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Laboratory Investigation

Cannabinoids selectively inhibit proliferation and induce death of cultured human glioblastoma multiforme cells

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Summary

Normal tissue toxicity limits the efficacy of current treatment modalities for glioblastoma multiforme (GBM). We evaluated the influence of cannabinoids on cell proliferation, death, and morphology of human GBM cell lines and in primary human glial cultures, the normal cells from which GBM tumors arise. The influence of a plant derived cannabinoid agonist, Δ^9-tetrahydrocannabinol (Δ^9-THC), and a potent synthetic cannabinoid agonist, WIN 55,212-2, were compared using time lapse microscopy. We discovered that Δ^9-THC decreases cell proliferation and increases cell death of human GBM cells more rapidly than WIN 55,212-2. Δ^9-THC was also more potent at inhibiting the proliferation of GBM cells compared to WIN 55,212-2. The effects of Δ^9-THC and WIN 55,212-2 on the GBM cells were partially the result of cannabinoid receptor activation. The same concentration of Δ^9-THC that significantly inhibits proliferation and increases death of human GBM cells has no significant impact on human primary glial cultures. Evidence of selective efficacy with WIN 55,212-2 was also observed but the selectivity was less profound, and the synthetic agonist produced a greater disruption of normal cell morphology compared to Δ^9-THC.

Abbreviations: CB – cannabinoid; ERK – extracellular signal regulated kinases; FBS – fetal bovine serum; GBM – glioblastoma multiforme; MANOVA – multivariate analysis of variance; MAPK – mitogen-activated protein kinases; Δ^9-THC – Δ^9-tetrahydrocannabinol; TLM – time lapse microscopy

Introduction

Cannabinoids have been shown to control cell growth and death in multiple types of cancer [1]. The endocannabinoid system was discovered through research focusing on the primary active component of *Cannabis sativa*, Δ^9-THC, and other synthetic cannabinoids [2]. To date, two G-protein coupled receptors, CB_1 and CB_2, have been demonstrated to mediate a majority of the pharmacological effects of the compounds [3]. There is, however, evidence for the existence of subtypes of cannabinoid receptors [4]. The present literature suggests that cannabinoids could provide a two-tiered approach for inhibiting tumor growth [5]. First, cannabinoids have been shown to produce direct antiproliferative and apoptotic effects in cell lines derived from many types of cancers [6]. This direct antitumor activity has been primarily attributed to the activation of both CB_1 and CB_2 receptors but there is also data suggesting that cannabinoids can directly inhibit the growth of cancer through other mechanisms [7,8]. The possibility that cannabinoids might also inhibit tumor growth indirectly is supported by studies demonstrating that cannabinoid compounds can decrease angiogenesis through modulation of proangiogenic factors [9,10].

Of potential clinical relevance is the selective efficacy apparent with cannabinoids. Compounds activating the endocannabinoid system can induce apoptosis in rat C6 glioma cells *in vitro* as well as inhibit growth and eradicate tumors *in vivo* [11]. Importantly, cannabinoid-induced cell death was observed in C6 glioma cells but this phenomenon was not seen in rodent primary astrocytes and neuronal cultures [12]. This finding was further supported by *in vivo* data showing cannabinoids could inhibit, and in some cases eradicate, C6 glioma tumors but there was no discernable tissue damage in surrounding healthy tissue [11]. The potential of these compounds to inhibit growth of gliomas has prompted a human clinical trial [13].

The prior studies indicating selective efficacy against glioma tumors in rodent models prompted us to evaluate the effects of cannabinoids in human cells. We compared the responses of normal human glial cells with those of human cell lines derived from the highest grade glioma tumor, glioblastoma muliforme (GBM). The use of time lapse microscopy (TLM) in addition to

end-point assays allowed a more complete evaluation of the cellular responses to cannabinoids. Here we directly quantify cannabinoid-induced changes in cell proliferation, death, and morphology. We also document indirect evidence for an influence of cannabinoids on cell motility. Detailed analysis using both a plant derived cannabinoid agonist, Δ^9-THC, and a potent synthetic agonist, WIN 55,212-2, are presented.

Materials and methods

Cell culture and treatments

Human cell lines derived from brain tumor biopsy specimens from patients with GBM were maintained at 37 °C and 5% CO_2. The human GBM cell lines used were SF126, U87-MG, U251, SF188, and U373-MG. In experiments determining the sensitivity of multiple human GBM cell lines to WIN 55,212-2, cells were first cultured in RPMI media containing 10% fetal bovine serum (FBS). The serum concentration was reduced to 0.1% FBS on the first day of the treatment with cannabinoid compounds. Primary human mixed glial cultures were isolated from second trimester elective abortion fetal brain by established methods [14] and were confirmed to be glia by uniform staining (data not shown) with an antiglial fibrillary acid protein (GFAP) antibody (Boehringer, Mannheim). In the TLM experiments, SF126 GBM cells and normal glial cells were grown in 6 well plates in Eagle's Basal Medium (EBM) with 0.02 μg/ml human epidermal growth factor, 0.025 mg/ml insulin, 0.025 μg/ml progesterone, 0.05 mg/ml transferrin, 0.05 mg/ml gentamicin, and 10% FBS. The EBM-based media assured viable growth of the primary glial cultures. Prior to the first day of treatment with cannabinoid compounds, the cells were allowed to grow uninterrupted for three days. The serum concentration was reduced to 0.1% FBS on the first day of treatment with cannabinoid compounds because high concentrations of serum have been shown to inhibit the antiproliferative effects of Δ^9-THC [15]. Δ^9-THC, SR141716A, and SR144528 were obtained from the National Institutes on Drug Abuse. WIN55,212-2 was purchased from Sigma/RBI (St. Louis, MO). The media with the appropriate compounds was replaced every 24 h.

