IMUNE on Marijunana
The total story

How Marijuana Affects the Brain
THC, a key ingredient in marijuana, attaches to cannabinoid receptors throughout the body. Several areas of the brain have high densities of these receptors, which helps explain the different effects of the drug.

How the receptors work
Nerve cells communicate by passing chemical messages across contact points called synapses.

The most active ingredient in marijuana, THC, attaches to cannabinoid receptors and modifies nerve action.

<table>
<thead>
<tr>
<th>Synapse</th>
<th>THC</th>
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<td>Cannabis receptor</td>
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Sources: Igor Grant, University of California Center for Medicinal Cannabis Research; WSJ research

Estimated percentage of people in a national survey who used a substance at least once and became dependent

- Tobacco: 32%
- Heroin: 23%
- Cocaine: 17%
- Alcohol: 15%
- Stimulants (other than cocaine): 11%
- Anxiolytic sedative hypnotic drugs: 9%
- Marijuana: 9%
- Anxiogenic drugs: 8%
- Psychedelic drugs: 5%
- Inhalant drugs: 4%

Sources: The National Comorbidity Survey, which included 28,000 participants and was supported by the National Institute on Drug Abuse; results published in Experimental and Clinical Psychopharmacology, 1994.
Marijuana Cuts Cancer Tumor Growth

Marijuana is not really a hallucinogenic but more a euphoric. It is called psychoactive substance because it makes one think that they are expanding consciousness, some do some don’t expand their minds, but all THINK they do, it is a profoundly good herb with amazing healing properties, it should be legal but countries fear that free use will lead to over use and this will erode the work force, just like alcohol already does, they tried prohibition on alcohol and it failed just like the prohibition of marijuana is failing.

Freedom of choice is always better, but this kind of freedom needs information freedom and evidence to be true freedom. And of course peer pressure to use drugs before the prefrontal cortex of the brain develops at 18 years old must be prohibited. And I mean really prohibited.

Use of the oil has medicinal value. Smoking has the side effect of loss of motivation for most users. Few can resist this. The oil is only destroyed by extreme heat cooking or long microwave use. Normal and low to moderate heat cooking have little effect on the medicinal quality of the herb.

The Prefrontal Cortex of the Brain is Responsible for Not Being Able to Resist Peer Pressure, Since this does Not fully Develop till age 20 it is Hard for Children to Resist Peer Pressure
Cannabinoids offer cancer patients a therapeutic option in the treatment of highly invasive cancers. The medical science is strongly in favor of hemp oil as a primary cancer therapy, not just in a supportive role to control the side effects of chemotherapy. According to Dr. Robert Ramer and Dr. Burkhard Hinz of the University of Rostock in Germany medical marijuana can be an effective treatment for cancer.[2] Their research was published in the Journal of the National Cancer Institute Advance Access on December 25th of 2007 in a paper entitled Inhibition of Cancer Cell Invasion by Cannabinoids via Increased Expression of Tissue Inhibitor of Matrix Metalloproteinases-1.[3]

Marijuana cuts lung cancer tumor growth in half, a 2007 Harvard Medical School study shows.[4]. The active ingredient in marijuana cuts tumor growth in common lung cancer in half and significantly reduces the ability of the cancer to spread, say researchers at Harvard University who tested the chemical in both lab and mouse studies. "The beauty of this study is that we are showing that a substance of abuse, if used prudently, may offer a new road to therapy against lung cancer," said Anju Preet, Ph.D., a researcher in the Division of Experimental Medicine. Researchers reported in the August 15, 2004 issue of Cancer Research, the journal of the American Association for Cancer Research, that marijuana's constituents inhibited the spread of brain cancer in human tumor biopsies.[5] Researchers at the University of Milan in Naples, Italy, reported in the Journal of Pharmacology and Experimental Therapeutics that non-psychoactive compounds in marijuana inhibited the growth of glioma cells in a dose-dependent manner, and selectively targeted and killed malignant cells through apoptosis. "Non-psychoactive CBD produce[s] a significant anti-tumor activity both in vitro and in vivo, thus suggesting a possible application of CBD as an antineoplastic agent."[6]
Marijuana Science: Why Pot Heads Are Slackers

By Denise Chow, Staff Writer  |  July 01, 2013 04:53pm ET

The stereotype of pot smokers as lackadaisical loafers is supported by new research: People who smoke marijuana regularly over long periods of time tend to produce less of a chemical in the brain that is linked to motivation, a new study finds. Researchers in the United Kingdom scanned the brains of 19 regular marijuana users, and 19 nonusers of the same sex and age, using positron emission tomography (PET), which helps measure the distribution of chemicals throughout the brain.

They found that the long-term cannabis users tended to produce less dopamine, a "feel good" chemical in the brain that plays an important role in motivation and reward-driven behavior.
Use Hemp Oil for Medicine

The medical science is strongly in favor of hemp oil as a primary cancer therapy. But contrary to popular belief, smoking cannabis does not assist a great deal in treating disease within the body as therapeutic levels cannot be reached through smoking. Creating oil from the plant or eating the plant is the best way to go about getting the necessary ingredients, the cannabinoids.

**SMOKING MARIJUANA CAN HURT YOUR ABILITY TO . . .**

- concentrate
- remember what you learned
- focus on projects or tasks
- react quickly while driving
- perform in sports owing to effects on coordination

The following studies are on the anticancer Effects of Natural Marijuana Oil
Marijuana Cuts Lung Cancer Tumor Growth In Half, Study Shows

Date:
April 17, 2007
Source:
American Association for Cancer Research
Summary:
The active ingredient in marijuana cuts tumor growth in common lung cancer in half and significantly reduces the ability of the cancer to spread, say researchers at Harvard University who tested the chemical in both lab and mouse studies.

The active ingredient in marijuana cuts tumor growth in common lung cancer in half and significantly reduces the ability of the cancer to spread, say researchers at Harvard University who tested the chemical in both lab and mouse studies.
They say this is the first set of experiments to show that the compound, Delta-tetrahydrocannabinol (THC), inhibits EGF-induced growth and migration in epidermal growth factor receptor (EGFR) expressing non-small cell lung cancer cell lines. Lung cancers that over-express EGFR are usually highly aggressive and resistant to chemotherapy.
THC that targets cannabinoid receptors CB1 and CB2 is similar in function to endocannabinoids, which are cannabinoids that are naturally produced in the body and activate these receptors. The researchers suggest that THC or other designer agents that activate these receptors might be used in a targeted fashion to treat lung cancer.
"The beauty of this study is that we are showing that a substance of abuse, if used prudently, may offer a new road to therapy against lung cancer," said Anju Preet, Ph.D., a researcher in the Division of Experimental Medicine.
Acting through cannabinoid receptors CB1 and CB2, endocannabinoids (as well as THC) are thought to play a role in variety of biological functions, including pain and anxiety control, and inflammation. Although a medical derivative of THC, known as Marinol, has been approved for use as an appetite stimulant for cancer patients, and a small number of U.S. states allow use of medical marijuana to treat the same side effect, few studies have shown that THC might have anti-tumor activity, Preet says. The only clinical trial testing THC as a treatment against cancer growth was a recently completed British pilot study in human glioblastoma.
In the present study, the researchers first demonstrated that two different lung cancer cell lines as well as patient lung tumor samples express CB1 and CB2, and that non-toxic doses of THC inhibited growth and spread in the cell lines. "When the cells are pretreated with THC, they have less EGFR stimulated invasion as measured by various in-vitro assays," Preet said.
Then, for three weeks, researchers injected standard doses of THC into mice that had been implanted with human lung cancer cells, and found that tumors were reduced in size and weight by about 50 percent in treated animals compared to a control group. There was also about a 60 percent reduction in cancer lesions on the lungs in these mice as well as a significant reduction in protein markers associated with cancer progression, Preet says.
Although the researchers do not know why THC inhibits tumor growth, they say the substance could be activating molecules that arrest the cell cycle. They speculate that THC may also interfere with angiogenesis and vascularization, which promotes cancer growth.
Preet says much work is needed to clarify the pathway by which THC functions, and cautions that some animal studies have shown that THC can stimulate some cancers. "THC offers some promise, but we have a long way to go before we know what its potential is," she said.
Marijuana ingredient inhibits VEGF pathway required for brain tumor blood vessels

Cannabinoids, the active ingredients in marijuana, restrict the sprouting of blood vessels to brain tumors by inhibiting the expression of genes needed for the production of vascular endothelial growth factor (VEGF).

According to a new study published in the August 15, 2004 issue of the journal Cancer Research, administration of cannabinoids significantly lowered VEGF activity in laboratory mice and two patients with late-stage glioblastoma.

"Blockade of the VEGF pathway constitutes one of the most promising antitumoral approaches currently available," said Manuel Guzmán, professor of biochemistry and molecular biology, with the Complutense University in Madrid, Spain, and the study's principal investigator.

"The present findings provide a novel pharmacological target for cannabinoid-based therapies."

Glioblastoma multiforme, the most aggressive form of glioma, strikes more than 7,000 Americans each year and is considered one of the most malignant and deadliest forms of cancer, generally resulting in death within one to two years following diagnosis.

The disease is usually treated with surgery, followed by conventional radiation alone or in combination with chemotherapy. However, the main tumor often evades total destruction, surviving and growing again, eventually killing the patient. For this reason, researchers are actively seeking other therapeutic strategies, some of which might be considered novel.

In this study, the investigators chose to work with cannabinoids which, in previous studies, have been shown to inhibit the growth of blood vessels, or angiogenesis, in laboratory mice. However, little was known about the specific mechanisms by which cannabinoids impair angiogenesis, or whether the chemical might do the same in human tumors.

To answer the first part of the question, the scientists induced gliomas in mice, which were subsequently inoculated with cannabinoids. Using DNA array analysis, the team examined 267 genes associated with the growth of blood vessels in tumors and found that cannabinoids lowered the expression of several genes related to the VEGF pathway, critical for angiogenesis.

The researchers also discovered that cannabinoids apparently worked by increasing the activity of ceramide, a lipid mediator of apoptosis, resulting in the functional inhibition of cells needed for VEGF production. The ability of cannabinoids to alter VEGF production was significantly stifled following the introduction of a ceramide inhibitor.
"As far as we know, this is the first report showing that ceramide depresses VEGF pathway by interfering with VEGF production," according to Guzmán.

To answer the second part of the question relating to clinical tests, the scientists obtained tumor biopsies from two patients with glioblastomas who had failed standard therapy, including surgery, radiotherapy and chemotherapy. The biopsied tissue was analyzed before and after local injection of a cannabinoid.

"In both patients, VEGF levels in tumor extracts were lower after cannabinoid inoculation," said Guzmán.

The results, he added, suggest a potential new approach toward the treatment of these otherwise intractable brain tumors.

"It is essential to develop new therapeutic strategies for the management of glioblastoma multiforme," the scientists wrote, "which will most likely require a combination of therapies to obtain significant clinical results."

Also participating in the study were Cristina Blázquez and Amador Haro, from Complutense University; Luis González-Feria, from University Hospital, Tenerife, Spain; Luis Álvarez, from La Paz University Hospital in Madrid; and M. Llanos Casanova, from the Project on Cellular and Molecular Biology and Gene Therapy, CIEMAT, also in Madrid.

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Founded in 1907, the American Association for Cancer Research is a professional society of more than 22,000 laboratory, translational, and clinical scientists engaged in all areas of cancer research in the United States and in more than 60 countries. AACR's mission is to accelerate the prevention and cure of cancer through research, education, communication, and advocacy. Its principal activities include the publication of five major peer-reviewed scientific journals: Cancer Research; Clinical Cancer Research; Molecular Cancer Therapeutics; Molecular Cancer Research; and Cancer Epidemiology, Biomarkers & Prevention. AACR's Annual Meetings attract more than 15,000 participants who share new and significant discoveries in the cancer field. Specialty meetings, held throughout the year, focus on the latest developments in all areas of cancer research.
Antitumor Effects of Cannabidiol, a Nonpsychoactive Cannabinoid, on Human Glioma Cell Lines

Abstract

Recently, cannabinoids (CBs) have been shown to possess antitumor properties. Because the psychoactivity of cannabinoid compounds limits their medicinal usage, we undertook the present study to evaluate the in vitro antiproliferative ability of cannabidiol (CBD), a nonpsychoactive cannabinoid compound, on U87 and U373 human glioma cell lines. The addition of CBD to the culture medium led to a dramatic drop of mitochondrial oxidative metabolism \[3-(4,5\text{-dimethyl-2-thiazolyl})-2,5\text{-diphenyl-2H tetrazoliyum bromide test}\] and viability in glioma cells, in a concentration-dependent manner that was already evident 24 h after CBD exposure, with an apparent IC\(_{50}\) of 25 μM. The antiproliferative effect of CBD was partially prevented by the CB2 receptor antagonist \(N\-(1S)-\text{endo-1,3,3-trimethylbicyclo[2,2,1]heptan-2-yl}-5-(4\text{-chloro-3-methylphenyl})-1-(4\text{-methylbenzyl})-\text{pyrazole-3-carboxamide (SR144528; SR2)}\) and α-tocopherol. By contrast, the CB1 cannabinoid receptor antagonist \(N\-(piperidin-1-yl)-5-(4\text{-chlorophenyl})-1-(2,4\text{-dichlorophenyl})-4\text{-methyl-1H-pyrazole-3-carboximide hydrochloride (SR141716; SR1)}\), capsazepine (vanilloid receptor antagonist), the inhibitors of ceramide generation, or pertussis toxin did not counteract CBD effects. We also show, for the first time, that the antiproliferative effect of CBD was correlated to induction of apoptosis, as determined by cytofluorimetric analysis and single-strand DNA staining, which was not reverted by cannabinoid antagonists. Finally, CBD, administered s.c. to nude mice at the dose of 0.5 mg/mouse, significantly inhibited the growth of subcutaneously implanted U87 human glioma cells. In conclusion, the nonpsychoactive CBD was able to produce a significant antitumor activity both in vitro and in vivo, thus suggesting a possible application of CBD as an antineoplastic agent.

Marijuana and its derivatives have been used in medicine for many centuries, and currently there is a renewed interest in the study of the therapeutic effects of cannabinoids. Cannabinoids produce their effects by binding to specific plasma membrane G protein-coupled receptors. To date, two cannabinoid receptors have been characterized: the CB1 receptor, expressed primarily in the brain and in some peripheral tissues, and CB2 receptors, expressed by cells of the immune system (Howlett et al., 2002; Pertwee and Ross, 2002). Ongoing research is determining whether cannabinoid ligands may be effective agents in the treatment of pain, glaucoma, the wasting and emesis associated with cancer.
chemotherapy and AIDS, and neurodegenerative disorders such as multiple sclerosis (Goutopoulos and Makriyannis, 2002). Among the potential therapeutic activities, one of the most exciting and promising areas of current cannabinoid research is the demonstrated ability of these compounds to affect a number of pathways involved in the cell survival/death decision (Bifulco and Di Marzo, 2002; Guzman et al., 2002).

Both natural and synthetic as well as endogenous cannabinoids have been found to affect the rate of cell proliferation in cell lines derived from the central nervous system. Very intriguing was the demonstration that THC and WIN-55,212-2 have been demonstrated to suppress the growth of rat glioma C6 cells inoculated intracerebrally in the rat or subcutaneously in immune-deficient mice, through a cannabinoid receptor-dependent mechanism (Sanchez et al., 1998; Galve-Roperh et al., 2000; Guzman et al., 2002). These observations were of the utmost interest for their possible impact on the clinical management of malignant gliomas, which represent the most common form of brain tumor associated with an unfavorable prognosis and refractoriness to surgical, radiological, and pharmacological treatment. However, the well known psychotropic effects of THC and related compounds raise a number of clinical and ethical considerations, thus limiting their medicinal usage. A subsequent work also highlighted that CB2 selective agonist can induce either in vitro or in vivo a significant tumor regression (Sanchez et al., 2001). However, the application of CB2 compounds is limited by their intrinsic immunosuppressive effects that would be expected to inhibit host antitumor immunity. As a matter of fact, Zhu et al. (2000) have recently reported that THC injection led to an accelerated growth of lung tumor implants in immune-competent mice through an involvement of CB2 receptors. Therefore, one alternative therapeutic approach is represented by the use of nonpsychoactive cannabinoids. Among the bioactive constituents of marijuana, cannabidiol (CBD) does not have significant intrinsic activity over cannabinoid receptors (Howlett et al., 2002) and, thus, does not produce psychotropic and adverse side effects, which makes it one of the bioactive constituents with the highest potential for therapeutic use. Moreover, recent reports indicate that CBD can act as a neuroprotective agent in both in vivo (Braida et al., 2003) and in vitro studies (Hampson et al., 2000). Regarding the potential effects of CBD on the immune system, they appear somehow different and/or weaker, as compared with the classical cannabinoid effects. Although the work of Malfait et al. (2000) and Faubert Kaplan et al. (2003) suggested anti-inflammatory and/or immunosuppressive properties of CBD, other studies were not unequivocal. Smith et al. (1997) reported no effect of CBD in affecting the mortality of mice sublethally infected with Legionella, and a recent paper by Killestein et al. (2003) reported in multiple sclerosis patients, treated orally with a combination of THC/CBD, an increase in plasma interleukin-12 level, thus suggesting a pro- rather than anti-inflammatory effect of the therapy. Moreover, in accordance with these data and with our unpublished observations, Srivastava et al. (1998) found stimulation and inhibition in some cytokine levels induced by CBD. Thus, the nonpsychotropic cannabinoid seems to possess a minor impact on immune function. Therefore, the present study was undertaken to investigate, in vitro and in vivo, the possible antiproliferative effect of CBD on two glioma cell lines of human origin and characterize its mechanism of action.

