

# $\Delta^9$ -Tetrahydrocannabinol induces apoptosis in C6 glioma cells

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**Abstract**  $\Delta^9$ -Tetrahydrocannabinol (THC), the major active component of marijuana, induced apoptosis in C6.9 glioma cells, as determined by DNA fragmentation and loss of plasma membrane asymmetry. THC stimulated sphingomyelin hydrolysis in C6.9 glioma cells. THC and *N*-acetyl sphingosine, a cell-permeable ceramide analog, induced apoptosis in several transformed neural cells but not in primary astrocytes or neurons. Although glioma C6.9 cells expressed the CB1 cannabinoid receptor, neither THC-induced apoptosis nor THC-induced sphingomyelin breakdown were prevented by SR141716, a specific antagonist of that receptor. Results thus show that THC-induced apoptosis in glioma C6.9 cells may rely on a CB1 receptor-independent stimulation of sphingomyelin breakdown.

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**Key words:** Cannabinoid; Apoptosis; Sphingomyelin hydrolysis; Glioma cell

## 1. Introduction

Cannabinoids, the active components of marijuana, exert a wide spectrum of effects such as alterations in cognition and memory, analgesia, anticonvulsion, antiinflammation, and alleviation of both intraocular pressure and emesis [1]. It is currently established that cannabinoids exert their effects by binding to specific plasma-membrane receptors [1]. To date, two different cannabinoid receptors have been characterized and cloned from mammalian tissues, viz. CB1 [2] and CB2 [3]. The recent discovery of a family of endogenous ligands of cannabinoid receptors [4–6] as well as the potential therapeutic applications of a number of cannabinoid ligands [7,8] have focused a lot of attention on cannabinoids during the last few years.

Glial cells play a major role in brain function since they are involved in processes such as the homeostasis of the neuronal microenvironment, the formation of the blood-brain barrier, the guidance of neuron migration in the developing embryo, and the secretion of neurotrophic factors for neuron healing or development [9]. A potential direct and specific action of cannabinoids on glial cells is supported by some recent observations. Thus, the CB1 receptor mRNA is expressed in astrocytes and astrocytoma cells [10]. Astrocytes in culture have

also been shown to bind and take up anandamide, a putative endogenous ligand of the CB1 receptor [11]. In astrocytoma cells, cannabinoids lead to the stimulation of the mitogen-activated protein kinase and to the induction of the immediate-early gene *krox-24* [12]. In the course of our studies on the metabolic effects of cannabinoids on C6 glioma cells [13] we have observed that cannabinoids inhibit the growth of these cells. Hence, the present work was undertaken to further characterize the anti-proliferative effect of cannabinoids on C6 glioma cells.

## 2. Materials and methods

### 2.1. Materials

THC and 3,4,5-dimethylthiazol-2,5-diphenyltetrazolium bromide thiazol blue (MTT) were from Sigma (St. Louis, USA). The mouse neuroblastoma N18 TG2 cell line and the human astrocytoma U373 MG cell line were kindly given by Dr. V. Di Marzo (CNR, Naples, Italy) and Dr. P. Casellas (Sanofi Recherche, Montpellier, France), respectively. The anti-CB1 cannabinoid receptor antibody was kindly given by Dr. Allyn Howlett (St. Louis University, USA). SR141716 was kindly given by Sanofi Recherche (Montpellier, France). [*Me*-<sup>3</sup>H]Choline and the ECL detection kit were from Amersham (Bucks, UK).

### 2.2. Cell cultures

The C6.9 cell subclone (10–20 passages) was cultured in F12 medium supplemented with 10% fetal calf serum. Twenty four hours before the experiment, cells were transferred to serum-free medium consisting of F12 medium supplemented with insulin (5 µg/ml), transferrin (5 µg/ml), sodium selenite (5 ng/ml), and defatted and dialyzed bovine serum albumin (1.0 mg/ml) [14]. The astrocytoma U373 MG [12], the neuroblastoma N18 TG2 [15], newborn-rat cortical astrocytes [16] and newborn-rat cortical neurons [17] were cultured as previously described.