MTT assay

To quantify cell proliferation the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrasodium bromide (MTT) assay was used (Chemicon, Temecula, CA). Cells were seeded in 96 well plates at 1000 cells/well and in 6 well flat-bottomed dishes at 8000 cells/well to obtain optimal cell density throughout the experiment. In all assays cells were first incubated at 37 °C with MTT for four hours and then isopropanol with 0.04 N HCl was added and absorbance was measured. The absorbance was read at one hour in a plate reader with a test wavelength of 570 nm.

The IC_{50} and E_{max} values with corresponding 95% confidence limits were compared by analysis of logged data (GraphPad Prism, San Diego, CA). Significant differences were determined using analysis of variance (ANOVA) or the unpaired Student's *t*-test, where suitable (GraphPad Prism, San Diego, CA). Bonferroni–Dunn post-hoc analyses were conducted when appropriate. *P*-values < 0.05 defined statistical significance.

Time lapse microscopy (TLM)

For each experiment, cell cultures were transferred from the incubator to a time-lapse microscope equipped with a heated stage and a plexi-glass environmental chamber (Axiovert 200; Zeiss, Gottingen, Germany). Cell cultures were maintained at routine incubation settings (37 °C, 5% CO_2) and optimum humidity. Temperature and CO_2 concentration were independently maintained using digital controlling units (Zeiss, Gottingen, Germany). Two sets of phase contrast images from each well were taken in 300 s intervals using a Cohu 2600 Series compact monochrome interline transfer CCD camera. An Openlab software automation (Improvision, Lexington, MA) drove the camera and stage movements, and compiled the acquired phase images. Each TLM experiment ran for a total of 72 h. Images were subsequently processed as Quicktime movies using Openlab (Improvision, Lexington, MA).

TLM data analysis

Every cell in the initial microscopic field was identified and numbered. We counted the initial number of cells in each of 12 microscopic fields and then followed each cell individually to quantify divisions, deaths and emigrations. Emigration was measured as the rate that cells initially in the microscopic field moved out of the field and were no longer able to be tracked. All numbered cells and their progeny were tracked for the duration of their onscreen viability using compiled Quicktime movies. Each cell's life events were identified using a modified version of a previously described cell pedigree system [16]. Cells identified as dead at the start of the video or that entered the microscopic field after the initial frame were not included in this analysis. Cataloged data was entered into a Microsoft Excel spreadsheet for further analysis.

TLM statistical analysis

Division counts during 4-h periods of observation were converted to rates per cell by the following formula:

$$\text{rate}(t) = ln[N(t-1) + b(t)] - ln[N(t-1)],$$

where $N(t-1)$ is the number of cells at the start of the time period and $b(t)$ is the number of divisions during the time period. These estimates are based on an assumption that counts are dependent on the number of cells present. Similar formulas were used to convert counts of deaths and emigrations to rates.

There were three replicate experiments for each treatment/cell type combination. We plotted average rates as well as a locally smooth function, called lowess, against time period to get a visual picture of changes in rates over time. Because of the small numbers of replicates at each time point, statistical power is low for detecting differences in rates at any individual time point. Also, there was no consistent shape for smoothed rate functions over time, so that parametric models could not easily be fit to the observed data. Thus the most practical method for making inferences about division rates was to use multivariate analysis of variance (MANOVA) with rates at each time point considered as a separate variable. This method of analysis does not allow modeling of changes as a smooth function of time, but does provide a test of whether rates differed, by treatment, over a collection of time points (e.g., the first 24 h). We used STATA version 8 to perform MANOVA. This program calculates four multivariate statistics that are commonly used in MANOVA: Wilks' lambda, Pillai's trace, Lawley–Hotelling trace and Roy's largest root. Each is optimal under certain conditions and none is under all conditions. The advantages of using MANOVA over a collection of time points rather than testing individual time points separately are stability in estimating variance and allowance for correlations among pairs of time points. The advantage of using four MANOVA statistics rather than relying on one is that there will be more sensitivity in detecting treatment differences.

MANOVA was not appropriate for analyzing death (and emigration) rates because the counts of cell death during 4-h observation periods were often zero. We pooled data over 24-h periods, from each of the three experiments for each treatment (Vehicle, WIN55,212-2 or Δ^9-THC), and then used the nonparametric Kruskal–Wallis (KW) test to determine whether death rates differed by treatment. If the KW test was significant (i.e. $P < 0.05$) pairwise treatment differences were tested using the ranksum (Mann–Whitney) test.

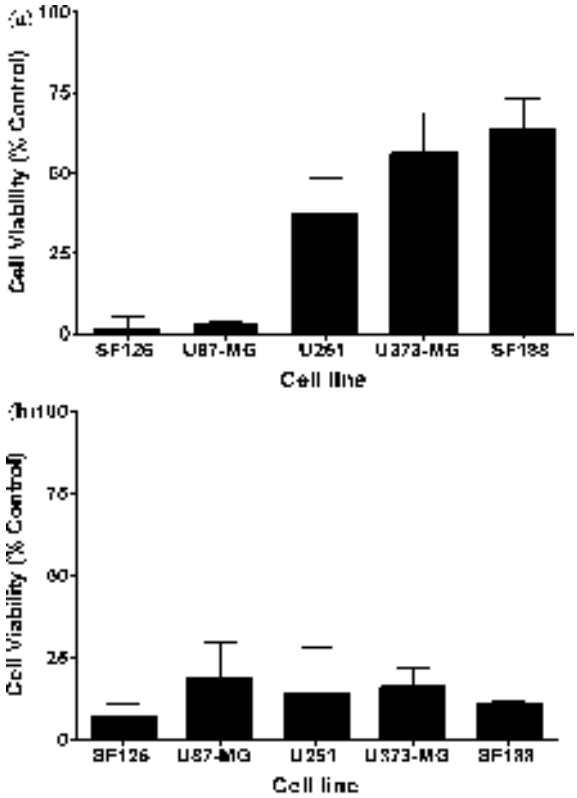

Figure 1. The cannabinoid agonists, WIN 55,212-2 and Δ^9-THC, inhibit the proliferation of multiple human GBM cell lines. Cells were treated for 7 days with vehicle (ethanol, 1 μl/ml), or (a) 1.25 μM WIN55,212-2 or (b) 2 μM Δ^9-THC. The MTT assay was used to quantify cell proliferation. The absorbance of the media alone at 570 nm was subtracted, and % control was calculated as the absorbance of the treated cells/control cells × 100. Data are the mean of at least three independent experiments; bars, ±SE.

