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Antitumor effects of ajulemic acid (CT3), a synthetic non-psychoactive cannabinoid

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Abstract

One of the endogenous transformation products of tetrahydrocannabinol (THC) is THC-11-oic acid, and ajulemic acid (AJA; dimethylheptyl-THC-11-oic acid) is a side-chain synthetic analog of THC-11-oic acid. In preclinical studies, AJA has been found to be a potent anti-inflammatory agent without psychoactive properties. Based on recent reports suggesting antitumor effects of cannabinoids (CBs), we assessed the potential of AJA as an antitumor agent. AJA proved to be approximately one-half as potent as THC in inhibiting tumor growth *in vitro* against a variety of neoplastic cell lines. However, its *in vitro* effects lasted longer. The antitumor effect was stereospecific, suggesting receptor mediation. Unlike THC, however, whose effect was blocked by both CB_1 and CB_2 receptor antagonists, the effect of AJA was inhibited by only the CB_2 antagonist. Additionally, incubation of C6 glioma cells with AJA resulted in the formation of lipid droplets, the number of which increased over time; this effect was noted to a much greater extent after AJA than after THC and was not seen in WI-38 cells, a human normal fibroblast cell line. Analysis of incorporation of radiolabeled fatty acids revealed a marked accumulation of triglycerides in AJA-treated cells at concentrations that produced tumor growth inhibition. Finally, AJA, administered p.o. to nude mice at a dosage several orders of magnitude below that which produces toxicity, inhibited the growth of subcutaneously implanted U87 human glioma cells modestly but significantly. We conclude that AJA acts to produce significant antitumor activity and effects its actions primarily via CB_2 receptors. Its very favorable toxicity profile, including lack of psychoactivity, makes it suitable for chronic usage. Further studies are warranted to determine its optimal role as an antitumor agent.

Keywords: Cannabinoids; Chemotherapeutic agents; Ajulemic acid; Glioma cell lines; Lipid droplets

1. Introduction

Several years ago, a number of reports noted *in vitro* and *in vivo* antineoplastic activity of cannabinoids but concluded that their efficacy was limited by both the development of tolerance and psychoactivity [1–4]. Recently, however, there has been renewed interest in cannabinoids as anticancer agents. For example, recent studies demonstrated an increased rate of apoptosis of C6 glioma cells exposed to THC [5] and inhibition of human breast and prostate cancer cell proliferation by the endogenous cannabinoid agonist anandamide [6,7]. More intriguing is another study that demonstrated *in vivo* efficacy of THC and similar agonists when administered intratumorally to rats harboring intracranial C6 gliomas [8]. The mechanisms underlying the antineoplastic actions of cannabinoids remain unclear, however; possible etiologies include blockade of growth factor stimulation and sustained ceramide accumulation [5,6,8].

Because the psychoactivity of THC limits its medicinal usage, great effort has been directed toward finding an agent that separates these activities. One such strategy is based on

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Abbreviations: AJA, ajulemic acid, dimethylheptyl-THC-11-oic acid; DG, diglyceride; FBS, fetal bovine serum; MEM, Minimum Essential Medium; MTT, microculture methyl-thiazolyl tetrazolium; PKC, protein kinase C; TEM, transmission electron microscopy; TG, triglyceride; and THC, tetrahydrocannabinol.

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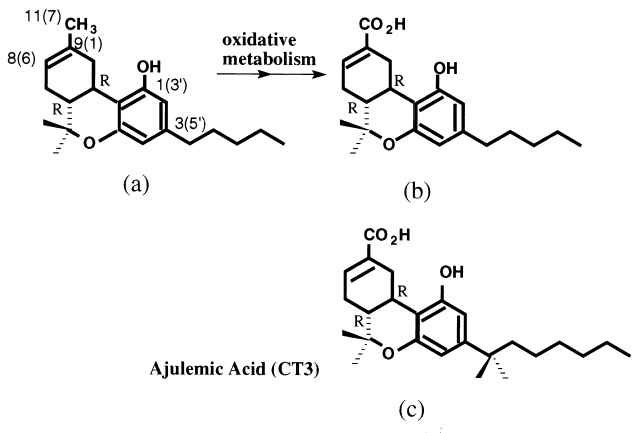

Fig. 1. Cannabinoid metabolism and structures. (a) THC (Δ^8-tetrahydrocannabinol). (b) THC-11-oic acid. (c) Ajulemic acid (AJA; dimethylheptyl-THC-11-oic acid). The unbracketed numbers refer to the currently used system; bracketed numbers to an older system. R indicates the absolute configuration of the chiral centers. THC (a) is biotransformed in two successive oxidations to its principal metabolite, THC-11-oic acid (b). AJA (c) is a synthetic analog of THC-11-oic acid (b).

the properties of the acid metabolites of THC, which show little or no psychoactivity and bind less avidly to cannabinoid receptors [9–11]. Several recent studies indicated efficacy for these acids and suggest that they might contribute to the analgesic properties of THC and attenuate its psychotropic effects [12,13]. A more potent synthetic analog, AJA (dimethylheptyl-THC-11-oic acid), was therefore synthesized by modifying the pentyl side chain (Fig. 1); this compound is both analgesic [14,15] and anti-inflammatory [16] and has no detectable psychoactive properties.