### 2.3. MTT assay

Cells were incubated with 5 µg/ml MTT for 4 h at 37°C. The medium was then aspirated, the formazan crystals were solubilized with 60 mM HCl in isopropanol and the absorbance at 570 nm was monitored [14].

### 2.4. DNA isolation and analysis

Cells (floating plus attached) were pooled, pelleted, lysed and incubated at 50°C for 1 h in a medium containing 50 mM Tris-HCl, pH 8.0, 50 mM EDTA, 0.5% (w/v) sodium lauryl sarcocylate, and 0.5 mg/ml proteinase K. After treatment for 1 h with DNase-free RNase A at 50°C, samples were mixed with 1% low-gelling temperature agarose at 62°C and subjected to electrophoresis in 1.5% agarose gels.

### 2.5. Binding of fluorescent annexin V

Cells grown in slide flasks coated with poly-D-lysine were washed twice with PBS and then with binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl and 2.5 mM CaCl<sub>2</sub>) prior to incubation with fluorescein isothiocyanate-conjugated annexin V for 20 min at 4°C. After cell fixation with paraformaldehyde, permeabilization with ethanol/acetic acid and digestion with RNase A, propyl iodine was added and samples were subject to fluorescence microscopy [18].

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**Abbreviations:** MTT, 3,4,5-dimethylthiazol-2,5-diphenyltetrazolium bromide thiazol blue; THC,  $\Delta^9$ -tetrahydrocannabinol

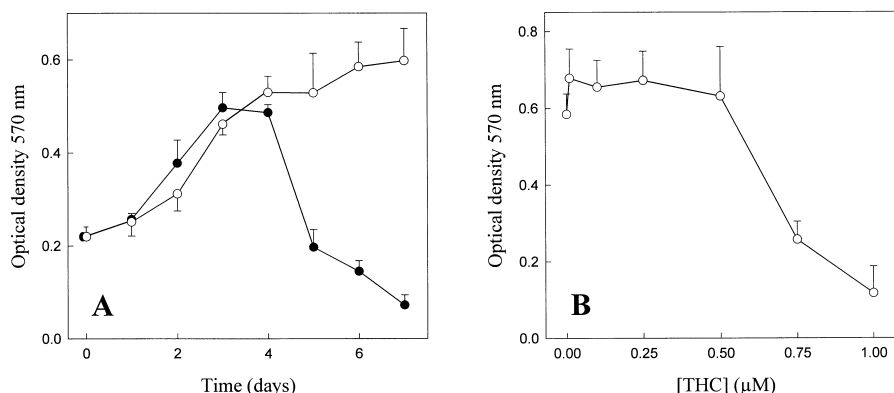


Fig. 1. THC-induced depression of mitochondrial oxidative metabolism in C6.9 glioma cells. A: Cells were cultured in serum-free medium in the absence (○) or in the presence (●) of 1 μM THC for the times indicated. B: Cells were cultured in serum-free medium in the presence of the indicated concentrations of THC for 5 days. In all cases, the medium was renewed every 48 h. The mitochondrial redox state was assessed by the MTT test as the optical density at 570 nm. Results correspond to 4 different experiments.

#### 2.6. Determination of sphingomyelin hydrolysis

Cells were cultured in serum-containing medium and then transferred to serum-free medium for 24 h as described above. Then the medium was changed and cells were incubated for a further 48-h period in chemically-defined medium supplemented with 1 μCi of [<sup>3</sup>H-Me]choline per well. Reactions were started by the addition of the agonists and sphingomyelin was subsequently extracted with chloroform/methanol/water as described before [19].