Results

Treatment of multiple human GBM cell lines with cannabinoids

WIN 55,212-2 and Δ^9-THC significantly reduced the growth of all of the human GBM cell lines examined (Figure 1). Five human GBM cell lines were treated for seven days with WIN 55,212-2 and Δ^9-THC to evaluate their sensitivity to the antiproliferative effects of a cannabinoid. We chose this time point to take into account the variable sensitivities of different cell lines [12,17]. Compared to controls, the percentage of growth for each cell line in the presence of the cannabinoid agonist WIN 55,212-2 was SF126 = 2% (±0.4), U87-MG = 3% (±1.0), U251 = 37% (±11), U373-MG = 56% (±12) and SF188 = 64% (±10). In the presence of Δ^9-THC the values were SF126 = 7% (±4), U87-MG = 19% (±11.0), U251 = 14% (±13), U373-MG = 10% (±1), and SF188 = 16 % (±6). SF126 was the most sensitive to the antiproliferative effects of WIN 55,212-2 and Δ^9-THC. This cell line was used for further studies.

Concentration response analysis with WIN 55,212-2 and Δ^9-THC

The initial experiments with SF126 suggested the antiproliferative effects of WIN 55,212-2 began to occur by 48 h. Accordingly, we shortened the agonist treatment periods during the detailed concentration response analysis experiments. Cells were treated for three days with multiple concentrations of Δ^9-THC and WIN 55,212-2 ranging from 0.1 nM to 2 μM (Figure 2). Cell proliferation was compared between groups and IC_{50} values for the antiproliferative effects of each compound were calculated. The IC_{50} values for Δ^9-THC and WIN 55,212-2 were 0.60 μM (0.39–0.91) and 0.98 μM (0.77–1.2), respectively. A biphasic effect on cell proliferation was observed with Δ^9-THC but not WIN 55,212-2. Compared to control, 100 nM Δ^9-THC produced a slight increase in cell growth, 129% (104–153). This was not seen in the presence of 100 nM WIN 55,212-2, 98% (81–111).

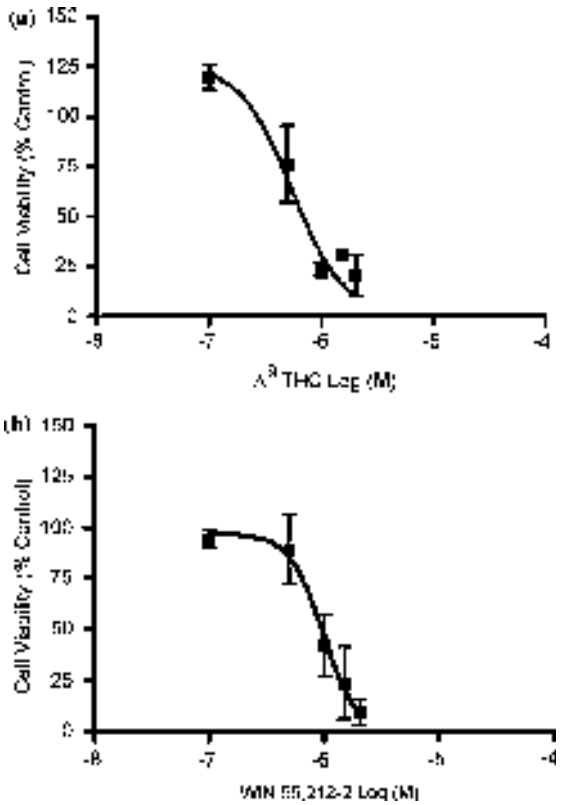

Figure 2. The cannabinoid agonists, Δ^9-THC and WIN 55,212-2, inhibit the proliferation of human GBM cells. Cells were treated for 3 days with vehicle (ethanol, 1 μl/ml), or increasing concentrations of (a) Δ^9-THC or (b) WIN55,212-2. The MTT assay was used to determine cell proliferation. The absorbance of the media alone at 570 nm was subtracted, and % control was calculated as the absorbance of the treated cells/control cells × 100. Data are the mean of at least three independent experiments; bars, ±SE.

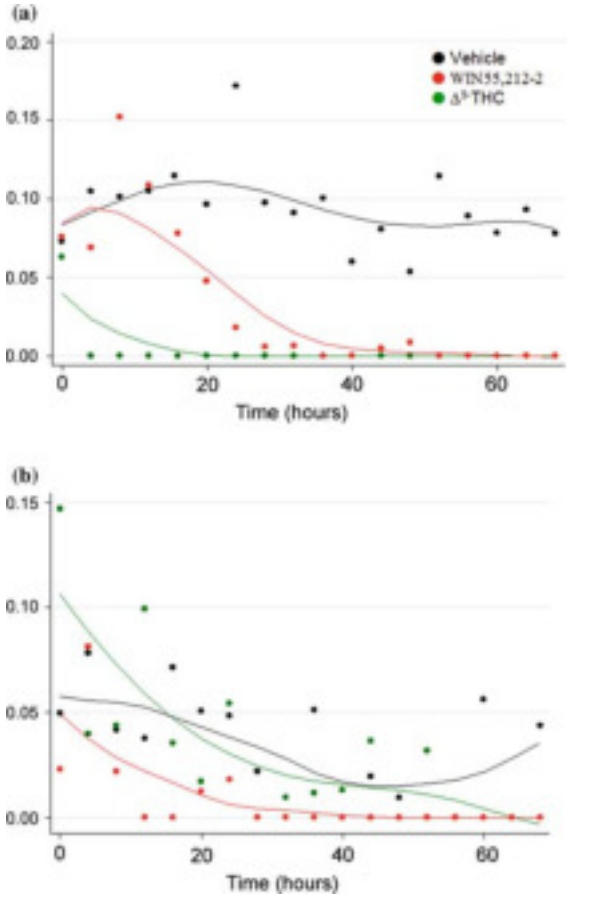

Figure 3. In the presence of cannabinoids, cell division rates of human GBM cells are selectively decreased compared to normal human glial cells. (a) Treatment of SF126 GBM cells with 1 μM Δ^9-THC or 1.25 μM of WIN 55,212-2 for three days. (b) Treatment of normal glial cells with the same concentrations of the agonists. Phase contrast images from each well were taken from three independent experiments. Cell division rates are plotted per every 4 h. Rates are depicted as individual points and smooth lines represent the locally weighted least squared fit.

