

ORIGINAL ARTICLE

Δ^9 -Tetrahydrocannabinol inhibits cell cycle progression by downregulation of E2F1 in human glioblastoma multiforme cells

GIL GALANTI^{1,2}, TAMAR FISHER², IRIS KVENTSEL², JACOB SHOHAM¹, RUTH GALLILY³, RAPHAEL MECHOULAM⁴, GAD LAVIE⁵, NINETTE AMARIGLIO², GIDEON RECHAVI² & AMOS TOREN²

¹The Mina and Everard Goodman Faculty of Life Science, Bar-Ilan University, Ramat-Gan, Israel, ²Department of Pediatric Hemato-Oncology, Safra Children's Hospital, Sheba Medical Center, Tel Hashomer, Israel, ³The Lautenberg Center for General and Tumor Immunology, The Hebrew University Medical Faculty, Ein Kerem Campus, Jerusalem, Israel, ⁴Department of Medicinal Chemistry and Natural Products, Hebrew University Medical Faculty, Jerusalem, Israel and ⁵Blood Center, Sheba Medical Center, Tel-Hashomer, Israel

Abstract

Background. The active components of *Cannabis sativa* L., Cannabinoids, traditionally used in the field of cancer for alleviation of pain, nausea, wasting and improvement of well-being have received renewed interest in recent years due to their diverse pharmacologic activities such as cell growth inhibition, anti-inflammatory activity and induction of tumor regression. Here we used several experimental approaches, which identified *delta-9-tetrahydrocannabinol* (Δ^9 -THC) as an essential mediator of cannabinoid antitumoral action. **Methods and results.** Administration of Δ^9 -THC to glioblastoma multiforme (GBM) cell lines results in a significant decrease in cell viability. Cell cycle analysis showed G_{0/1} arrest and did not reveal occurrence of apoptosis in the absence of any sub-G₁ populations. Western blot analyses revealed a THC altered cellular content of proteins that regulate cell progression through the cell cycle. The cell content of E2F1 and Cyclin A, two proteins that promote cell cycle progression, were suppressed in both U251-MG and U87-MG human glioblastoma cell lines, whereas the level of p16^{INK4A}, a cell cycle inhibitor was upregulated. Transcription of thymidylate synthase (TS) mRNA, which is promoted by E2F1, also declined as evident by QRT-PCR. The decrease in E2F1 levels resulted from proteasome mediated degradation and was prevented by proteasome inhibitors. **Conclusions.** Δ^9 -THC is shown to significantly affect viability of GBM cells via a mechanism that appears to elicit G₁ arrest due to downregulation of E2F1 and Cyclin A. Hence, it is suggested that Δ^9 -THC and other cannabinoids be implemented in future clinical evaluation as a therapeutic modality for brain tumors.

Glioblastoma multiforme (GBM) is considered to be the most aggressive form of brain tumors [1]. Standard therapeutic arsenal available for treatment of GBM is relatively ineffective, disease remissions remain short and the median survival of these patients is less than one year [2]. Two discrete subsets of glioblastoma derived from astrocytic lineage have been recognized: De novo glioblastomas represent the most frequent presentation with an initial diagnosis of glioblastoma without evidence of preexistent lower grade tumor. These patients are commonly of older age and have a high rate of epidermal growth factor receptor (EGFR) amplification and phosphatase and tensin homolog (PTEN)

mutations [3]. Another form of this disease is secondary glioblastomas which develop in younger patients and arise after a preceding diagnosis of lower grade tumors. High rates of TP53 and RB mutations have been reported in these tumors [4]. GBM remains incurable, which led us to look for novel therapies.

Cannabinoids, the active components of *Cannabis sativa* L. (marijuana; hashish) have been used in folklore medicine for many centuries to alleviate pain, depression, amenorrhea, inflammation and lack of appetite. A renewed interest currently exists in understanding the mechanisms by which cannabinoids exert their therapeutic effects and in their

utilization for additional applications. Ongoing research has shown that cannabinoid ligands may be effective agents in the treatment of pain, inflammation, neurodegenerative disorders such as multiple sclerosis and Parkinson's disease, and the wasting and emesis associated with AIDS and cancer chemotherapy [5]. In recent years, there is a renewed interest in the role of cannabinoids in cancer therapy. Antitumoral effects of cannabinoid on various types of tumor xenografts including lung adenocarcinoma [6], glioma [7], and thyroid epithelioma [8] in animal models have been identified.

Although cannabinoids induce apoptosis or cell cycle arrest in different transformed cells *in-vitro* [9,10], knowledge regarding its mechanisms is limited. In this study we examined the effect of Δ^9 -THC, which accounts for the majority of the known pharmacological effects of Marijuana, on viability and proliferation of two human GBM cell lines; U251-MG and U87-MG. As cell cycle analyses showed that treatment of these cells with Δ^9 -THC culminates in G_{0/1} arrest, we evaluated the effects of Δ^9 -THC on various cellular mediators involved in the transition from G_{0/1} to S.

Materials and methods

Cell lines and culture conditions

The human glioblastoma cell lines U87-MG (with wild type p53) obtained from the American Type Culture Collection (ATCC) and U251-MG (bearing mutant p53) developed in the laboratory of Dr. J. Ponten, University of Uppsala, Sweden. Cells were maintained in Dulbecco's Modified Eagle's Medium (GIBCO, IL) containing 4 mM glutamine, 100 units/mL penicillin, and 100 μ g/mL streptomycin, supplemented with 10% heat-inactivated fetal bovine serum (FBS). Cell lines were maintained at 37°C in a humidified atmosphere containing 5% CO₂.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Cells (2.5×10^4) were plated in triplicates in flat bottom 96-well plates in medium DMEM as mentioned above. The cells were allowed to adhere to the plate surface overnight and then cultured with Δ^9 -THC (0-50 μ g/ml) for 24 and 48 h. Cell viability was then determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which measures reduction of MTT to formazan by mitochondria of viable cells. Formazan was measured spectrophotometrically by absorption at 560 nm in a PowerWaveXTM (BioTek) plate reader. All experiments were repeated at least three times.

