

Anti-tumoral action of cannabinoids: Involvement of sustained ceramide accumulation and extracellular signal-regulated kinase activation

ISMAEL GALVE-ROPERH¹, CRISTINA SÁNCHEZ¹, MARÍA LUISA CORTÉS²,
TERESA GÓMEZ DEL PULGAR¹, MARTA IZQUIERDO² & MANUEL GUZMÁN¹

¹Department of Biochemistry and Molecular Biology I, School of Biology, Complutense University,
28040-Madrid, Spain

²Department of Molecular Biology, Severo Ochoa Molecular Biology Center, School of Sciences,
Autónoma University, 28049-Madrid, Spain

I.G.-R. and C.S. contributed equally to this work

Correspondence should be addressed to M.G.; email: mgp@solea.quim.ucm.es

Δ^9 -Tetrahydrocannabinol, the main active component of marijuana, induces apoptosis of transformed neural cells in culture. Here, we show that intratumoral administration of Δ^9 -tetrahydrocannabinol and the synthetic cannabinoid agonist WIN-55,212-2 induced a considerable regression of malignant gliomas in Wistar rats and in mice deficient in recombination activating gene 2. Cannabinoid treatment did not produce any substantial neurotoxic effect in the conditions used. Experiments with two subclones of C6 glioma cells in culture showed that cannabinoids signal apoptosis by a pathway involving cannabinoid receptors, sustained ceramide accumulation and Raf1/extracellular signal-regulated kinase activation. These results may provide the basis for a new therapeutic approach for the treatment of malignant gliomas.

Δ^9 -Tetrahydrocannabinol (THC), the main active component of marijuana, and other cannabinoids produce a wide spectrum of central and peripheral effects, such as alterations in cognition and memory, analgesia, anticonvulsion, anti-inflammation, and alleviation of both intraocular pressure and emesis. Cannabinoids produce their effects by binding to specific plasma membrane G protein-coupled receptors^{1,2}. So far, two different cannabinoid receptors have been characterized and cloned from mammalian tissues: CB₁ (ref. 3) and CB₂ (ref. 4). The CB₁ receptor is mainly distributed in the central nervous system, whereas the CB₂ receptor is mostly expressed in cells of the immune system. The recent discovery of a family of endogenous ligands of cannabinoid receptors^{5–7} has focused much attention on cannabinoids during the last few years. The importance of the endogenous cannabinoid system is supported by the finding of high levels of cannabinoid receptors in brain⁸; the specific mechanisms of endocannabinoid synthesis, uptake and degradation^{2,9}; and the neuromodulatory properties of endogenous cannabinoids⁹. Moreover, ongoing research is determining whether cannabinoid ligands may be effective agents in the treatment of chronic pain, glaucoma, spasms, and the wasting and emesis associated with acquired immunodeficiency syndrome and cancer chemotherapy^{10,11}. The potential therapeutic application of cannabinoids is, however, controversial and constitutes a widely debated issue with ample scientific and social relevance.

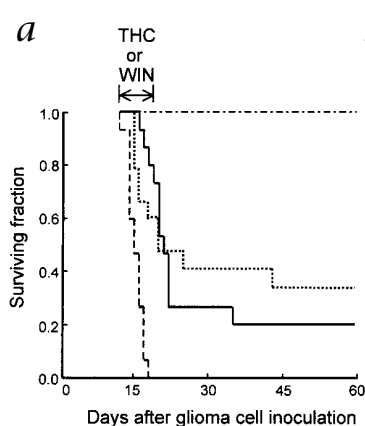
One of the most intriguing and unexplored actions of cannabinoids is their ability to inhibit the growth of transformed cells in culture. The endogenous cannabinoid anandamide and other cannabinoid agonists inhibit human breast cancer cell proliferation¹². THC also induces apoptosis of trans-

formed neural cells¹³. The potential biological and therapeutic implications of the antiproliferative action of cannabinoids are hampered, however, by the absolute lack of knowledge of the molecular mechanism responsible for this effect. In addition, so far the inhibition of cell growth by cannabinoids has not been investigated *in vivo*. Therefore, we sought to determine whether cannabinoids inhibit the growth of transformed cells *in vivo* and the mechanism by which they inhibit the growth of transformed cells.

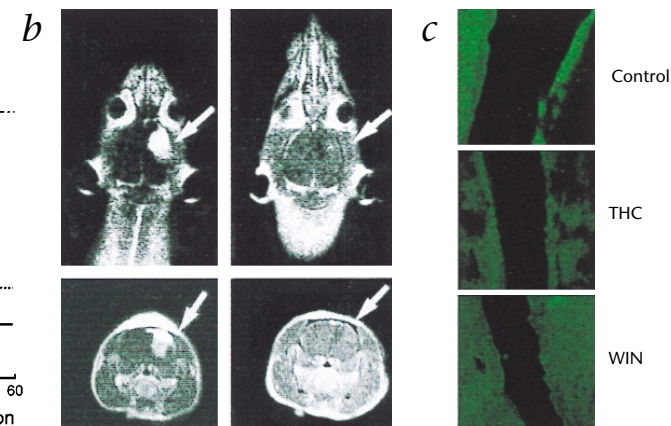
Regression of malignant gliomas *in vivo*