Cell division rates in GBM and glial cultures during cannabinoid treatments

We next determined how Δ^9-THC and WIN 55,212-2 would alter the proliferation of human GBM cells compared to human primary glial cultures. We used human primary glial cultures as a model of normal cells since they are not transformed or derived from a tumor. 1 μM Δ^9-THC or 1.25 μM of WIN 55,212-2 reduced the growth of SF126 cells by approximately 75% (Figure 2). We therefore treated human primary glial cultures and SF126 cells with these same concentrations and analyzed both groups with TLM. Beginning on day one, a minimum of 87 cells was tracked for each treatment (Δ^9-THC or WIN 55,212-2) in both groups.

Treatment of SF126 cells with each agonist inhibited or completed blocked cell division. There were clear examples of cell division in control SF126 cell experiments. This event was observed as the rounding up of a single cell followed by the emergence of two daughter cells. Division rates were more or less constant over time for untreated cells. In contrast, Δ^9-THC and WIN 55,212-2 significantly inhibited the rate of division of SF126 cells (Figure 3a). The plot of the observed division rates by time suggests different patterns of inhibition for each treatment. The rates demonstrate that the onset of this inhibition occurred more rapidly in the presence of Δ^9-THC as compared to WIN 55,212-2. The inhibition of cell division produced by Δ^9-THC begins during the first 4 h of treatment whereas significant inhibition in the presence of WIN 55,212-2 is not observed until after day one. Analysis of variance (MANOVA) on the data from the first day (24 h) showed significant differences among the three treatments ($P = 0.026$ for Wilks' lambda, $P = 0.01$ for Lawley-Hotelling trace, $P = 0.001$ for Roy's largest root and $P = 0.17$ for Pillai's trace). During the first day, post-hoc pairwise comparisons showed no significant difference between vehicle and WIN 55,212-2 ($P = 0.35$ for all four statistics); significant inhibition of divisions by Δ^9-THC ($P = 0.006$ vs. vehicle for all four statistics); and significant differences between Δ^9-THC and WIN 55,212-2 ($P = 0.027$ for all four statistics).

During the second and third days there were significant differences among the three treatments ($P < 0.001$ for all statistics except Pillai's trace, which had $P = 0.62$ for day two and $P = 0.58$ for day three). These differences were attributable to continued

divisions in vehicle in contrast to none in Δ^9-THC and very few in WIN 55,212-2 treated cells after the first day (Figure 3a). The response to both cannabinoid agonists differed significantly from vehicle ($P < 0.001$ for all four statistics). There was no difference between each agonist for the rate of cell division ($P = 0.69$ for day two and $P = 0.97$ for day three for all four statistics). The high P-Value for Pillai's trace statistic when comparing all three treatment is attributable to the preponderance of zero values for Δ^9-THC and WIN 55,212-2 during the second and third days. Pillai's trace statistic has been reported to produce more conservative P-values when data are not multivariate normal. To further verify our findings, we performed rank tests (which do not require assuming a normal distribution) to compare the division rates during the second and third days. These tests confirmed that division rates for both treatments were significantly lower than those for vehicle, as is evident in the plot (Figure 3a).

In comparison, the same experimental protocol was followed using human primary glial cultures. 1 μM of Δ^9-THC and 1.25 μM of WIN 55,212-2 did not significantly inhibit the division rate of the cells after one day (Figure 3b). MANOVA revealed no differences among the three treatments during the first day ($P = 0.26$ for Wilks, $P = 0.16$ for Pillai, $P = 0.41$ for Lawley–Hotelling and $P = 0.14$ for Roy statistics). During the second day there was some evidence to support reduced rates of division in WIN 55,212-2 treated cells compared to vehicle ($P = 0.07$ for Wilks, $P = 0.22$ for Pillai, $P = 0.04$ for Lawley–Hotelling and $P = 0.006$ for Roy's largest root). Again, the conservative P-value for the Pillai statistic is attributable to a preponderance of zero division rates during the second day. There were few divisions during the third day under any of the experimental conditions, so comparisons had to be based on rank tests of division rates over the 24-h period. Overall, the KW test showed no differences among the treatments ($P = 0.31$). Only the comparison of WIN (no divisions during day three) with control almost demonstrated a significant difference ($P = 0.06$).

