of endocannabinoid metabolizing enzymes in glial cells in normal brain. FAAH is increased in hypertrophic astrocytes surrounding AD plaques or inflammatory infiltrates but is not detected in other types of glial cells (Pazos et al. 2005). MGL can be found in oligodendrocytes at different developmental stages, however in cultured oligodendrocyte progenitor cells (OPCs) MGL expression was lower than during maturation stages, suggesting that the increased levels of 2-AG are required for OPCs differentiation (Gomez et al. 2010).

Cannabinoids elicit a wide range of central and peripheral effects, which are mediated mostly through cannabinoid receptors (Howlett et al. 2002). There are two types of specific seven-transmembrane, $G_{i/o}$-protein-coupled receptors cloned so far, called CB1 and CB2 (Figs. 11.2 and 11.3), although an existence of additional cannabinoid-binding receptors has been suggested (Howlett et al. 2002; Stella 2004). CB1 and CB2 differ in their predicted amino acid sequence, tissue distribution and expression pattern. Overall homology between CB1 and CB2 is remarkably low (e.g. 44 % for human and 68 % for murine receptors) with significant disparities in the domains, which interact with G proteins and effector proteins. Thus, quite expectedly two cannabinoid receptors were shown to play distinct physiological roles and share only some common signaling mechanisms.

Many of the effects of cannabinoids rely on the CB1 receptor activation. CB1 is particularly abundant in discrete areas of the brain, such as cortex, hippocampus, basal ganglia and cerebellum, as well as in peripheral nerve terminals, where it mediates inhibition of neurotransmitter release and is involved in the control of motor activity, memory, cognition appetite and sensory perception. CB1 is also present in some extra-neural sites, such as testis, uterus, vascular endothelium, eye, spleen, and tonsils, controlling processes such as vascular tone, intraocular pressure and immune response (Howlett et al. 2002). Glial cells have been shown to express CB1 receptors, although their precise function in astrocytes, oligodendrocytes and microglia has been only partly unveiled.

By contrast, the CB2 receptor is predominantly expressed in cells and organs of the immune system (Howlett et al. 2002). The role of peripheral CB2 receptor activation under physiologic conditions is not well defined. CB2 signaling is involved in B-cell differentiation and modulation of immune response. Increased levels of CB2 are reported in tissues during development, inflammation, injury and cancer, revealing a critical role for the CB2 receptor in regulating these processes (Howlett et al. 2002). The CB2 receptor was believed to be absent from healthy brain, however, its expression has been detected in microglia – brain macrophages (Gong et al. 2006; Stella 2004), as well as in a small subpopulation of neurons (Gong et al. 2006; Van Sickle et al. 2005). Animal experiments show that CB2-selective agonists do not induce widespread psychoactive effects attributed to activation of the CB1 receptor (Guzman 2003; Valenzano et al. 2005).

Extensive molecular studies have demonstrated that activation of the CB1 and CB2 cannabinoid receptors upon agonist binding is canonically linked to inhibition of adenylyl cyclase via the α-subunit of $G_{i/o}$-protein. The consequent decrease in cyclic AMP (cAMP) production leads to down-regulation of protein kinase A (PKA) and impedes PKA – dependent signaling. The CB1 receptor, acting as a guardian on presynaptic membranes, is coupled to ion channels, inducing for example inhibition of voltage-gated L, N- and P/Q Ca^{2+} channels and activation of G-protein activated inwardly rectifying K^+ channels (Howlett et al. 2002). Cannabionoids have been also reported to affect several pathways that are more directly involved in the control of cell proliferation, differentiation and survival. Depending on a cell type and treatment conditions, signaling via cannabinoid receptors is linked to activation or inhibition of phosphatidylinositol 3-kinase (PI3K)/Akt pathway. Stimulation of either CB1 or CB2 receptor results in activation of three classes of mitogen-activated protein kinases (MAPKs): extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 MAPK. Cannabinoid receptors are also coupled to activation of phospholipase C and a subsequent release of Ca^{2+} from inositol-1,4,5-trisphosphate (IP_3)-sensitive stores, as well as to modulation of the sphingomyelin cycle (Guzman 2003; Howlett et al. 2002).