**Materials and Methods**

**Materials.** CBD was a generous gift from GW Pharmaceuticals (Salisbury, UK). It was initially dissolved in ethanol to a concentration of 250 mM and stored at −20°C. CBD was further diluted with tissue culture medium for in vitro studies or PBS in in vivo studies to the desired concentration, keeping the ethanol
concentration below 0.001%. SR141716 and SR144528 were kindly given by Dr. F. Barth (Sanofi Synthélabo Recherche, Montpellier, France).

L-Cycloserine, phorbol 12-myristate 13-acetate (PMA), fumonisin B₁, desipramine, α-tocopherol, capsazepine, and pertussis toxin were purchased from Sigma-Aldrich (St. Louis, MO). Tissue culture media and all supplements were obtained from Sigma-Aldrich.

**Cell Culture.** U87 and U373 human glioma cells were used. Cells were maintained at 37°C in a humidified atmosphere with 5% CO₂ and 95% air. Cells were cultured in 75-cm² culturing flasks in DMEM supplemented with 4 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 1% sodium pyruvate, 1% nonessential amino acids, and 10% heat-inactivated fetal bovine serum. For in vitro studies, cells were seeded in serum-free medium, consisting of DMEM supplemented with 5 μg/ml insulin, 5 μg/ml transferrin, and 5 ng/ml sodium selenite, in multiwell plates or Petri dishes according to experimental protocol. After a 24-h incubation, the medium was removed and new culture medium, containing the compounds to be tested, was added.

**Analysis of Cell Viability.** To determine the effects of CBD upon cell viability, we carried out the MTT colorimetric assay ([3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide]; Sigma-Aldrich). Briefly, glioma cells were seeded in a 96-well flat bottom multiwell at a density of 6000 cells/well for U373 and 8000 cells/well for U87 cells. After 24 h, cells were treated with CBD and/or cannabinoid antagonists/inhibitors at the indicated concentrations and times. At the end of the incubation with the drugs, MTT (0.5 mg/ml final concentration) was added to each well and the incubation was then continued for 4 h. The insoluble formazan crystals were solubilized by the addition of 100 μl of 100% dimethyl sulfoxide. The plates were read at 570 nm using an automatic microtiter plate reader.

The viability of the cells was also estimated by the trypan blue dye-exclusion method. Cells were seeded in Petri dishes (40,000 cells/dish). After 24 h, cells were treated with CBD for the desired time. At the end of the exposure, the cells were harvested and incubated with 0.1% trypan blue for 2 to 5 min. The percentage of cells that excluded the vital dye trypan blue was determined microscopically.

**Flow Cytometric Analysis.** Tumor cells were cultured in 12-well plates in the presence or absence of CBD and/or cannabinoid antagonists for 24 h, as described above. The percentage of apoptotic cells on the total cell population (adhering + detached cells) was evaluated as previously described (Ceruti et al., 2000). Briefly, cells were collected, washed, and centrifuged at 200g. The cell pellet was gently resuspended in 1 ml of hypotonic fluorochrome solution [propidium iodide (PI), 50 μg/ml in 0.1% sodium citrate plus 0.1% Triton X-100; Sigma-Aldrich].

Cells were analyzed after a minimum of 30 min of incubation in the dark at room temperature, and apoptosis was detected in individual cells using a flow cytometer (equipped with a single 488-nm argon laser; BD Biosciences, San Jose, CA) by reduced fluorescence of PI in apoptotic nuclei.

**Detection of Single-Strand DNA (ssDNA).** To further confirm the presence of apoptosis and discriminate between necrotic and apoptotic cell death, we detected ssDNA fragments in the nuclei using an ELISA detection kit with a mouse monoclonal antibody to ssDNA (Chemicon International, Temecula, CA). This antibody does not recognize DNA in double-stranded conformation and provides specific detection of apoptosis (Frankfurt and Krishan, 2001).

Briefly, following the manufacturer’s instructions, cells were seeded at 8000 cells/well in a 96-multiwell plate. After the incubation with the drug, the plates were centrifuged at 200g for 5 min and the cells were fixed with 80% methanol in PBS for 30 min at room temperature. The plates were dried and cells were incubated with formamide for 10 min at room temperature for an additional 10 min at 75°C, and then 5 min at 4°C. Cells were incubated for 1 h with 3% nonfat dry milk and then incubated with the
antibody mixture (containing a primary monoclonal antibody to ssDNA and horseradish peroxidase-labeled secondary antibody) for 30 min. The addition of 2-2′-azino-bis[3-ethylbenziazoline-6-sulfonic acid solution for 40 min permitted the reading of the plates at 405 nm in a standard microtiter reader. As positive control, ssDNA was used, and as negative control, necrotic cells were obtained by hyperthermia by heating the cells at 56°C for 1 h and then incubating them for 1 h at 37°C.

**Nude Mouse Xenograft Model of Human Glioma.** Athymic female CD-1 nude (nu/nu) mice (Charles River Laboratories, Calco, Italy), 8 weeks old, were used. The animals were injected subcutaneously on the left flank with 3 × 10⁶ U87 human glioma cells in 0.1 ml of PBS. Tumors were measured using an external caliper, twice a week, and volume was calculated by the formula: 4π/3 × (length/2) × (width/2)².

Seven days after the inoculation, when the tumor volume had reached an average volume of about 70 mm³, mice were randomly divided into two groups. The mice were treated peritumorally with CBD (dissolved in 0.1 ml of sterile PBS supplemented with 5 mg/ml defatted and dialyzed bovine serum albumin) or its vehicle, at a dose of 0.5 mg/mouse (day 0 of treatment). The injection was repeated once a day, 5 days per week, and the tumor volumes were checked twice a week until the animals were sacrificed.

Mice were monitored daily for health status, and 23 days after the beginning of the treatment, when the control group tumor burden was exceeding 10% of the host weight, the experiments were stopped and the animals sacrificed. This protocol was conducted in accordance with the Italian regulation for the welfare of animals in experimental neoplasia (Permission no. 94/2000A) and met the European Community directives regulating animal research.

**Statistics.** Statistical analysis for cell proliferation data was performed using one-way analysis of variance followed by the post hoc analysis Dunnett’s t test.

In apoptotic studies, the differences between the groups were analyzed by Bonferroni’s t test. All biochemical data are presented as the mean ± S.E.M. of at least three separate experiments. In vivo data were analyzed by Mann-Whitney test to compare medians for nonparametric data. All statistical analyses were undertaken using GraphPad Prism 3.00 (GraphPad Software, San Diego, CA).

**Results**

**Inhibition of Human Glioma Cell Proliferation by CBD.** The aim of initial experiments was to investigate whether CBD could affect the viability of the U87 and U373 human glioma cell lines. The addition of CBD to the culture medium of both human glioma cell lines for 24 h resulted in a concentration-dependent inhibition of the mitochondrial oxidative metabolism, as determined by MTT test. The range of concentrations tested was from 5 μM to 40 μM. For both cell lines, the concentration that started to be significant was 15 μM, with a reduction in O.D. values of 20 ± 0.6% (n = 12) and 15 ± 0.8% (n = 12) for U87 and U373, respectively, as compared with the control. Further inhibition in the MTT test was observed, with 20 μM (28 ± 2% for U87 and 40 ± 3.2% for U373, n = 12), 30 μM (70 ± 3.5% for U87 and 70 ± 3.43 for U373, n = 12), and 40 μM (96 ± 10% for U87 and 94 ± 10.34 for U373, n = 12), with IC₅₀ values of 26.2 ± 2.8 μM in U87 cells and 24.1 ± 2.16 μM for U373.

In a subsequent series of experiments, we tested the effect of a single administration of CBD (using the mean IC₅₀ concentration of 25 μM) to the cells following their growth during a 4-day period. When the MTT test was performed daily for 4 days, we found that the growth inhibition for both cell lines (Fig. 1, A–C) was still present during this period, with a maximum effect seen at 4 days. Interestingly, these results were positively correlated with the drop in cell number in a time-dependent manner (Fig. 1, B–
as assessed by counting the cells by trypan blue exclusion. Taken together, these findings indicated that CBD induced its effects on gliomas in a concentration- and time-dependent manner, suggesting a specific mechanism by which CBD could affect the viability of glioma cell lines.

Fig. 1.

Time-dependent inhibition of U87 and U373 cell proliferation induced by CBD. Cells were cultured in serum-free medium in the absence (□) or presence (•) of 25 μM CBD, added at a day “0,” for the time indicated. A and B, MTT and trypan blue tests on U87 glioma cell line; C and D, MTT and trypan blue tests on U373 glioma cell line. Results correspond to three different experiments and values are expressed as mean (O.D. or number of cells) ± S.E.M. ★, p < 0.05; ★★, p < 0.01; ★★★, p < 0.001 versus untreated cells (□), Dunnett’s t test.

Effects of Cannabinoid, Vanilloid Receptor Antagonists, and Pertussis Toxin upon Antiproliferative Effects of CBD. Most of the effects of cannabinoids on the central nervous system described so far are believed to be mediated by cannabinoid receptors. CBD has been reported to bind cannabinoid receptors with weak affinity, and recently, it has been demonstrated that it can also bind vanilloid receptors (Bisogno et al., 2001). Hence, we next studied whether the effects of CBD described in the present report were dependent on the stimulation of these receptors. Thus, we performed studies with the specific antagonist SR1, selective for CB1 receptor, SR2, selective for CB2 receptor, and capsazepine (CPZ), selective for the vanilloid receptor VR1, using in vitro concentrations that did not, per se, affect cell viability (data not shown).

As shown in Fig. 2 for U87 cells, the CBD-induced growth inhibition over a 24-h period was never prevented by SR1 and CPZ; by contrast, the SR2 antagonist appeared to significantly antagonize this effect, although in a noncomplete manner. Curiously, under the same experimental condition, the combination of SR2 with the other selective antagonists did not block the effect induced by CBD. However, the ability of SR2 in blocking the CBD growth inhibition was lost after 4 days of exposure to CBD (Fig. 2). Similar results were also found in U373 (Fig. 2), where, however, we observed only a nonsignificant trend for SR2 in limiting the CBD-inhibitory effect during 24 h of exposure.
Effect of concomitant treatment of U87 and U373 glioma cells cultured in serum-free medium with a combination of the selective antagonists 0.5 μM SR1, 0.5 μM SR2, and/or 0.625 μM CPZ upon the sensitivity to the antiproliferative effects of CBD (MTT test). Cells were treated for 24 h or 4 days with 25 μM CBD and the indicated antagonist compounds as reported in the figure. Results correspond to at least three different experiments and values are expressed as mean O.D. ± S.E.M ★★★, p < 0.001; ★★, p < 0.01, versus untreated cells (control); †, p < 0.05 versus 25 μM CBD, Dunnett’s t test.

Cannabinoid receptors are coupled to heterotrimeric G\textsubscript{i}/G\textsubscript{o} proteins that can be inactivated by pretreatment with pertussis toxin (PTX). We therefore compared the antiproliferative effect of CBD in the presence or absence of PTX. Pretreatment of U87 and U373 cells with 100 ng/ml PTX for 18 h was unable to limit the antimitotic effect of CBD (Fig. 3).

Absence of an effect of PTX on CBD-induced antiproliferation. U87 and U373 cells were preincubated for 18 h with 100 ng/ml PTX and treated with different concentrations of CBD for 24 h. Viability was determined by MTT test. Results correspond to three different experiments and values (O.D.) are expressed as mean ± S.E.M.

Apoptosis Induced by CBD. To verify whether the CBD-induced reduction in glioma cell growth was indeed due to apoptotic cell death, both flow-cytometric analysis and ssDNA detection assay have been utilized. Flow-cytometric analysis was carried out on the total cell population (i.e., adhering + detached cells) and apoptosis was assessed as appearance of a hypodiploid DNA peak after PI staining of nuclei. In U87 control culture, after 24 h of incubation, only 2.66 ± 1.38% of the cells underwent a spontaneous
apoptosis (Fig. 4). The exposure to the ineffective concentration of CBD in the MTT test at 10 μM caused an induction of apoptosis overlapping that obtained in control cells (7.25 ± 4.03%). By contrast, culturing the cells with CBD for 24 h with the IC\textsubscript{50} concentration caused an induction of apoptosis in 51.58 ± 4.82% of the total cell population. Similar results were observed in the U373 cells, where in the control group the percentage of apoptosis was 2.09 ± 0.16%, and the exposure to CBD was found to increase the apoptotic rate from 8.66% ± 7.77 with 10 μM to 41.36 ± 11.8 with 25 μM CBD (data not shown). The addition of CB1 and CB2 cannabinoid antagonists never reversed the CBD-induced cell death (data not shown).

Fig. 4. CBD-induced apoptosis in U87 glioma cells. Cultures were grown in either serum-free medium alone (control) or in a medium containing 10 μM or 25 μM CBD. After 24 h of exposure, cells were detached, centrifuged, resuspended, and incubated with PI solution, and apoptosis was quantified as reduced fluorescence by flow cytometry on the total cell population (adhering + detached cells). A representative experiment is shown in the figure. Y values represent the relative cell number and X values represent the DNA content (PI fluorescence). Numbers on the graphs represent the percentage of apoptotic cells. Histograms represent the mean values ± S.E.M. of the percentage of apoptotic cells obtained in four independent experiments. ★★★, p < 0.001 versus control (C), Student’s t test. Similar results were obtained with U373 glioma cells (data not shown).

The presence of apoptosis was further confirmed by an ELISA apoptosis detection with a specific monoclonal antibody to ssDNA, which represents a specific and sensitive marker of apoptosis. As reported in Fig. 5, CBD at the concentration of 25 μM induced in both cell lines a significant increase in the O.D. values of apoptosis, as compared with the untreated cells. As expected, cells treated with hyperthermia and used as negative necrotic control did not show any degree of apoptosis.

Fig. 5.
ELISA-ssDNA monoclonal antibody detection of apoptosis induced by CBD on glioma cells. Cells were untreated (C) or exposed to CBD (10 μM and 25 μM) for 24 h, as previously described (see *Materials and Methods*). Cells treated with hyperthermia (HT; 56°C for 1 h, followed by an incubation at 37°C for 1 h) were used as necrotic cells-negative control of apoptosis. Data are expressed as O.D. (405 nm) and represent the mean ± S.E.M of at least three experiments. ★★, p < 0.01; ★★★, p < 0.001 versus control (C), Student’s *t* test.

**Mechanism of Action of CBD.** Since ceramide accumulation has been reported to mediate the apoptosis induced in C6 glioma cells by THC (*Galve-Roperh et al., 2000; Gomez del Pulgar et al., 2002; Jacobsson et al., 2001*), we started investigating whether CBD also acted through a similar pathway. We performed experiments with selective inhibitors of ceramide synthesis, using concentrations previously reported to be effective in antagonizing cannabinoid effects (*Gomez del Pulgar et al., 2002,* and not affecting, under our conditions, the cell viability. In our hands, L-cycloserine and fumonisin B₁ (inhibitors of serine-palmitoyl-transferase and ceramide synthase, respectively) had no effect on the cellular response evoked by CBD (*Table 1*). In addition, neither desipramine (an inhibitor of acid sphingomyelinase) nor PMA (an inhibitor of neutral sphingomyelinase via protein kinase C activation), prevented CBD-induced inhibition of cell viability in U87 cells (*Table 1*).

View this table:

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<td>Effect of inhibitors of ceramide synthesis upon the antiproliferative effects of CBD (25 μM) on U87 cell viability (MTT test)</td>
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U87 cells were incubated in the absence or presence of CBD and different concentrations of inhibitors. The values are expressed as mean O.D. ± S.E.M. of at least three independent experiments. To evaluate whether events of oxidative stress were implicated in the mechanism of CBD action, we also tested the effect of a coincubation with the antioxidant agent α-tocopherol. We found that this compound at the concentration of 10 μM significantly prevented, although in a partial manner, the antiproliferative effect of CBD (*Fig. 6*).

![Fig. 6.](image)

**Fig. 6.**

Effect of increasing concentrations of α-tocopherol upon the antiproliferative effect of 25 μM CBD in U87 glioma cells. Cell viability was determined by MTT assay after 24 h of treatment. Results correspond to three different experiments, and values are expressed as mean O.D ± S.E.M. ★, p < 0.05; ★★, p < 0.01, versus untreated cells (control); OO, p < 0.01 versus CBD alone, Dunnett’s *t* test.
**In Vivo Studies.** Since tumor regression in animal experimental tumor models represents an important endpoint of clinical relevance, in a final set of experiments we evaluated the ability of in vivo CBD to reduce tumor growth. To address this point, tumors were induced in athymic nude mice by a subcutaneous inoculation of U87 cells into the flank region of the animals. We found that between 12 and 23 days of treatment, CBD-treated mice had significantly smaller tumors than did control mice (Fig. 7). The regression was of about 70% at day 18 (572 ± 147 mm³, n = 7, in CBD-treated mice, versus 1765 ± 259 mm³, n = 7, in control mice), although at 23 days of treatment this effect appeared weaker, with an inhibition of the tumor growth of about 50%, as compared with control animals (1210 ± 210 mm³, n = 7, in CBD-treated mice, versus 2212 ± 256 mm³, n = 7, in control mice).