Cell cycle analysis

Cells (1×10^6) were seeded into a 90 mm plate in standard medium. After an overnight incubation for the cells to adhere, Δ^9 -THC at doses of 5, 7, 10 and 20 μ g/ml were administered and the cells incubated at 37°C for 24h and 48h, washed twice with ice-cold PBS, detached with 0.25% trypsin-EDTA and pelleted at 128 \times g.

The pellet was resuspended in cold PBS and the cells fixed with absolute ethanol for 1 h at 4°C. The cells were washed twice with cold PBS. Distribution of the cells in G1, S and G2/M phases of the cell cycle was monitored following staining of nuclei with 50 μ g/mL propidium iodide (Sigma) containing 125 units/mL pretease-free RNase, 1% NP-40, and PBS buffered Na-citrate for 30 min in the dark. The pellet was analyzed with Coulter EPICS XL-MCL flow cytometer using the Multicycle program (Phoenix Inc) for data analysis.

Western blot analysis

Nuclear extracts were prepared from GBM cells using buffer C [20 mM HEPES (pH 7.9), 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and complete Protease Inhibitor mixture 40 μ g/ml]. The protein content was calibrated using the BCA protein assay reagent kit (Pierce, Rockford, IL). Samples were separated on 10–15% SDS-PAGE and transblotted onto nitrocellulose filters (Schleicher & Schuell, Dassel, Germany). Equal protein loading was verified by staining with Ponceau S (Sigma, St. Louis, MO). The membranes were probed at 4°C over night with the following primary antibodies: mouse monoclonal Abs anti E2F1, anti p16^{INK4A}, rabbit polyclonal anti Cyclin A, and goat polyclonal anti actin (Santa Cruz Biotechnology). Proteins were visualized by staining with horseradish peroxidase-conjugated anti-mouse IgG, anti-rabbit IgG, and anti-goat IgG secondary antibodies for 1 hour and developed on Fuji Scientific Imaging Film by enhanced chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate; Pierce) according to manufacturer's instructions.

Quantifications of separated proteins were performed by densitometric analyses using the OptiQuant version 4.0, image-analysis software (Packard Co., Meriden, CT). Briefly, the developed photographic films were scanned on a flatbed scanner, using standard settings. The images were converted to gray-scale negatives using Photoshop version. 8.0 software and Optiquant was then used to estimate the intensity of each band. Results were presented as digital light units (DLU).

Quantitative RT-PCR

Real-time quantitative reverse transcription-PCR was carried out using the LightCycler SYBR Green PCR Master Mix (Applied Biosystems) and performed by using ABI 7900 Sequence Detection System (Applied Biosystems). Two μg of total RNA were reverse-transcribed using Superscript II RNase H RT (Invitrogen) and random hexamers (Invitrogen) per manufacturer's instructions. The reaction mixture was initially heated to 95°C for 10 min followed by 40 reaction cycles of denaturation at 95°C for 15 s, and annealing/extension at 60°C for 1 min. The following sense and antisense primers were used for QRT-PCR amplification: human E2F1, 5'-GCCACTGACTCTGCCACCA – sense and 5'-GGACAACAGCGGTTCTTGCT – antisense, human thymidylate synthase, 5'-GAAGAATCATCATGTGCGCTTG – sense and 5'-GCGCCATCAGAGGAAGATCTC – antisense; human Cyclin A, 5'-GAAGACGAGACGGGTTGCA – sense, and 5'-AGGAGGAACGGTGACATGCT – antisense. Gene expression levels were normalized against the endogenous control gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-TGGACCTCATGGCCACA – sense, and 5'-TCAAGGGGTCTACATGGCAA – antisense. Quantification was carried out in triplicate. All primers were obtained from Daniel Biotech (Rehovot, IL).

Immunofluorescence staining

Cells (3×10^5) were seeded on glass coverslips, incubated for 24 and 48 h at 37°C with or without $20 \mu\text{g/ml}$ Δ^9 -THC, washed twice with PBS and fixed *in situ* with absolute methanol at -20°C for 20 min. The cells were immunostained with a mouse monoclonal E2F1 antibody (Santa Cruz Biotechnology) for 2 h at 37°C . Unbound antibody was removed by washing with Tris buffered saline (TBS) containing 10% Tween 20 (Sigma) and the cells stained with Rhodamine Red-X-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) for 1 h at 37°C in the dark. Nuclei were simultaneously counterstained with 4,6-diamino-2-phenylindole (DAPI, Sigma) solution. Slides were mounted and examined under a fluorescence microscope (Olympus Provis AX70).

Statistical analyses

All data were presented as mean \pm SD from at least three sets of independent experiments. The two-

tailed Student *t* test was used in statistical analyses. $P \leq 0.05$ were considered statistically significant.

Results

Δ^9 -THC inhibits proliferation of human glioblastoma multiforme cell lines

Cells from the GBM U251-MG and U87-MG lines were incubated with Δ^9 -THC at doses of 0–50 $\mu\text{g/ml}$ for 24 h and 48h, and reductions in cell viability were estimated using the MTT assay. As shown in Figure 1A, Δ^9 -THC induced loss of cell viability and cell death. We next addressed the question whether treatment with Δ^9 -THC has an anti proliferative activity with effect on cell cycle progression. We found increases in the percentage of cells sequestered in the G_0 - G_1 compartment from 59% in control cells to 76% in U87-MG during induction of Δ^9 -THC and statistical significant elevation from 55% in control cells to 73% post treatment with Δ^9 -THC in U251-MG ($*p < 0.05$) (addition of $\sim 20\%$ of cells arrested in G_1). Accordingly, the percentages of cells in G_2 and S phase ($*p < 0.05$ in U87-MG) were found to decrease (Figure 1B), indicating that these cell populations have undergone G_1 phase arrest.