#### 2.7. Western blot analysis of the CB1 cannabinoid receptor

Cells were scraped and sonicated (2 × 5 s) on ice, and the particulate fraction was obtained after centrifugation at 40 000 × g for 60 min [16]. Western blot analysis of the CB1 receptor was performed as described by Song and Howlett [20]. Blots were finally subjected to luminography with an ECL detection kit.

#### 2.8. Statistical analysis

Results shown represent the means ± S.D. of the number of experiments indicated in every case. Every experimental condition was routinely assayed in quadruplicate. Statistical analysis was performed by ANOVA. A post hoc analysis was made by the Student-Neuman-Keuls test.

### 3. Results and discussion

#### 3.1. THC induces apoptosis in C6.9 glioma cells

Gliomas often show a gross variability characterized by the presence of morphologically distinct cell types within the same tumor [21]. Moreover, glioma cell lines such as C6 have been reported to be heterogenous [14,22]. Hence we used in the present study a subclone of C6 cells (the C6.9 subclone) that has been previously characterized in terms of programmed cell death [14]. As shown in Fig. 1A, addition of THC to the culture medium led to a dramatic drop of mitochondrial oxidative metabolism in C6.9 cells as determined by the MTT test. This effect of THC occurred 4–5 days after the beginning of cell challenge to the cannabinoid (Fig. 1A), was dose-dependent (Fig. 1B) and was accompanied by the death of the cells, which rounded up, detached from their solid support and took up trypan blue (Fig. 2 and data not shown). Interestingly, the THC-induced decrease in optical density at 570 nm in the MTT test showed a non-linear, cooperative-like pattern (Fig. 1A) that may be indicative of an apoptotic process. Experiments were subsequently performed to characterize the THC-induced cell death.

(i) THC-induced glioma cell death was linked to a loss of

plasma membrane asymmetry, as determined by the fluorescent annexin V binding assay (Fig. 2).

(ii) THC induced ladder-patterned DNA fragmentation in C6.9 glioma cells (Fig. 3), a process that is typical of cell death by apoptosis.

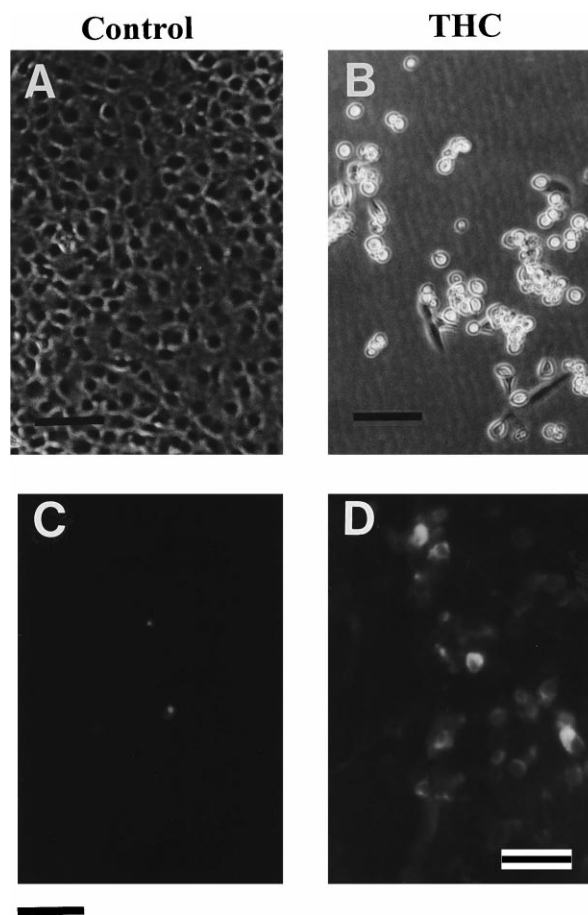


Fig. 2. Micrographs of vehicle- and THC-treated C6.9 glioma cells. A, B: Phase contrast micrographs. C, D: Binding of fluorescent annexin V. In all cases, cells were treated with or without 1 μM THC for 5 days as in Fig. 1. Bar: 25 μm.