There is emerging evidence suggesting additional pharmacological targets for cannabinoids. Peroxisome proliferator-activated receptors (PPARs) and transient receptor potential cation channel subfamily V member 1 (TRPV1, also known as capsaicin or vanilloid receptor) have been shown to be activated by cannabinoids, including Δ^9-THC and endogenous AEA. However, the precise role of these receptors in cannabinoid signaling is still unclear (Mackie and Stella 2006).

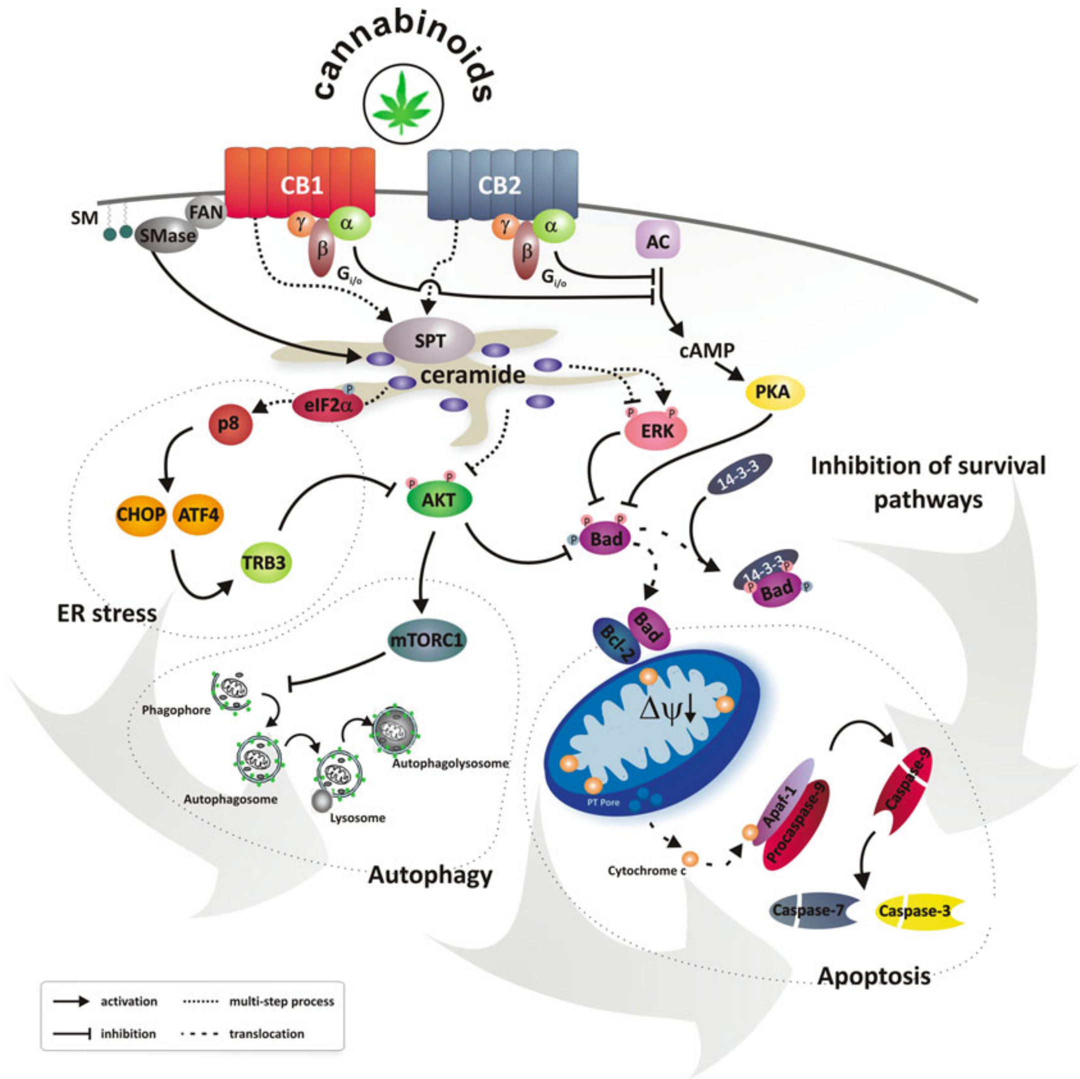

Fig. 11.3 Mechanisms of anti-tumoral action of cannabinoids. Increased ceramide synthesis *de novo* via induction of serine palmitoyltransferase (SPT) plays a central role in cannabinoid-induced cell death. Signals from CB1 via adaptor protein FAN (factor associated with neutral sphingomyelinase activation) trigger also the ceramide production from sphingomyelin breakdown, catalysed by neutral sphingomyelinase (SMase). Stimulation of cannabinoids receptors leads to inhibition of adenylyl cyclase (AC), reduction of cAMP levels and decreased protein kinase A (PKA) activity. Inhibition of PKA and pro-survival pathways (Akt and ERK signaling) stimulates translocation of Bad to the outer mitochondrial membrane and its pro-apoptotic function. Interaction between Bad and Bcl-2 triggers a decrease of the mitochondrial membrane potential (ΔΨ) and release of pro-apoptotic factors (such as cytochrome c) to the cytosol, where apoptosis is executed by caspase cascade. Alternatively, induction of apoptosis by cannabinoids can be mediated by ER (endoplasmic reticulum)-stress and autophagy. Cannabinoid-induced phosphorylation of eIF2α (eukaryotic translation initiation factor 2α) and subsequent up-regulation of the stress-regulated protein p8 and ER-stress-related downstream targets: ATF4 (activating transcription factor 4), CHOP (the C/EBP-homologous protein) and TRB3 (tribbles homologue 3) leads to inhibition of Akt, an upstream activator of mTORC1. Decreased activity of Akt/mTORC1 pathway contributes to initiation of autophagy, that precedes apoptosis of glioma cell

11.3 Cannabinoid System in Gliomas