Given the inhibition by THC of the growth of transformed neural cells in culture¹³, we evaluated the functionality of the system *in vivo* using an intracerebral tumor model. We used THC as well as WIN-55,212-2, a potent synthetic agonist of cannabinoid receptors that could be used at lower doses than THC and therefore produce less-substantial side effects^{1,2}. We injected C6 glioma cells directly into the right cerebral hemispheres of rats to induce malignant tumors, and visualized them by magnetic resonance imaging (MRI). All the rats left untreated uniformly died 12–18 days after glioma cell inoculation (Fig. 1a). To test the anti-tumoral effect of cannabinoids, 12 days after cell injection we connected an osmotic pump delivering THC or WIN-55,212-2 for 7 days to a brain infusion cannula placed at the site of tumor inoculation. In preliminary experiments, we used a wide dose range of THC (50–3000 μ g) and WIN-55,212-2 (5–300 μ g). In further experiments, we used doses in the range of 500–2,500 μ g THC and 50–250 μ g WIN-55,212-2, depending on such factors as the size, aspect and infiltration of the tumor (that is, we used the highest doses to treat large, dense and invasive tumors).

Fig. 1 Cannabinoid-induced regression of malignant gliomas in Wistar rats. **a**, Kaplan-Meier survival curves of rats with brain tumors. Tumors were induced in 45 rats by intracerebral injection of C6 glioma cells (day 0); 15 rats were left untreated (---), 15 were treated with THC (—) and 15 were treated with WIN-55,212-2 (....) between days 12 and 19. Tumor-bearing rats treated with THC or WIN-55,212-2 lived significantly longer than those in the control group ($P < 0.01$). THC and WIN-55,212-2 were also given to rats ($n = 5$ for each) in which no tumor was induced (---).



b, Gadolinium-enhanced magnetic resonance views in axial (upper) and coronal (lower) projections of a rat brain before (left) and after (right) THC treatment. A 100-mm³ glioma (arrows) was completely eradicated by treatment with 500 µg THC. The image was obtained 7 days after THC delivery ended. **c**, Absence of apoptosis in the subventricular zone of rat brain after



treatment with THC or WIN-55,212-2 for 7 d. The lateral ventricle of the right cerebral hemisphere (of cannabinoid inoculation) is flanked by the lateral septal nucleus (left) and the caudate putamen (right). The labeling in control rat (top) shows basal apoptosis in the caudate putamen. Data represent results obtained in two other rats of each type.

Cannabinoid-treated rats survived significantly longer than control rats (Fig. 1a). THC administration was ineffective in three rats, which died by days 16–18. Nine of the THC-treated rats surpassed the time of death of untreated rats, and survived up to 19–35 days. Moreover, the tumor was completely eradicated in three of the treated rats. Likewise, administration of WIN-55,212-2 was ineffective in six rats, which died by days 15–18; increased the survival time of four rats up to 19–43 days; and completely eradicated the tumor in five rats. An MRI scan of one of the THC-treated rats (Fig. 1b) showed that after cannabinoid administration there was a total absence of tumoral mass, and in its place, a residual hypointense image interpreted as a fibrous scar appeared at the site of injection (Fig. 1b, arrow). There has been no recurrence in any of the eight surviving rats, which have been monitored periodically by MRI.

Inoculation of C6 glioma cells into rat brain is a well-characterized model to induce malignant tumors. Nevertheless, the use of this model might allow for immune responses to occur if the rat were provided with an immune enhancement regime¹⁴. To distinguish between direct cannabinoid actions on glioma cells and potential immune-related responses induced by cannabinoid treatment, we studied mice deficient in recombination activating gene 2 ($Rag2^{-/-}$), which lack mature T and B cells¹⁵. We inoculated C6 glioma cells subcutaneously into the right flanks of $Rag2^{-/-}$ mice to induce malignant tumors. Tumor growth was considerably less in mice injected with 500 µg

THC/day or 50 µg WIN-55,212-2/day than in control mice (Fig. 2a). Photographs of tumor-bearing mice and histology of dissected tumors after cannabinoid treatment for 7 days (Fig. 2b), along with the above data, show that cannabinoids produce a direct anti-tumoral action, although most likely a certain immune competence favors tumor eradication.

Safety of cannabinoid administration

Although it is widely believed that cannabinoids are not neurotoxic^{2,8,9}, we determined whether cannabinoids, particularly THC at high doses, produce adverse effects to the rat brain. Using rats in which no tumor was induced, we monitored toxicity to normal brain tissue by delivering 2,500 µg THC or 250 µg WIN-55,212-2 for 7 days. The survival of these rats was not affected at all by cannabinoids (Fig. 1a). As with the eight aforementioned ‘cannabinoid-cured’ rats, careful MRI analysis of all those tumor-free rats showed no sign of damage related to necrosis, edema, infection or trauma. To rule out the possibility that cannabinoids are toxic to dividing neural cells, we used TUNEL staining of the subventricular zone of rat brain, which undergoes continued proliferation in the adulthood. Cannabinoid administration did not induce any substantial apoptotic effect in the brain *in vivo* (Fig. 1c). Moreover, the weak labeling in the caudate putamens of control rats was not evident in cannabinoid-treated rats.