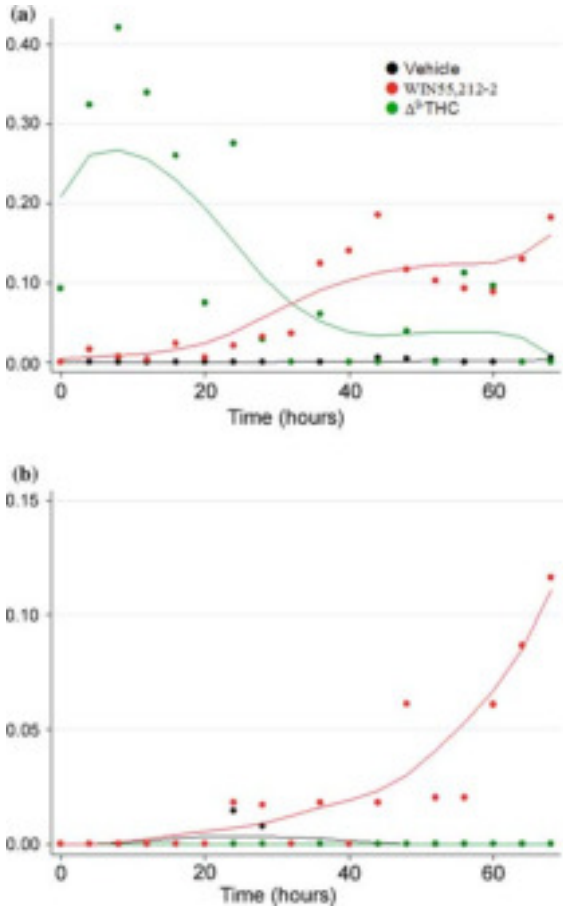

Figure 4. In the presence of cannabinoids, cell death rates of human GBM cells are selectively decreased compared to normal human glial cells. (a) Treatment of SF126 GBM cells with 1 μM Δ^9-THC or 1.25 μM of WIN 55,212-2 for three days. (b) Treatment of normal glial cells with the same concentrations of the agonists. Phase contrast images from each well were taken from three independent experiments. Cell death rates are plotted per every 4 h. Rates are depicted as individual points and smooth lines represent the locally weighted least squared fit.

Cell death rates in GBM and glial cultures during cannabinoid treatments

Treatment of SF126 cells with both cannabinoids significantly increased the death rate of SF126 cells (Figure 4a). This event was observed as the rounding up of a cell followed by the rupture of the membrane. Death rates following treatment with Δ^9-THC reached a maximum during the first day and were significantly greater than those for WIN 55,212-2 ($P = 0.0001$ for Kruskal–Wallis test for equality of all three, $P < 0.001$ for each pairwise comparison). Following treatment with WIN 55,212-2, death rates increased throughout the treatment period and were consistently greater than those for controls on all three days ($P < 0.001$ for each day). This is in contrast to the response of the GBM cells to Δ^9-THC which only differed from controls during the first day ($P = 0.0003$ for day one, $P = 0.051$ for day two and $P = 0.40$ for day three). This was the result of a large decrease in number of cells that could be evaluated since a majority of the cell population was dead by day two.

In normal glial cultures, the death rates for all treatments were zero during the first day (Figure 4b). Toward the end of the second day, treatment with WIN 55,212-2 resulted in significantly higher death rates than treatments with Δ^9-THC and vehicle ($P = 0.04$ and 0.07, respectively). Δ^9-THC and vehicle did not differ significantly during this time period. During the third day, treatments with WIN 55,212-2 produced significantly higher death rates than those with Δ^9-THC or vehicle, both of which were zero during this time period ($P < 0.001$ for each comparison).

Cell emigration rates in GBM and glial cultures during cannabinoid treatments

Treatment with Δ^9-THC reduced emigration ($P = 0.0008$) of SF126 cells during the first day, while there was no significant reduction in the presence of WIN 55,212-2 relative to vehicle ($P = 0.98$) (Figure 5a).

Emigration was measured as the rate that cells migrated out of the microscope field and were no longer able to be tracked. Highly motile cells are more likely to move out of the microscopic field during the course of the experiment and their emigration rates will be high. In contrast, less motile cells will have low emigration rates. During the second and third days, SF126 cells treated with both Δ^9-THC and WIN 55,212-2 showed significantly lower rates of emigration compared to control ($P < 0.001$ for each pairwise comparison).

Δ^9-THC only marginally reduced the emigration rate of the normal glial cells, compared to vehicle, during the first day ($P = 0.06$) (Figure 5b). No significant differences were observed during the second and third day of treatment with Δ^9-THC. Treatment with WIN 55,212-2 caused a significant reduction in emigration rates of glial cells during the first and second day ($P < 0.001$) and reductions continued into the third day. Emigration rates following Δ^9-THC treatments were between those for WIN 55,212-2 and vehicle i.e. Δ^9-THC did not differ from WIN 55,212-2 nor from vehicle.

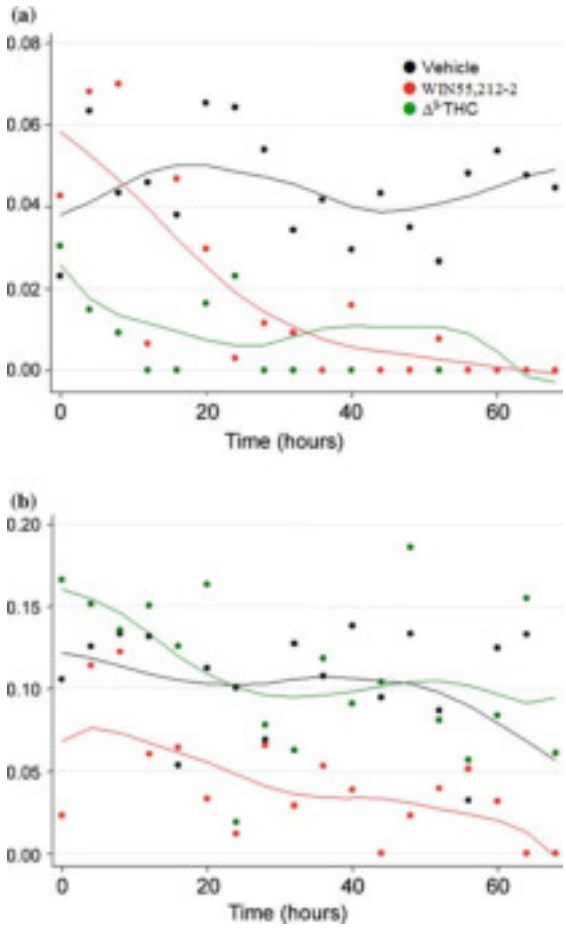

Figure 5. In the presence of cannabinoids, cell emigration rates of human GBM cells are selectively decreased compared to normal human glial cells. (a) Treatment of SF126 GBM cells with 1 μM Δ^9-THC or 1.25 μM of WIN 55,212-2 for three days. (b) Treatment of human primary glial cells with 1 μM Δ^9-THC or 1.25 μM of WIN 55,212-2 for three days. Phase contrast images from each well were taken from three independent experiments. Cell emigration rates are plotted per every 4 h. Rates are depicted as individual points and smooth lines represent the locally weighted least squared fit.