No sub- G_1 population was detected apparently pointing away from the possibility that the treatment with Δ^9 -THC induced apoptosis. Furthermore, the treatment of the GBM cells with Δ^9 -THC appears to have triggered morphological changes after 24 h of exposure to the opiate (Figure 1C II and IV) relative to untreated controls (Figure 1C I and III). Microscopic examination demonstrated cytoplasmic swelling and formation of vesicles in the treated cells

Δ^9 -THC modulates cellular expression of the E2F1 transcription factor at the protein and mRNA levels

In order to elucidate the mechanism of Δ^9 -THC action, we studied the effects of cell treatment with Δ^9 -THC on expression of several proteins that are involved in the control of cell proliferation. These analyses found that the transcription factor E2F1 was a primary target most significantly affected by Δ^9 -THC. Nuclear E2F1 protein levels in U251-MG were decreased by 30% 24 h post treatment with Δ^9 -THC and to progressively decrease at 48 h (Figure 2A, left panel). Similarly, E2F1 levels in U87-MG significantly declined by 40% at the 48 h post treatment time point relative to the control group (Figure 2A right panel). To determine whether the decreased expression of E2F1 results from downregulated expression of the gene at the

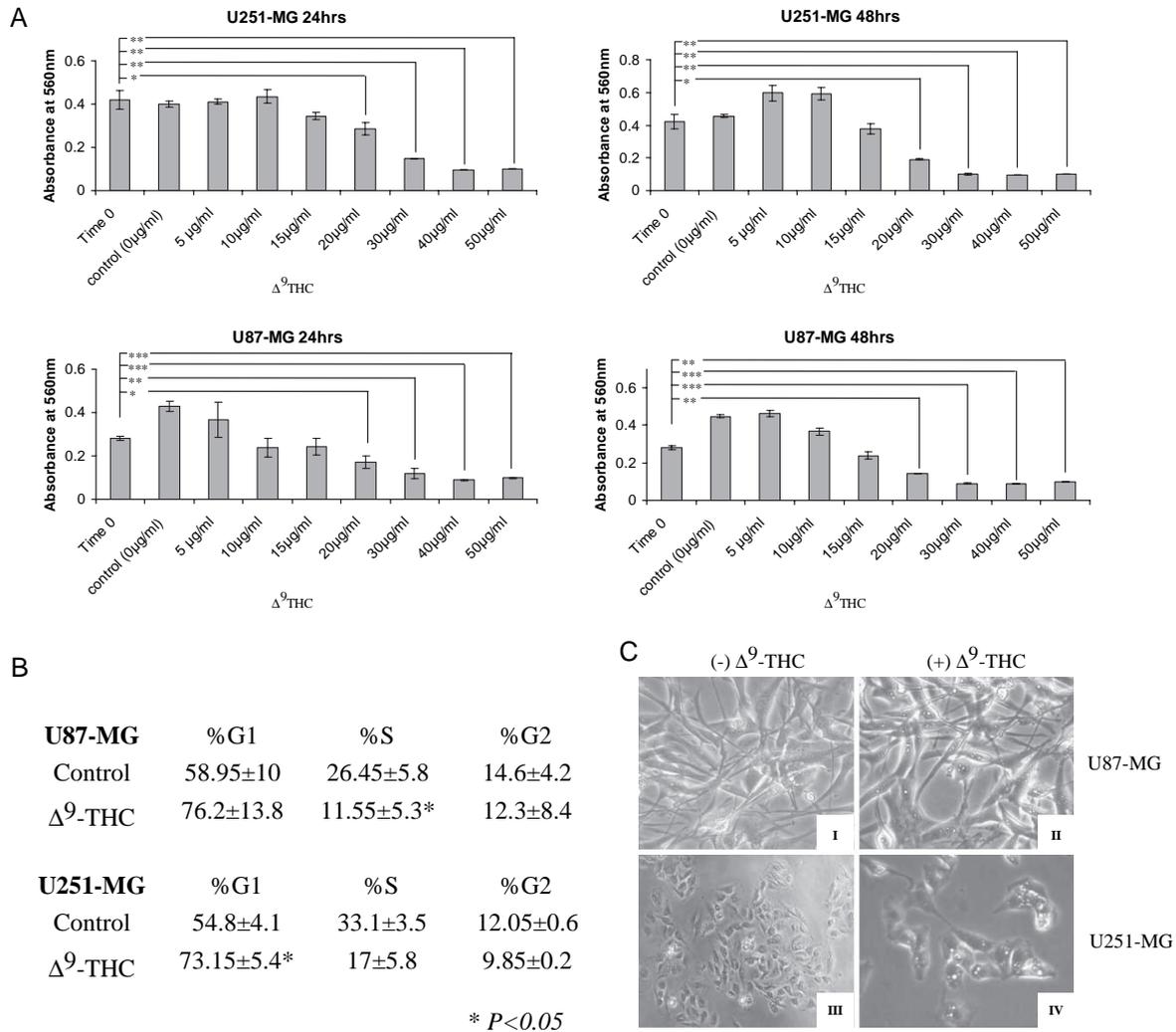


Figure 1. Δ^9 -THC induce cell death in human glioblastoma multiforme cell lines through G_0 - G_1 transition (G_1 arrest). *A*, dose-dependent effect of Δ^9 -THC on cell viability is shown by the MTT assay. U251-MG (*upper*) and U87-MG (*lower*) cells were treated with increasing concentration of Δ^9 -THC (0, 5, 10, 15, 20, 30, 40, and 50 $\mu\text{g/ml}$) compared with untreated cells ("Time 0") for 24 (*left*) and 48 h (*right*). Columns, mean of three different measurements; bars, \pm SD; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ versus untreated cells. *B*, effect of Δ^9 -THC on induction of G_0/G_1 arrest. Cell cycle profile was evaluated by propidium iodide staining and FACS analysis (as indicated in Materials and methods). The fractions of cells in G_1 phase increased in both GBM cell lines with the reduction of cells in S phase and G_2 phase after treatment with 10 $\mu\text{g/ml}$ Δ^9 -THC for 24 h in U251-MG and 20 $\mu\text{g/ml}$ Δ^9 -THC for 48 h in U87-MG. The percentages of gated cells are indicated, \pm SD; *, $p < 0.05$. *C*, representative image of GBM cell cultures captured from each well by using a camera attached to an inverted microscope (Leica DMIL, Germany). U87-MG and U251-MG were incubated for 0 h (I, III) and for 24 h with 20 $\mu\text{g/ml}$ Δ^9 -THC (II, IV). Cytoplasm swelling and formations of membrane bound vesicles are observed post treatment with Δ^9 -THC.

transcriptional level, E2F1 mRNA levels were quantified in the cells following treatment with Δ^9 -THC by QRT-PCR. Significant decreases in E2F1 mRNA levels were noted after treatment with Δ^9 -THC for 24 h and after 48 h in both cell lines compared to the corresponding untreated control cells. Immunofluorescence staining of cells with an antibody to E2F1 after treatment with Δ^9 -THC for 24 and 48 h confirmed these results (Figure 2C and D). The control cells were stained with DAPI alone. Both GBM cell lines presented significant whole cell decrease in E2F1 after 24 h and rose after 48 h.

The effect of Δ^9 -THC on several cell cycle mediators that regulate G_1 -S transition

Cell cycle progression is also regulated via irreversible transitions propelled by CDKs and cyclins [11]. Therefore, we reasoned that an observed G_1 arrest could be due to a decrease in kinase activity of CDKs, cyclins, and increase in kinase inhibitors (CKIs) [12]. The function of cyclin A gene has been shown to be essential for the induction of S phase [13]. Here, we show distinct decline in protein levels of cyclin A after treatment with Δ^9 -THC for 24 and 48 h (Figure 3A) and in its mRNA levels (Figure 3B).

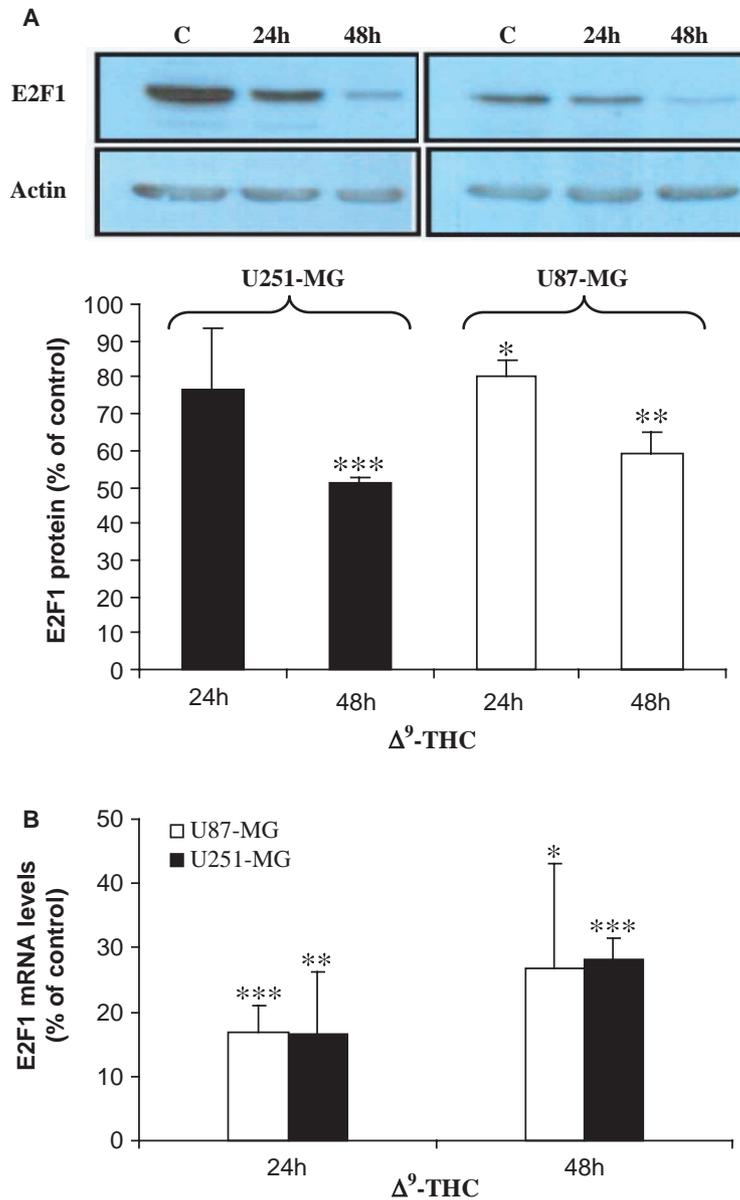


Figure 2 (Continued)

In addition, we examined the level of p16^{INK4A}, a cyclin dependent kinase inhibitor (CDKI) that act as tumor suppressor. Inactivation of p16^{INK4A} is critical for the formation or progression of many human cancers. Restoration of p16^{INK4A} expression in glioma cells inhibits cell growth by blocking cell cycle progression in G₁ phase [14]. We found elevated levels of p16^{INK4A} in U251-MG after 24 and 48 h of Δ^9 -THC application, and after 24 h in U87-MG (Figure 3A).