We also examined other potential side effects of cannabinoid administration. In both tumor-free and tumor-bearing rats,

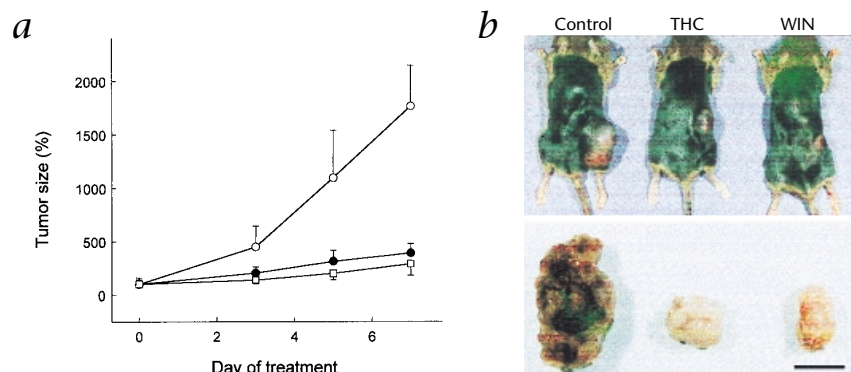
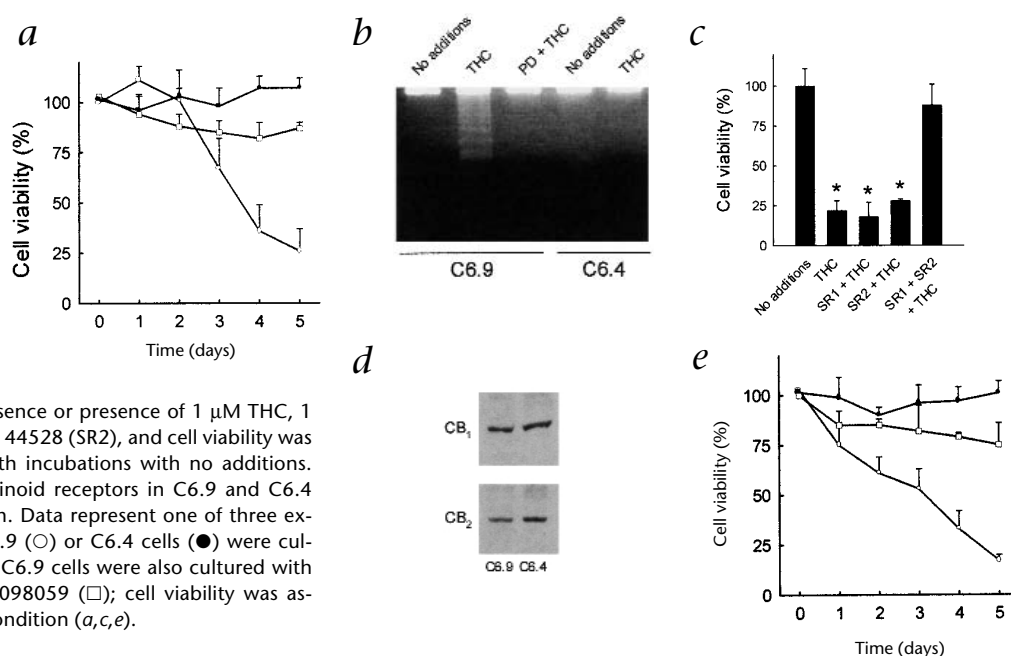


Fig. 2 Cannabinoid-induced regression of malignant gliomas in $Rag2^{-/-}$ mice. **a**, Growth of tumors in control and cannabinoid-treated mice. C6 glioma cells were injected subcutaneously into 18 mice. When tumors reached the desired size (day 0), six mice were treated with vehicle (○), six were treated with THC (●) and six were treated with WIN-55,212-2 (□) for up to 7 d. The tumors of the mice treated with THC or WIN-55,212-2 were significantly smaller than those of control mice at all times ($P < 0.01$). **b**, Subcutaneous gliomas in the right flanks of mice (top row) and after dissection (bottom row; scale bar represents 1 cm) after treatment for 7 d with vehicle (Control), THC or WIN-55,212-2.

Fig. 3 Different sensitivity of C6.9 and C6.4 glioma cells to THC-induced death. **a**, C6.9 (○) or C6.4 cells (●) were cultured with 1 μ M THC, and C6.9 cells were also cultured with 1 μ M THC and 25 μ M PD098059 (□); cell viability was assessed. **b**, Apoptotic DNA fragmentation in C6.9 but not in C6.4 glioma cells. Cells were cultured for 5 d in the absence or presence of 1 μ M THC and/or 25 μ M PD098059 (PD). Data represent one of three experiments with similar results. **c**, C6.9 cells were cultured for 5 d in the absence or presence of 1 μ M THC, 1 μ M SR141716 (SR1) and/or 1 μ M SR144528 (SR2), and cell viability was assessed. *, $P < 0.01$, compared with incubations with no additions. **d**, Presence of CB₁ and CB₂ cannabinoid receptors in C6.9 and C6.4 glioma cells, shown in a luminogram. Data represent one of three experiments with similar results. **e**, C6.9 (○) or C6.4 cells (●) were cultured with 10 μ M C₂-ceramide, and C6.9 cells were also cultured with 10 μ M C₂-ceramide and 25 μ M PD098059 (□); cell viability was assessed. $n = 6$ experiments for each condition (a,c,e).