End-point assay determination of cell proliferation during cannabinoid treatments

At the completion of the TLM experiments, all samples were analyzed with the MTT assay to quantify changes in cell proliferation. Treatment of SF126 cells with 1 μM of Δ^9-THC or 1.25μM WIN 55,212-2 for three days caused a dramatic reduction in cell growth (Figure 6). Compared to controls, the proliferation of SF126 cells in the presence of Δ^9-THC or WIN 55,212-2 was 26 % (±4) and 30% (±9), respectively. Treatment of normal glial cultures with the same concentrations of agonists caused significantly less inhibition of cell proliferation. The percentage of proliferation in glial cultures in the presence of Δ^9-THC or WIN 55,212-2 was the 84% (±0.4) and 71% (±1.0), respectively.

Evaluation of cannabinoid receptor involvement

To determine the involvement of CB_1 and CB_2 receptors in the agonist effects that were observed with SF126 cells, we repeated the experiments in the presence of specific antagonists (Figure 7). When applied alone, the agonists Δ^9-THC and WIN 55,212-2 reduced cell growth to 24% (±7) and 23% (±3), respectively. In the presence of the CB_1 antagonist SR141716A, the reduction in cell growth produced by both agonists was significantly reversed: Δ^9-THC = 59% (±4) and WIN 55,212-2 = 56% (±7) ($P < 0.01$). In the presence of the CB_2 antagonist SR144528, the reduction in cell growth produced by both agonists was also significantly reversed: Δ^9-THC = 61% (±4) and WIN 55,212-2 = 59% (±8) ($P < 0.01$). Increase blockade, however, did not occur when the antagonists were administered in combination. In the combined presence of SR141716A and SR144528, the reduction in cell growth produced by Δ^9-THC and WIN 55,212-2 was 54% (±3) and 55% (±3),

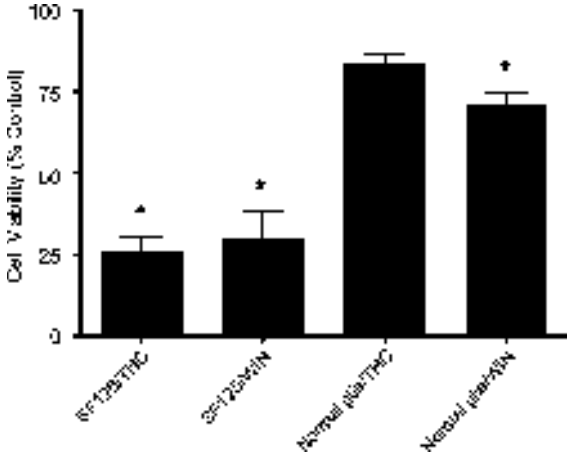

Figure 6. End point analysis confirms selective inhibition of human GBM cells observed by TLM. Treatment of SF126 GBM cells or normal glial cells with 1 μM Δ^9-THC or 1.25 μM of WIN 55,212-2 for three days. The MTT assay was used to quantify cell proliferation. The absorbance of the media alone at 570 nm was subtracted, and % control was calculated as the absorbance of the treated cells/control cells × 100. Data are the mean of at least three independent experiments; bars, ±SE. The asterisk (*) indicates statistically significant differences from control ($P < 0.05$).

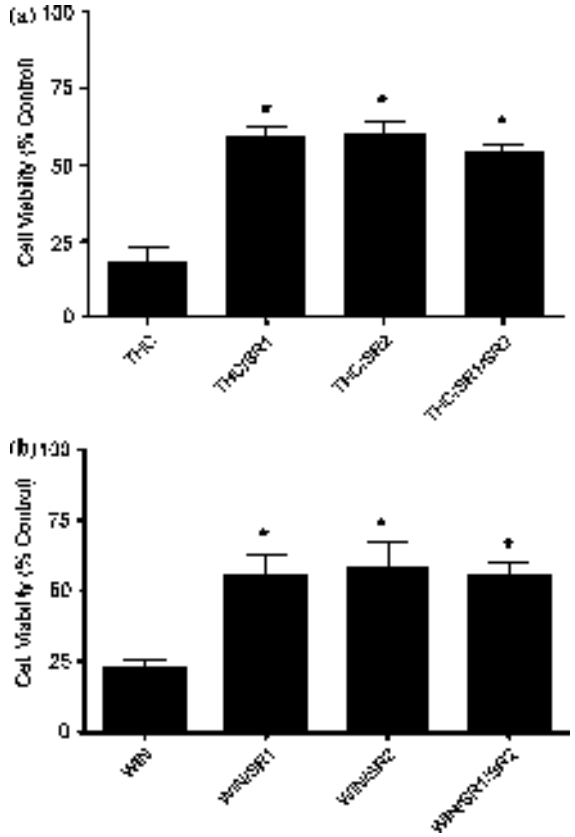

Figure 7. CB_1 and CB_2 receptor antagonists partially block the antiproliferative effects of Δ^9-THC and WIN 55,212-2. Human SF126 GBM cells were treated with 1 μM Δ^9-THC or 1.25 μM WIN 55,212-2 in the presence or absence of 0.5 μM SR141716A, 0.5 μM SR144528, or a combination of both 0.5 μM SR141716A and 0.5 μM SR144528. The absorbance of the media alone at 570 nm was subtracted, and % control was calculated as the absorbance of the treated cells/control cells × 100. Data are the mean of at least three independent experiments; bars, ±SE. The asterisk (*) indicates statistically significant differences from control ($P < 0.05$).

respectively. Neither antagonist significantly influenced cell proliferation when given alone or in combination (data not shown).