Endocannabinoids, their receptors and specific machinery involved in biosynthesis, uptake and degradation, constitute the endocannabinoid system (ECS). A growing array of data suggests that alterations of a balance in the cannabinoid system between the levels of endogenous ligands and their receptors occur during malignant transformation in various types of cancer, including gliomas. While non-transformed astrocytes express only the CB1 cannabinoid receptor, both types of functional cannabinoid receptors have been found in several established human glioblastoma cell lines, as well as in primary cultures derived from the most malignant brain tumor, *glioblastoma multiforme* (GBM) (Galve-Roperh et al. 2000; Howlett et al. 2002; Sanchez et al. 2001). Immunohistochemical analysis of low and high grade human glioma surgical specimens revealed increased CB2 receptor expression in tumor cells, invading microglia/macrophages and endothelial cells of the tumor blood vessels, as compared to non-tumor brain samples (Ellert-Miklaszewska et al. 2007; Sanchez et al. 2001; Schley et al. 2009). We detected the presence of CB2 receptors in all analyzed biopsies of astrocytomas and glioblastomas. The proportion of malignant tumors expressing high levels of CB2 (10 out of 16, 62.5 %) was over 2-fold higher than that seen in the tumors of lower grade (7 out of 29, 24 %) (Ellert-Miklaszewska et al. 2007). Thus, the extent of CB2 expression correlated with the tumor malignancy grade. Interestingly, some benign pediatric astrocytic tumors, such as subependymal giant cell astrocytoma (SEGA), which may occasionally cause mortality owing to progressive growth in some patients, also displayed high CB2-imunoreactivity. Moreover, as observed by Sanchez et al., CB2 receptor immunoreactivity markedly prevailed over detected CB1 receptor levels in grade IV astrocytomas (Sanchez et al. 2001). The levels of CB1 receptor expression in tumor and tumor-associated endothelial cells were not significantly different from the control tissue and showed no dependence on tumor grade (Sanchez et al. 2001; Schley et al. 2009). However, in the recent study the decreased CB1 receptor expression was detected in high grade glioblastomas as compared to non-tumoral brain tissue (De Jesus et al. 2010).