cannabinoid administration induced no substantial change in behavioral parameters such as motor coordination or physical activity. Food and water intake as well as body weight gain were unaffected during and after cannabinoid delivery. Likewise, the general hematological profiles of cannabinoid-treated rats were normal. Thus, neither biochemical parameters (glucose, urea, uric acid, creatinine, cholesterol and bilirubin) nor markers of tissue damage (alanine and aspartate aminotransferases, γ -glutamyltransferase, creatine kinase and lactate dehydrogenase) changed substantially during the 7-day delivery period or for at least 2 months after cannabinoid treatment ended.

Signaling through cannabinoid receptors and ERK

Gliomas often show a gross variability characterized by the presence of morphologically distinct cell types within the same tumor^{16,17}. Moreover, glioma cell lines such as C6 may be heterogeneous^{18,19}. To study the mechanism of THC action, we subcloned the C6 glioma cell line by limiting dilution¹⁹, and used two subclones: the C6.9 subclone, which has been characterized in terms of THC-induced apoptosis¹³, and the C6.4 subclone, which is resistant to THC-induced apoptosis and therefore con-

stitutes an excellent reference for comparison (Fig. 3a and b).

We first determined the possible involvement of cannabinoid receptors in THC-induced death of C6.9 glioma cells. Potent synthetic agonists such as WIN-55,212-2, CP-55,940 and HU-210 induced C6.9 glioma cell death at lower doses than did THC, as expected from their higher affinity for cannabinoid receptors^{1,2}. Thus, after 5 days of cannabinoid challenge, there was a 50% decrease in C6.9 glioma cell viability with 20 nM WIN-55,212-2, 45 nM CP-55,940, 10 nM HU-210 and 480 nM THC ($n = 4$ experiments for each). Neither SR141716 (a selective CB₁ receptor antagonist) nor SR144528 (a selective CB₂ receptor antagonist) was able to prevent THC-induced cell death by itself. However, when the two antagonists were added simultaneously, there was an efficient prevention of THC action (Fig. 3c). Accordingly, C6.9 glioma cells expressed both the CB₁ and the CB₂ cannabinoid receptor (Fig. 3d). The insensitivity of the C6.4 subclone to THC-induced death was not because of a lack of expression of cannabinoid receptors (Fig. 3d).

We next studied modulators of the activity of various protein kinases involved in the control of cell growth, to determine whether the effect of THC could be attributed to any of those kinases. THC-induced C6.9 glioma cell death was not prevented by either the adenylyl cyclase activator forskolin or the protein kinase A inhibitor H-7 ($n = 3$ experiments for each; data not shown). Neither the phorbol ester phorbol 12-myristate 13-acetate nor the protein kinase C inhibitor GF109203X antagonized the effect of THC ($n = 3$ experiments for each; data not shown). The p38

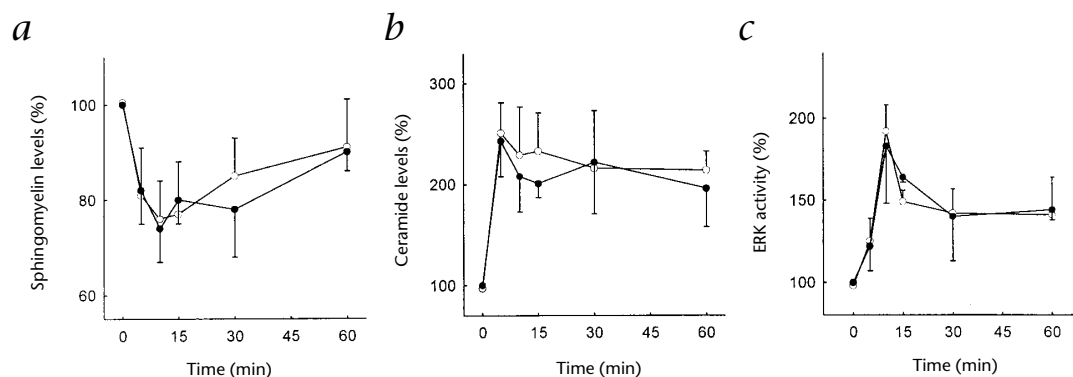


Fig. 4 Short-term effect of THC on sphingomyelin hydrolysis, ceramide levels and ERK activity in C6.9 and C6.4 glioma cells. C6.9 (○) or C6.4 cells (●) were incubated with 1 μ M THC (times, horizontal axes). $n = 6$ experiments for each condition. **a**, Sphingomyelin levels. **b**, Ceramide levels. **c**, ERK activity.