Morphology of treated GBM and glial cultures

Unique characteristics within the cytoplasm of both cell types were noted for each agonist when compared to control cells. Δ^9-THC produced the formation of small round vacuoles in both SF126 cells and primary glial cultures (Figure 8b and f). These vacuoles were concentrated around the cell nucleus. The morphology of the glial cultures was comparable to controls over the three day treatment period except for the presence of the small vacuoles in the Δ^9-THC treated cells. Approximately 69% (±13) of the primary glial cells contained vacuoles during the first day of treatment. By the last day of treatment 55% (±9) demonstrated this characteristic. The rapid effect of Δ^9-THC on SF126 cell division and death precluded us from quantifying the amount of cells containing these vacuoles.

Large webbed compartments within the cytoplasm of both cell types were apparent during treatments with WIN 55,212-2 (Figure 8c and g). During the first day of treatment, a greater percentage of glial cells (91% ± 6) compared to SF216 cells (66% ± 3) were affected in this manner ($P < 0.02$). By the last day of treatment, 80 % (±10) of the primary glial cultures demonstrated this morphological characteristic. After one day of treatment, this observation could not be followed in SF126 cell because a majority of the cells had rounded up or began to die. We also treated both cell populations with the receptor inactive enantomer of WIN 55,212-2, similarly named WIN 55,212-3, and studied them with TLM (Figure 8d and H). In these experiments, the morphological characteristics of the treated cells were similar to controls.

Discussion

We observed that both WIN 55,212-2 and Δ^9-THC could inhibit the proliferation of multiple human GBM cell lines. The most sensitive of these to the

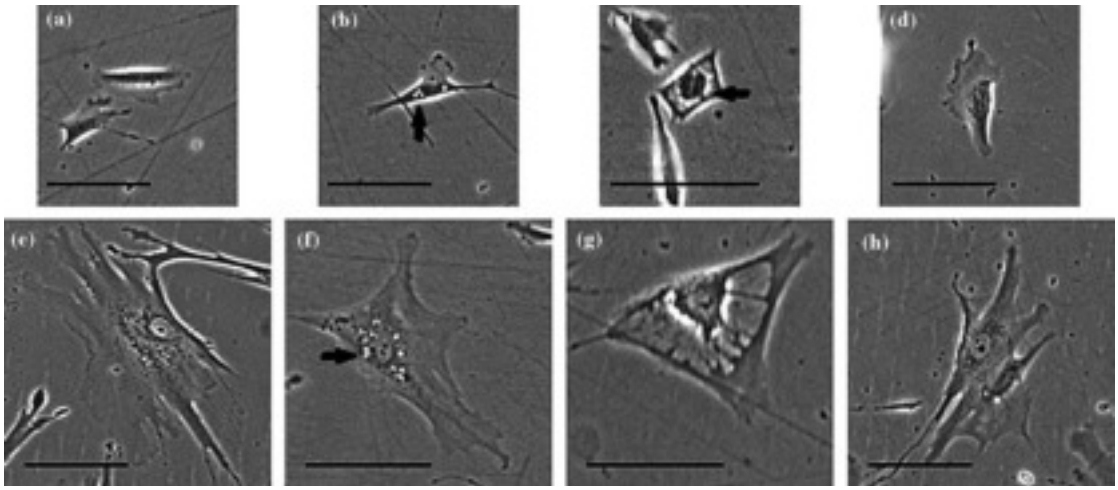

Figure 8. Δ^9-THC and WIN 55,212-2 produce distinct morphological alterations in human GBM and normal human glial cells. Vehicle-treated control cultures of S126 GBM cells (a) and normal glial cells (e) are shown for reference. Lipid body formation, denoted by black arrows, was observed in SF126 cells (B) and normal glial cells (f) as a result of treatment with Δ^9-THC. Large webbed compartments in SF126 cells (black arrow in c) and normal glial cells (g) were seen during treatments with WIN55,212-2. The morphology of SF126 cells (d) and normal glial cells (h) treated with the inactive enantomer of WIN 55,212-2, WIN55,212-3, was similar to controls. Scale bar represents 100 μm.

antiproliferative effects was the GBM cell line SF126. Further analysis with SF126 cells using TLM revealed distinct responses to different cannabinoid agonists. Δ^9-THC rapidly inhibited the division of SF126 cells within the first four hours of treatment. In the presence of WIN 55,212-2, the division of SF126 cells was not significantly inhibited until the second day of treatment. Analysis of cell death also revealed differences in the responses to the agonists. Most of the cell death following Δ^9-THC treatment occurred during the first day. WIN 55,212-2 produced a more delayed effect with cell death beginning on the second day of treatment and continuing throughout the duration of the experiment.

Δ^9-THC proved a more potent agonist compared to WIN 55,212-2 in our experiments and the onset of action for Δ^9-THC was more rapid. The observed potencies were surprising because the synthetic aminoalkylindole WIN 55,212-2 has a significantly higher affinity for both CB_1 and CB_2 receptors compared to the classical cannabinoid Δ^9-THC [18]. Similarly, WIN55,212-2 is consistently more potent and efficacious compared to Δ^9-THC when cannabinoid receptor pathways are evaluated [19], including effects on cAMP, mitogen activated protein kinases (MAPK), and ion channel activity [3]. It has been shown that cannabinoid receptor-mediated generation of ceramide, leading to long-term stimulation of extracellular signal regulated kinase (ERK), plays a pivotal role in inducing GBM cell death [1]. The assessment of cannabinoid activities through this signal transduction pathway may explain the apparent discrepancy in potencies because long-term effects are being evaluated. Another potential explanation may be the activation of additional receptors other than CB_1 and CB_2 [8,20]. This explanation is supported by our observation that the antiproliferative effects of both Δ^9-THC and WIN 55,212-2 were only partially reversed when CB_1 or CB_2 antagonists were given alone or in combination. We cannot, however, rule out the possibility that the conditions of the experiment were not optimal to produce full blockade.