Along with E2F1 we also tested mRNA levels of its regulated enzyme that is essential for DNA synthesis and repair; thymidylate synthase (TS) [15]. We have shown that treatment with Δ^9 -THC decreased TS mRNA levels more than 1.5 fold in

U87-MG and 3.5 fold in U251-MG for 24 h and by 2 fold and 5 fold respectively after 48 h (Figure 3B).

Δ^9 -THC represses E2F1 via the Proteasome System

The regulatory mechanism for E2F1 gene expression has not yet fully understood. In the course of this study, we have investigated the mechanism responsible for the suppression of E2F1 expression by Δ^9 -THC. Δ^9 -THC was shown to suppress E2F1 expression through the proteasome pathway. This was shown by employing the proteasome inhibitor MG132 (Calbiochem, Mercury, Israel). U251-MG and U87-MG were treated with 3 μ M of MG132 for 8 h. Results show decreased protein levels after

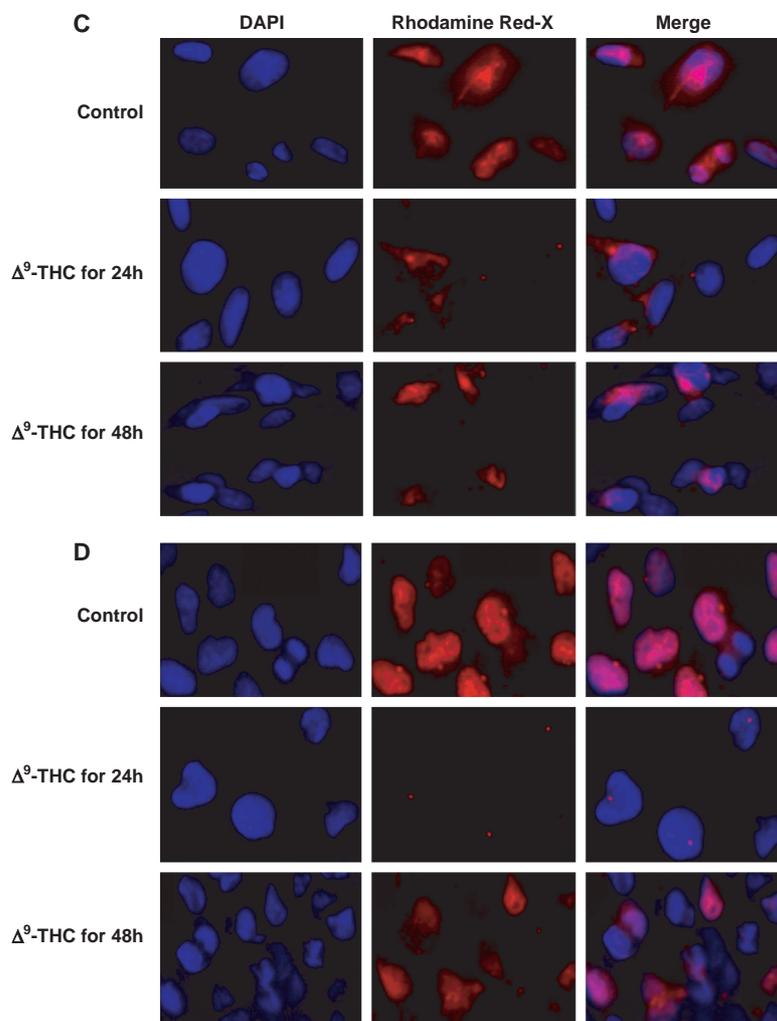


Figure 2. Δ^9 -THC decreases E2F1 protein and RNA expression levels in both human GBM cell lines. *A*, western blot analysis shows a significant decrease of nuclear E2F1 resulting from treatment with 20 $\mu\text{g}/\text{ml}$ Δ^9 -THC for 24 and 48 h when compared to untreated cells (set as 100%). Nuclear extract was prepared from buffer C (as mentioned in the Material and methods), and the membrane was probed overnight at 4°C with primary monoclonal E2F1 antibody. Actin serves as loading control. Results were analyzed by densitometry and expressed as a percentage of the intensity value of untreated cells. *Columns*, mean of four different measurements; *bars*, $\pm\text{SD}$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ versus untreated cells. *B*, verification by QRT-PCR confirms the decrease in E2F1 protein expression levels, by significant decrease in its RNA expression levels, after treatment of Δ^9 -THC for 24 and 48 h using the SYBR Green PCR Master Mix. E2F1 gene expression was normalized to GAPDH gene and presented as a percentage to corresponding control (set as 100%). *Columns*, mean of three different measurements; *bars*, $\pm\text{SD}$; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ statistical significant differences. *C*, U87-MG and *D*, U251-MG cells were plated on glass coverslips with or without 20 $\mu\text{g}/\text{mL}$ Δ^9 -THC for 24 and 48 h then fixed with methanol, incubated for 2 h with monoclonal E2F1 antibody, washed extensively with TBS, and incubated for 1 h with E2F1-secondary Ab (Rhodamine Red-X). Cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Slides were then mounted and examined using a fluorescence microscope. Photographs were taken at the same magnification ($\times 40$) and then transported to Photoshop.

administering Δ^9 -THC for 24 and 48 h and elevated protein levels resulting from the addition of MG132 along with Δ^9 -THC for 24 and 48 h in both GBM cell lines (Figure 4).