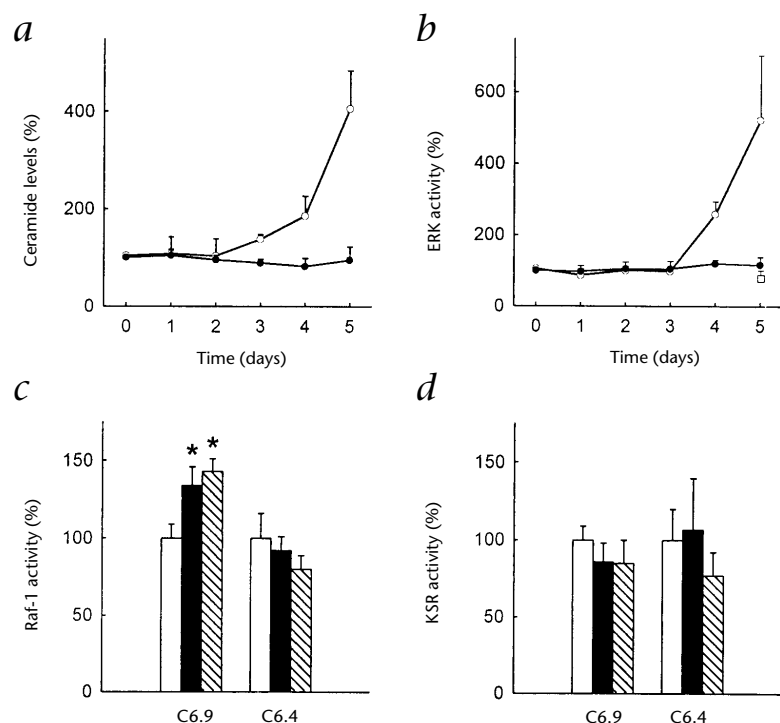


Fig. 5 Long-term effect of THC on ceramide levels and ERK, Raf1 and KSR activity in C6.9 and C6.4 glioma cells. **a** and **b**, C6.9 (○) or C6.4 cells (●) were incubated with 1 μ M THC, and C6.9 cells were also cultured with 1 μ M THC and 25 μ M PD098059 (**b** only; □) (times, horizontal axes). Ceramide levels (**a**) and ERK activity (**b**) were determined. $n = 6$ experiments for each condition. **c** and **d**, Cells were cultured with no additions (open bars), 1 μ M THC (filled bars) or 10 μ M C₂ ceramide (hatched bars) for 5 d, and Raf1 activity (**c**) and KSR activity (**d**) were determined. $n = 4$ experiments for each condition. *, $P < 0.01$, compared with incubations with no additions.

mitogen-activated protein kinase (MAPK) inhibitor SB203580 was also ineffective ($n = 4$ experiments; data not shown). In contrast, the tyrosine kinase inhibitor genistein ($n = 4$ experiments; data not shown) as well as the extracellular signal-regulated kinase (ERK) cascade inhibitor PD098059 (Fig. 3*a* and *b*) were able to suppress THC-induced death of C6.9 glioma cells.

Role of sustained ceramide accumulation and ERK activation

Cannabinoids induce accumulation of the pro-apoptotic lipid ceramide in primary astrocytes^{20,21}. Thus, we determined whether the cell-permeable ceramide analog C₂-ceramide produced different effects on C6.9 and C6.4 glioma cells. Like THC, C₂-ceramide induced the death of C6.9 but not of C6.4 cells. Moreover, the C₂-ceramide-induced death of C6.9 cells was prevented by PD098059 (Fig. 3*e*). We next investigated whether THC produced different effects on ceramide concentration in the two cell subclones. Rapid increases of intracellular ceramide levels in response to pro-apoptotic ligands such as tumor necrosis factor, Fas ligand and interleukin 1 are usually attributed to sphingomyelin breakdown^{22,23}. Likewise, short-term incubation with THC induced sphingomyelin hydrolysis in C6.9 and C6.4 glioma cells (Fig. 4*a*). This was concomitant with an increase in intracellular ceramide levels (Fig. 4*b*). The magnitude of the effect of THC on ceramide and sphingomyelin levels was quantitatively similar in the two subclones. Because PD098059 prevents THC- and C₂-ceramide-induced death of

C6.9 glioma cells, we determined whether ERK was activated in these cells. Both THC (Fig. 4*c*) and C₂-ceramide (Table 1) were able to activate ERK acutely in C6.9 cells, but there was also a quantitatively similar effect in the C6.4 subclone. In addition to ERK, other protein kinase cascades, such as c-Jun N-terminal kinase (JNK; GDB designation, MAPK8) and p38 MAPK, may be potential targets of ceramide action in the induction of apoptosis in a number of cell types, including neural cells^{22,23}. Acute exposure to THC and C₂-ceramide activated JNK in C6.9 and C6.4 cells in a similar manner, whereas p38 MAPK activity remained unaffected (Table 1).