We hypothesized that since AJA proved equipotent or more potent than THC in several anti-inflammatory bioassays, that it would also have significant antitumor effects. This property would then make AJA an attractive substitute for THC based upon its lack of known psychoactive effects and favorable toxicity profile.[1] To test this hypothesis, we compared AJA with THC as an antineoplastic agent and found that it was nearly equipotent to THC *in vitro* and superior *in vivo.* Furthermore, we determined that its antineoplastic effect was medated primarily through actions on CB_2 receptors and was associated with a marked increase in cell DG levels.

2. Materials and methods

2.1. Reagents

Δ^8-THC and THC-11-oic acid were obtained from NIDA. AJA was obtained from Organix. SR141716a [*N*-(piperidin-1-yl)-5-(4-chlorophenyl-1*H*-pyrazole-3-carboximide] and SR144528 [*N*-[(1*S*)-endo-1, 3, 3-trimethylbicyclo[2.2.1]heptan-2-yl]-5-(4-chloro-3-methylphenyl) ([C3H]4-methylbenzyl)-pyrazole3-carboxamide] were obtained from Dr. Francis Barth (Sanofi Research). All drugs were stored as concentrated solutions in DMSO at 4°. The concentrations were such that 10 μL added to 1.0 mL of medium gave the desired final concentration.

2.2. Cells

C6 rat glioma, U87, U373, U118, and A172 human glioma, and WI-38 human fibroblast cells were obtained from the ATCC. HS758T (human breast cancer), HT1376 (human bladder cancer), J82 (human bladder cancer), CALU-6 (human lung cancer), DU-145 (human prostate cancer), and PC-3 (human prostate cancer) were obtained from Dr. T. Savarese (UMASS Cancer Center Tissue Bank). All cell lines were maintained in MEM supplemented with 10% FBS until used.

2.3. MTT assay

Cells were maintained in MEM containing 0.5% serum overnight. On the day of assay, 2×10^4 cells/well were placed in 24-well plates containing 1 mL MEM and 10% FBS for 2 hr after which substances to be tested were added in a total volume of 10 μL DMSO. After the designated incubation period, 100 μL of MTT ($C_{18}H_{16}N_5SBr$; Sigma Chemical Co.) solution (5 mg/mL in PBS) was added to each well and incubated for 2 hr at 37°. Then cells were triturated in 1.1 mL of isopropanol MTT solubilization solution (Sigma). Three hundred microliters of this mixture was transferred in duplicate to a 96-well plate, and the optical density was recorded using a ThermoMax microplate reader at 570/650 nm absorbance. Based on a standard curve with known numbers of cells, experimental readings were in the linear range for cell numbers.

2.4. Cell cycle analysis

Cells were maintained in 0.5% FBS/MEM for 48 hr to arrest them in G_0/G_1 after which they were placed in 10% FBS/MEM either with or without the experimental agent. Forty-eight hours later, cells were trypsinized, and a trypan blue exclusion test was performed to ensure that $>$ 70% of the cells were viable. Cells were then washed with PBS, fixed by placing on ice for 30 min in 80% EtOH, then washed again, and spun at 800 *g* for 5 min at 4°. Propidium iodide (50 μg/mL; Sigma) in PBS solution with Triton X-100 (0.05%, w/v), 0.1 mM EDTA, and 50 μg/mL of RNase was added for 30 min at 37°. Cells were then filtered through a 35 μm strainer cap to ensure single cell suspension and analyzed on a MoFlo cell sorter (Cytomation, Inc.).

[1] Atlantic Technology Ventures, Inc., unpublished data. Cited with permission.

2.5. Oil red O method

Cells (5.0×10^4) were incubated on coverslips in medium supplemented with THC, DMSO, or AJA for allotted time intervals. After incubations, wells were rinsed with 60% isopropanol and immersed in oil red O solution (Sigma) for 10 min. After a brief wash in 60% isopropanol, cells were stained in hematoxylin for 30 sec, washed in water, and fixed in 4% paraformaldehyde before coverslipping.

2.6. TEM

C6 glioma cells on coverslips in either standard medium (10% FBS/MEM) or medium supplemented with 25 μM AJA were fixed with 2.5% (v/v) glutaraldehyde in 0.5 M sodium cacodylate-HCl buffer (pH 7.0) for 30 min at 30°. The fixed samples were washed three times and left overnight in buffer after which the cells were post-fixed for 1 hr in 1% osmium tetroxide (w/v) in the same buffer. Coverslips were stained en bloc (20 min) with 1% aqueous uranyl-acetate (w/v), washed, dehydrated through a graded series of ethanol, and transferred through two changes of propylene oxide. Cultures were embedded upside down on a flat embedding mold filled with a mixture of LX 112/Araldite 502 epoxy resin and polymerized overnight at 70°. The polymerized blocks were freed from the glass coverslips by rapid immersion in liquid nitrogen. The epoxy blocks were then cut, oriented, and attached to blank epoxy stubs with a drop of Super Glue. Ultrathin sections (70 nm) were cut on a Reichart-Jung ultramicrotome using a diamond knife. Sections were collected and mounted on copper support grids, contrasted with lead citrate and uranyl acetate, and examined with a Philips CM 10 transmission electron microscope at 60 kV.