A link between CB2 expression and malignancy grade of the tumor has been reported also in prostate, breast and pancreatic cancer, and the level of CB2 expression in transformed cells was higher than in the respective normal tissue (Caffarel et al. 2006; Carracedo et al. 2006a; Sarfaraz et al. 2005). In examined human tumors, CB2 receptors were usually located in the areas of intense tissue proliferation and invading cells. The enhancement of cannabinoid receptor expression in malignant versus healthy tissues might suggest a possible role of the endocannabinoid system in the tonic suppression of cell divisions and cancer growth. This hypothesis is partly supported by the finding of increased levels of anandamide and decreased levels of endocannabinoid metabolizing enzymes (i.e. FAAH) in human glioblastoma compared to human non-tumor brain tissue (Petersen et al. 2005).

11.4 Action of Cannabinoids in Glioma Cells

Cannabinoids induce significant inhibition of cell growth in tumor cells, due to modulation of proteins and nuclear factors involved in the control of cell survival, transformation and cell death. The programmed cell death of glioma cells after cannabinoid treatment was first described by Manuel Guzman and his co-workers (Sanchez et al. 1998). They showed that Δ^9-THC is able to inhibit growth of rat C6 glioma cells *in vitro* and induce cell death with features typical for apoptosis, a programmed cell death process (Sanchez et al. 1998). We reported an apoptotic death triggered by a mixed CB1/CB2 synthetic agonist WIN 55,212-2 in rat glioma cells (Ellert-Miklaszewska et al. 2005). Recent studies (Gomez del Pulgar et al. 2002; Salazar et al. 2009) and our own observations show effectiveness of Δ^9-THC, WIN55,212-2 and a CB2-selective synthetic cannabinoid JWH133 to induce apoptosis of cultured human glioblastoma cells and tumor-derived primary cultures (unpublished).

Cannabinoids exert anti-tumor effects *in vivo* leading to a significant regression of malignant gliomas in cannabinoid-treated animals (Duntsch et al. 2006; Galve-Roperh et al. 2000; Massi et al. 2004; Sanchez et al. 2001). Local administration of Δ^9-THC or the synthetic cannabinoid, WIN55,212-2, 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 (Galve-Roperh et al. 2000). Studies performed in mouse xenograft models with intratumoral and intraperitoneal drug administration demonstrated that non-psychoactive phytocannabinoid cannabidiol (Massi et al. 2004), a CB2-selective agonist JWH133 (Sanchez et al. 2001) or a novel synthetic cannabinoid KM-233 (Duntsch et al. 2006) blocked the proliferation of human astrocytoma cells implanted subcutaneously in the flank of immune-deficient mice. Our preliminary data suggest that systemic cannabinoid administration can effectively hamper intracranial tumor growth in rats.

11.4.1 Mechanism of Cannabinoids Pro-Apoptotic Action – Inhibition of Pro-Survival Pathways

Several events and signal transduction pathways triggered by stimulation of the CB1 and CB2 receptors have already been described to participate in the cannabinoid-induced cell death in various tumor cells (Guzman 2003; Guzman et al. 2001). They include inhibition of PKA, activation of MAPK, superoxide generation, and a strong increase in intracellular calcium concentration (Howlett et al. 2002). However, the mechanisms underlying the antitumor effects of cannabinoid receptor activation are still not well understood, and experimental data suggest that these effects may be cell-type specific.

Sanchez and coworkers showed that Δ^9-THC was able to partially antagonize the forskolin-induced elevation of intracellular cAMP concentration, but did not affect basal cAMP levels in C6 glioma cells (Sanchez et al. 1997). As described in details in Sect. 3.5, glioma C6 cells are characterized by a very low constitutive level of cAMP, and rather increased, than decreased concentration of intracellular cAMP may be responsible for inhibition of cell proliferation. It suggest, that cannabinoid receptors may be coupled to inhibition of adenylyl cyclase in glioma cells, but down-regulation of cAMP levels is unlikely to play a role in the induction of apoptosis triggered by cannabinoids in these cells. Involvement of other signaling molecules or adaptor proteins (of still mostly unknown identity) in cannabinoid receptor signaling is a subject of ongoing studies.