Long-term ceramide accumulation and ERK activation may act in concert with short-term 'peaks' of these parameters in the control of cell fate^{23,24}. We therefore determined ceramide levels and ERK activity in our system during the 5-day experimental period. THC induced a considerable accumulation of ceramide (Fig. 5*a*) and activation of ERK (Fig. 5*b*) in C6.9 but not in C6.4 glioma cells after 4–5 days of challenge (that is, coinciding with the time at which C6.9 cell death occurs). The sustained activation of ERK induced by THC was fully prevented by PD098059 (Fig. 5*b*). C₂-ceramide also produced a sustained activation of ERK in C6.9 but not in C6.4 cells (Table 1). Likewise, long-term treatment with THC and C₂-ceramide induced in C6.9 but not in C6.4 cells a significant activation of Raf1 (Fig. 5*c*), a pivotal element in the control of cell fate by the ERK cascade²⁵. These changes in Raf1 activity were not mediated by kinase suppressor of Ras (KSR) (Fig. 5*d*), a ceramide-activated protein kinase that

Table 1 Effect of THC and C₂-ceramide on ERK, JNK and p38 MAPK activity in C6.9 and C6.4 glioma cells

Additions	ERK		JNK		p38 MAPK	
	C6.9	C6.4	C6.9	C6.4	C6.9	C6.4
Short-term experiments						
None	1.0 $\pm$ 0.2	1.0 $\pm$ 0.1	1.0 $\pm$ 0.2	1.0 $\pm$ 0.3	1.0 $\pm$ 0.2	1.0 $\pm$ 0.3
THC	1.9 $\pm$ 0.2*	1.8 $\pm$ 0.4*	2.2 $\pm$ 0.2*	1.7 $\pm$ 0.2*	1.1 $\pm$ 0.2	1.1 $\pm$ 0.2
C ₂ -ceramide	2.4 $\pm$ 0.3*	2.5 $\pm$ 0.5*	2.6 $\pm$ 0.3*	2.8 $\pm$ 0.3*	1.3 $\pm$ 0.2	1.4 $\pm$ 0.3
Long-term experiments						
None	1.0 $\pm$ 0.2	1.0 $\pm$ 0.1	1.0 $\pm$ 0.2	1.0 $\pm$ 0.2	1.0 $\pm$ 0.3	1.0 $\pm$ 0.3
THC	5.2 $\pm$ 1.8*	1.2 $\pm$ 0.2	3.6 $\pm$ 1.0*	2.3 $\pm$ 0.9*	4.1 $\pm$ 0.9*	2.5 $\pm$ 0.4*
C ₂ -ceramide	4.9 $\pm$ 1.2*	1.4 $\pm$ 0.4	3.4 $\pm$ 0.7*	2.4 $\pm$ 0.6*	4.3 $\pm$ 0.7*	2.2 $\pm$ 0.6*

Cells were cultured with no additions, 1 μ M THC or 10 μ M C₂-ceramide for 10 min (short-term) or 5 d (long-term), and kinase activation was measured. Values of the respective incubations with no additions were set at 1.0. Results represent four different experiments. *, $P < 0.01$, compared with the respective incubations with no additions.

may phosphorylate and activate Raf1 (ref. 26). In contrast to ERK, JNK and p38 MAPK were activated in the two subclones on long-term exposure to THC and C₂-ceramide, although the increase in C6.9 cells was higher than that in the C6.4 subclone (Table 1).

Discussion

Ceramide is believed to be important in the regulation of cell function in the central nervous system. Exposure of neural cells to stress stimuli may trigger ceramide accumulation, thereby producing changes in the cell survival/death decision^{22,23}. Ceramide may also induce apoptotic cell death during brain development as well as in neurodegenerative disorders such as Alzheimer disease, Parkinson disease, epilepsy and ischemia/stroke^{22,27,28}. Our experiments with two subclones of C6 glioma cells demonstrate a salient similarity of THC and ceramide action on cell fate, Raf1/ERK activation and JNK/p38 MAPK regulation, as well as a close correspondence of cell fate and ceramide levels as modulated by THC. Thus, THC may induce the death of glioma cells through sustained ceramide accumulation and Raf1/ERK activation. Our data also show that the THC- and ceramide-induced activation of Raf1/ERK in glioma cells occurs independently of KSR, a protein involved in the stimulation of Raf1 by the 55-kDa tumor necrosis factor receptor²⁶. Our observations are in line with those showing that ceramide directly binds to and activates Raf1 (ref. 29). Indeed, Raf1 has a ceramide-binding motif²³, thereby linking the ceramide pathway with the ERK cascade in the control of cell fate. Nevertheless, it has been reported that ceramide binding to Raf1 does not lead to Raf1 stimulation³⁰, and the activation of Raf1 by KSR is independent of the kinase activity of the latter³¹. The precise role of KSR as a modulator of the ERK cascade is still a matter of debate.

It is generally accepted that the activation of the ERK cascade leads to cell proliferation³². However, recent investigations have begun to define situations in which ERK mediates cell cycle arrest³³, antiproliferation³⁴, as well as apoptotic³⁵ and non-apoptotic³⁶ death in many cells, including neural cells. The relation between activation of the ERK cascade and cell proliferation depends on the duration of the stimulus²⁴. Our data show that the apoptotic action of THC relies on the long-term peak of ERK activation. Moreover, the lack of protection provided by the selective p38 MAPK inhibitor, the effective prevention produced by the selective ERK cascade inhibitor, and the induction of JNK and p38 MAPK in C6.4 cells indicate selective involvement of the ERK cascade in cannabinoid-induced cell death.