**Fig. 7.**

CBD effect on subcutaneous U87 glioma cell growth. CBD (0.5 mg/mouse) administration began 7 days after U87 cell inoculation into the left flank of the athymic nude mice (day 0 of the treatment). CBD was injected in the peritumoral area once a day, 5 days per week. Tumor diameters were measured twice a week. Results represent the mean of seven mice in each group and are expressed as mean volume ± S.E.M. ★, p < 0.05; ★★, p < 0.01, versus control mice; Mann-Whitney nonparametric test. On the right of the graph is reported an example of s.c. gliomas after dissection. Tumors were grown in the presence of vehicle (control) or CBD after 23 days of treatment.

**Discussion**

In the current study we demonstrated that the nonpsychoactive cannabinoid compound CBD can induce in vitro and in vivo inhibition of tumoral cell growth, and we also showed, for the first time, that CBD can trigger the apoptosis of human gliomas, a very aggressive tumor characterized by poor clinical prognosis and unsatisfactory response to the currently available pharmacological agents. Some very old data about the antiproliferative properties of CBD on transformed cells were first reported by Munson et al. (1975), who showed that the drug was ineffective, in vivo, in reducing the growth of Lewis lung adenocarcinoma or was a little effective in inhibiting DNA synthesis on Lewis lung cells and L1210 leukemia cells (Carchman et al., 1976). After that, to our knowledge, the only report that has studied CBD was the work of Jacobsson et al. (2000), demonstrating that in C6 rat glioma cells, CBD had a modest effect only evident after 6 days of incubation with the drug and only in a serum-free condition. At that time, no further investigation or discussion was put forward about the ability of this compound to alter the proliferation rate of glioma cells.
In our study, we demonstrated that CBD caused a concentration-related inhibition of the glioma cell viability under serum-free conditions to exclude any interaction with the reported direct interaction between serum proteins, such as albumin, and cannabinoids (Zheng et al., 1993). A question could be raised about the relatively high values of IC\textsubscript{50} of CBD found in this work, as compared with the reported more potent THC (Sanchez et al., 1998). It is well known that nonpsychotropic cannabinoids are usually used at higher concentrations either in vitro or in vivo to obtain pharmacological effects (Malfait et al., 2000; Recht et al., 2001), probably due to the relatively low affinity of these compounds for CB1 and CB2 cannabinoid receptors (Showalter et al., 1996; Bisogno et al., 2001). Importantly, however, our results revealed that compared with THC, which caused an inhibition of cell viability during a 2- to 3-day or 4- to 5-day period (Sanchez et al., 1998; Ruiz et al., 1999), the inhibitory effects of CBD already became apparent after 24 h of exposure to the drug. The different potency of CBD versus THC was also confirmed in our experimental protocol, where THC affected the viability of our cell lines after 4 days of exposure and with an IC\textsubscript{50} of about 3 to 4 μM (data not shown).

In the present study we showed for the first time the ability of CBD to induce programmed cell death in human glioma cells through two independent methods based on the appearance of a hypodiploid DNA peak after PI staining of nuclei and detection of apoptotic DNA with monoclonal antibody to ssDNA. These results demonstrated that CBD was capable of inducing cell death and that this mechanism correlated with the reported inhibition of cell growth found in both in vitro and in vivo studies. These results are in accordance with previous reports demonstrating cannabinoids as agents able to cause apoptosis in both in vitro and in vivo experiments (Guzman et al., 2002). In fact, THC has been reported to trigger cell death in C6 glioma cells (Sanchez et al., 1998; Galve-Roperh et al., 2000), cortical neurons (Campbell, 2001), and human prostate PC-3 cells (Ruiz et al., 1999), mainly through a receptor-mediated mechanism. Thus it appears that nonpsychoactive compounds such as CBD also can share these apoptotic properties with THC.

The exact mechanism by which CBD induces apoptosis and antiproliferative effects remains partially unclear. CBD has been reported to bind with relatively low-affinity CB1 and CB2 receptors, and recently, it has been demonstrated that it can also bind vanilloid receptors (Bisogno et al., 2001). However, because of its lipophilic properties, it cannot be ruled out that CBD can also act through an aspecific intercalation into the cell membrane. In our hands, neither the CB1 antagonist nor capsazepine was able to block the effect of CBD. By contrast, the CB2 antagonist appeared to affect the inhibition of mitochondrial oxidative metabolism induced by CBD during 24 h of exposure, but only to a partial extent. In any case, this antagonism was not observed in apoptotic studies. Thus, despite the presence of cannabinoid receptors in glioma cell lines, CBD appeared to exert its action through a small involvement of these receptors and no stimulation of vanilloid receptors. In accordance with the CBD insensitivity to antagonists, the inefficacy of PTX pretreatment in reversing CBD effects reinforces the notion of a cannabinoid G\textsubscript{i}/G\textsubscript{o}-coupled receptor-independent mechanism.

On the other hand, the role of cannabinoid receptors in THC-induced cell death also is controversial: whereas early reports seemed to point to an unidentified CB receptor-independent mechanism (Sanchez et al., 1998; Ruiz et al., 1999), further investigations have shown that both CB1 and CB2 receptors can contribute to this cytotoxic effect (Galve-Roperh et al., 2000), although in an unclear manner. Similar conflicting results were also found for AEA, which resulted in triggering cellular events through no involvement (Sancho et al., 2003; Sarker and Maruyama, 2003) or a partial involvement (Maccarrone et al., 2000; Jacobsson et al., 2001) of cannabinoid/vanilloid receptors. In addition, another very intriguing hypothesis, that cannot be ruled out, is that CBD could act through the stimulation of a novel non-CB1, non-CB2 receptor that could be present in these cells, although this hypothesis appears
unlikely since CBD was insensible to SR1 compound (Jarai et al., 1999). Also, the hypothesis that CBD could act indirectly, enhancing the AEA level through an inhibition of anandamide amidase activity (Watanabe et al., 1996), appears improbable since the SR1 compound and/or capsazepine, alone or in combination, did not reverse the effects of CBD.

Since apoptosis by THC and AEA has been demonstrated to be primarily related to ceramide generation (Galve-Roperh et al., 2000; Jacobsson et al., 2001; Gomez del Pulgar et al., 2002), in a first attempt to investigate through which mechanism CBD could induce its effects, we investigated whether CBD could also exert its effects through ceramide accumulation. We did not find any involvement of this intracellular messenger, thus suggesting that the CBD mechanism is clearly different from that described for THC and AEA. The protective effect of α-tocopherol we found suggests an implication of an oxidative stress mechanism in the antiproliferative effects of CBD and argues against a simple cell-toxic mechanism. On the other hand, similar results were also reported by Jacobsson et al. (2001), who described that the inhibition of cell growth induced by AEA on C6 cells was prevented by α-tocopherol. In accordance with this evidence, also, Sarker et al. (2000) found that AEA-induced apoptosis in PC-12 cells caused a rise in intracellular superoxide levels, and this effect was prevented by the antioxidant agent N-acetyl cysteine. These results led us to hypothesize that the effect of CBD could be attributed, in some way, to reactive oxygen species production that, in turn, can mediate the cell death in human glioma cells. To further clarify this point, experiments are now in progress to evaluate the mechanism underlying CBD-induced oxidative stress. Another possibility, as already reported for THC (Chan et al., 1998), is that CBD could induce cell death by a signal transduction cascade related to an increase in intracellular arachidonic acid induced by an activation of phospholipase A2. A further possibility that we have to take into account is that CBD could induce an uncoupling of mitochondrial potential, as already reported for THC and AEA (Maccarrone et al., 2000; Sarafian et al., 2003).

Finally, a very important point of this work is the demonstration of the in vivo efficacy of CBD in reducing tumor growth. This evaluation of in vivo effect of CBD is essential, with the aim to develop a cannabinoid-based therapeutic strategy for gliomas devoid of CB-mediated psychotropic side effects. In our study, we observed a significant inhibition of the in vivo tumor growth over a 23-day period with a dose of CBD of 0.5 mg/mouse. In this study, we did not attempt to find the ideal dose for the treatment. Nevertheless, a significant delay in tumor growth was observed. Currently, it is not clear whether the apparent slighter effect induced by CBD seen at the end of the protocol is related to development of tolerance of tumor cells to the growth inhibition induced by CBD. This effect could in part reflect increased drug metabolism, drug regimen, dose used, or drug resistance. Nevertheless, the fact that this agent is not psychoactive to any degree and that it can be administered at high doses without apparent toxicity encourages further studies.

In conclusion, a cannabinoid-based therapeutic strategy for neural diseases devoid of undesired psychotropic side effects could find in CBD a valuable compound in cancer therapies along with the perspective of evaluating a synergistic effect with other cannabinoid molecules and/or with other chemotherapeutic agents as well as with radiotherapy. Whatever the precise mechanism underlying the CBD effects, the present results suggest a possible application of CBD as a promising, nonpsychoactive, antineoplastic agent.
We are indebted to GW Pharmaceuticals for kindly providing CBD; we are also grateful to Dr. F. Barth (Sanofi Synthélabo Recherche) for providing the compounds SR141716A and SR144528. We thank Dr. E. Monti (University of Insubria, Italy) for U87 and U373 cell lines.

**Footnotes**

- This work was supported by a grant from the Cannabinoid Research Institute, affiliated with GW Pharmaceuticals, Oxford, UK, and by a grant from the Italian Ministry for University and Scientific and Technological Research (FIRST 2001).

**ABBREVIATIONS:** CB, cannabinoid; CBD, cannabidiol; THC, Δ9-tetrahydrocannabinol; VR, vanilloid receptor; AEA, N-arachidonylethanolamide (anandamide); MTT, 3-(4,5-dimethyl-2-thiazoly)-2,5-diphenyl-2H tetrazolium bromide; PMA, phorbol 12-myristate 13-acetate; WIN 55,212-2, R-(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazinyl]-[1-napthanelnyl] methanone mesylate; SR141716A (SR2), N-[(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3 carbboximide hydrochloride; SR144528 (SR1), N-[(1S)-endo-1,3,3-trimethylbicyclo[2,2,1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide; PBS, phosphate-buffered saline; PI, propidium iodide; ssDNA, single-strand DNA; ELISA, enzyme-linked immunosorbent assay; CPZ, capsazepine; PTX, pertussis toxin.

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**References**


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Combining the two most common cannabinoid compounds in Cannabis may boost the effectiveness of treatments to inhibit the growth of brain cancer cells and increase the number of brain cancer cells that die off. That's the finding of a new study published in the latest issue of the journal *Molecular Cancer Therapeutics*.

Researchers at the California Pacific Medical Center Research Institute (CPMCRI) combined the non-psychoactive Cannabis compound, cannabidiol (CBD), with Δ9-tetrahyrdocannabinol (Δ9-THC), the primary psychoactive active ingredient in Cannabis. They found the combination boosts the inhibitory effects of Δ9-THC on glioblastoma, the most common and aggressive form of brain tumor and the cancer that claimed the life of Sen. Ted Kennedy last year.

“Our study not only suggests that combining these two compounds creates a synergistic effect,” says Sean McAllister, Ph.D., a scientist at CPMCRI and the lead author of the study. “but it also helps identify molecular mechanisms at work here, and that may lead to more effective treatments for glioblastoma and potentially other aggressive cancers.”

Previous studies had shown that Δ9-THC was effective in inhibiting brain cancer growth in cell cultures and in animal models and prompted a small clinical trial in Spain. There is also evidence that other compounds in Cannabis might prove effective against tumors, but limited scientific evidence is available. The CPMCRI researchers screened a number of different cannabis-based compounds before settling on CBD as the most active one.

“Compared to using Δ9-THC alone against glioblastoma cell lines, the combination therapy of Δ9-THC and CBD showed a significant improvement in activity, both in slowing down the growth of those cells and also, and perhaps more importantly, in doubling the number of cancer cells which underwent apoptosis or programmed cell death,” says Dr. McAllister.

The next step in the research is to carry out similar studies in animal models of aggressive brain cancer. Even if the synergistic effect is not evident in those studies, the combination treatments may allow for stronger doses to be given to patients due to non-overlapping toxicities and decrease development of resistance to the activity of Δ9-THC or CBD alone.

Despite the promising findings of the study the researchers point out that they are not a recommendation for people with brain cancer to smoke marijuana. They say it is highly unlikely that effective concentrations of either Δ9-THC or CBD could be reached by smoking cannabis.

The study was funded by the National Institute of Health and the SETH group.

California Pacific Medical Center. Beyond Medicine.

At San Francisco’s California Pacific Medical Center, we believe in the power of medicine. We research the most up-to-date treatments, hire the most qualified individuals, and practice the most modern, innovative medicine available. We deliver the highest quality expert care, with kindness and compassion, in acute, post-acute and outpatient services, as well as preventive and complementary medicine. But we also believe that medicine alone is only part of the solution. That’s why we look intently at each individual case and treat the whole person, not just the illness. It’s why we go beyond medical care and provide our patients with things like disease counseling, family support and wellness treatments. As one of California’s largest private, community-based, not-for-profit, teaching medical centers, and a Sutter Health affiliate, we are able to reach deep into our community to provide education, screening and financial support in some of the city’s most underserved neighborhoods. Medicine can transform a body. But going beyond medicine can transform a life. [www.cpmc.org](http://www.cpmc.org)
Molecular Biologist Explains How THC Completely Kills Cancer Cells

February 18, 2014 by Arjun Walia. 195 Comments.


Below is a video of Dr. Christina Sanchez, a molecular biologist at Complutense University in Madrid, Spain, clearly explaining how THC (the main psychoactive constitute of the cannabis plant) completely kills cancer cells.

Not long ago, we published an article examining a case study recently published where doctors used cannabis to treat Leukemia, you can read more about that here. To read more articles and view studies about how cannabis is an effective treatment and cure for cancer, click here.

Cannabinoids refer to any of group of related compounds that include cannabiol and the active constituents of cannabis. They activate cannabinoid receptors in the body. The body itself produces compounds called endocannabinoids and they play a role in many processes within the body that help to create a healthy environment. I think it's also important to note that cannabis has been shown to treat cancer without any psychoactive effects.

Cannabinoids have been proven to reduce cancer cells as they have a great impact on the rebuilding of the immune system. Although not every strain of cannabis has the same effect, more and more patients are seeing success in cancer reduction in a short period of time by using cannabis. Contrary to popular belief, smoking cannabis does not assist a great deal in treating disease within the body as therapeutic levels cannot be reached through smoking. Creating oil from the plant or eating the plant is the best way to go about getting the necessary ingredients, the cannabinoids.

The world has come a long way with with regards to accepting this plant as a medicine rather than a harmful substance. It’s a plant that could benefit the planet in more ways than one. Cannabis is not something offered in the same regard as chemotherapy, but more people are becoming aware if it, which is why it’s so important to continue to spread information like this. Nobody can really deny the tremendous healing power of this plant.

34 MEDICAL STUDIES PROVING CANNABIS CURES CANCER

(Maybe not cures but helps)

Posted by R & S in Health On October 7, 2013

There’s still a lot of confusion across the nation about whether or not marijuana is effective for cancer patients. Odds are you’ve heard something about it but weren’t sure whether the information was reliable or definitive. So, in order to help clear things up, here is a list of 34 studies showing that marijuana cures cancer, categorized by the type of cancers being cured in each study. As you sort through the articles, note that the consistent theme between them is that cannabis shrinks tumors and selectively targets cancer cells. As bills and voter initiatives to legalize medical marijuana spread from state to state, remember that we’re not just talking about mitigating the side effects of chemo (though this is another viable use), we’re talking about curing the cancer itself as well as preventing its spread. I’ve taken the liberty of only including articles from credible scientific journals, removing any biased or otherwise improperly cited studies. Enjoy!