2.7. Lipid incorporation assay

Monolayers of C6 or WI-38 cells were prepared in 24-well culture dishes as described above. Carboxy-labeled [^{14}C]oleic acid (150,000 dpm/well) obtained from ARC (specific activity: 55 mCi/mmol) was added to each monolayer and incubated for 2 hr at 4°. Treatment with the indicated cannabinoid was then begun by the addition of the drug in 10 μL of DMSO to 1 mL of the culture medium covering each monolayer. Treatment was continued for 48 hr except where indicated otherwise at which time the medium was removed and discarded, since preliminary examination showed that little if any radiolabeled lipid was present. After washing with PBS (1 mL), the cellular lipids were extracted for 0.5 hr with 0.5 mL of 95% ethanol at room temperature. All treatments were performed in quadruplicate; vehicle-treated cells served as a control.

Prior to evaporation under vacuum, [^{14}C]cholesterol (50,000 dpm) was added as a recovery marker (ARC; specific activity: 50 mCi/mmol). The sample residues were then dissolved in 30 μL of methanol containing 10 μg each of steroyl-arachidonoyl diglyceride, triolein, and phosphatidylcholine (PC) and applied to 0.25-mm silica gel thin layer plates. A first elution was performed with dichloromethane: acetone (90:10) for the analysis of neutral lipids. The R_f values of the standards were: PC = 0, cholesterol = 0.38, DG = 0.64, and TG = 0.81. Following the quantitation of the neutral lipids, a second elution was carried out using chloroform:methanol:acetic acid:water (50:25:8:2) as the eluent for the analysis of phospholipid. The R_f value of PC was 0.33; DG and TG moved to the solvent front. All standards were detected by exposure to iodine vapor.

The zones of radioactivity were detected by exposure of the TLC plates to x-ray film for 48 hr. A TIFF computer file of the film was generated using the Fluor-S System (Bio-Rad). The chromatograms were quantified using NIH Image software. Peak height values of the display were used since the zones all showed narrow sharp peaks. These were adjusted for recovery using the individual cholesterol standard values for each zone. The values obtained were then divided by the numbers of cells in each well, and the results were expressed as an index/million cells.

2.8. In vivo subcutaneous model

U87MG cells (1×10^6) were suspended in 0.2 mL PBS and injected s.c. into the right flank of male nu/nu BalbC mice, 6 weeks of age (Charles River). Two days after the inoculation of the tumor cells, mice were randomized to receive either 0.1 mg/kg of AJA in safflower oil base, 20 mg/kg of THC, or an equal volume of safflower oil on Mondays, Wednesdays, and Fridays. When macroscopic tumors appeared, diameters were measured in two dimensions, and then the average was recorded as the mean diameter. Mice were followed until tumor diameters averaged 25 mm at which point they were killed humanely. This protocol for evaluating flank tumors in mice has been approved by our Institutional Animal Care and Use Committee (IACUC).

2.9. Statistical analyses

Cell proliferation data were plotted, and IC_{50} values were obtained using Kaleidagraph with curve fitting done using the Hill expression. A Sigmastat (SPSS Science) statistics package was used to perform statistical analysis of the data. One-way ANOVA tests were used to compare multiple groups, and Chi-square analysis was used to compare proportions. A P value < 0.05 was considered to be significant.

3. Results

After 48 hr, C6 glioma cell growth was inhibited in a concentration-dependent fashion by THC (IC_{50} 5.8 μM). By comparison, the IC_{50} of THC-11-oic acid was almost an order of magnitude higher (49.5 μM). The inhibitory effect

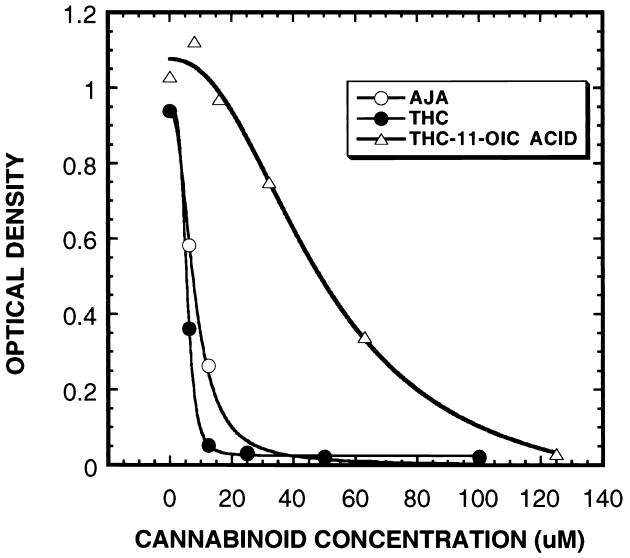

Fig. 2. Inhibition of glioma cell growth by cannabinoids. Comparison of the effects of THC, THC-11-oic acid, and AJA on C6 glioma cell growth for 48 hr. Cells were plated (2×10^4/well) and experiments were performed under conditions as described in "Materials and methods." All points represent the averages of four experiments; the curves represent a best fit type of analysis. Data were plotted using Kaleidagraph and curve fitted using the Hill expression.

of AJA was greater than that of THC-11-oic acid, but after 48 hr was approximately one-half as potent as THC (IC_{50} 16.0 μM) (Fig. 2). The character of the inhibition curves suggested that AJA and THC were nearly equipotent and that both inhibited these cells more potently than THC-11-oic acid.