Gliomas constitute the most frequent class of primary brain tumors and are among the most malignant forms of cancer, resulting in the death of affected patients within months. So far, the therapeutic treatment of gliomas is only palliative, and includes surgical removal, radiotherapy, chemotherapy and immunotherapy^{16,37}. Gene therapy is now being tested as a targeted strategy against gliomas^{38,39}. However, the success of all these treatments is usually hampered by such factors as the rapid growth, remarkable heterogeneity, high degree of infiltration and extreme resistance to chemotherapy (most likely related to the expression multi-drug resistance genes) shown by gliomas^{16,17,37,40}. Likewise, cultured glioma cells are fairly resistant to multiple proapoptotic stimuli such as cancer chemotherapy drugs, gamma irradiation and tumor necrosis factor^{16,19}.

The development of new therapeutic strategies is therefore essential for the management of malignant gliomas⁴¹. The antiproliferative effect of cannabinoids reported here might provide the

basis for a new therapeutic approach for the treatment of gliomas. This could be particularly useful for synthetic cannabinoid ligands such as WIN-55,212-2, which led to better curative results than THC at 10% the dosages. The involvement of both the CB₁ and the CB₂ receptor in the anti-tumoral action of cannabinoids might have clinical relevance because, unlike CB₁ receptor agonists, CB₂ agonists are not expected to produce psychotropic side effects. Although the presence and functionality of the CB₁ receptor in the central nervous system is firmly established^{1,2,8}, we know of only one report describing expression of the CB₂ receptor in the central nervous system⁴². Nonetheless, it is possible that non-differentiated cells such as glioma cells express proteins not expected to be present in well-differentiated neural cells.

Both *in vitro* evidence¹³ and *in vivo* evidence (our results) show that the apoptotic effect of cannabinoids is selective, occurring in transformed but not in non-transformed neural cells. In addition, in our study cannabinoid administration produced neither necrotic brain damage (as evidenced by MRI results) nor alterations of behavioral and hematological parameters. Moreover, in keeping with our TUNEL staining data, cannabinoids protect cultured neurons from glutamate-induced excitotoxicity^{42,43}, from ischemic death⁴⁴, and, because of their antioxidant properties, from hydroperoxide-induced oxidative damage⁴⁵. Cannabinoids are being tested as therapeutic agents in the treatment of neurodegenerative diseases such as multiple sclerosis and Parkinson disease^{10,11,46}. Current research is aimed at exploring the potential use of cannabinoids as antiproliferative agents.

Methods

Glioma cell culture and viability. C6 glioma cells (10–20 passages) were cultured in F12 medium supplemented with 10% fetal calf serum. For *in vitro* studies, 24 h before the experiment, cells were transferred to serum-free medium consisting of F12 medium supplemented with 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml sodium selenite, and 1 mg/ml defatted and dialyzed bovine serum albumin (BSA). The latter medium was renewed every 48 h. Cell viability was determined by the MTT test¹³. Cells were incubated with 5 µg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-5-diphenyltetrazolium bromide thiazol blue) for 4 h at 37 °C. The medium was aspirated, the formazan crystals were solubilized with 60mM HCl in isopropanol, and the absorbance at 570 nm was monitored. For DNA isolation and analysis, cell lysates were centrifuged through columns containing a glass fiber fleece to which nucleic acids bind (Boehringer). Samples were further treated with DNase-free RNase A and separated by electrophoresis in 1.5% agarose gels¹³.

Anti-tumoral action of cannabinoids in rats. Male Wistar rats (250–300 g in body weight) were anesthetized with 3% isoflurane in a mixture of oxygen (at a rate of 0.8 l/min) and protoxide (at a rate of 0.4 l/min). C6 glioma cells (5 × 10⁵) in phosphate-buffered saline (PBS) supplemented with 0.1% glucose were injected stereotactically into the fronto-parietal lobe of the right cerebral hemisphere (4 mm to the right of bregma, 4.5 mm deep from the skull)⁴⁷. Rats received 2 mg/l dexamethasone and about 75 mg tetracycline/kg body weight in the drinking water for 3 d before and 7 d after the surgery. The tumor was closely monitored by MRI (refs. 47,48). Axial and coronal views of the skull were made with a Cpflex small coil around the rat. T1-weighted projection images were obtained using spin echo, with a time of repetition (TR) of 650 ms; time to echo (TE), 17.0ms; two acquisitions; slice, 3 mm in thickness; field of view, 50mm × 100mm; matrix, 256 × 512 pixels; and high-resolution sequence post-enhancement with gadolinium (0.2 ml/kg body weight) with magnetization transfer.

Cannabinoid administration to the rats started 12 d after glioma cell inoculation. At that time, the average size of tumors was 70 mm³ (range, 25–100 mm³) as estimated by MRI (ref. 48). Cannabinoids were delivered by a brain infusion cannula located at the site of tumor inoculation and fixed to the skull with dental cement; a small stainless steel screw secured

the cannula and the dental cement. The brain infusion cannula was connected subcutaneously by a catheter tube to a mini-osmotic pump (Alzet 2001), which operated at a flux of 1 μ l/h for 7 d. The osmotic pump was filled with either 500–2,500 μ g THC or 50–250 μ g WIN-55,212-2 in PBS supplemented with 5 mg/ml defatted and dialyzed BSA.