Cures Brain Cancer

http://www.nature.com/bjc/journal/v95/n2/abs/6603236a.html
Cures Mouth and Throat Cancer

Cures Breast Cancer
http://jpet.aspetjournals.org/content/early/2006/05/25/jpet.106.105247.full.pdf+html
http://www.molecular-cancer.com/content/9/1/196
http://www.pnas.org/content/95/14/8375.full.pdf+html

Cures Lung Cancer
http://www.nature.com/onc/journal/v27/n3/abs/1210641a.html

Cures Uterine, Testicular, and Pancreatic Cancers
http://www.cancer.gov/cancertopics/pdq/cam/cannabis/healthprofessional/page4
http://cancerres.aacrjournals.org/content/66/13/6748.abstract

Cures Prostate Cancer
http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3339795/?tool=pubmed
Cures Colorectal Cancer

Cures Ovarian Cancer
http://www.aacrmeetingabstracts.org/cgi/content/abstract/2006/1/1084

Curse Blood Cancer
http://molpharm.aspetjournals.org/content/70/5/1612.abstract

Cures Skin Cancer

Cures Liver Cancer

Cures Biliary Tract Cancer

Cures Bladder Cancer
Cures Cancer in General


About the Author

Michael Taillard, professional economist, published author and board member of NORML Nebraska.
Δ9-Tetrahydrocannabinol-Induced Apoptosis in Jurkat Leukemia T Cells Is Regulated by Translocation of Bad to Mitochondria

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Abstract

Plant-derived cannabinoids, including Δ9-tetrahydrocannabinol (THC), induce apoptosis in leukemic cells, although the precise mechanism remains unclear. In the current study, we investigated the effect of THC on the upstream and downstream events that modulate the extracellular signal-regulated kinase (ERK) module of mitogen-activated protein kinase pathways primarily in human Jurkat leukemia T cells. The data showed that THC down-regulated Raf-1/mitogen-activated protein kinase/ERK kinase (MEK)/ERK/RSK pathway leading to translocation of Bad to mitochondria. THC also decreased the phosphorylation of Akt. However, no significant association of Bad translocation with phosphatidylinositol 3-kinase/Akt and protein kinase A signaling pathways was noted when treated cells were examined in relation to phosphorylation status of Bad by Western blot and localization of Bad to mitochondria by confocal analysis. Furthermore, THC treatment decreased the Bad phosphorylation at Ser\(^{112}\) but failed to alter the level of phospho-Bad on site Ser\(^{136}\) that has been reported to be associated with phosphatidylinositol 3-kinase/Akt signal pathway. Jurkat cells expressing a constitutively active MEK construct were found to be resistant to THC-mediated apoptosis and failed to exhibit decreased phospho-Bad on Ser\(^{112}\) as well as Bad translocation to mitochondria. Finally, use of Bad small interfering RNA reduced the expression of Bad in Jurkat cells leading to increased resistance to THC-mediated apoptosis. Together, these data suggested that Raf-1/MEK/ERK/RSK-mediated Bad translocation played a critical role in THC-induced apoptosis in Jurkat cells. (Mol Cancer Res 2006;4(8):549–62)

Keywords:

Introduction

Cannabinoids, the biologically active constituents of marijuana (Cannabis sativa), produce a wide spectrum of central and peripheral effects, such as alterations in cognition and memory, analgesia, anticonvulsion, anti-inflammation, and alleviation of both intraocular pressure and pain relief (1). There has been a growing interest in cannabinoids since the cloning of two subtypes of the cannabinoid receptors, CB1 (2) and CB2 (3). The CB1 receptor is mainly expressed in the central nervous system, whereas the CB2 receptor is predominantly expressed in immune cells (4). Both cannabinoid receptors are coupled to heterotrimeric G\(_{i/o}\) proteins and interact with the mitogen-activated protein kinases (MAPK), particularly the extracellular signal-regulated kinase (ERK; ref. 5).
At almost the same time, endogenous ligands for these receptors, capable of mimicking, to some extent, the pharmacologic actions of marijuana’s psychoactive principle Δ9-tetrahydrocannabinol (THC), have been discovered (6). Numerous studies have shown that THC can modulate the functions of immune cells (7). More recently, we reported that the immunosuppressive property of THC can be attributed, at least in part, to its ability to induce apoptosis in T cells and dendritic cells through ligation of CB2 receptors and that the latter was regulated by activation of nuclear factor-κB (8), recruiting both intrinsic and extrinsic pathways of apoptosis. Interestingly, we also found that THC and other cannabinoids could induce apoptosis in transformed murine and human T cells (9), including primary acute lymphoblastic human leukemia cells, and furthermore that the treatment of mice bearing a T-cell leukemia with THC could cure ∼25% of the mice (10). These findings are consistent with studies showing that THC and other cannabinoids can induce apoptosis in a variety of tumor cell lines, thereby raising the possibility of the use of cannabinoids as novel anticancer agents (11).

The precise mechanism through which cannabinoids induce apoptosis is under active investigation and may vary based on cell type. In normal and transfected neural cells, vascular endothelial cells, and Chinese hamster ovary cells, cannabinoid treatment was shown to induce activation of ERK (12), c-Jun NH2-terminal kinase (JNK), and p38 (13,14). In contrast, it was shown that cannabinoids were cytotoxic in leukemic cells and that they inhibited neuronal progenitor cell differentiation through attenuation of the ERK pathway (15). In glioma cells, THC was shown to induce apoptosis via ceramide generation (16). However, cannabinoids can also block ceramide-induced apoptosis of normal astrocytes (17). Together, such studies suggest that the precise signaling pathways that are evoked by cannabinoid receptor activation in normal and transformed cells may vary based on cell type and that such activation could lead to the generation of survival or death signals.

In the current study, we investigated the molecular mechanisms underlying apoptosis induced by THC, specifically addressing the role of MAPK signaling. Treatment with THC caused interruption of the MAPK/ERK kinase (MEK)/ERK signaling module that was required for apoptotic lethality, and this event possibly played an important functional role in mediating THC-induced translocation of Bad to mitochondria.

Results

Treatment of Jurkat Cells with THC Induces Suppression of the Raf-1/MEK/ERK Cytoprotective Signaling Pathway through the Signaling of the Cannabinoid Receptors

To gain insights into the functional role of cannabinoid receptor pathway in THC-mediated lethality, Jurkat cells were pretreated with either SR141716 (CB1 antagonist) or SR144528 (CB2 antagonist) in the absence or presence of THC for a designated period (6-30 hours), after which the percentage of cells displaying the morphologic features of apoptosis was determined by the Wright-Giemsa-stained cytospin preparation. THC caused dose-dependent apoptosis in Jurkat cells (data not shown) with a very substantial increase in cell death detected at 10 μmol/L (Fig. 1A). Time course analysis revealed that exposure to SR141716 (CB1 antagonist) or SR144528 (CB2 antagonist) individually at the concentration of 1 or 2 μmol/L were minimally toxic over 30-hour treatment interval, whereas treatment with 10 μmol/L THC alone caused ∼50% apoptosis (Fig. 1A). However, when cells were pretreated with either SR141716 or SR144528 followed by exposure to THC, there was a significant reduction in apoptosis by 6 hours and a very substantial reversal in lethality after 12 hours (Fig. 1A). Very similar results were obtained when THC-treated cells were evaluated for combined early and late apoptotic cells by Annexin V/propidium iodide (PI) analysis (Fig. 1B). In this assay, cells stained for Annexin V alone are considered to be early apoptotic cells and those stained for Annexin V and PI represent late apoptotic cells. We also analyzed the cells for loss of mitochondrial membrane potential (MMP; Δψm) as described (10) and designated them as cells with “low” 3,3-dihexyloxacarbocyanine iodide uptake (Fig. 1B). Next, we measured the levels of CB1 and CB2 receptors in Jurkat cells and compared them with other tumor cell lines, such as the T-cell lymphoma Hut78 and the glioma U251 known to express CB1 receptors. As shown in Fig. 1C, Jurkat and Hut78 but not U251 cells expressed significant levels of CB2 mRNA. In addition, when CB1 mRNA levels were analyzed, Jurkat cells were found to express low levels. Interestingly, when all cells were cultured with THC, CB1 and CB2 receptor expression was significantly increased. These data
explained why in Jurkat cells not only CB2 antagonist but also CB1 antagonist were able to partially block THC-induced apoptosis.

FIGURE 1.

Effects of THC on Raf-1/MEK/ERK signaling in Jurkat cells. A. Jurkat cells were pretreated with either 1 μmol/L SR141716 or 2 μmol/L SR144528 in medium containing 10% FBS in the absence or presence of 10 μmol/L THC for a designated period (6-30 hours), after which the percentage of apoptosis was determined by examining Wright-Giemsa-stained cytospin preparations as described in Materials and Methods. Points, mean percent apoptosis of at least three separate experiments done in triplicate; bars, SD. B. Cells were treated as described in A for 12 hours, after which the extent of apoptosis and loss of MMP were monitored by Annexin V/PI staining and 3,3-dihexyloxacarbocyanine iodide uptake, respectively. Columns, mean percent apoptosis of three separate experiments done in triplicate; bars, SD. C. Expression of CB1 and CB2 was determined by reverse transcription-PCR analysis. Total RNA was isolated from Jurkat, Hut78, and U251 tumor cells, which were either left untreated or treated with THC. mRNA was reverse transcribed and amplified by PCR with primers specific for CB1 and CB2. Photograph of ethidium bromide–stained amplicons. D. Cells were treated as described in A for 12 hours, after which the Western analysis was used to monitor expression of phospho-Raf-1, Raf-1, phospho-MEK1/2, MEK1/2, phospho-ERK1/2, ERK1/2, phospho-RSK (Thr359/363), phospho-RSK (Ser380), phospho-RSK (Thr573), RSK, phospho-p38, p38, phospho-JNK, and JNK. β-Actin served as loading control. E. Mol4, Hut78, and SupT1 cells were either left untreated or treated with THC at designated concentrations (7.5, 5, and 10 μmol/L, respectively) in medium containing 10% FBS for 18 hours, after which the levels of phosphor-ERK1/2 and total ERK1/2 were monitored by Western blot. ERK1/2 served as loading control. F. Jurkat cells were either left untreated or treated with 10 μmol/L THC for a designated interval (from 0.5 to 12 hours), after which an ERK kinase assay was done by immunoprecipitation of ERK using a monoclonal phospho-ERK1/2 (Thr202/Tyr204). The immunoprecipitate was subsequently incubated with an Elk-1 fusion protein in the presence of ATP, which allowed precipitated phospho-ERK to phosphorylate Elk-1, a major substrate of phospho-ERK. D, DMSO. Elk-1 served as loading controls. Representative of a minimum of three separate experiments.
observed in levels of total p38 or JNK. Consequently, we evaluated the levels of phospho-p90RSK (Thr359/Ser363), phospho-p90RSK (Ser380), phospho-p90RSK (Thr356), and total p90RSK following exposure of Jurkat cells to THC in the absence or presence of CB1/2 antagonists (Fig. 1D). THC caused a reduction in p90RSK phosphorylation involving both Thr359 and Ser363-specific phosphorylation sites. However, when cells were pretreated with CB1 or CB2 antagonist, the effects of THC on both phosphorylation sites of this protein were significantly reversed. No changes were detected in levels of total p90RSK or phospho-p90RSK on sites Ser380 and Thr573. Attempts also were made to extend ERK inactivation to other human tumor cell lines. As shown in Fig. 1E, an 18-hour exposure of Molt4, Hut78, and SupT1 cells to THC resulted in a marked reduction in the levels of phospho-ERK1/2, with the total ERK1/2 expression remaining unchanged. Finally, essentially similar results were obtained when the effects of THC were examined in kinase activity. A decrease in ERK activity was detectable in Jurkat cells after treatment with THC beginning at hour 1, and this effect was even more pronounced at 12 hours (Fig. 1F). Taken together, these data suggest that suppression of the Raf-1/MEK/ERK cytoprotective signaling pathway by THC may play an important functional role in the induction of apoptosis in Jurkat cells as well as in other tumor cell lines tested.

**Caspase-Independent Events in Raf-1/MEK/ERK Signaling**

To determine the role caspase activation in THC-mediated perturbations in ERK signaling, Jurkat cells were pretreated with pan-caspase inhibitor Z-VAD-FMK in the presence or absence of THC. As shown in Fig. 2A, caspase inhibition failed to alter the THC-mediated down-regulation of phosphorylation of Raf-1, MEK1/2, ERK1/2, and RSK (Thr359/Ser363). No changes were noted in the levels of total Raf-1, MEK1/2, ERK1/2, and RSK or phospho-p90RSK on sites Ser380 and Thr573. These results indicated that in Jurkat cells down-regulation of the Raf-1/MEK/ERK/RSK axis represents an early consequence of the treatment of cells with THC and that it proceeds in a caspase-independent manner. As shown in Fig. 2B, exposure of Jurkat cells to Z-VAD-FMK significantly inhibited THC-induced procaspase-10, procaspase-2, procaspase-8, procaspase-9, procaspase-3, and Bid processing as well as poly(ADP-ribose) polymerase (PARP) degradation. It should be noted that treatment of Jurkat cells with Z-VAD-FMK alone did not alter the expression of any of the molecules under investigation (data not shown).

**Antiapoptotic Effect of Phorbol 12-Myristate 13-Acetate Depends on ERK Activation**

Effect of exposure of Jurkat cells to pan-caspase inhibitor, Z-VAD-FMK. Jurkat cells were pretreated with 20 μmol/L Z-VAD-FMK in medium containing 10% FBS in the presence or absence of 10 μmol/L THC for a total 12 hours, after which Western analysis was done to monitor expression of (A) phospho-Raf-1, Raf-1, phospho-MEK1/2, MEK1/2, phospho-ERK1/2, ERK1/2, phospho-RSK (Thr359/Ser363), phospho-RSK (Ser380), phospho-RSK (Thr573), and RSK and after 24 hours to monitor expression of (B) procaspase-10, procaspase-2, procaspase-8, Bid, procaspase-9, procaspase-3, and PARP or cleaved fragments (CF). β-Actin served as a loading control. Representative experiment. Two additional studies yielded similar results.
To confirm the possible role of ERK activation in THC-induced apoptosis, Jurkat cells were pretreated with 10 nmol/L phorbol 12-myristate 13-acetate (PMA), a potent activator of ERK (18, 19). Next, the cells were treated with 10 μmol/L THC for a total of 12 hours or left untreated. Parallel studies were done on cells precultured with 25 μmol/L U0126, a MEK inhibitor. The extent of apoptosis was then determined by terminal deoxynucleotidyl transferase–mediated dUTP end labeling (TUNEL) assay. Treatment of cells with THC alone for 12 hours caused ~43% apoptosis, which was partially blocked in the presence of either CB1 or CB2 antagonist, thereby confirming the involvement of CB1 and CB2 receptors in apoptosis (Fig. 3A). In addition, THC-induced apoptosis was also partially blocked by PMA (Fig. 3A). Moreover, treatment of cells with U0126 alone caused significant levels of apoptosis consistent with our hypothesis that MEK inhibition in Jurkat cells can lead to cell death. Furthermore, the combination of THC plus U0126 caused a marked increase in apoptosis, which was more than that seen when either of these compounds was used alone. As expected, treatment with PMA plus U0126 caused a significant inhibition in apoptosis when compared with the use of U0126 alone. To characterize interaction between THC and U0126 more rigorously and over a range of drug concentrations at a fixed ratio (1:2.5), median dose effect analysis was used. When the extent of apoptosis was determined, combination index values considerably less than 1.0 were obtained (Fig. 3B), corresponding to highly synergistic interaction. These data together suggest that THC-induced ERK inactivation may play a critical role in apoptosis in Jurkat cells.

FIGURE 3.
Role of ERK action in the regulation of THC-induced apoptosis. A. Jurkat cells were first cultured to 1 μmol/L SR141716, 2 μmol/L SR144528, 10 nmol/L PMA, or 25 μmol/L U0126 in medium containing 10% FBS in the absence or presence of 10 μmol/L THC for a total 12 hours, after which the extent of apoptosis was analyzed by TUNEL assay. TUNEL-positive cells were quantified by flow cytometric analysis. B. Jurkat cells were exposed to varying concentration of THC and U0126 at a fixed ratio (1:2.5) for 24 hours, after which the percentage of apoptosis was determined as described in Fig. 1A. Median dose effect analysis was used to determine the combination index for each fraction effect. Combination indices < 1.0 correspond to synergistic interactions. Representative experiment; three independent experiments done in triplicate. C. Lysates prepared from cells pretreated with 10 nmol/L PMA or 25 μmol/L U0126 in medium containing 10% FBS in the absence or presence of 10 μmol/L THC for 12 hours were blotted and probed for phospho-ERK1/2. Representative experiment. Two additional studies yielded similar results. D. Quantitative changes in ERK1/2 phosphorylation were determined by densitometric analysis of immunoblots. Columns, mean of triplicate determinations in five separate experiments; bars, SD. *, P < 0.05, **, P < 0.005. E. Samples collected from cells cultured as described in C for 24 hours in medium containing 10% FBS were monitored for protein levels of procaspase-10, procaspase-2, procaspase-8, Bid, procaspase-9, procaspase-3, PARP, and cleaved fragments. Representative of a minimum of three separate experiments.
Next, we determined protein levels of phospho-ERK to see what protective action was mediated by PMA in THC-treated cells. As shown in Fig. 3C, incubation with PMA alone induced increased phosphorylation of ERK1/2. When cells were exposed to PMA followed by THC, there was significant reversal in phospho-ERK1/2 levels. In contrast, treatment with U0126 alone caused a significant decrease in phospho-ERK1/2, whereas the combination of U0126 plus THC resulted in essentially the complete disappearance of phospho-ERK1/2. PMA was a potent activator of ERK as has also been reported by others (18, 19), inasmuch as it reversed the effect of the combined treatment of U0126 plus THC on ERK1/2. These data were corroborated determining the levels of the phosphorylation of ERK1/2 by densitometric analysis (Fig. 3D). Finally, the effect of these agents, alone and in combination, were examined in relation to caspase cascades in THC-treated cells (Fig. 3E). Whereas U0126 alone had a demonstrable effect on the activation of all caspases tested as well as on the cleavage of caspases and PARP, treatment with THC alone caused much more cleavage of each of these proteins, consistent with the increased levels of apoptosis observed previously. Treatment with PMA blocked the THC-induced activation of caspases and other markers of apoptosis. Caspase analysis using U0126 alone, PMA plus U0126, and U0126 plus THC was all consistent with the above observation on the ability of U0126 to enhance and PMA to block apoptosis in THC-treated Jurkat cells.