Fig. 3. THC induces DNA fragmentation in C6.9 glioma cells. Cells were treated with or without 1  $\mu$ M THC for 5 days as in Fig. 1. A representative gel is shown. Similar results were obtained in 2 other experiments.

These observations thus indicate that THC-induced glioma cell death is a process that differs from necrotic death and displays several features characterizing an apoptotic, programmed cell death event.

### 3.2. THC-induced apoptosis may result from sphingomyelin hydrolysis, and occurs in transformed but not in non-transformed neural cells

Sphingomyelin hydrolysis is a key process in the control of many physiological events related to signal transduction and cellular regulation [23]. In the context of the present study, the involvement of ceramide (the product of sphingomyelin breakdown) in the induction of apoptosis is widely documented [23,24]. Hence we investigated the possible effect of THC on sphingomyelin hydrolysis. As shown in Fig. 4, the addition of THC to the cell culture medium produced a significant breakdown of cellular sphingomyelin. As previously shown for other mediators that induce sphingomyelin hydrolysis (e.g. tumor necrosis factor  $\alpha$  and interleukin-1) (cf. [23–25]), the effect of THC was rapid, transient and accounted for ca. 25% of total cellular sphingomyelin (Fig. 4).

The possibility that THC-induced cell death is not restricted to C6.9 glioma cells but may be a more general process was studied by using a number of neural cell types. Thus, the astrocytoma U373 MG and the neuroblastoma N<sub>18</sub> TG<sub>2</sub> cell lines ensued THC-mediated death, although they were not as sensitive as the C6.9 glioma cell line (Fig. 5). In contrast, neither astrocytes nor neurons in primary culture were sensitive to the apoptotic action of THC (Fig. 5) even after at least 15 days of challenge to the cannabinoid (data not shown). *N*-Acetylsphingosine, a cell-permeable analog of ceramide, displayed a similar pattern of cell-death induction as THC, i.e. it produced the death of the different transformed neural cell lines studied but not of primary astrocytes or primary neurons (Fig. 5). Results therefore suggest that ceramide might be the mediator of THC-induced apoptosis, although we are aware that further research is necessary to demonstrate this possibility.

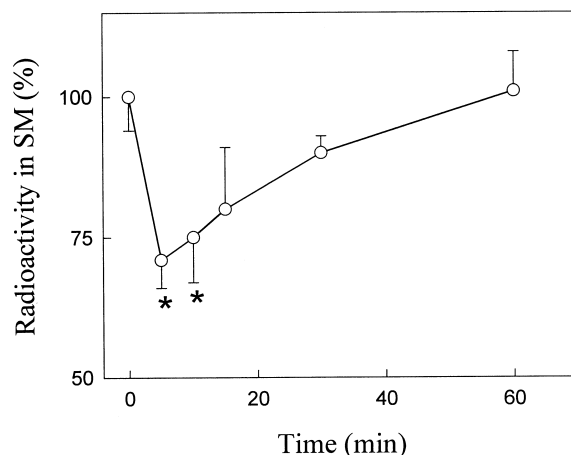


Fig. 4. Stimulation of sphingomyelin hydrolysis by THC in C6.9 glioma cells. Cells were exposed to 1  $\mu$ M THC for the times indicated and the radioactivity in cellular sphingomyelin (SM) was determined. Results are expressed as percentage of incubations with no additions and correspond to 6 different experiments. \*Significantly different ( $P < 0.01$ ) vs. incubations with no additions.

### 3.3. THC-induced apoptosis is not mediated by the CB1 cannabinoid receptor

Most of the effects of cannabinoids on the central nervous system described so far are believed to be mediated by the CB1 cannabinoid receptor [1]. Hence we next studied whether the effects of THC described in the present report were also dependent on this receptor. As shown in Fig. 6, the CB1 receptor was present in C6.9 glioma cells in amounts comparable to those observed in neurons and neuroblastoma N<sub>18</sub> TG<sub>2</sub> cells. Astrocytes and astrocytoma U373 MG cells also contained detectable, though lower, amounts of the CB1 receptor. A particulate fraction from skeletal muscle was used as a negative control.