We also screened a number of human cell lines derived from a variety of cancer types (brain, breast, bladder, lung, and prostate), as well as a fibroblast cell line (WI-38), for their sensitivity to THC and AJA. All cell lines were inhibited after being exposed to both cannabinoids for 48 hr. THC (range, 5.8 to 16.0 μM) was approximately twice as potent as AJA (range, 14.5 to 33.2 μM) in terms of IC_{50} values for these cell lines (Table 1).

The effectiveness of AJA was maintained, however, for a longer duration *in vitro*. Thus, when MTT assays were performed daily for 5 days, growth inhibition was significantly ($P < 0.05$, *t*-test) greater for AJA after 3 days and continued to be greater than 50% after 5 days, while it had decreased to only 33% for THC. This effect was noted in both the C6 and U87 cell lines (Fig. 3).

To assess whether the effect of AJA was receptor mediated, we examined its stereospecificity by comparing it with its enantiomer *S,S*-AJA [17]; this material contains 5% *R,R*-AJA (chiral HPLC analyses were done by S. Abidi, Atlantic Technology Ventures, Inc.). Growth inhibition was decreased markedly after incubation with 25 μM *S,S*-AJA compared with the stereochemically pure AJA ($P < 0.001$, *t*-test) (Fig. 4). This strongly suggests that the effect of AJA is stereospecific and, therefore, receptor mediated.

Table 1
Inhibition of human cell line growth by THC and AJA

Line	Source	IC_{50} (μM)	
		THC	AJA
U87	Glioma	7.4	16.6
U373	Glioma	12.8	33.2
A172	Glioma	9.3	14.5
U118	Glioma	9.7	33.2
CALU-6	Lung cancer	5.8	19.5
HS758T	Breast cancer	14.2	23.8
HT1376	Bladder cancer	10.3	20.5
J82	Bladder cancer	16.0	28.9
PC-3	Prostate cancer	7.3	29.6
DU-145	Prostate cancer	7.3	24.5
WI-38	Fibroblast	8.1	29.7

Cells (2×10^4) were incubated in quadruplicate in concentrations of cannabinoids ranging from 0 to 100 μM for 48 hr before MTT assays were performed, as described in "Materials and methods." The IC_{50} values were calculated from Hill plots.

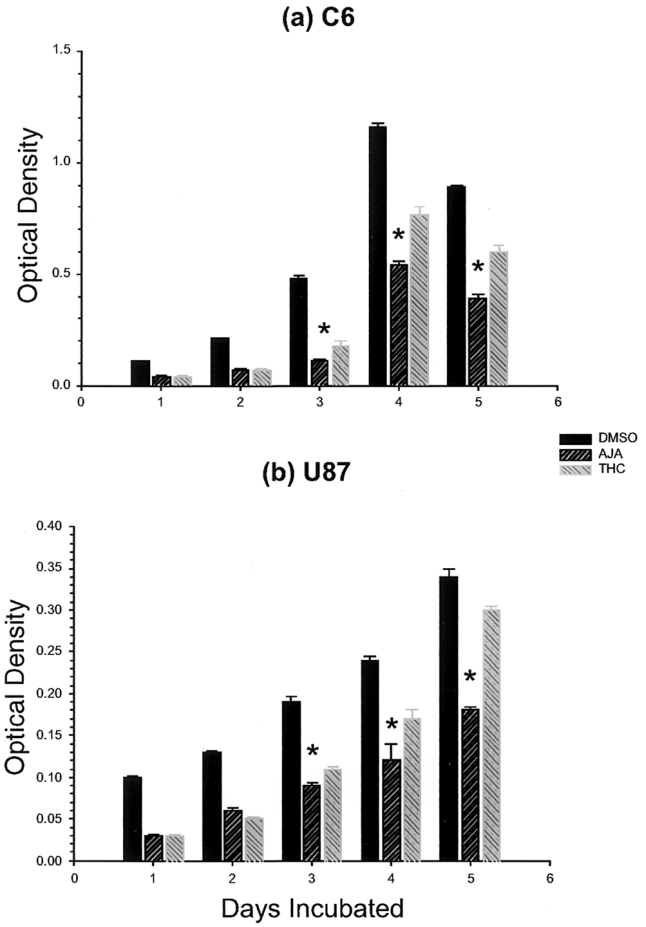

Fig. 3. Assessment of the duration of action of THC and AJA in two glioma cell lines. MTT assays were carried out for the days recorded for both cannabinoids at a concentration of 25 μM, as well as for the DMSO vehicle. Values are expressed as mean OD ± SEM. Key: (*) $P < 0.05$, one-way ANOVA for AJA versus both DMSO and THC.