The best characterized mechanism of cannabinoid-induced cell death of glioma cells involves sustained accumulation of pro-apoptotic sphingolipid ceramide (Fig. 11.3), which modulates signaling pathways crucial in the control of tumor cell growth and survival (Galve-Roperh et al. 2000; Sanchez et al. 2001). Activation of the CB1 receptor triggers two peaks of ceramide generation in glioma cells (Galve-Roperh et al. 2000; Gomez del Pulgar et al. 2002; Sanchez et al. 2001). Treatment with Δ^9-THC or another CB1 receptor agonist produces a rapid release of ceramide *via* enzymatic hydrolysis of sphingomyelin from the cell membrane, catalyzed by neutral sphingomyelinase (Fig. 11.3). This effect is G-protein independent and involves the adaptor protein FAN (factor associated with neutral sphingomyelinase activation). The second ceramide peak is generated within hours or days after receptor activation and depends on increase of ceramide synthesis *de novo* via induction of serine palmitoyltransferase, a regulatory enzyme of sphingolipid biosynthesis (Gomez del Pulgar et al. 2002). Selective CB2 receptor agonists, such as JWH133, are supposed to stimulate only the ceramide synthesis process, which is sufficient to turn on the cell death program (Gomez del Pulgar et al. 2002; Sanchez et al. 2001). Thus, enhanced production of ceramide *de novo* is considered as an important event in cannabinoids-induced apoptosis. However, still little is known about the signaling pathways underlying the promotion of ceramide synthesis through cannabinoid receptor activation.

Galve-Roperh and co-workers postulated that the increased ceramide levels reported upon cannabinoid challenge led to prolonged activation of Raf-1/MEK/ERK signaling cascade and thus mediated

glioma cell cycle arrest and cell death (Galve-Roperh et al. 2000). The same authors showed also that pharmacological inhibition of ceramide synthesis *de novo* prevented the inhibition of protein kinase B/Akt triggered by cannabinoids (Gomez del Pulgar et al. 2002). Our studies revealed that rather down-regulation of ERK activity, together with inhibition of PI3K/Akt pathway, contributed to rat C6 glioma cell death induced by WIN55,212-2 (Ellert-Miklaszewska et al. 2005). The serine/threonine protein kinase Akt, activated downstream of PI3K, as well as the Ras-activated Raf1/MEK/ERK pathway are widely recognized as key mediators of growth factor-promoted cell survival in gliomas (Kapoor and O'Rourke 2003). Both survival pathways converge on a small pro-apoptotic member of a Bcl-2 family of proteins, Bad. Bad is also a substrate for PKA, which is negatively linked to both cannabinoid receptors via canonical $G_{i/o}\alpha$-mediated inhibition of adenylyl cyclase and subsequent decrease of cAMP levels. Phosphorylation of Bad by Akt, ERK and PKA retains the protein in the cytosol, where it is recognized by 14-3-3 regulatory proteins and sequestered (Zha et al. 1996). Otherwise, Bad translocates to mitochondria, and formation of heterodimers between non-phosphorylated Bad and anti-apoptotic proteins, such as Bcl-X_L or Bcl-2, may result in a loss of integrity of the outer mitochondrial membrane (Zha et al. 1996). The release of cytochrome c and other pro-apoptotic proteins from mitochondria triggers the executive phase of programmed cell death. We proposed a mechanism, in which the decrease of mitogenic/pro-survival signaling evoked by the synthetic cannabinoid WIN55,212-2 promotes the pro-apoptotic function of Bad (Fig. 11.3). Accordingly, we demonstrated changes in Bad phosphorylation level followed by collapse of the mitochondrial membrane potential in C6 glioma cells treated with WIN55,212-2. These events preceded activation of caspase 9 by factors released from disrupted mitochondria, subsequent processing of effector caspases and finally oligonucleosomal DNA fragmentation.