The effect of SR141716, a specific high affinity CB1 receptor

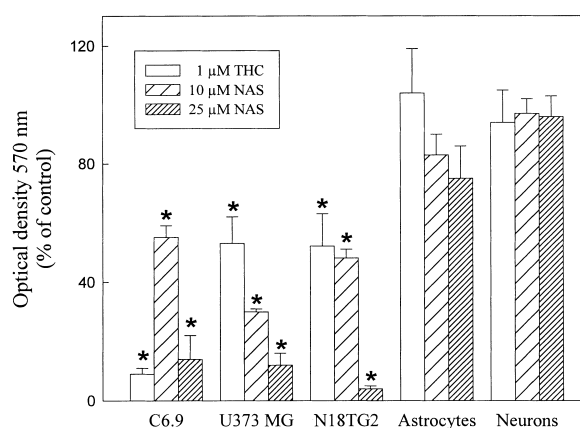


Fig. 5. THC and *N*-acetylsphingosine induce the death of transformed but not of non-transformed neural cells. C6.9 glioma cells, astrocytoma U373 MG cells, neuroblastoma N<sub>18</sub> TG<sub>2</sub> cells, primary astrocytes and primary neurons were cultured with 1  $\mu$ M THC for 5 (C6.9 glioma) or 10 days, with 10  $\mu$ M *N*-acetylsphingosine (NAS) for 3 days, or with 25  $\mu$ M NAS for 3 days. Results are expressed as percentage of cell survival with respect to the respective control incubations as determined by the MTT test and correspond to 4 different cultures for each cell type. \*Significantly different ( $P < 0.01$ ) vs. incubations with no additions.

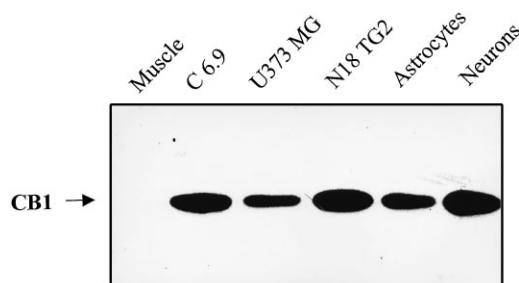


Fig. 6. Presence of the CB1 cannabinoid receptor in transformed and non-transformed neural cells. Total membrane fractions (10  $\mu$ g total protein per lane) from the different transformed and non-transformed neural cell lines were subjected to Western blotting with an antibody raised against the rat CB1 cannabinoid receptor. A representative luminogram is shown. Similar results were obtained in 2 other experiments.

antagonist [26], was tested on THC-induced apoptosis and THC-induced sphingomyelin breakdown in C6.9 glioma cells. As shown in Fig. 7A, the THC-induced glioma cell death, as determined by the MTT test, was not prevented by SR141716. Likewise, SR141716 was unable to antagonize the THC-induced sphingomyelin hydrolysis (Fig. 7B). These data therefore indicate that in spite of the presence of the CB1 receptor in glioma C6.9 cells the apoptotic effect of THC is not mediated by this receptor. It is worth noting that the THC-induced metabolic stimulation of C6 glioma cells has been shown to be prevented by SR141716 [13], indicating that cannabinoids exert CB1 receptor-dependent and CB1 receptor-independent actions on those cells. Anyway, the possible existence in glial cells of a SR141716-insensitive cannabinoid receptor distinct from CB1 and CB2 has been put forward [27].