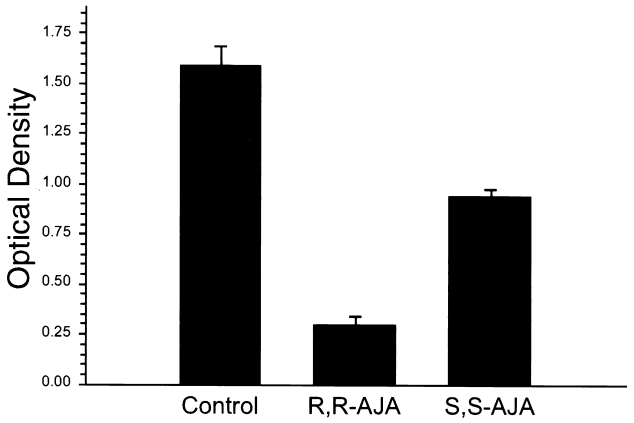

Fig. 4. Comparison of enantiomeric AJAs. Cells were plated (2×10^4/well) and experiments were performed under conditions as described in "Materials and methods." Values are expressed as mean OD ± SEM. Results represent the averages of three experiments. In a 48-hr MTT assay, there was a marked decrease of potency of a 25 μM concentration of *S,S*-AJA ($P < 0.001$, *t*-test).

We next confirmed that the actions of AJA are mediated by the cannabinoid receptors. Co-incubation of C6 glioma cells with either 10 μM SR141716a (a CB_1 receptor antagonist) or 1 μM SR144528 (a CB_2 receptor antagonist) for 48 hr resulted in a significant reversal of THC potency (33 and 46%, respectively). By contrast, only incubation with the CB_2 antagonist diminished the effect of AJA significantly; at the dosage of AJA tested, inhibition was reversed markedly by co-administration of the CB_2 receptor antagonist (63%) (Table 2). This result suggests that the antitumor effect of THC is exerted through both CB_1 and CB_2 receptors, whereas the effect of AJA appears to occur predominantly through CB_2.

One distinguishing feature of the antitumor action of AJA compared with that of THC was marked cell enlargement. To investigate whether this effect was due to a mitotic block, we assessed the effect of AJA on the kinetics of reentry into the cell cycle. After incubating C6 glioma cells for 24 hr in 0.5% serum to synchronize cells in G_1/G_0 and

Table 2
Inhibition of antiproliferative effects of THC and AJA by CB_1 and CB_2 antagonists

	% Inhibition	
	SR141716a (CB_1)	SR144528 (CB_2)
THC	33*	46*
AJA	19	63*

Cb glioma cells (4×10^4) were incubated for 2 days with or without antagonist, and the MTT assay was performed as described in "Materials and methods." Concentrations of THC and AJA were 12.5 and 25 μM, respectively. Concentrations of the cannabinoid (CB) antagonists were 10 and 1 μM for CB_1 and CB_2 antagonists, respectively. Results represent the means of eight experiments.

*$p < 0.05$, one-way ANOVA vs control.

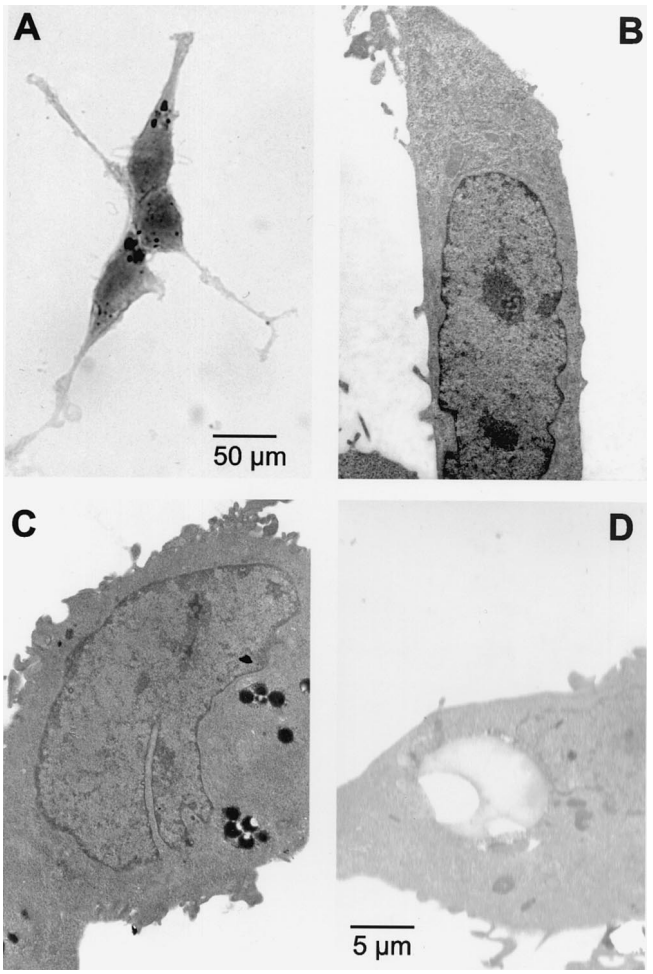

Fig. 5. Induction by AJA of lipid droplet formation in C6 glioma cells. (A) C6 cells treated with 25 μM AJA for 2 days. Oil red O stain (bar = 50 μm). (B) TEM of a C6 cell under basal conditions. (C) TEM of a C6 cell treated with 25 μM AJA for 48 hr. Note the increased size of the cell and the appearance of dark inclusions. (D) Cells treated with sodium periodate. As shown, the droplets were bleached, confirming their lipid nature. For (B), (C), and (D), the bar = 5 μm.

then exposing them to 10% serum either with or without 25 μM AJA for 2 days, we found a modest increase of treated cells in S phase (from 4.4 to 13.0%, $P = 0.003$, *t*-test), but did not note a change in the overall character of the profile. Interestingly, the rate of apoptosis (as assessed by the lack of sub-G_0 cells in both control and treated cells) was not increased. We conclude, based on these effects, that the effect of AJA on cell cycle kinetics is relatively minimal.