Our findings differ somewhat from one report of cannabinoid treatment in rat C6 glioma cell lines but are consistent with another [11,21]. In one study it was reported that when CB_1 and CB_2 antagonists were given alone, they could not reverse the antiproliferative effects of Δ^9-THC [11]. Only when the two antagonists were used in combination could full reversal of the antiproliferative effects be produced. This suggested that both receptors would need to be activated to limit cell growth. On the other hand, another investigation using rat C6 glioma cells found that either antagonist could partially reverse the antiproliferative effects of Δ^9-THC [21]. The data suggest that a common pathway could be activated through either CB_1 or CB_2 receptors and this leads to reductions in cell growth and increases in cell death. Consistent with this model involving a common pathways an *in vivo* study demonstrated a selective CB_2 agonist could inhibit C6 glioma tumor growth as effectively as a mixed CB_1 and CB_2 agonist [22].

Although Δ^9-THC could inhibit the proliferation of SF126 cells with an IC_{50} of 600 nM, we did observe a small mitogenic effect when cells were treated with 100 nM of Δ^9-THC. This biphasic effect was not observed during treatments with WIN 55,212-2. Recent reports have demonstrated that Δ^9-THC and the endocannabinoid methanadamide can stimulate cancer cell growth at concentrations of 100–200 nM [23–25]. A general hypothesis has been proposed that cannabinoid receptor activation of short term or low levels of ERK stimulation vs. long-term and high levels of ERK stimulation is the switch between cell growth and cell death [13]. Two pathways have been described that lead to stimulation of cell growth by cannabinoids. The first is linked to direct CB_1 and CB_2 receptor activation of the PKC-PI3K-ERK pathway and subsequent up-regulation of androgen and nerve growth factor receptors [23,24]. Alternatively, the mitogenic effects of cannabinoids might be the result of tumor necrosis factor alpha-converting enzyme (TACE/ADAM17)-mediated trans-activation of the epidermal growth factor receptor leading to stimulation of ERK [25]. This biphasic modulation of cancer cell proliferation has also been observed with the effective anticancer agent retinoic acid [26].

Mitogenic activity was not evident when SF126 cells were treated with WIN 55,212-2. Past investigations studying cannabinoid receptor activation demonstrated that Δ^9-THC acts as partial agonists whereas WIN 55,212-2 is a full agonist [27,28]. At lower concentrations, partial agonists at CB_1 and/or CB_2 receptors may promote cell proliferation in cancer cell lines. The aminoalkylindoles (ex. WIN 55,212-2) have also been shown to have a unique binding site and activation profile in cannabinoid receptors [29,30]. It is possible that distinct initial interactions of each class of agonist at CB_1 and CB_2 could lead to a divergence of down-stream signaling; this could produce altered responses in cell growth. Our data suggest that cannabinoid anti-tumor agents could be designed to lack this stimulatory phase.

The activity of cannabinoid compounds, especially Δ^9-THC, was markedly different in SF126 cells compared to normal glial cells. In GBM cells, compared to the primary glial cultures, the inhibition of cell division and increases in cell death where much greater. There are several potential explanations for this selective efficacy. The apoptotic actions of Δ^9-THC in rat C6 gliomas require the long-term up-regulation of ERK [11]. Glioma cell lines may be more susceptible to this up-regulation compared to normal glial cells because of differences in signal transduction in apoptotic and cell survival pathways [13]. Increased CB_2 receptor expression has been reported in highly malignant human glioma tumors [22]. This increase in CB_2 receptor expression may facilitate cannabinoids in producing long-term up-regulation of ERK in GBM cells.

In both SF126 cells and normal glial cultures, Δ^9-THC and WIN 55,212-2 produced distinct alterations in cell morphology. Treatment of both SF126 cells and primary glia with Δ^9-THC resulted in an increase production of small vacuoles concentrated around the nucleus of the cell within the cytoplasm. The appearance of these vacuoles has been previously described in rat C6 glioma cells during treatment with Δ^9-THC [21]. Noted also in this study was that the appearance of the vacuoles was

restricted to neoplastic cells compared to a non-neoplastic model (fibroblasts). In contrast, we observed these vesicles in both our primary glial cultures as well as in SF126 cells. Recht et al. [21] demonstrated the vacuoles within the rat C6 glioma cells contained triglycerides or cholesteryl oleate. A direct link between the appearance of these lipid bodies in the presence of Δ^9-THC and decrease in cell proliferation has not been made. There is the potential that metabolites of Δ^9-THC may inhibit enzymes responsible for lipid metabolism and could lead to the formation of the vacuoles [31]. We did not observe these lipid bodies in the presence of WIN 55,212-2, providing evidence against cannabinoid receptor activation as a potential mechanism for their formation.

WIN 55,212-2 produced significant disruption in the cytoplasmic region of both cell types. This was observed as the formation of sizeable webbed compartments surrounding the nucleus and extending to the plasma membrane. These were especially evident in the large normal glial cells. The intracellular disruptions were not apparent following treatment with the receptor inactive enantomer of WIN 55,212-2. This indicated that the disruptions may be cannabinoid receptor mediated. However, Δ^9-THC did not produce these cytoplasmic disruptions suggesting that the effect of WIN 55,212-2 on cell morphology is the result of additional receptor interactions unique to the ligand.

Since normal tissue toxicity limits the efficacy of current GBM treatment, the selectivity we observed in human tissues with both cannabinoid agonists is of clinical importance. Δ^9-THC is currently being used in clinical trials to treat recurrent GBM [13,32]. Consistent with previous reports our results suggest that high concentrations of cannabinoid agonists would be required to achieve anti-tumor activity. It is likely that higher concentrations could be achieved due to recent technological advances that enable delivery of high concentrations of compounds directly to the site of GBM tumors [33]. The unexpected reversal of potencies observed with Δ^9-THC and WIN 55,212-2 suggest the development of future cannabinoid-based anticancer agents using past structure–activity relationship at CB_1 and CB_2 receptors may not be appropriate. A more detailed structure-activity analysis of the antiproliferative and apoptotic effects of cannabinoids is warranted to test this hypothesis.

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