Discussion

Therapeutic treatment modalities currently being used to treat glioblastoma multiform (GBM) (e.g., chemotherapy, radiotherapy and surgery) continue to yield unsatisfactory results. Median survival

remains within the range of one year [1,2] and the search for novel therapeutics thus continues. We studied the effect of Δ^9 -THC, a plant-derived cannabinoid, on the viability and cell cycle progression of GBM cell lines in culture. Cannabinoids have previously been shown to affect various types of tumor xenografts [6–8] and to induce apoptosis or cell cycle arrest [9,10] in those cells.

Results presented herein show that Δ^9 -THC is capable of blocking GBM cells from progressing through the various phases of the cell cycle. We also

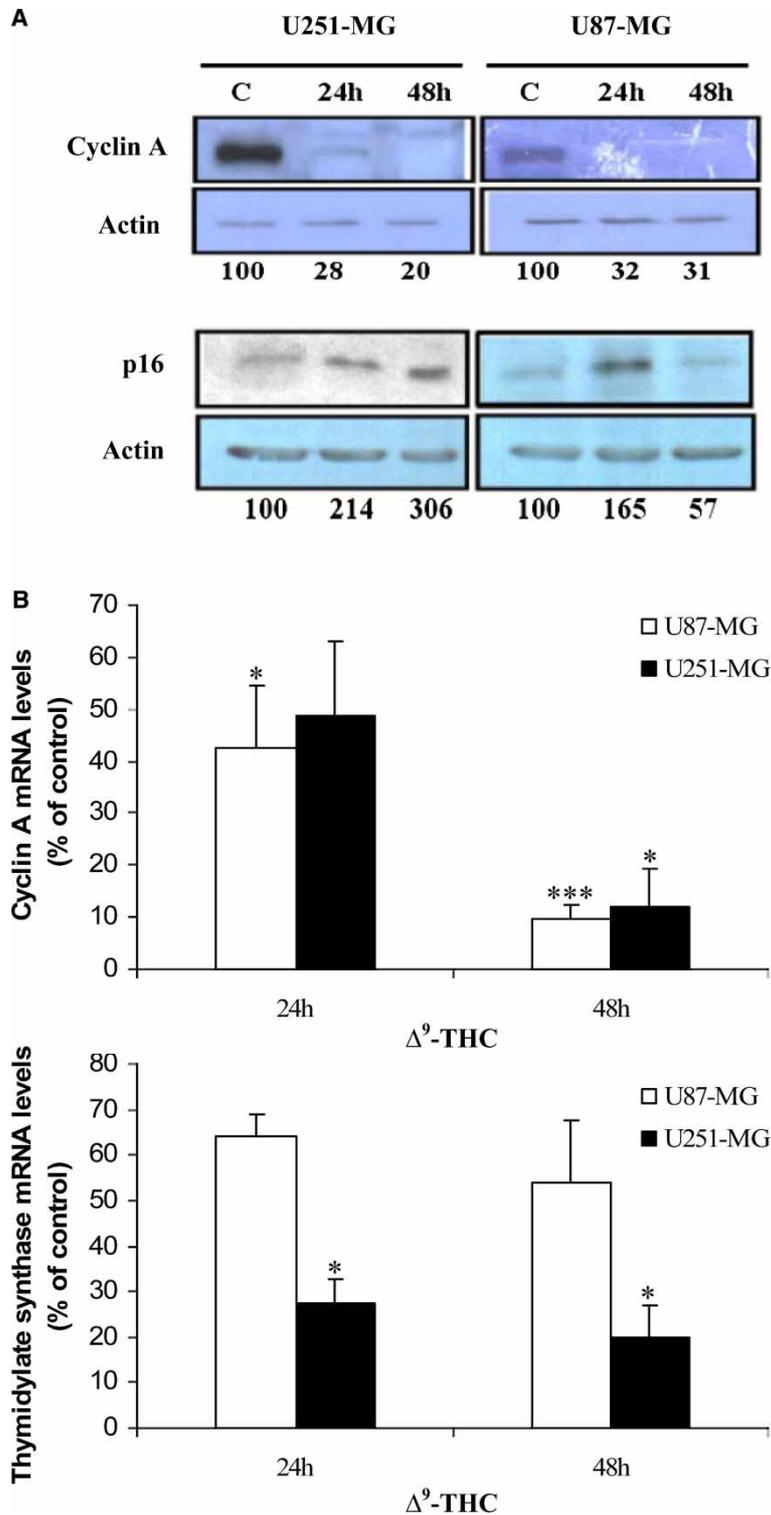


Figure 3. Δ^9 -THC effect on protein levels and on genes that are involved in cell cycle progression. *A*, Cyclin A protein levels in U251-MG and U87-MG cells (*upper*) were prominently downregulated due to 20 $\mu\text{g/ml}$ of Δ^9 -THC for 24 and 48 h when compared to untreated cells. p16^{INK4A} levels in nuclear fractions of the cells were elevated after 24 h of treatment in both cell lines (*lower*). Actin was used as a loading control. Analyses were repeated at least three times. Expressions of the different proteins are represented as O.D. relative to respective controls at the designated time-points (set at 100). *B*, verification of expressions of cell cycle related genes using QRT-PCR. *Upper*, Cyclin A RNA levels were significantly decreased in both cell lines, respectively to the Western results. Thymidylate synthase (*lower*), E2F1 upregulated enzyme, expression levels were downregulated in U87-MG and significantly in U251-MG after 24 and 48 h of treatment with 20 $\mu\text{g/ml}$ Δ^9 -THC corresponding to E2F1 RNA levels as indicated previously. All genes expression levels were normalized to GAPDH gene and presented as a fold increase relative to corresponding control. Columns, mean of three different measurements; bars, \pm SD; * $p < 0.05$, *** $p < 0.001$ statistical significant differences.