**Enforced Activation of MEK1/ERK Substantially Blocks THC-Mediated Caspase Activation, DNA Fragmentation, and Apoptosis**

To further define the functional role of MEK/ERK, a Jurkat cell line that inducibly expresses a constitutively active MEK1 construct (Mek/30) under the control of a doxycycline-responsive promoter was employed. As shown in Fig. 4A, exposure to THC in the absence of doxycycline resulted in apoptosis in ∼50% of cells, whereas apoptosis was markedly reduced in the presence of doxycycline at 12 hours (P < 0.05), and these effects were even more pronounced at 24 hours (P < 0.005; data not shown). Similar results were obtained with a second MEK1-inducible clone (Jurkat Mek/6; data not shown). Western analysis revealed that cells cultured in the absence of doxycycline displayed minimal expression of a hemagglutinin tag and modest basal expression of phospho-MEK (Fig. 4B). However, when cells were cultured in the presence of doxycycline, a pronounced increase in expression of the hemagglutinin tag was noted along with substantial increases in expression of phospho-MEK, phospho-ERK, and phospho-RSK (Thr<sup>359</sup>/Ser<sup>363</sup>). Enforced activation of MEK also diminished THC-mediated activation of procaspase-8, procaspase-9, procaspase-3, and Bid processing as well as PARP degradation. Analogous to results obtained with the inducible MEK system, in the absence of doxycycline, THC treatment alone resulted in the induction of DNA fragmentation (Fig. 4C). When cells were cultured in the presence of doxycycline, DNA degradation was significantly blocked. Together, these data suggest that Raf-1/MEK/ERK/RSK pathways play an important functional role in THC-induced apoptosis.
Enforced activation of MEK/ERK blocks THC-mediated apoptosis. A. Jurkat cells inducibly expressing a constitutively active, hemagglutinin-tagged MEK1 vector under the control of a tetracycline-responsive promoter were exposed to either 1 μmol/L SR141716 or 2 μmol/L SR144528 followed by 10 μmol/L THC for 12 hours in the presence or absence of 2 μg/mL doxycycline (DOX). Percentage of apoptotic cells was then determined as described in Fig. 1A. Columns, mean of three separate experiments; bars, SD. *, P < 0.05, significantly less than values obtained for the treated cells in the absence of doxycycline. B. Cells were treated as described in A. Proteins (prepared from cells in the absence and presence of doxycycline) were transferred onto a single piece of nitrocellulose following fractionation using the same gel, and Western analysis was used to monitor expression of the hemagglutinin (HA)–tagged MEK, phospho-MEK, phospho-ERK, and phospho-RSK (Thr359/Ser363) at exactly same incubation time. Levels of caspases and PARP were immunoassayed by culturing Jurkat cells for 24 hours as described in A. Arrows, THC-induced breakdown products or active caspases. β-Actin served as a loading control. Representative experiment. An additional study yielded equivalent results. C. Cells were treated for 24 hours as described in A, after which DNA was isolated and subjected to agarose gel electrophoresis.

**Pertussis Toxin Pretreatment Prevents THC-Induced Cell Death**

Next, we examined the relationship between receptor-mediated activation of the G protein and THC-mediated apoptosis. To this end, Jurkat cells were exposed to either 50 or 100 ng/mL pertussis toxin (PTX) for 16 hours in the absence or presence of 10 μmol/L THC for an additional 12 hours. As shown in Fig. 5A, pretreatment with PTX caused a significant inhibition in THC-induced apoptosis. In related studies, the effect of PTX was also monitored with respect to MAPK signaling and the activation of apoptotic regulatory proteins induced by THC. Exposure of cells to PTX reversed the THC-mediated reduction in expression of phospho-Raf-1 as well as phosphorylation of MEK1/2, ERK1/2, and RSK (Thr359/Ser363, Fig. 5B). Similarly, PTX treatment reversed other THC-mediated effects, including cleavage of procaspase-10, procaspase-2, procaspase-8, procaspase-9, procaspase-3, and Bid and degradation of PARP (Fig. 5B). Together, these data suggest a role for G-protein signaling in THC-induced MAPK signaling, caspase activation, and apoptosis.

**FIGURE 5.**

THC-induced apoptosis is blocked by PTX. A. Log-phase Jurkat cells were pretreated with either 50 or 100 ng/mL PTX for 16 hours in the presence or absence of 10 μmol/L THC for an additional 12 hours. The extent of apoptosis was determined as described in Fig. 1A. Columns, mean of three separate experiments done in triplicate; bars, SD. B. Cells were cultured with 50 ng/mL PTX for 16 hours in the presence or absence of 10 μmol/L THC for an additional 12 hours followed by immunoblotting of whole-cell lysates with antibodies that recognize phospho-Raf-1, Raf-1, phospho-MEK1/2, MEK1/2, phospho-
ERK1/2, ERK1/2, phospho-RSK (Thr^{359}/Ser^{363}), RSK, phospho-p38, p38, phospho-JNK, and JNK. Levels of caspases and PARP were immunoassayed by culturing Jurkat cells with 50 ng/mL PTX for 16 hours in the presence or absence of 10 μmol/L THC for an additional 24 hours in medium containing 10% FBS. β-Actin served as a loading control. Representative of three independent experiments.

**THC Down-Regulates the Raf-1/MEK/ERK/RSK Signaling Pathway and Triggers Mitochondrial Localization of Bad**

Recently, the MAPK-activated RSK was shown to promote cell survival through phosphorylation and inactivation of the proapoptotic Bcl-2 family member, Bad (18). In addition, mitochondrial membrane-based protein kinase A (PKA) and Akt, a kinase activated by growth factors through a phosphatidylinositol 3-kinase (PI3K)-dependent mechanism, could also be implicated in Bad phosphorylation. To this end, we examined whether Bad played a role in THC-induced apoptosis involving Akt and PKA signaling pathways. First, Jurkat cells were cultured with either 15 μmol/L LY294002, a PI3K inhibitor, or 2 μmol/L H-89, a PKA inhibitor. Next, the cells were treated with 10 μmol/L THC for a total of 12 hours or left untreated. The percentage of apoptotic cells was then determined by examining Wright-Giemsa-stained cytospin preparations. As shown in Fig. 6A, coadministration of LY294002 at a concentration of 15 μmol/L, which was minimally toxic alone, resulted in apoptosis in majority of THC-treated cells. A very similar pattern was noted when the extent of apoptosis was determined by TUNEL assay (data not shown). However, no involvement of the PKA event was noted when cells were exposed to THC in combination with H-89 (Fig. 6A). Western blot analysis (Fig. 6B) revealed that combined treatment with THC and LY294002 (12 hours) resulted in a down-regulation in Akt phosphorylation involving both Thr^{308} and Ser^{473}-specific phosphorylation sites, which was more than that seen when either of these compounds was used alone. No changes were observed in the levels of total Akt with any treatments.

**FIGURE 6.** Effects of THC on mitochondrial localization of Bad in THC-stimulated cells. A. Jurkat cells were treated with either 15 μmol/L LY294002 (LY) or 2 μmol/L H-89 in medium containing 10% FBS in the absence or presence of 10 μmol/L THC for a total 12 hours, after which the percentage of apoptotic cells were determined as described in Fig. 1A. Columns, mean of three separate experiments done in triplicate; bars, SD. *, P < 0.05, significantly less than values obtained for the treated cells with THC + LY294002. B. Cells were cultured with either 15 μmol/L LY294002 or 25 μmol/L U0126 in medium containing 10% FBS in the absence or presence of 10 μmol/L THC for a total 12 hours, after which Western analysis was used to monitor expression of phospho-Akt (Thr^{308}), phospho-Akt (Ser^{473}), and Akt. Akt served as a loading control. C. Cells were cultured as described in B, after which precleared cell lysates were incubated overnight with mouse monoclonal anti-Bad IgG conjugated to protein A-agarose beads. Immunoprecipitates were subsequently subjected to Western analysis to monitor the phosphorylation status of phospho-Bad (Ser^{112}), phospho-Bad (Ser^{136}), and phospho-Bad (Ser^{155}). All sites were analyzed on a single blot. Cos cells were used as a positive control. Representative experiment. Two additional studies yielded equivalent results. D. Quantitative changes in Bad phosphorylation were
determined by densitometric analysis of immunoblots. Columns, mean of three independent experiments done in triplicate; bars, SD. E. Jurkat cells treated as described in B, after which cells were adhered to slides by cytoospin and subjected to double staining with anti-Bad antibodies and Cy2-labeled secondary antibodies (green) followed by a mitochondrion-specific dye (MitoTracker Deep Red 633) and then analyzed by confocal microscopy. Similar results were obtained in three independent experiments. To examine whether the Akt pathway suppressed by THC may play a role in dephosphorylation of Bad with the cell death, Jurkat cells were stimulated with either LY294002 or U0126 at designated concentrations. Subsequently, the cells were treated with 10 μmol/L THC for a total 12 hours or left untreated. The phosphorylation status of Bad was monitored by the immunoprecipitation following by Western analyses. As shown in Fig. 6C, THC and U0126 alone diminished the levels of phosphorylation of Bad on site Ser136, whereas the levels of phospho-Bad on site Ser155 remained unchanged. When cells were exposed to THC in combination with U0126, there was a significant reduction and essentially the complete disappearance of phospho-Bad (Ser136) levels. In contrast, coadministration of THC plus LY294002 did not enhance the degree of the dephosphorylation of Bad on site Ser136 when this was compared with LY294002 treatment alone. Furthermore, treatment of Jurkat cells with THC alone at the concentration (5-15 μmol/L) of THC and the time course analyses (30 minutes to 30 hours) did not alter the level of phospho-Bad on site Ser136 under investigation (only one data point shown). No significant changes were observed in the levels of phospho-Bad on site Ser155 with any treatments (Fig. 6C). These results were confirmed using densitometric analysis (Fig. 6D), which distinguishes the changes of Bad phosphorylation after designated treatment.

Lastly, the effects of combined exposure to THC were examined in relation to the localization of Bad. Generally, Bad resides in the cytosol but translocates to the mitochondria following death signaling. We therefore used confocal microscopy to study the translocation of Bad following exposure of Jurkat cells to THC alone or in combinations with other treatments. Double-immunofluorescence analysis with anti-Bad antibody and mitochondria-specific dye (MitoTracker Deep Red 633) showed a strong association of Bad with mitochondria in THC-treated cells compared with vehicle-treated cells (Fig. 6E). To further establish a link between the MEK/ERK pathway and the translocation of Bad to the mitochondria in THC-treated Jurkat cells, we used U0126, a potent inhibitor of this pathway. In addition, we used LY294002 to see whether combined treatment with LY294002 and THC could enhance the translocation of Bad to the mitochondria. The data shown in Fig. 6E indicated that exposure to U0126 significantly enhanced THC-induced translocation of Bad to the mitochondria and that a combination of THC plus U0126 caused marked increase in Bad translocation to mitochondria. In addition, treatment of Jurkat cells with LY294002 alone induced minimal Bad translocation and THC plus LY294002 did not augment this process when compared with THC alone (Fig. 6E). These findings together suggested that THC induced the translocation of Bad to mitochondria without significant association of Bad with PI3K/Akt pathway. To further determine whether THC induced translocation of Bad via the interruption of Raf-1/MEK/ERK/RSK pathway, Jurkat cells were stably transfected with a constitutively active MEK1 construct (Mek/30). As shown in Fig. 7A, exposure to either THC or U0126 in the absence of doxycycline resulted in dephosphorylation of Bad on site Ser136, and these effects were even more pronounced when cells were treated with THC plus U0126. Similar results were obtained with a second MEK1-inducible clone (Jurkat Mek/6; data not shown). However, when cells were cultured in the presence of doxycycline, THC-induced down-regulation of Bad on this site was blocked. No changes were detected in levels of phosphorylation of Bad on other sites when cells were cultured in the absence or presence of doxycycline. Confocal analysis revealed that cells cultured in the absence of doxycycline displayed a strong association of Bad with mitochondria in THC plus U0126–treated cells, which was more than that seen when either of these compounds was used alone (Fig. 7B). However, when cells were cultured in the presence of doxycycline, enforced activation of MEK/ERK/RSK significantly blocked THC-mediated translocation of Bad to the mitochondria, analogous to results obtained with a second MEK1-inducible clone (Jurkat Mek/6; data not shown). Together, these findings suggest that suppression of Raf-1/MEK/ERK/RSK signaling pathway by THC plays an important functional role in the induction of Bad translocation to the mitochondria.
FIGURE 7.

Enforced expression of constitutively active MEK blocks THC translocation of Bad to the mitochondria. **A.** Jurkat cells expressing constitutively active MEK (Mek/30) were exposed to U0126 (25 μmol/L) and THC (10 μmol/L) alone or in combination for 12 hours, after which proteins (prepared from cells in the absence and presence of doxycycline) were loaded onto the same SDS-PAGE followed by transferring onto a single piece of nitrocellulose, and the immunoprecipitates were subjected to Western analysis to monitor the phosphorylation status of phospho-Bad (Ser\(^{112}\)), phospho-Bad (Ser\(^{133}\)), and phospho-Bad (Ser\(^{155}\)) at same incubation time. Representative experiment. Two additional studies yielded equivalent results. **B.** Cell lysates were prepared from clones (Mek/30) of Jurkat cells as described in **A**, after which cells were adhered to slides by cytospin and subjected to double staining with anti-Bad antibodies and Cy2-labeled secondary antibodies (green) followed by a mitochondrion-specific dye (MitoTracker Deep Red 633) and then analyzed by confocal microscopy. Representative of three independent experiments.

To confirm the requirement of Bad in THC-induced apoptosis of Jurkat cells, we used a RNA interference approach. It has been shown that small interfering RNA (siRNA) consisting of 21-bp dsRNA can mediate RNA interference effect in mammalian cells. We used two types of Bad siRNA designated Bad siRNA-I and Bad siRNA-II as described in Materials and Methods. Both were able to significantly reduce Bad protein expression, whereas control siRNA had no significant effect (Fig. 8A and C). Depletion of Bad in Jurkat cells was found to significantly inhibit THC-mediated apoptosis when compared with Jurkat cells transfected with control siRNA (Fig. 8B and D). These data therefore corroborated our earlier results showing the crucial role of Bad in THC-mediated downstream signaling in induction of apoptosis of Jurkat cells.
Discussion

Recently, cannabinoids were shown to inhibit the proliferation of several human cancer cell lines, including leukemia (5, 10), breast (19), prostate (20), and gliomas (16). Studies from our laboratory showed that activation of cannabinoid receptors on normal and transformed T cells triggers apoptosis (10, 21). The data presented in the current study provide new insights into the functional roles of cannabinoid receptors and MAPK cascades in cannabinoid-mediated mitochondrial injury, caspase activation, and cell death. MAPK pathways consist of three parallel serine/threonine kinase (ERK, JNK, and the p38 MAPK) modules involved in the regulation of diverse cellular events, including proliferation, differentiation, apoptosis, etc. (22). In the current study, we investigated the effect of THC treatment on all three modules of MAPK signaling pathways in Jurkat cells and noted that THC-mediated cell death was associated with interruption of the Raf-1/MEK/ERK signaling pathway without significantly altering the p38 and JNK modules. Consequently, we further investigated the effect of THC on the upstream and downstream events that modulate the ERK module of MAPK. The pronounced down-regulation of phospo-Raf-1 was also associated with a marked reduction in phosphorylation of MEK, a major Raf-1 substrate (23), phosho-ERK, the primary MEK target (24), and RSK, the first substrate of ERK (25). This process occurred early, was dependent on cannabinoid receptor ligation, and proceeded upstream of caspase activation. THC also decreased the phosphorylation of Akt. However, no significant association of Bad translocation with PI3K/Akt and PKA signaling pathways was noted.

The regulation of mitochondrial membrane function and the release of apoptotic regulatory factors from mitochondria are key components of the apoptotic repertoire, tightly controlled by the Bcl-2 family proteins (26, 27). There is accumulated evidence suggesting that the MAPK pathway, through Bad phosphorylation, plays a significant functional role in cell survival (26). The family of RSK was among the first cytoplasmic MAPK substrates identified (28). RSK phosphorylate a variety of substrates and regulate a diverse array of cellular functions, such as gene transcription, protein synthesis, and cell cycle regulation (29). Thus, MAPK activates RSK, which in turn catalyzes the phosphorylation of Bad (26). Cell survival is associated with the phosphorylation of Bad, which, by interacting with the scaffold protein 14-3-3 in the cytosol, prevents its translocation to the mitochondria (30). In the absence of survival signals, Bad is dephosphorylated, translocates to the mitochondria, and interacts with Bcl-2 and Bcl-XL, thereby preventing the survival function of Bcl-2 and Bcl-XL (30, 31). Data from the present study showed that THC inactivated the cytoprotective serine/threonine kinase pathway (Raf-1/MEK/ERK/RSK) that plays an important functional role in mediating Bad translocation. This may have caused the localization of Bad to the mitochondria as seen following THC treatment.