A second distinctive feature of the antitumor effect of AJA was the appearance of refractile bodies that could be easily visualized with phase microscopy; these refractile bodies stained readily with oil red O stain, which is specific for TGs and cholestryl oleate (Fig. 5A) [18]. Both the number of cells that had refractile bodies and the number of refractile bodies/cell increased over time. By 48 hr of treatment, significantly more AJA-treated cells had refractile bodies than did those treated with THC (83 compared to 27%, $P < 0.001$, χ^2 analysis). Furthermore, the mean num-

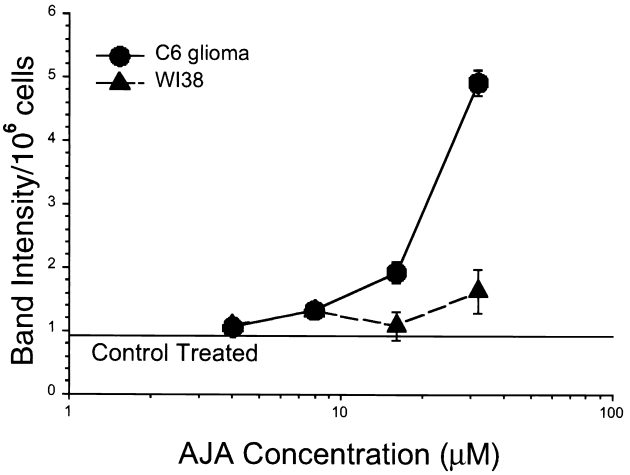

Fig. 6. Incorporation of [^{14}C]oleic acid into TGs. C6 and WI-38 human fibroblast cells were incubated with AJA for 24 hr The y-axis represents the average band intensity per 10^6 cells. Each point represents the values from three experiments ± SD. The "control treated" line corresponds to the TG values obtained when cells were incubated in DMSO vehicle alone. Values for the control treatment were essentially superimposable for C6 and WI-38 cells. Conditions were as described in "Materials and methods."

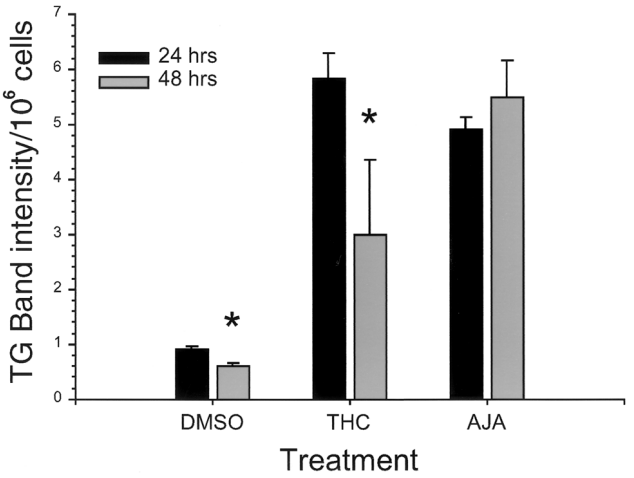

Fig. 7. Continuation of [^{14}C]oleic acid incorporation into TGs in AJA-treated C6 glioma cells. TG band intensities were measured in triplicate and are expressed as OD ± SD. Key: (*) $P < 0.05$, *t*-test, 24-hr compared with 48-hr time points. Conditions were as described in "Materials and methods."

ber of refractile bodies per cell was also much higher in cells treated with AJA compared with THC for 48 hr (1.78 ± 1.6 vs 0.22 ± 0.5 per cell, $P < 0.001$, *t*-test). Interestingly, no refractile bodies were noted in normal human fibroblasts (cell line WI-38) treated with AJA.

We next examined AJA-treated and untreated cells using TEM. AJA-treated cells were neither apoptotic nor necrotic; this is consistent with our observation that although cell proliferation was inhibited markedly, the remaining cells had a high (>95%) viability rate, as assessed by trypan blue exclusion, and recovered quickly upon drug removal. Moreover, AJA-treated cells were enlarged and contained large lipid droplets (Fig. 5, B and C). These droplets could be bleached with sodium periodate (Fig. 5D), confirming their lipid nature.

Since these observations suggested an abnormality in the synthesis, uptake, or breakdown of lipids, we examined the incorporation of labeled fatty acids into C6 glioma and WI-38 fibroblast cells, using TLC methodology. Incorporation of [^{14}C]oleic acid into bands corresponding to TGs in C6 cells increased in a concentration-related fashion after AJA treatment. At the highest concentration utilized (32 μM), incorporation into TG was increased over 5-fold (Fig. 6). By contrast, no consistent increase in TG incorporation was noted in the WI-38 cells, suggesting a differential effect on tumor cells.

We found a transient increase in [^{14}C]oleic acid incorporation into the TGs of C6 cells after THC treatment. At 48 hr post-incubation, however, TG bands in both control and THC-treated cells had decreased by 34 and 46%, respectively ($P < 0.05$, *t*-test), while the band in the AJA-treated cells increased 11% (P = NS) (Fig. 7). Thus, there appeared to be a more sustained increase in incorporation of oleic acid into TG after AJA administration. Interestingly, this result reflects that of the appearance of refractile bodies; at 24 hr, there were essentially equal numbers of fat droplets for THC-treated and AJA-treated cells, whereas a big difference was apparent by 48 hr.