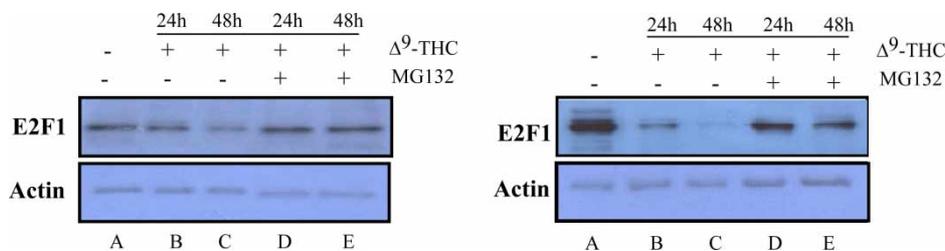


Figure 4. E2F1 protein levels might be mediated via the proteasome degradation. We assessed the protein expression levels of E2F1 by Western blot analysis on two different GBM cell lines; U87-MG (*left*) and U251-MG (*right*). Cells were treated in the absence (*lane A*) or presence of 20 $\mu\text{g/ml}$ Δ^9 -THC for 24 h (*lane B*) and 48 h (*lane C*). To determine whether decrease in E2F1 levels is mediated by the proteasome machinery, we employed MG132, a proteasome inhibitor. 3 μM MG132 were added for 8 h to cell culture kept in presence of Δ^9 -THC for 24 h (*lane D*) and 48 h (*lane E*). Increased levels of E2F1 are shown in lanes D and E. E2F1 protein levels were determined from the nuclear extract. Actin was used as loading control.

provide insight into the possible mechanism for the downregulated cell replication induced in GBM cells by Δ^9 -THC.

Analyses of cellular expression of cell cycling regulators involved in the control of G₁-S transition revealed decreases in mRNA and protein levels of the E2F1 transcription factor following treatment of GBM cells with Δ^9 -THC for 24 and 48 h. E2F1 is an essential mediator for cell proliferation and cell survival [16]. A decrease in E2F1 after cell treatment with Δ^9 -THC was also evident by immunofluorescent staining of the cells with an anti E2F1 antibody. Approximately 20% elevation in the percentage of cells sequestered in the G₁ phase and, in parallel, decreases in the proportion of cells proceeding to the S and G₂ phases of the cell cycle resulted from the treatment with Δ^9 -THC. These are the actual types of responses that would be expected from inactivation of E2F1.

The prolonged interruption in cell proliferation by Δ^9 -THC has also been found to impact on the overall viability of the GBM cell lines. Viability decreased to levels lower than would be expected from purely cytostatic effects, indicating that cell death did occur, although it could not be assigned to apoptosis.

The importance of the Δ^9 -THC mediated decrease in E2F1 may be in the finding that thymidylate synthase (TS), the expression of which is controlled by E2F, is also found to be down regulated by the Δ^9 -THC treatment of cells. This decrease may have the potential of being clinically relevant as several clinical studies have indeed shown occurrence of elevated levels of thymidylate synthase at the mRNA and protein levels in several human cancers [17–19]. Furthermore, these malignancies demonstrate active cellular proliferation which leads to tumor invasiveness and metastasis [17,18].

The data presented here also shows that Δ^9 -THC induces downregulation of Cyclin A mRNA and protein levels in the two GBM cell lines. Cyclin A is

a cell cycle regulatory protein that functions in S phase and G₂ and it can bind and activate cyclin-dependent kinase 2 (cdk2) and cyclin-dependent kinase-1 (cdk1) [13].

We also examined the cellular levels of expression of cyclin dependent kinase (cdk) inhibitors. One such protein is p16^{INK4A} which inhibits the binding of the CDK4 protein to cyclin D1/2, thus preventing phosphorylation of the RB protein, and arresting the cell cycle in the G₁ phase. In gliomas, p16^{INK4A} is inactivated in over 50% of the cases either by homozygous deletion [20], mutations [21] or methylation [22]. Moreover, overexpression of the p16^{INK4A} gene considerably reduces the ability of glioma cells to invade normal brain [23]. Replacement of the wild-type p16^{INK4A} gene may be a valid molecular approach to control the growth of malignant gliomas. In this study p16^{INK4A} protein was elevated 24 h post Δ^9 -THC therapy.

Taken together, these findings strongly suggest that the anti-cancer effects of these cell cycle regulators are modulated in ways leading to cell cycle arrest.

In the second part of this study, we analyzed potential mechanisms which can induce these effects. Recently, several studies demonstrated that regulation of E2F1 protein levels could be mediated via the ubiquitin-proteasome-dependent degradation. The ubiquitin-proteasome system controls the destruction of the cell cycle regulatory proteins such as cyclins and CDK-inhibitors. These proteins have been found to undergo ubiquitin dependent proteolysis during the progression of the cell cycle [24]. Similarly, E2F1 has also been shown to be regulated through this degradation system [25]. We found that Δ^9 -THC suppresses E2F1 level-through the proteasome pathway.

The suppression of cell cycle progression via E2F1 seems to be multifactorial: p16^{INK4A} suppresses cell cycle progression by phosphorylation of Rb thereby maintaining E2F1 in a transcriptional

inactive complex with Rb, in addition to proteasome-mediated degradation of the protein as well as a decrease in E2F1 mRNA levels in response to Δ^9 -THC.

In conclusion, this study supports the renewed interest in cannabinoids as anti-cancer substances in addition to their well known supportive effect. Our observations provide new insights into the proteasome machinery, and also clarify the role of E2F1 and other G₁-S phase proteins induced by Δ^9 -THC therapy. We suggest that Δ^9 -THC and other cannabinoids should now be implemented in future studies of GBM and possibly other malignancies therapy.