Based on these results, the effect of THC treatment on the PI3K/AKT and PKA signaling pathways was also examined in the regulation of Bad and cell death (32-34). Our findings indicated that THC caused the marked reduction in the level of phospho-Bad on site Ser112 without significantly altering the PKA module. The effect on Bad Ser112 was more pronounced when cells were exposed to THC in combination with U0126 consistent with recent studies that the MAPK-activated RSK phosphorlates Bad on
Ser$^{112}$ (30, 35). Although there is down-regulation of phospho-AKT on both sites Thr$^{308}$ and Ser$^{472}$ following exposure of Jurkat cells to THC, no change was detected in phospho-Bad on the site Ser$^{159}$, which was reported to be implicated in this pathway (32, 33). This finding was confirmed by THC dose response and time course analysis when treated cells were examined in phosphorylation status of Bad on this site. Moreover, confocal analysis indicated that LY294002, an inhibitor of PI3K, failed to enhance the degree of Bad translocation following exposure of Jurkat cells to THC. In view of evidence linking the dysregulation of the PI3K/Akt progression to apoptosis, it is tempting to invoke this mechanism to explain the ability of THC and LY294002 to induce cell death in Jurkat cells. However, identification of the specific events responsible for PI3K inhibitor-mediated lethality remains an elusive goal. Akt is implicated in post-translational modification of Bad (32), regulation of the expression of antiapoptotic proteins, including Bcl-2 and XIAPs (36), and modulation of diverse pathways governing cell survival decisions, including those associated with GSK (37), mammalian target of rapamycin (38), nuclear factor-$\kappa$B (39), etc. (40-42). In this regard, the finding that LY294002, an inhibitor of PI3K, failed to enhance THC-mediated translocation of Bad to mitochondria argues against a critical role for this pathway in regulation of Bad phosphorylation. It has long been known that cannabinoids, including THC, can stimulate the MAPK cascade in Chinese hamster ovary cells (13), rat and mouse hippocampus (14), rat primary astrocytes (43), human astrocytoma cells (44), transformed neural cells (16, 45) human breast cancer cells (19), and the striatum (46). Furthermore, a recent report has shown evidence indicating that THC-induced apoptosis in certain types of leukemic cells is mediated by down-regulation of ERK (5). Our findings represented THC-mediated ERK inactivation and cell death in a variety of human tumor cell lines, which are consistent with this report in the literature (5). However, the precise role of cannabinoid receptor as a modulator of the ERK cascade is still a matter of debate. It is generally accepted that the activation of the ERK cascade leads to cell proliferation (47). However, recent investigations have begun to define situations in which ERK mediates cell cycle arrest (48), antiproliferation (49), and apoptotic (50) or nonapoptotic (51) death in many cell lines. In most but not all systems studied, inactivation of MEK and ERK is associated with the promotion of cell death (52, 53). The mechanism by which this occurs is not known with certainty but may vary based on the cell type. Except this, it may be related to perturbations in downstream ERK targets, including the Elk (54), CREB family of transcription factors (35), etc. Alternatively, inactivation of ERK may prevent phosphorylation of Bad and in so doing preserve its proapoptotic capacity through interaction with Bcl-2/Bcl-X (26, 27). To further define the functional role of the MEK/ERK/RSK pathway in the translocation of Bad to mitochondria, Jurkat cells that were stably transfected with a constitutively active MEK construct were employed. Consistent with this model, enforced expression of constitutively active MEK overcame the suppression of ERK/RSK activation and the reduction in the levels of phospho-Bad on Ser$^{112}$ and substantially protected cells from THC/U0126 lethality. In fact, the protection effects of enforced ERK activation plays an important role in blocking the localization of Bad to mitochondria. However, given the pleiotropic nature of THC action, the possibility that other downstream targets of this agent contribute to lethality cannot be excluded. The relation between activation of the ERK cascade and cell proliferation/antiproliferation depends on various stimuli (55) and varies between diverse types of cells (56, 57).

In a recent study, it was shown that C6 glioma cells exposed to a synthetic cannabinoid, WIN 55,212-2, exhibited down-regulation of the Akt and ERK signaling pathways before induction of apoptosis (58). Exposure to WIN 55,212-2 caused a decrease in phospho-Bad. In addition, Powles et al. (5) showed that THC-induced cell death in leukemic cells was preceded by significant changes in the expression of genes involved in MAPK signal transduction pathways. The current study further extends these observation in a leukemia model by identifying Raf-1/MEK/ERK/RSK-mediated localization of Bad to mitochondria as a critical mechanism regulating cannabinoid-induced lethality. This was supported by the observation that enforced expression of constitutively active MEK/ERK resulted in the inhibition of mitochondrial Bad localization, caspase activation, and apoptosis. Moreover, down-regulation of Bad expression by siRNA led to marked resistance of Jurkat cells to THC-mediated apoptosis. Although the ERK pathway has been shown to play a pivotal role in regulating cell growth and differentiation to growth factors, cytokines, and phorbol esters (59), it is also weakly activated by stress (60, 61). In contrast, JNK and p38 are weakly activated by growth factors but are highly activated in response to stress signals, including tumor necrosis factor, ionizing and UV irradiation, and hyperosmotic stress, which lead to induction of apoptosis (53, 62). For example, there is mixed evidence for the role of ERK in influencing cell survival of cisplatin-treated cells. Some studies have suggested that ERK activation is associated with enhanced survival of cisplatin-
treated cells (63, 64), whereas others have shown that elevated expression of Ras, an upstream component of the ERK signaling pathway, leads to increased sensitivity to the drug (65). THC and other cannabinoids can induce apoptosis in a variety of tumor cell lines, thereby raising the possibility of the use of cannabinoids as novel anticancer agents (11). However, the use of cannabinoids that activate CB1 receptors is severely limited by their psychoactive effects. The fact that malignant cells of the immune system express CB2 receptors that can be targeted to induce apoptosis offers a novel approach to use CB2 select agonists as anticancer drugs with no psychoactive properties. Because CB2 receptors are almost exclusively expressed on immune cells, the use of CB2 select agonists should not exert generalized toxicity that is common to other modes of treatment, such as radiation or chemotherapy. One possible drawback could be that use of select CB2 agonists to kill tumor cells may also cause immunosuppression. Thus, further studies are necessary to address the relative sensitivity of normal and transformed immune cells to CB2 agonists in vivo. Identifying the molecular pathways that trigger apoptosis following ligation of cannabinoid receptors is critical in understanding how endogenous and exogenous cannabinoids may regulate the growth of normal and transformed immune cells. The current study provides useful and novel information on developing a new class of anticancer drugs by targeting cannabinoid CB2 receptors.

Materials and Methods

Cells
Jurkat, Molt4, SupT1, Hut78, and U251 glioma cell lines were purchased from American Type Culture Collection (Rockville, MD). The cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS; Atlanta, Norcross, GA). They were maintained in a 37°C, 5% CO₂, fully humidified incubator.

Reagents
THC was obtained from the National Institute of Drug Abuse (Rockville, MD). SR141716 and SR144528 were obtained from Sanofi Recherche (Montpellier, France). PTX and LY294002 were purchased from Sigma (St. Louis, MO). 3,3-Dihexyloxacarbocyanine iodide, U0126, PMA, and the PKA inhibitor H-89 were purchased from Calbiochem (San Diego, CA). Annexin V/PI was purchased from BD PharMingen (San Diego, CA). The pan-caspase inhibitor Z-VAD-FMK was purchased from R&D Systems (Minneapolis, MN) and all agents were initially dissolved in DMSO (Sigma) as stock solution and stored at −20°C.

Detection of THC-Induced Apoptosis In vitro
Jurkat cells (4 × 10⁵ per well) were cultured in 24-well plates in the presence or absence of designated concentrations of THC for 12 to 24 hours. After cytopin, the cell preparations were stained with Wright-Giemsa and viewed by light microscopy to evaluate the extent of apoptosis as described previously (52). The percentage of apoptotic cells was determined by evaluating ≥500 cells per treatment in triplicate. To confirm the results of morphologic analysis, TUNEL method was used as described elsewhere (66).

Flow Cytometry to Detect Apoptosis by TUNEL
Following each treatment, the cells were harvested, washed twice with PBS, and fixed with 4% paraformaldehyde for 30 minutes at room temperature. The cells were then permeabilized on ice for 2 minutes, incubated with FITC-dUTP and terminal deoxynucleotidyl transferase (Boehringer Mannheim, Indianapolis, IN) for 1 hour at 37°C and 5% CO₂, and analyzed using a Beckman Coulter Cytofluorometer FC 500 (Fullerton, CA).

Determination of MMP
MMP was monitored using 3,3-dihexyloxacarbocyanine iodide as described (9). For each condition, 4 × 10⁵ cells were incubated in 1 mL 3,3-dihexyloxacarbocyanine iodide (40 nmol/L) at 37°C in for 15 minutes and subsequently analyzed using a Becton Dickinson FACScan cytofluorometer (Mansfield, MA) with excitation and emission settings of 488 and 525 nm, respectively. Control experiments documenting the loss of MMP were done by exposing cells to 5 μmol/L carbamoyl cyanide m-chlorophenylhydrazone (Sigma; 15 minutes, 37°C), an uncoupling agent that abolishes the MMP.

Annexin V/PI
Annexin V/PI staining was done as described previously (10). The cells were analyzed using a Becton Dickinson FACScan flow cytometer.
RNA Isolation and Reverse Transcription-PCR
RNA was isolated from ~1 × 10⁷ cells using the RNeasy Mini kit (Qiagen, Valencia, CA) according to the recommendations from the manufacturer. DNA was removed by the RNase-free DNase set (Qiagen) following RNA isolation. RNA concentration was determined spectrophotometrically, and the integrity of the each preparation was verified by agarose gel electrophoresis. cDNA was synthesized by reverse transcription of 50 ng total RNA as template for first-strand synthesis using the Sensiscript RT kit (Qiagen). All PCR was prepared using MasterAmp PCR Premix F (Epicerent Technologies, Madison, WI) according to the recommendations from the manufacturer and using Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA). Human CB1 was amplified using primers HCB1U (5'-CGTGGGGAGCCTGTCCCTCA-3') and HCB1L (5'-CATGCGGGTGGTGCTGGG-3'), which yield a product of 403 bp. Human CB2 was amplified using primers HCB2U (5'-CGCCGAAGCCCTCATACC-3') and HCB2L (5'-CCTTTCGGGATTTCCCTG-3'), which yield a product of 522 bp. β-Actin was used as a positive control, with primers BAU (5'-AAGGCCAACCGTGGAAAGATGGACC-3') and BAL (5'-ACCGCTCGTTGCCAATAGTGATGA-3'), with a product size of 427 bp. PCR was carried out using the following variables: 95°C for 10 seconds, 59°C for 20 seconds, and 72°C for 45 seconds for 35 cycles followed by a final 1 minute at 72°C in an Applied Biosystems GeneAmp 9700 (Foster City, CA). The resulting PCR products were separated on a 1.2% agarose gel.

Immunoblot Analysis
Immunoblotting was done as described previously (52). The source of antibodies was as follows: caspase-2 (Alexis, San Diego, CA); Bid, phospho-ERK1/2 (Thr202/Tyr204), JNK, phospho-JNK, phospho-38, phospho-p38 MAPK, phospho-Akt (Thr308), phospho-Akt (Ser473), Akt, and Bad (C-7; Santa Cruz Biotechnology, Santa Cruz, CA); Bad, phospho-Bad (Ser112), phospho-Bad (Ser155), caspase-3, caspase-8, caspase-9, caspase-10, ERK1/2, PARP, MEK1/2, phospho-MEK1/2, p90RSK, phospho-p90RSK (Thr357/Ser367), phospho-p90RSK (Ser380), and phospho-p90RSK (Thr577; Cell Signaling Technology, Danvers, MA); c-Raf-1, phospho-c-Raf-1 (Biosource, Camarillo, CA); and β-actin (Sigma). Cell lysates were prepared and the concentration of the protein was measured by using standard Bradford assays. The proteins were fractionated in SDS-PAGE and transferred onto polyvinylidene difluoride membranes using a dry blot apparatus (Bio-Rad, Hercules, CA). The membrane was incubated in blocking buffer for 1 hour at room temperature followed by incubation in primary antibody at 4°C overnight. The membrane was then washed thrice (10-15 minutes) with washing buffer (PBS-0.2% Tween 20) and incubated for 1 hour in horseradish peroxidase–conjugated secondary antibody (Cell Signaling Technology, Inc.) in blocking buffer. The membranes were then washed several times and incubated in developing solution (equal volume of solutions A and B; Enhanced Chemiluminescence Western Blotting Detection Reagents, Amersham Biosciences, Little Chalfont, United Kingdom) and signal was detected using ChemiDoc System (Bio-Rad). Densitometric analyses of the Western blots were done with UN-SCAN-IT (Silk Scientific, Orem, UT) software digitizer technology.

Immunoprecipitation
After drug treatments, cells were washed with PBS and incubated for 10 minutes in lysis buffer [150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 8.0), 1 mmol/L EDTA, 1% (v/v) NP40, 0.25% (w/v) sodium deoxycholate, 1 mmol/L NaF, 1 mmol/L sodium pyrophosphate, 100 μmol/L Na3VO4, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin]. Precleared by incubation with protein A-agarose bead slurry, cell lysates (2 μg/mL total cell protein) were incubated overnight with mouse monoclonal anti-Bad IgG or rabbit polyclonal anti-Bad IgG conjugated to agarose beads. Immunoprecipitates were captured by addition of protein A-agarose. The agarose beads were collected by centrifugation, washed twice in ice-cold lysis buffer, boiled in Laemmli sample buffer, and subjected to SDS-PAGE and subsequent immunoblot analysis.

Tet-On-Inducible Jurkat Cell Lines
Stably transfected Jurkat clones that inducibly expressed constitutively active MEK1 were generated as described (52). To test for the induced expression of the hemagglutinin-tagged MEK1, stable clones were left untreated or were treated for designated periods with 2 μg/mL doxycycline (Sigma), harvested, and analyzed for expression of the appropriate protein by Western blot as described above.

Transfection of Jurkat Cells with Mouse Bad siRNA
Jurkat cells (5 × 10⁶) were transfected with 1.7 μmol/L human siRNA (5'-GAAGGGACUUCUCUGGCGCTTT-3'; 5'-CGGCGAGAUGGCCUUUCCCTT-3'; designated siRNA-1; Cell Signaling Technology, Inc., Danvers, MA) using nucleofection of Jurkat cells with Transfection Reagent kit and Nucleofactor II electroporation system following the protocols of the company (Amaza, Inc.,...
Gaithersburg, MD). Jurkat cells were also transfected with human-specific control siRNA (1.7 μmol/L) conjugated with fluorescein (Cell Signaling Technology, Inc., Danvers, MA) and pmaxGFP plasmid (2 μg; Amaxa). In a separate experiment, 5 × 10⁶ Jurkat cells were also transfected with human Bad siRNA (siGENOME SMARTpool reagent M-003870-02; 3 μg; Dharmacon RNA Technologies, Lafayette, CO; designated siRNA-II) using nucleofection of Jurkat cells with Transfection Reagent kit and Nucleofactor II electroporation system following the protocols of the company. As a control, 5 × 10⁶ Jurkat cells were transfected with human-specific negative control SiGLO RISK-free siRNA (3 μg; designated control siRNA-II) unconjugated or conjugated with Cy-3 (Pool D-001206-13-05; Dharmacon RNA Technologies) and pmaxGFP plasmid (2 μg). Transfected Jurkat cells were cultured for 48 hours in complete medium at 37°C and 5% CO₂. We observed >90% cell viability after transfection. To examine the efficiency of transfection, we observed pmaxGFP plasmid-transfected Jurkat cells under fluorescent microscope and observed >50% cells expressing green fluorescent protein. We also examined the expression of green fluorescent protein by performing flow cytometry. Jurkat cells, untransfected or transfected with control siRNA or human Bad siRNA, were treated with vehicle or THC, and 24 hours after treatment, cells were harvested and apoptosis was determined by performing TUNEL assay and using flow cytometry.

**Immune Complex Kinase Assay (a Nonradioactive Method)**

ERK activity was measured in THC-treated cell lysates using p44/42 MAPK Assay kit (Cell Signaling Technology, Inc., Danvers, MA).

**DNA Fragmentation Assay**

For qualitative assessment of internucleosomal DNA fragmentation, DNA was extracted from cell lysates after the appropriate treatment and subjected to agarose gel electrophoresis as described previously (67).

**Confocal Microscopy**

Confocal microscope images were captured on a LSM 510 microscope with a ×60, 1.3 NA Apochromat objective (Carl Zeiss, Inc., Thornwood, NY). Jurkat cells were grown as described above on a 24-well plate (Corning, Corning, NY). After treatment, confocal microscopy of cells was done after double staining with primary antibody against Bad and mitochondrion-specific dye (MitoTracker Deep Red 633; Molecular Probes, Inc. Eugene, OR) as indicated in individual experiments. The excitation wavelength for Bad and MitoTracker Deep Red 633 were 490 and 644 nm, respectively.