Based on the observation that AJA exerts a cytostatic effect on tumor cells, we next sought to determine whether AJA had any *in vivo* activity. To address this, we assessed growth of the human glioma cell line U87, after subcutaneous injection into nude mice. We elected to compare a dose of AJA (0.1 mg/kg) with a dose of THC (20 mg/kg); the dose of THC had been noted previously to be effective in studies examining different actions of THC [19], while the dose of AJA was that determined to be effective in a previous study examining its anti-inflammatory effects [16].

A delay in both the appearance and the size of the tumors was noted only for the AJA group (Fig. 8). Between days 12 and 19 post-inoculation, AJA-treated mice had smaller tumors than either control or THC-treated mice ($P < 0.05$, Dunnett's test). However, by 22 days post-implantation, AJA-treated tumors were no longer significantly smaller, although their mean diameters continued to be less than those in the vehicle control group.

4. Discussion

Prompted by reports that marihuana smoke alters DNA synthesis in lung cell cultures [4] and inhibits both bone marrow leukopoiesis [20] and lymphocyte activity [21], a number of reports in the late 1970s examined the potential of the active principle of *Cannabis*, THC, as an antineoplastic agent. These reports demonstrated that THC could inhibit both cancer cell DNA synthesis and certain murine

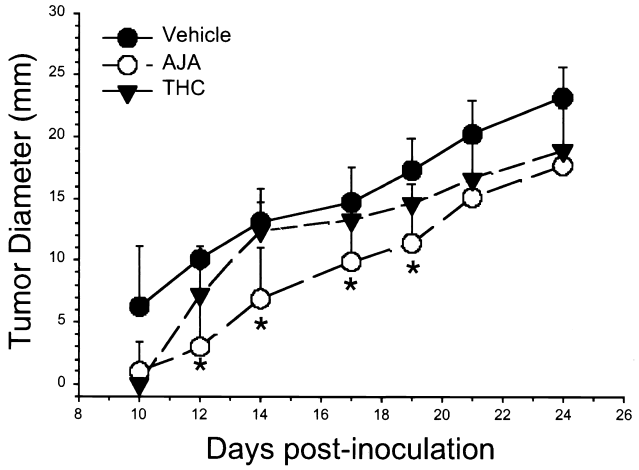

Fig. 8. Cannabinoid effect on subcutaneous glioma growth. AJA (0.1 mg/kg) and THC (20 mg/kg) administration began 1 day after U87MG cell inoculation into the right flank. Tumor diameters were measured thrice weekly. Results represent the means of six mice in each group and are expressed as average diameter ± SD. Key: (*) $P < 0.05$, Dunnett's test vs both THC and vehicle.

neoplasms *in vivo* [1–3], although tolerance tended to develop [2]. Recently, there has been renewed interest in the antitumor effects of cannabinoids. *In vitro* studies, for example, demonstrated that THC induces apoptosis of C6 rat glioma cells through stimulation of sphingomyelin hydrolysis [5] and that the endogenous cannabinoid anandamide blocks human breast and prostate cancer cell proliferation through a CB_1-like receptor-mediated inhibition of endogenous prolactin and nerve growth factor actions [6,7]. More recently, Galve-Roperh and colleagues [8] demonstrated an *in vivo* effect of THC against glioma cells implanted both intracranially and subcutaneously. The authors concluded that THC induced apoptosis via both CB_1 and CB_2 cannabinoid receptors that triggered a sustained ceramide accumulation and a Raf1/ERK activation.

Δ^9-THC is metabolized rapidly to a number of oxygenated products; the most important route involves oxidation of the allylic methyl group, ultimately leading to a carboxy group [22]. Acid metabolites of undetermined structure were first reported in 1970 [23] and since then, have been substantiated in a number of species including humans [24, 25]. This terminal carboxy metabolite has no psychotropic effect in humans [26] and does not produce the behavioral responses typical of Δ^9-THC in laboratory animals [10,11]. Nevertheless, it does retain analgesic and anti-inflammatory properties [12,13]. AJA is a synthetic analog formed by modifying the pentyl side chain of Δ^9-THC-11-oic acid. AJA is analgesic [14,15], can inhibit certain THC effects and is a potent anti-inflammatory agent [16]. It is also nontoxic in both rodent and canine models[2] and could conceivably be administered on a chronic basis without producing significant toxicity.

Because the unique pharmacologic profile of AJA makes it an attractive antitumor agent, we compared its antitumor activity with that of THC. In our MTT assay, we noted that THC exerted an inhibitory effect on a wide range of human cell lines with IC_{50} values in the range of 6–16 μM. AJA is more potent than THC-11-oic acid but is on average about 2-fold less potent than THC *in vitro* after a 48-hr incubation (although it, too, inhibited growth in all cell lines tested). Importantly, however, our results also revealed that compared with THC, the inhibitory effects of AJA were more sustained in at least two glioma cell lines and that this effect became apparent after 3 days of treatment.