Acknowledgements

This work was performed in partial fulfillment of the requirements for M.Sc degree of G.G. from the Mina and Everard Goodman Faculty of Life Science, Bar-Ilan University, Israel. G.R. holds the Djerassi Chair in Oncology at the Sackler School of Medicine, Tel Aviv University. Gil Galanti and Tamar Fisher contributed equally to this paper.

References

- [1] Collins VP. Gliomas. *Cancer Surv* 1998;32:37–51.
- [2] Kraemer DF, Fortin D, Neuwelt EA. Chemotherapeutic dose intensification for treatment of malignant brain tumors: Recent developments and futures directions. *Curr Neurol Neurosci* 2002;2:216–24.
- [3] Smith JS, Tachibana I, Passe SM, Huntley BK, Borell TJ, Iturria N, et al. PTEN mutation, EGFR amplification, and outcome in patients with anaplastic astrocytoma and Glioblastoma Multiform. *J Natl Cancer Inst* 2001;15:93:1246–56.
- [4] Kleihues P, Ohgaki H. Primary and secondary glioblastomas: From concept to clinical diagnosis. *Neurooncol* 1999;1:44–51.
- [5] Porter AC, Felder CC. The endocannabinoid nervous system: Unique opportunities for therapeutic intervention. *Pharmacol Ther* 2001;90:45–60.
- [6] Munson AE, Harris LS, Friedman MA, Dewey WL, Carchman RA. Antineoplastic activity of cannabinoids. *J Natl Cancer Inst* 1975;55:597–602.
- [7] Galve-Roperh I, Sánchez C, Cortés ML, del Pulgar TG, Izquierdo M, Guzmán M. Anti-tumoral action of cannabinoids: Involvement of sustained ceramide accumulation and extracellular signal-regulated kinase activation. *Nat Med* 2000;6:313–9.
- [8] Bifulco M, Laezza C, Portella G, Vitale M, Orlando P, De Petrocellis L, et al. Control by the endogenous cannabinoid system of ras oncogene-dependent tumor growth. *FASEB J* 2001;15:2745–7.
- [9] Guzmán M, Sánchez C, Galve-Roperh I. Control of the cell survival/death decision by cannabinoids. *J Mol Med* 2001;78:613–25.
- [10] Caffarel MM, Sarrio D, Palacios J, Guzman M, Sanchez C. Delta9 tetrahydrocannabinol inhibits cell cycle progression in human breast cancer cells through Cdc2 regulation. *Cancer Res* 2006;1;66(13):6615–21.
- [11] Grana X, Reddy EP. Cell cycle control in mammalian cells: Role of cyclins, cyclin dependent kinases (CDKs), growth suppressor genes and cyclin-dependent kinase inhibitors (CKIs). *Oncogene* 1995;11:211–9.
- [12] Morgan DO. Principles of CDK regulation. *Nature* 1995;374:131–4.
- [13] Pagano M, Pepperkok R, Verde F, Ansorge W, Draetta G. Cyclin A is required at two points in the human cell cycle. *EMBO J* 1992;11:961–71.
- [14] Arap W, Nishikawa R, Furnari FB, Cavenee WK, Huang H-SS. Replacement of the p16/CDKN2 gene suppresses human Glioma cell growth. *Cancer Res* 1995;55:1351–4.
- [15] DeGregori J, Kowalik T, Nevins JR. Cellular targets for activation by the E2F1 transcription factor include DNA synthesis- and G₁/S-regulatory genes. *Mol Cell Biol* 1995;15:4215–24.
- [16] Dyson N. The regulation of E2F by pRB-family proteins. *Genes Dev* 1998;12:2245–62.
- [17] Mizutani Y, Wada H, Yoshida O, Fukushima M, Nonomura M, Nasao M, et al. Significance of thymidylate synthase activity in renal cell carcinoma. *Clin Cancer Res* 2003;9:1453–60.
- [18] Nomura T, Nakagawa M, Fujita Y, Hanada T, Mimata H, Nomura Y. Clinical signification of thymidylate synthase expression in bladder cancer. *Int J Urol* 2002;9:368–76.
- [19] Shintani Y, Ohta M, Hirabayashi H, Tanaka H, Iuchi K, Nakagawa K, et al. New prognostic indicator for non-small-cell lung cancer, quantitation of thymidylate synthase by real-time reverse transcription polymerase chain reaction. *Int J Cancer* 2003;104:790–5.
- [20] Schmidt EE, Ichimura K, Reifenberger G, Collins VP. CDKN2 (p16/MTS1) gene deletion or CDK4 amplification occurs in the majority of glioblastomas. *Cancer Res* 1994;54:6321–4.
- [21] Kyritsis AP, Zhang B, Zhang W, Xiao M, Takeshima H, Bondy ML, et al. Mutations of the p16 gene in gliomas. *Oncogene* 1996;12:63–7.
- [22] Costello JF, Berger MS, Huang H-JS, Cavenee WK. Silencing of p16/CDKN2 expression in human gliomas by methylation and chromatin condensation. *Cancer Res* 1996;56:2405–10.
- [23] Chintala SK, Fueyo J, Gomez-Manzano C, Venkaiah B, Bjerkvig R, Yung WKA, et al. Adenovirus-mediated p16/CDKN2 gene transfer suppress Glioma invasion in vitro. *Oncogene* 1997;15:2049–57.
- [24] Yew PR. Ubiquitin-mediated proteolysis of vertebrate G₁- and S phase regulators. *J Cell Physiol* 2001;187:1–10.
- [25] Campanero MR, Flemington EK. Regulation of E2F through ubiquitin-proteasome-dependent degradation: Stabilization by the pRB tumor suppressor protein. *Proc Natl Acad Sci USA* 1997;94:2221–6.