**Statistical Analysis**

The significance of differences between experimental conditions was determined using the two-tailed Student's t-test. To characterize synergistic or antagonistic interactions between agents, median dose effect analysis (68) was employed using a commercially available software program (Calcusyn, Biosoft, Ferguson, MO).

**Footnotes**

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The College Student and Marijuana: Research Findings concerning Adverse Biological and Psychological Effects

10.1080/07448481.1983.9936145

**Armand M. Nicholi Jr, M.D.**

pages 73-77

**Abstract**

Next to alcohol, marijuana is the most widely used drug among college students. Recent surveys of high reliability indicate that within the college age group approximately 21 million have smoked marijuana, and several millions smoke it daily. Research findings document clearly that marijuana has distinct adverse biological and psychological effects, even when smoked in moderate doses, and for short periods of time. The short-term and long-term adverse effects of the drug have important implications for the college community—especially the effects on short-term memory and learning, on psychomotor coordination, on behavior, and on reproduction. **Moderate doses of marijuana impair intellectual functioning in several areas including the ability to read with comprehension, to acquire, to store and to recall information, and to communicate clearly. Moderate doses also impair motor coordination—especially driving skills, concentration, and judgment—a significant finding in light of the high incidence of auto fatalities among**
the college age group. The long-term effects of marijuana on sperm, on the menstrual cycle, and on the human fetus also have relevance to this young age group. A detailed knowledge of these adverse effects of marijuana will prove helpful to all those who work within college health facilities.
Marijuana’s Effects on the Brain

When marijuana is smoked, its active ingredient, THC, travels throughout the body, including the brain, to produce its many effects. THC attaches to sites called cannabinoid receptors on nerve cells in the brain, affecting the way those cells work. Cannabinoid receptors are abundant in parts of the brain that regulate movement, coordination, learning and memory, higher cognitive functions such as judgment, and pleasure.
In the early 1960s, a young postdoctoral student stumbled onto something that puzzled him. After reading the literature on cannabis, he was surprised to see that while the active compound in morphine had been isolated from opium poppies 100 years before and cocaine isolated from coca leaves around the same time, the active component of marijuana was still unknown. This simple observation launched his life's work. That young Israeli researcher, Raphael Mechoulam, is now a heavily decorated scientist, recently nominated for the prestigious Rothschild Prize. More than 50 years ago, however, he had trouble starting his scientific journey.
For starters, he needed cannabis to study and didn't know how to obtain it. Eventually, he obtained his research supply from friends in the police department. The young scientist was in a hurry, and didn't want to wait to cut through the red tape required by Israel's Health Ministry. "Yes, I broke the law," he told me when I met with him in Tel Aviv last year, "but I apologized and explained what I was trying to do."

It's a good thing the Israeli government didn't stall his progress, because Mechoulam was moving at breakneck speed.

**Dr. Sanjay Gupta: "Doubling down" on medical marijuana**

By 1963, he determined the structure of cannabidiol (CBD), an important component of marijuana. A year later, he became the first person to isolate delta-9 tetrahydrocannabinol (THC), the psychoactive ingredient in marijuana. Over the ensuing decades, Mechoulam and his team continued to isolate numerous compounds from the cannabis plant.

Their work also went a long way toward illuminating how the drug works in the brain. When Mechoulam's team identified the first known endogenous cannabinoid, a chemical actually made by the brain itself, he named it "anandamide." In the Sanskrit language, ananda means "supreme bliss," which gives us some insight into what Mechoulam thinks of cannabinoids overall.

It was halfway through our long afternoon discussion that Mechoulam, now 83, pulled out a paper he had written in 1999, describing something known as "the entourage effect."
Think of it like this: There are more than 480 natural components found within the cannabis plant, of which 66 have been classified as "cannabinoids." Those are chemicals unique to the plant, including delta-9-tetrahydrocannabinol and cannabidiols. There are, however, many more, including:

- Cannabigerols (CBG);
- Cannabichromenes (CBC);
- other Cannabidiols (CBD);
- other Tetrahydrocannabinols (THC);
- Cannabinol (CBN) and cannabinodiol (CBDL);
- other cannabinoids (such as cannabicyclol (CBL), cannabielsoin (CBE), cannabinol (CBT) and other miscellaneous types).

Other constituents of the cannabis plant are: nitrogenous compounds (27 known), amino acids (18), proteins (3), glycoproteins (6), enzymes (2), sugars and related compounds (34), hydrocarbons (50), simple alcohols (7), aldehydes (13), ketones (13), simple acids (21), fatty acids (22), simple esters (12), lactones (1), steroids (11), terpenes (120), non-cannabinoid phenols (25), flavonoids (21), vitamins (1), pigments (2), and other elements (9).

Minister’s daughter: Cannabis "a gift from God"

Here is the important point. Mechoulam, along with many others, said he believes all these components of the cannabis plant likely exert some therapeutic effect, more than any single compound alone.

While science has not yet shown the exact role or mechanism for all these various compounds, evidence is mounting that these compounds work better together than in isolation: That is the "entourage effect."

Take the case of Marinol, which is pure, synthetic THC. When the drug became available in the mid-1980s, scientists thought it would have the same effect as the whole cannabis plant. But it soon became clear that most patients preferred using the whole plant to taking Marinol.

Researchers began to realize that other components, such as CBD, might have a larger role than previously realized.

To better understand the concept of the entourage effect, I traveled to the secret labs of GW Pharmaceuticals, outside London. In developing Sativex, a cannabis-based drug to treat multiple sclerosis, the company's chairman, Dr. Geoffrey Guy, told me the company ran into some of the same obstacles that Marinol faced.

More than a decade of experiments revealed that a whole plant extract, bred to contain roughly the same amounts of THC and CBD in addition to the other components in the plant, was more effective in reducing the pain and spasms of MS than a medication made of a single compound.

It could be that multiple individual compounds play a role, or it could be due to their interaction in the body; it could also be combination of both, Guy said.

Now, maybe this all sounds obvious. After all, eating real fruits, vegetables and other plants provides better nutrition than just taking vitamin pills with one nutrient or mineral in each. Science is showing us that we can likely say the same about cannabis.

As we move forward with creating medicines, like Charlotte’s Web, for the patients who can benefit from cannabis -- this is an important point to keep in mind.

Unlike other drugs that may work well as single compounds, synthesized in a lab, cannabis may offer its most profound benefit as a whole plant, if we let the entourage effect flower, as Mechoulam suggested more than a decade ago.

"This was our only hope": Medical marijuana refugees
Barak Obama and his U.S. deputy Director of Drug Policy say “Pot is less hazardous than alcohol”

President Obama’s statements of support have marijuana advocates applauding.

by Jonas Paulauskas Medical Expose’ Reporter and Professor of Medicine Desire’ Dubounet, D. Sc. L.P.C.C. (2-24-2014)

Soon afterwards President Barack Obama’s comments that marijuana is no more dangerous than alcohol, his deputy drug specialist has fully agreed.

During a House Oversight Committee hearing Tuesday, Michael Botticelli, deputy director of the White House’s Office of National Drug Control Policy, was grilled about the harms of marijuana and mixed signals about the drug coming out of the Obama administration.

Botticelli’s office must consider marijuana dangerous and harmful, and the federal Drug Enforcement Administration reluctantly consider it illegal.

"The administration continues to oppose attempts legalize marijuana and other drugs," Botticelli said during the hearing.

“How many people die from marijuana overdoses every year?” Connolly probed of Botticelli. NONE is the answer.

“I don’t know that I know,” Botticelli responded. "It is very rare." “Very rare. Now just contrast that with prescription drugs, unintentional deaths from prescription drugs; one American dies every 19 minutes,” Connolly replied. “Nothing comparable to marijuana. Is that correct?” Botticelli agreed. “Hundreds of thousands of people die every year from alcohol related deaths. Automobile, liver disease, esophageal cancer, blood poisoning,” Connolly continued. "Is it not a scientific fact that there is nothing comparable with marijuana? I’m not saying it is good or bad, but when we look at deaths and illnesses, alcohol, other hard drugs are certainly — even prescription drugs — are a threat to public health in a way that just isolated marijuana is not. Isn’t that a scientific fact? Or do you dispute that fact?” “I don’t dispute that fact,” Botticelli said. In an interview with the New Yorker magazine published last month, President Obama stated that he views marijuana as a "bad habit" and "a vice" but no more dangerous than alcohol. “As has been well documented, I smoked pot as a kid, and I view it as a bad habit and a vice, not very different from the cigarettes that I smoked as a young person up through a big chunk of my adult life," Obama said. "I don’t think it is more dangerous than alcohol."

The president also alleged marijuana is less dangerous than alcohol “in terms of its impact on the individual consumer." Those comments, coupled with the legalization of recreational marijuana in Colorado and Washington, have emboldened marijuana advocates and stirred similar efforts in other states. Meanwhile, dozens of petitions related to marijuana
legalization have been popping up on online petition sites. One is urging the NFL to stop punishing players for marijuana use. Another wants Obama to remove pot from the Drug Enforcement Agency's list of top-tier illegal drugs.

"President Obama, if marijuana is safer than alcohol," the petition reads, "remove it from the DEA's schedule of drugs." However, that doesn't seem possible, at least in the short term.

James Capra, chief of operations for the DEA, told a Senate panel last month that "going down the path to legalization in this country is reckless and irresponsible.

"I'm talking about the long-term impact of legalization in the United States," Capra continued. "It scares us. The treatment people are afraid, the education people are afraid. Law enforcement is worried what is going to happen. In every part of the world where this experiment has been tried, it has failed, time and time again."
Here is the truth of the problem with Marijuana  WATCH
http://www.youtube.com/watch?v=fuJPrjEAzeE

DARE TO TELL THE TRUTH ABOUT Marijuana
Marijuana vs. Alcohol

<table>
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<th>Marijuana</th>
<th>Alcohol</th>
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<td>Does use contribute to...</td>
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<tr>
<td><strong>NO</strong> Overdose Deaths?</td>
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<td><strong>NO</strong> Long-term Health Problems?</td>
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<td><strong>NO</strong> Violent Crimes?</td>
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<td><strong>NO</strong> Serious Injuries?</td>
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Introduction

To say that marijuana has been given a bad rap over the past few decades is an understatement. If you’re like most Americans, you have been led to believe that marijuana is a dangerous and addictive drug that has destroyed the lives of millions of teens and adults. You have been encouraged to believe that marijuana causes lung cancer and is a “gateway” to harder drugs. The government has even tried to convince you that most people who use marijuana are losers who sit around on couches all day doing nothing.

What we would like to do is wipe the slate clean and start over. Forget everything you have heard in the past and be open-minded to the truth about marijuana. We are not here to tell you that it is without harms or is some kind of miracle drug. We simply hope you will come to understand that it is far, far less harmful than what your government has told you.

Part of the problem is that many people are simply unfamiliar with marijuana. They have never tried it (or perhaps only tried it a time or two decades ago) and assume the worst. They have been conditioned to think that marijuana use is bad and that people who use it are dangerous or strange or maybe even dirty. They have visions of people using marijuana and being totally zonked out, unable to maintain a regular conversation.

The truth is that marijuana is widely used in a manner quite similar to alcohol. Adults might consume it before enjoying a dinner party with friends. Friends might have a little before engaging in a spirited game of ultimate Frisbee. And spouses – yes, even some couples you know – might imlibe a bit while enjoying a romantic evening together. Concert-goers have even been known to have a puff or two before or during a show – which more likely than not results in them dancing or otherwise enjoying the music, not lying on the ground like lumps.

None of this is “bad” or “wrong” or “immoral.” It is simply something that these responsible adults choose to do. And frequently it is something they choose to do specifically instead of alcohol. And for good reason! Alcohol is more toxic, more addictive, more harmful to the body, more likely to result in injuries, and more likely to lead to interpersonal violence than marijuana.
Below are just a few facts that highlight the very different impacts of these two popular substances on those who consume them and on the broader community. A vast amount of additional information can be found in the book, *Marijuana is Safer: So why are we driving people to drink?* (Chelsea Green, 2009), which can be purchased on [Amazon.com](http://www.amazon.com) or accessed for free on-line at [Scribd.com](http://www.scribd.com).

**Safer for the Consumer**

- **Many people die from alcohol use. Nobody dies from marijuana use.** The U.S. Centers for Disease Control and Prevention (CDC) reports that more than 37,000 annual U.S. deaths, including more than 1,400 in Colorado, are attributed to alcohol use alone (i.e. this figure does not include accidental deaths). On the other hand, the CDC does not even have a category for deaths caused by the use of marijuana.

- **People die from alcohol overdoses. There has never been a fatal marijuana overdose.** The official publication of the Scientific Research Society, *American Scientist*, reported that alcohol is one of the most toxic drugs and using just 10 times what one would use to get the desired effect could lead to death. Marijuana is one of – if not the – least toxic drugs, requiring thousands of times the dose one would use to get the desired effect to lead to death. This “thousands of times” is actually theoretical, since there has never been a case of an individual dying from a marijuana overdose. Meanwhile, according to the CDC, hundreds of alcohol overdose deaths occur the United States each year.

- **The health-related costs associated with alcohol use far exceed those for marijuana use.** Health-related costs for alcohol consumers are eight times greater than those for marijuana consumers, according to an assessment recently published in the *British Columbia Mental Health and Addictions Journal*. More specifically, the annual cost of alcohol consumption is $165 per user, compared to just $20 per user for marijuana. This should not come as a surprise given the vast amount of research that shows alcohol poses far more – and more significant – health problems than marijuana.

- **Alcohol use damages the brain. Marijuana use does not.** Despite the myths we’ve heard throughout our lives about marijuana killing brain cells, it turns out that a growing number of studies seem to indicate that marijuana actually has neuroprotective properties. This means that it works to protect brain cells from harm. For example, one recent study found that teens who used marijuana as well as alcohol suffered significantly less damage to the white matter in their brains. Of course, what is beyond question is that alcohol damages brain cells.

- **Alcohol use is linked to cancer. Marijuana use is not.** Alcohol use is associated with a wide variety of cancers, including cancers of the esophagus, stomach, colon, lungs, pancreas, liver and prostate. Marijuana use has not been conclusively associated with any form of cancer. In fact, one study recently contradicted the long-time government claim that marijuana use is associated with head and neck cancers. It found that marijuana use actually reduced the likelihood of head and neck cancers. If you are concerned about marijuana being associated with lung cancer, you may be interested in the results of the largest case-controlled study ever conducted to investigate the respiratory effects of marijuana smoking and cigarette smoking. Released in 2006, the study, conducted by Dr. Donald Tashkin at the University of California at Los Angeles, found that marijuana smoking was not associated with an increased risk of developing lung cancer. Surprisingly, the researchers found that people who smoked marijuana actually had lower incidences of cancer compared to non-users of the drug.
• **Alcohol is more addictive than marijuana.** Addiction researchers have consistently reported that marijuana is far less addictive than alcohol based on a number of factors. In particular, alcohol use can result in significant and potentially fatal physical withdrawal, whereas marijuana has not been found to produce any symptoms of physical withdrawal. Those who use alcohol are also much more likely to develop dependence and build tolerance.

• **Alcohol use increases the risk of injury to the consumer. Marijuana use does not.** Many people who have consumed alcohol or know others who have consumed alcohol would not be surprised to hear that it greatly increases the risk of serious injury. Research published this year in the journal *Alcoholism: Clinical & Experimental Research*, found that 36 percent of hospitalized assaults and 21 percent of all injuries are attributable to alcohol use by the injured person. Meanwhile, the *American Journal of Emergency Medicine* reported that lifetime use of marijuana is rarely associated with emergency room visits. According to the British Advisory Council on the Misuse of Drugs, this is because: "Cannabis differs from alcohol ... in one major respect. It does not seem to increase risk-taking behavior. This means that cannabis rarely contributes to violence either to others or to oneself, whereas alcohol use is a major factor in deliberate self-harm, domestic accidents and violence." Interestingly enough, some research has even shown that marijuana use has been associated with a decreased risk of injury.

### Safer for the Community

• **Alcohol use contributes to aggressive and violent behavior. Marijuana use does not.** Studies have repeatedly shown that alcohol, unlike marijuana, contributes to the likelihood of aggressive and violent behavior. An article published in the *Journal of Addictive Behaviors* reported that "alcohol is clearly the drug with the most evidence to support a direct intoxication-violence relationship," whereas "cannabis reduces the likelihood of violence during intoxication."

• **Alcohol use is a major factor in violent crimes. Marijuana use is not.** The National Institute on Alcohol Abuse and Alcoholism estimates that 25-30% of violent crimes in the United States are linked to the use of alcohol. According to a report from the U.S. Dept. of Justice, that translates to about 5,000,000 alcohol-related violent crimes per year. By contrast, the government does not even track violent acts specifically related to marijuana use, as the use of marijuana has not been associated with violence. (Of course, we should note that marijuana prohibition, by creating a widespread criminal market, is associated with acts of violence.)