Our further studies, as well as some published data suggest that human glioma cells treated with cannabinoids enter the suicide cell death using the same mitochondria-dependent pathway (Carracedo et al. 2006b). This mechanism contributes to the induction of apoptosis by cannabinoids also in other types of tumor cells (Velasco et al. 2007). However, involvement of an alternative, death receptor–dependent pathway in the apoptotic process triggered by these compounds cannot be ruled out.

11.4.2 The Role of ER Stress and Autophagy in Cannabinoid-Induced Cell Death

Different experimental approaches showed that the pro-apoptotic and tumor growth-inhibiting activity of cannabinoids relies on the accumulation of *de novo*-synthesized ceramide, an event that occurs in the endoplasmic reticulum (ER) and eventually leads to execution of cell death via mitochondrial pathway. A recent study by Carracedo et al. suggested a new link between the two events (Carracedo et al. 2006b). They showed that Δ^9-THC treatment of glioma cells leads to up-regulation of the transcription co-activator p8 and its ER stress-related downstream targets: ATF4 (activating transcription factor 4), CHOP (the C/EBP-homologous protein) and TRB3 (pseudo-kinase tribbles homologue 3). Selective knockdown of ATF4 and TRB3 blocked cannabinoid-induced apoptosis in glioma cells. Inhibition of ceramide synthesis *de novo* prevented Δ^9-THC-induced p8, ATF4, CHOP and TRB3 up-regulation as well as ER dilation, indicating that ceramide accumulation is an early event in the cannabinoid-triggered ER stress and apoptosis in glioma cells (Carracedo et al. 2006b). Further studies by the same group indicated that Δ^9-THC induced Ser51-phosphorylation of eukaryotic translation initiation factor 2α (eIF2α) (Salazar et al. 2009). Activated eIF2α (after phosphorylation of Ser51 by a protein kinase-like endoplasmic reticulum kinase, PERK) is known to attenuate general protein translation, while enhancing the expression of several genes related to the ER stress response (Schroder and Kaufman 2005). Experiments performed with eIF2α S51A mutant cells have shown that phosphorylation of this factor is required for the up-regulation of the stress protein p8, as well as ATF4, CHOP and TRB3, implicated in cannabinoid-evoked ER stress and glioma cell death (Fig. 11.3).

Irradiation and chemotherapeutic drugs kill cancer cells typically through induction of apoptosis. However, most cancers (including gliomas) are resistant to therapies that induce apoptosis. Autophagy is an evolutionarily conserved catabolic process, where a cell self-digests its cytoplasmic contents. Autophagy is accompanied by the progressive development of vesicle structures from autophagosomes to autolysosomes, and could be activated in response to multiple stress stimuli during cancer progression, such as nutrient starvation, the unfolded protein response (the major ER stress pathway) and hypoxia. A detailed analysis of human astrocytoma cell lines and a primary culture of human glioma cells indicated that Δ^9-THC treatment led to formation of autophagosomes in tumor cells (Salazar et al. 2009). Autophagy was preceded by ER stress and followed by apoptosis in cannabinoid-treated human and mouse cancer cells. Autophagy-mediated cell death was associated with inhibition of the Akt/mTORC1 pathway. Mammalian target of rapamycin, complex 1 (mTORC1) is the best characterized cellular stress sensor controlling some of core autophagy pathway components and considered a key step in the early triggering of autophagy (Klionsky and Emr 2000). Treatment with Δ^9-THC induced TRB3 expression and promoted its interaction with Akt. This led to decreased phosphorylation of Akt, as well as of its direct substrates TSC2 (tuberous sclerosis protein 2, tuberin) and PRAS40 (the proline-rich Akt substrate of 40 kDa), which in turn resulted in mTORC1 inhibition (Salazar et al. 2009). Δ^9-THC-treatment decreased phosphorylation of p70S6 kinase (a well-established mTORC1 downstream target) and its substrate phospho-S6 ribosomal protein. Altogether, Δ^9-THC treatment triggered the following cascade of intracellular events: up-regulation of ER-stress-related TRB3, mTORC1 inhibition and induction of autophagy, which led to glioma cell death (Fig. 11.3). Moreover, this pathway was shown to be essential for cannabinoid anti-tumoral action *in vivo*. Administration of Δ^9-THC to mice bearing tumors derived from human astrocytoma cells resulted in increased TRB3 expression, inhibition of mTOR signaling pathway, appearance of autophagy markers and caspase-3 activation. These findings indicate that cannabinoid promotes the autophagy-mediated cell death through stimulation of ER stress in human glioma cells (Salazar et al. 2009).