Because its stereospecificity suggested that its antineoplastic effects were receptor mediated, we compared the effects of antagonists of both CB_1 and CB_2 receptors on growth inhibition. We found, in agreement with previous studies [8], that THC exerted its effects via both receptors. By contrast, however, we noted an effect of only the CB_2 antagonist on the antitumoral effect of AJA. Other studies using non-neoplastic models have suggested that the effects of AJA are primarily CB_2 mediated [9]. This observation is consistent with AJA's lack of psychoactivity because, unlike the CB_1 receptor which is present in brain and peripheral tissues, the CB_2 receptor is expressed mainly in extraneural, primarily immune, tissues [27]. Thus, an agent that preferentially acted via this receptor subtype might be ideal for anticancer treatments if tumors were found to preferentially express CB_2 receptors. To date, such information is not available, and further studies will be needed to resolve this question.

The occurrence of lipid-containing vacuoles is also of interest and suggests possible mechanisms of action. We initially noted that AJA-treated cells were enlarged, which led us to suspect that cell division was hampered. However, this was not confirmed by cell kinetic analysis; in these studies, the propidium iodide labeling profiles were not altered visibly (although there was a significant increase in S phase cells), suggesting that the effect of AJA did not occur primarily via the cell cycle. Furthermore, there was little evidence of an increased rate of apoptosis, as assessed by the amount of cells in sub-G_0 and the high rate of cell viability in the treated cells.

Further analysis indicated that the enlargement of C6 glioma cells resulted from the development of distinctive lipid droplets. These droplets became more prominent over time and disappeared after removal of AJA. Unlike THC, where the number of droplets decreased between 24 and 48 hr, droplet formation continued to increase in AJA-treated cancer cells. This appearance seemed to be restricted to neoplastic cells, since they were not observed in the human fibroblast cell line, WI-38 (although WI-38 cells were inhibited by both THC and AJA).

We also observed that these droplets contained either TGs or cholesteryl oleate as evidenced by their positive oil

[2] Atlantic Technology Ventures, Inc., unpublished data. Cited with permission.

red O staining [18]. To investigate this further, we assessed the character of these lipids by measuring the incorporation of [^{14}C]oleic acid, using TLC methodology. We noted that AJA administration was associated with a markedly enhanced incorporation of labeled fatty acid into the non-polar lipid fractions.

Whether the increase in TGs is linked to the antiproliferative effects of AJA remains to be determined. However, there are several possible ways in which these two characteristics can be linked. Although TGs are mainly utilized by cells for metabolic reasons, their formation proceeds through steps in which an increase occurs in DGs, certain species of which are signal transducers and known physiological activators of the PKC cascade. Thus, one possible mechanism of action for AJA would be through modifications of PKC signaling. In general, PKC activation is associated with increased glioma cell growth [28–30]. However, increased PKC activity can sometimes be associated with antiproliferative effects. For example, the PKC isoenzymes that are stimulated may be those that slow growth, such as PKC δ [31]. Alternatively, increased DG may activate some other downstream mediator such as β-chimaerin to a greater extent than PKC, such that the balance is towards antiproliferation [32]. Further studies are needed to address this relationship.

It is important to note, moreover, that differences exist in the ability of DG to induce PKC activity based on such characteristics as its particular structure and degree of unsaturation; thus, in the absence of species identification, the presence of increased DG is not enough to establish this link [33,34]. It may be possible that the antitumor effect of AJA is a result of the increased lipid content, yet still be PKC independent. The appearance of visible oil droplets is one of the most prominent features of disordered cells and results from an imbalance in the ratio of TGs to phospholipid. Globular TG can appear secondary to either an excess beyond the amount that can be solubilized in lipoprotein micelles or lack of phospholipid proteins that participate in micellar formation [35]. It is therefore possible that the increase in TG itself is signaling the cell to cease proliferation. Previous studies have noted that in order for mitosis to occur, there must be enough phosphatidylcholine, which is produced by the reaction of DG and CDP-choline [36,37]. One could postulate, therefore, that AJA inhibits this reaction, which then leads to a cessation of cell cycle progression due to the lack of this essential membrane component. Further studies are in progress that address these issues.

Finally, the *in vivo* results of this study point to a modest effect of very low doses of AJA (0.1 mg/kg three times per week) on subcutaneous tumors. We chose to examine U87 tumors implanted s.c. in nude mice because of several recent reports demonstrating a consistent growth pattern in this model [38]. Unlike the studies of Galve-Roperh *et al.* [8], we administered both AJA and THC p.o. on a thrice weekly basis instead of by continuous intratumoral infusion. We did not attempt to find the ideal dose for AJA; instead, we chose to compare a dose of AJA that had been determined to be effective in a previous study examining its anti-inflammatory effects [16] with a dose of THC that had been noted previously to be effective in studies examining its cataleptic action [19]. The dosage of AJA used was over 6000-fold less than the maximum tolerated dose determined by prior studies.[3] Therefore, we do not know if the dose used was the optimal one. Nevertheless, a significant delay in tumor growth in only AJA-treated mice was seen. Although a delay was noted, this was only temporary. It is not clear whether this represented the development of tolerance of tumor cells to the growth inhibition or whether changing the dose or route of administration (i.e. intratumoral) would be more effective. Nevertheless, the fact that this agent is not psychoactive to any degree and that it can be administered at high doses without apparent toxicity suggest that further study of the antitumor capabilities of AJA is warranted.

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