### 3.4. Possible pathophysiological implications

The sphingomyelin cycle has been shown to play a pivotal role in the regulation of cell function in the central nervous system [25]. Thus, changes in the activity of the sphingomyelin cycle, which may in turn be related to the induction of apop-

totic cell death, have been shown to occur during brain development as well as in neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, epilepsy and ischemia/stroke [25,28]. Likewise, exposure of neural cells to physical (e.g. ultraviolet radiation), chemical (e.g. tumor necrosis factor  $\alpha$ ), bacterial (e.g. lipopolysaccharide) or viral (e.g. human immunodeficiency virus 1) stimuli may trigger sphingomyelin breakdown and therefore evoke changes in the cell survival/cell death decision [23–25]. A finely controlled balance between different ceramide-regulated pathways may determine the survival or the death of a particular neural cell [25]. The possibility that cannabinoids may control the activity of the sphingomyelin cycle (the present report) and the mitogen-activated protein kinase cascade [10,12] points therefore to a general role of these compounds as modulators of cell fate. In this respect, it is worth noting that oleoylethanolamide, a potential endogenous cannabinoid [1,6], is able to produce a sustained increase in intracellular ceramide levels by inhibiting acid ceramidase [29]. Further research is necessary to understand the mechanism of control of the sphingomyelin cycle by cannabinoids.

A number of studies are currently focused on potential therapeutic applications of cannabinoid ligands such as analgesia, antiemesis, antiinflammation and alleviation of intraocular pressure in glaucoma [7,8]. Cannabinoids are also being tested as therapeutic agents in the treatment of neurodegenerative diseases such as multiple sclerosis and Parkinson's disease [7,8]. The antiproliferative effect of THC described in the present report might provide the basis for a new therapeutic application of cannabinoids, especially since primary astrocytes and neurons are resistant to the apoptotic action of THC. In addition, our data suggest that the challenge of C6.9 cells to cannabinoids may be a useful model to study the molecular mechanisms involved in apoptosis in cells of glial origin.

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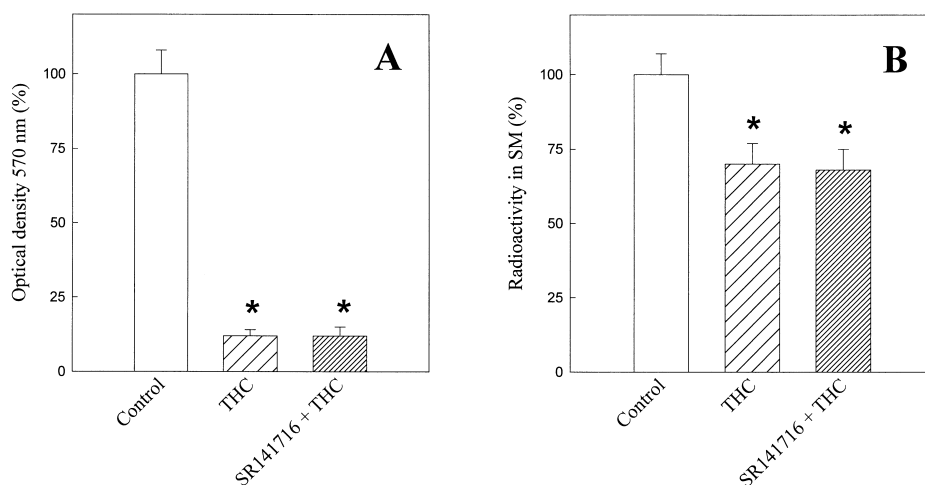


Fig. 7. Lack of effect of SR141716 on THC-induced death and sphingomyelin hydrolysis in glioma C6.9 cells. A: MTT test. Cells were cultured for 5 days with vehicle, 1  $\mu$ M THC, or 1  $\mu$ M THC plus 2  $\mu$ M SR141716. B: Sphingomyelin (SM) hydrolysis. Cells were treated with or without 2  $\mu$ M SR141716 for 30 min prior to the 10-min incubation with 1  $\mu$ M THC. Results correspond to 3 different experiments. \*Significantly different ( $P < 0.01$ ) vs. incubations with no additions.

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