TUNEL staining. Cannabinoids (2,500 μ g THC or 250 μ g WIN-55,212-2) were administered to tumor-free rats for 7 d as described above. Brains were fixed with 4% paraformaldehyde in PBS. Apoptotic cell death was determined in cryostat sections 40 μ m in thickness, using TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end-labeling) staining according to manufacturer's instructions (Boehringer). Positive controls consisted of brain sections treated with 10 μ g/ml DNase I for 10 min at room temperature. Fluorescein-dUTP labeling of DNA strand breaks was visualized with a Zeiss confocal laser scanning microscope using fluorescein optics (excitation, 488 nm; emission, 525 nm). The intensity of the laser beam and the sensitivity of the photodetector were kept constant to allow comparison between treatments. At least five optical fields per rat were examined.

Anti-tumoral action of cannabinoids in immune-deficient mice. Tumors were induced in Rag2^{-/-} mice by inoculation into the subcutaneous flank of 5×10^6 C6 glioma cells in PBS supplemented with 0.1% glucose. About 10 d after inoculation, when tumors had reached an average volume of 250 mm³ (range, 200–300 mm³), mice were assigned randomly to three groups and injected intratumorally for 7 d with vehicle, 500 μ g THC/day or 50 μ g WIN-55,212-2/day in 100 μ l PBS supplemented with 5 mg/ml defatted and dialyzed BSA. Tumors were measured with external caliper and volume was calculated as $(4\pi/3) \times (\text{width}/2)^2 \times (\text{length}/2)$.

Western blot analysis of CB₁ and CB₂ cannabinoid receptors. Cells were washed, scraped, and the particulate fraction was obtained after centrifugation at 40,000g for 60 min (ref. 13). Samples were separated by SDS-PAGE and proteins were transferred from the gels onto polyvinylidene fluoride membranes. The blots were blocked with 1% defatted and dialyzed BSA, and incubated with an antibody raised against residues 1–14 of the rat CB₁ receptor (1:5,000 dilution) or with an antibody raised against residues 350–361 of the human CB₂ receptor (1:2,000 dilution). Finally, samples were subjected to luminography with an ECL detection kit.

Sphingomyelin levels. At 48 h before the experiment, cells were transferred to chemically defined medium supplemented with 1 μ Ci Me-³H-choline. Reactions were started by the addition of the different modulators. Lipids were extracted and saponified, and sphingomyelin was resolved exactly as described⁴⁹.

Ceramide levels. In short-term experiments (Fig. 4b), 48 h before the experiment cells were transferred to chemically defined medium supplemented with 1 μ Ci 9,10-³H-palmitate. Lipids were extracted and saponified, and ceramide was resolved by thin-layer chromatography²⁰. In long-term experiments (Fig. 5a), in which the culture medium had to be renewed, lipids were extracted, saponified and incubated with *Escherichia coli* diacylglycerol kinase in the presence of γ -³²P-ATP, and ceramide 1-phosphate was resolved by thin-layer chromatography⁴⁹. The reliability of the diacylglycerol kinase assay was confirmed by the observations that in C6.9 glioma cells the magnitude of ceramide accumulation induced by short-term exposure to THC was quantitatively similar to the value obtained with the palmitate labeling procedure, and there was no substantial effect of 5-day exposure to THC on diacylglycerol levels, as determined by the diacylglycerol kinase assay ($n = 3$; data not shown).

Mitogen- and stress-activated protein kinase activities. Cells were washed and lysed, and supernatants were obtained as described²⁰. ERK activity was determined as the incorporation of γ -³²P-ATP into a specific peptide substrate²⁰. JNK and p38 MAPK activity was monitored as the incorporation of γ -³²P-ATP into specific substrates (c-Jun 1-169 and MAPKAP kinase-2 46-600, respectively) after SDS-PAGE, autoradiography and measuring of the radioactivity in excised substrate bands according to manufacturer's instructions (Upstate Biotechnology, Lake Placid, New York).

Raf1 activity. Raf1 was immunoprecipitated from cell lysates as described²⁰. The kinase reaction mixture was incubated 30 min at 30 °C with 0.7 μ g kinase-negative MEK1[97A] (Upstate Biotechnology, Lake Placid, New York) and 2 μ Ci γ -³²P-ATP in assay buffer containing 25 mM Tris HCl, pH 7.4, 10 mM MgCl₂, 0.5 mM EDTA, 5 mM NaF, 1 mM NaVO₄, 1 mM 4-nitrophenylphosphate and proteinase inhibitors^{20,26}. Reactions were stopped with SDS sample buffer, and substrate phosphorylation in the excised bands was determined after SDS-PAGE and autoradiography.

KSR activity. KSR was immunoprecipitated from cell lysates with an antibody against KSR (Santa Cruz Biotechnology, Santa Cruz, California) bound to protein G-sepharose. The kinase reaction mixture was incubated 30 min at 30 °C with 0.3 mM synthetic Raf1 peptide²⁶ and 2 μ Ci γ -³²P-ATP in the assay buffer described above for Raf1. The phosphorylated peptide was resolved using P81 phosphocellulose paper.

Statistics. Results represent means $\pm$ s.d. Statistical analysis used ANOVA. A *post hoc* analysis was made by the Student-Neuman-Keuls test. Survival data of tumor-bearing rats are presented as a Kaplan-Meier plot and the log rank test was applied for statistical analysis.

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