11.5 Therapeutic Potential of Targeting Cannabinoid Signaling in Gliomas

Standard chemotherapeutics are a double edged sword; they eliminate cancer cells but affect severely healthy cells in the body. Based on evidence from *in vitro* and *in vivo* preclinical studies, as well as from a pilot phase I clinical trial in patients with recurrent *glioblastoma multiforme*, cannabinoids appear to have a favorable safety profile and do not produce the generalized toxic effects as most conventional chemotherapeutic drugs. Δ^9-THC induced apoptosis in several cancer cell lines but showed less efficacy in nontransformed cell counterparts (Galve-Roperh et al. 2000; Guzman 2003; McAllister et al. 2005). Cannabinoids promote survival of glial cells and neurons in different models of injury, suggesting that the anti-proliferative effect of cannabinoids is selective for brain tumor cells, while viability of normal brain cells remains unaffected or even favored by cannabinoid challenge (Guzman 2003; Molina-Holgado et al. 2002). Several mechanisms could be responsible for cannabinoid compounds targeting only the cancer cells, including differences in cannabinoid signaling in glioma and normal neural cells and selective over-expression of the CB2 receptor in tumor cells. In contrast to pro-apoptotic action of Δ^9-THC and WIN55,12-2 on transformed glial cells, treatment of primary cultured astrocytes with these CB1/CB2-activating cannabinoids does not trigger ceramide generation *de novo* or induction of ER stress-related genes. In our studies administration of the CB2-selective agonist JWH133 was effective toward tumor cells, and it did not affect survival or morphology of normal astrocytes (unpublished). Negligible CB2 receptor expression in the normal brain and its abundance in high grade gliomas confer a relative safety of CB2-selective agonists for targeted glioma therapy. Moreover, the CB2 selective compounds are devoid of undesirable psychodysleptic side-effects, attributed to marijuana abuse, which are mediated by the CB1 receptor.

Due to genetic and epigenetic alterations malignant glioblastomas are highly resistant to radiation and chemotherapy. Mainstream therapeutic strategies for the management of all primary brain tumors are still mostly palliative, known to leave survivors with devastating neurological deficits and frequently with a high risk of the disease recurrence. The potency of synthetic cannabinoids to induce apoptosis in glioblastoma cells has been tested by us and others on several cell lines and primary cell cultures derived from biopsies of human tumors, which to some extent may reflect the heterogeneity of glioma molecular characteristics (Duntsch et al. 2006; Ellert-Miklaszewska et al. 2005; Galve-Roperh et al. 2000; Guzman 2003; Massi et al. 2004; McAllister et al. 2005; Sanchez et al. 2001). Thus, cannabinoids were able to override alterations of growth regulatory and apoptotic pathways, caused by common mutations reported in primary and secondary glioblastomas. Pro-apoptotic action of cannabinoids relies on the generation of ceramide and disruption of signaling pathways crucial for regulation of cellular proliferation, differentiation or apoptosis (Ellert-Miklaszewska et al. 2005; Galve-Roperh et al. 2000; Gomez del Pulgar et al. 2002; Salazar et al. 2009). Their unique mechanism of action among standard oncology remedies justifies further research on their anti-tumoral properties. A promising field to explore is to prolong the effectiveness of endocannabinoids by interference with their biosynthesis, uptake and breakdown. Therefore future studies on cannabinoid signaling system in gliomas are clearly needed.

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