• **Alcohol use contributes to the likelihood of domestic abuse and sexual assault. Marijuana use does not.** Alcohol is a major contributing factor in the prevalence of domestic violence and sexual assault. This is not to say that alcohol causes these problems; rather, its use makes it more likely that an individual prone to such behavior will act on it. For example, a study conducted by the Research Institute on Addictions found that among individuals who were chronic partner abusers, the use of alcohol was associated with significant increases in the daily likelihood of male-to-female physical aggression, but the use of marijuana was not. Specifically, the odds of abuse were eight times higher on days when men were drinking; the odds of severe abuse were 11 times higher. According to the Rape, Abuse and Incest National Network (RAINN) website highlights alcohol as the "most commonly used chemical in crimes of sexual assault" and provides information on an array of other drugs that have been linked to sexual violence. Given the fact that
marijuana is so accessible and widely used, it is quite telling that the word "marijuana" does not appear anywhere on the page.

**Medical Marijuana for Epilepsy**

Cannabinoid (CBD) is one of the primary and non-psychoactive cannabinoids found naturally in marijuana. CBD appears to benefit some epileptic patients who ingest it to avoid seizure activity. For epileptic patients who cannot tolerate or do not benefit from their antiepileptic drugs, medical marijuana can be a good alternative to successfully control their seizures, without experiencing debilitating side effects.

Epilepsy is a brain disorder in which a person has repeated seizures over time. Seizures are episodes of disturbed brain activity that cause changes in attention or behavior.

**Medical Marijuana**

**However, if a multinational drug company grinds up that herb, extracts the cannabis sativa and creates synthetic delta-9-tetrahydrocannabinol, combines it with gelatin, glycerin, iron oxide red, iron oxide yellow, titanium dioxide, markets it to doctors and hospitals under the name Marinol and in the process makes a bunch of wealthy Wall Street investors even richer, then it’s legal.**
A TOKE A DAY...  KEEPS THE DOCTOR AWAY.

Cannabis helps with glaucoma.
Cannabis relieves migraine headaches.
Cannabis helps brain cells.
Cannabis can relieve skin diseases.
Cannabis helps ease asthma attacks.
Cannabis may block epileptic seizures.
Cannabis works as a back spasm medicine.
Cannabis treats depression and other mood disorders.
Cannabis may help emphysema patients to breathe better.
Cannabis alleviates pain associated with chemotherapy.
Cannabis has successfully reduced tumours (benign and malignant).
Cannabis can help multiple sclerosis patients control spasms.
Cannabis can easily be grown organically (free of toxic chemicals).
Cannabis assists in over coming insomnia.
Cannabis dilates the bronchi, to allow more oxygen into the blood.
Cannabis helps paraplegic and quadriplegic patients.
Cannabis may induce antibacterial effects.
Cannabis is the best way known to dry the mouth’s saliva in dentistry.
Cannabis relieves the pain of arthritis and rheumatism.
Cannabis alleviates the symptoms of withdrawal from alcohol and narcotics.
Cannabis seed oil has an omega6/omega3 ratio of about 4:1
Cannabis seeds have essential amino acids in ideal proportions.
Emerging Clinical Applications for Cannabis and Cannabinoids: A Review of the Recent Scientific Literature
Fourth Edition

- Gliomas
- Multiple Sclerosis
- Alzheimer's
- ALS
- Fibromyalgia
- Chronic Pain
- Dystonia
- Tourette's Syndrome
- Hepatitis C
- HIV
- Diabetes
- Hypertension
- Pruritis
- Sleep Apnea
- Osteoporosis
- GI Disorders
- MRSA
- Incontinence
- Rheumatoid Arthritis
9 Major Health Benefits of Medical Marijuana

1. Treats Migraines
   Cannabis healing has been very effective in the treatment of migraine headaches. Migraine headaches are vascular in source and are often preceded by an aura characterized by nausea, flashes of light, fearfulness or photophobia.

2. Slows Down Tumor Growth
   Studies have shown that cannabis help in slow down the facsimile and slow down the production of cancer cells in body. It is also a natural anti-convulsant, which makes it effective in plummeting the seizures and vomiting related with chemo and radiation therapies. So taking marijuana slows down the tumor growth too.

3. Relieves Symptoms of Chronic Diseases
   Marijuana is one of the best natural pain relievers that can help suffers of chronic pain live a more relaxed lives. The side effects are often much less severe than the other common pain medications.

4. Prevents Alzheimer’s
   Cannabis reduces the occurrence of depression in Alzheimer’s patients, which can help them to keep up a higher level of brain function. That is a powerful way to keep patients performance for a longer time after the first onset of Alzheimer’s disease.

5. Treats Glaucoma
   Some strains of this medicinal plant have been shown that, they are potentially decreasing the force that glaucoma can place on the optic nerve; thereby the patients can easily cut the critical condition by smoking or taking the marijuana edibles or medicines.

6. Prevents Seizures
   Seizures is a kind of epilepsy which almost affects more than 2 million of Americans and 30 million of people worldwide. Epilepsy is a condition when some of the brain cells become abnormally excitable. People using marijuana to control epilepsy should be alert when there is any removal of any tablets which controls seizures may leave you more susceptible to the patient. Marijuana is no exception. Patients with epilepsy are advised to exercise caution when using oral THC because there is no enough sufficient knowledge about the convulsant or anti-convulsant properties of the single compound.

7. For ADD and ADHD
   Many people who endure with ADD and/ or ADHD find that medical cannabis reduces their habits to habit and their level of needful with definite tasks. There are no clinical studies on humans but there are some beginner studies have done on animals that point to less hyperactivity and impulsivity with the use of cannabinoids (the active medicines in cannabis).

8. Relieve PMS
   Millions of women have an illness or Premenstrual Syndrome (PMS). PMS includes the symptoms of headaches, abdominal cramps, bloating and fluid retention. Many women report that they have tried several different medications but none as give any significant relief like Medical Marijuana. Cannabinoid medicine has shown to give symptomatic relief from all the unpleasant symptoms of PMS.

9. Calm those with Tourette’s and OCD
   Several psychological disorders have been known to be related with the medicinal benefits of marijuana as well. Taking wave of prescribed amount of regular base can slow down the tics for those who are suffering from tourette’s syndrome and Obsessive Compulsive Disorder (OCD). Yes some of the qualities in marijuana plant help the patient to calm themselves when any creation of intrusive thoughts which produces fear, uneasiness and abnormal behaviors.
CLINICAL RESEARCH STUDY

The Impact of Marijuana Use on Glucose, Insulin, and Insulin Resistance among US Adults

Elizabeth A. Penner, BS, a, b, Hannah Buettner, BA, a, Murray A. Mittelman, MD, DrPH b, c

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ABSTRACT

BACKGROUND: There are limited data regarding the relationship between cannabinoids and metabolic processes. Epidemiologic studies have found lower prevalence rates of obesity and diabetes mellitus in marijuana users compared with people who have never used marijuana, suggesting a relationship between cannabinoids and peripheral metabolic processes. To date, no study has investigated the relationship between marijuana use and fasting insulin, glucose, and insulin resistance.

METHODS: We included 4657 adult men and women from the National Health and Nutrition Examination Survey from 2005 to 2010. Marijuana use was assessed by self-report in a private room. Fasting insulin and glucose were measured via blood samples after a 9-hour fast, and homeostasis model assessment of insulin resistance (HOMA-IR) was calculated to evaluate insulin resistance. Associations were estimated using multiple linear regression, accounting for survey design and adjusting for potential confounders.

RESULTS: Of the participants in our study sample, 579 were current marijuana users and 1975 were past users. In multivariable adjusted models, current marijuana use was associated with 16% lower fasting insulin levels (95% confidence interval [CI], 26%–6%) and 17% lower HOMA-IR (95% CI, 27%–6%). We found significant associations between marijuana use and smaller waist circumferences. Among current users, we found no significant dose-response.

CONCLUSIONS: We found that marijuana use was associated with lower levels of fasting insulin and HOMA-IR, and smaller waist circumference.

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KEYWORDS: Glucose, Insulin, Insulin resistance, Marijuana use

SEE RELATED EDITORIAL p. xxx

Oregon Medical Marijuana Program
Under-Utilization of Cannabis for Non-Pain Related Disorders

NOTE: The Oregon Medical Marijuana Act allows the use of cannabis to treat these symptoms, and just the discovery listed in this graphic that cause these symptoms. For instance, if a patient has the condition called Crohn’s disease, the patient could use cannabis to deal with it, even if the most potent anti-inflammatory substances are used for the disease.
Laboratory/Animal/Preclinical Studies

Cannabinoids are a group of 21-carbon–containing terpenophenolic compounds produced uniquely by Cannabis species (e.g., Cannabis sativa L.).[1,2] These plant-derived compounds may be referred to as phytocannabinoids. Although delta-9-tetrahydrocannabinol (THC) is the primary psychoactive ingredient, other known compounds with biologic activity are cannabidiol, cannabidiol (CBD), cannabichromene, cannabigerol, tetrahydrocannabinvarin, and delta-8-THC. CBD, in particular, is thought to have significant analgesic and anti-inflammatory activity without the psychoactive effect (high) of delta-9-THC.

Antitumor Effects

One study in mice and rats suggested that cannabinoids may have a protective effect against the development of certain types of tumors.[3] During this 2-year study, groups of mice and rats were given various doses of THC by gavage. A dose-related decrease in the incidence of hepatic adenoma tumors and hepatocellular carcinoma (HCC) was observed in the mice. Decreased incidences of benign tumors (polyps and adenomas) in other organs (mammary gland, uterus, pituitary, testis, and pancreas) were also noted in the rats. In another study, delta-9-THC, delta-8-THC, and cannabiol were found to inhibit the growth of Lewis lung adenocarcinoma cells in vitro and in vivo.[4] In addition, other tumors have been shown to be sensitive to cannabinoid-induced growth inhibition.[5-8]

Cannabinoids may cause antitumor effects by various mechanisms, including induction of cell death, inhibition of cell growth, and inhibition of tumor angiogenesis invasion and metastasis.[9-12] One review summarizes the molecular mechanisms of action of cannabinoids as antitumor agents.[13] Cannabinoids appear to kill tumor cells but do not affect their nontransformed counterparts and may even protect them from cell death. These compounds have been shown to induce apoptosis in gliomacells in culture and induce regression of glioma tumors in mice and rats. Cannabinoids protect normal glial cells of astroglial and oligodendroglial lineages from apoptosis mediated by the CB1 receptor.[14]

The effects of delta-9-THC and a synthetic agonist of the CB2 receptor were investigated in HCC.[15] Both agents reduced the viability of HCC cells in vitro and demonstrated antitumor effects in HCC subcutaneous xenografts in nude mice. The investigations documented that the anti-HCC effects are mediated by way of the CB2 receptor. Similar to findings in glioma cells, the cannabinoids were shown to trigger cell death through stimulation of an endoplasmic reticulum stress pathway that activates autophagy and promotes apoptosis. Other investigations have confirmed that CB1 and CB2 receptors may be potential targets in non-small cell lung carcinoma[16] and breast cancer.[17]

An in vitro study of the effect of CBD on programmed cell death in breast cancer cell lines found that CBD induced programmed cell death, independent of the CB1, CB2, or vanilloid receptors. CBD inhibited the survival of both estrogen receptor–positive and estrogen receptor–negative breast cancer cell lines, inducing apoptosis in a concentration-dependent manner while having little effect on nontumorigenic, mammary cells.[18]
CBD has also been demonstrated to exert a chemopreventive effect in a mouse model of colon cancer.[19] In the experimental system, azoxymethane increased premalignant and malignant lesions in the mouse colon. Animals treated with azoxymethane and CBD concurrently were protected from developing premalignant and malignant lesions. In in vitro experiments involving colorectal cancer cell lines, the investigators found that CBD protected DNA from oxidative damage, increased endocannabinoid levels, and reduced cell proliferation.

Another investigation into the antitumor effects of CBD examined the role of intercellular adhesion molecule-1 (ICAM-1).[12] ICAM-1 expression has been reported to be negatively correlated with cancer metastasis. In lung cancer cell lines, CBD upregulated ICAM-1, leading to decreased cancer cell invasiveness. In an in vivo model using severe combined immunodeficient mice, subcutaneous tumors were generated by inoculating the animals with cells from human non-small cell lung carcinoma cell lines.[20] Tumor growth was inhibited by 60% in THC-treated mice compared with vehicle-treated control mice. Tumor specimens revealed that THC had antiangiogenic and antiproliferative effects. However, research with immunocompetent murine tumor models has demonstrated immunosuppression and enhanced tumor growth in mice treated with THC.[21, 22]

In addition, both plant-derived and endogenous cannabinoids have been studied for anti-inflammatory effects. A mouse study demonstrated that endogenous cannabinoid system signaling is likely to provide intrinsic protection against colonic inflammation.[23] As a result, a hypothesis that phytocannabinoids and endocannabinoids may be useful in the risk reduction and treatment of colorectal cancer has been developed.[24-27] CBD may also enhance uptake of cytotoxic drugs into malignant cells. Activation of the transient receptor potential vanilloid type 2 (TRPV2) has been shown to inhibit proliferation of human glioblastoma multiforme cells and overcome resistance to the chemotherapy agent carmustine.[28] In an in vitro model, CBD increased TRPV2 activation and increased uptake of cytotoxic drugs, leading to apoptosis of glioma cells without affecting normal human astrocytes. This suggests that coadministration of CBD with cytotoxic agents may increase drug uptake and potentiate cell death in human glioma cells.

**Appetite Stimulation**

Many animal studies have previously demonstrated that delta-9-THC and other cannabinoids have a stimulatory effect on appetite and increase food intake. It is believed that the endogenous cannabinoid system may serve as a regulator of feeding behavior. The endogenous cannabinoid anandamide potently enhances appetite in mice.[29] Moreover, CB1 receptors in the hypothalamus may be involved in the motivational or reward aspects of eating.[30]

**Analgesia**

Understanding the mechanism of cannabinoid-induced analgesia has been increased through the study of cannabinoid receptors, endocannabinoids, and synthetic agonists and antagonists. The CB1 receptor is found in both the central nervous system (CNS) and in peripheral nerve terminals. Similar to opioid receptors, increased levels of the CB1 receptor are found in regions of the brain that regulate nociceptive processing.[31] CB2 receptors, located predominantly in peripheral tissue, exist at very low levels in the CNS. With the development of receptor-specific antagonists, additional
information about the roles of the receptors and endogenous cannabinoids in the modulation of pain has been obtained.[32,33]

Cannabinoids may also contribute to pain modulation through an anti-inflammatory mechanism; a CB2 effect with cannabinoids acting on mast cell receptors to attenuate the release of inflammatory agents, such as histamine and serotonin, and on keratinocytes to enhance the release of analgesic opioids has been described.[34-36] One study reported that the efficacy of synthetic CB1- and CB2-receptor agonists were comparable with the efficacy of morphine in a murine model of tumor pain.[37]

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3. National Toxicology Program.: NTP toxicity and carcinogenesis studies of 1-trans-delta(9)-tetrahydrocannabinol (CAS No. 1972-08-3) in F344 rats and B6C3F1 mice (gavage studies). Natl Toxicol Program Tech Rep Ser 446 (): 1-317, 1996. [PUBMED Abstract]


Legalize it? Medical evidence on marijuana blows both ways

May 25, 2009 By Sam McManis

Sparked anew by Gov. Arnold Schwarzenegger's call for the state to study the legalization of marijuana, both sides in the smoldering pot debate point to research to bolster their positions.

Such recitation of conflicting marijuana studies can be manipulated and selected buffet-style to serve whatever political and health agenda is being touted.

Even governmental findings can be contradictory. In 1999, for instance, the Office of National Drug Control Policy asked the Institute of Medicine to review evidence. The institute found that, "except for the harms associated with smoking, the adverse effects of marijuana use are within the range of effects tolerated for other medications."

Yet in 2006, the Food and Drug Administration ruled that marijuana has no health benefits and has known and proven harms. It is classified a Schedule 1 drug -- the highest risk of addiction -- in the Controlled Substances Act.

Wading through the medical literature, though, makes those conclusions less cut and dried. "When I was a resident in Kaiser in San Francisco in 1978, I gave a lecture to physicians on marijuana, and I remember my conclusion at that time was that you can find in the literature whatever you were looking for," says Dr. Donald Abrams, a University of California, San Francisco, oncologist and leading medical marijuana researcher. "'Marijuana is good for asthma.' 'Marijuana's bad for asthma.' 'Marijuana causes schizophrenia.' 'Marijuana (decreases) schizophrenia.' And, you know, the evidence is still like that."

There are many factors, of course. As noted by UCLA pulmonologist Dr. Donald Tashkin, who has studied marijuana's effects on the lungs for three decades, "That's just the nature of medical science. You have to deal with variability. The population studied may be different or the methods used to study may differ."

Yet when the arguments for legalization of marijuana, both for medicinal and recreational use, are put forth, solid medical science often gets clouded in an ideological haze. "Although we like to say we separate politics from science, with medical marijuana, that's really difficult," Abrams says. "It depends on who does the study, where it's published and what their agenda is."

Bearing in mind those caveats, here is a look at the research on marijuana's effect in areas critical to health.

**Lungs**

UCLA's Tashkin studied heavy marijuana smokers to determine whether the use led to increased risk of lung cancer and chronic obstructive pulmonary disease, or COPD. He had hypothesized that there would be a definitive link between cancer and marijuana smoking, yet the results proved otherwise. "What we found instead was no association and even a suggestion of some protective effect," says Tashkin, whose research was the largest case-control study ever conducted. The study was funded by the National Institutes of Health.
Tobacco smokers in the study had as much as a 21-fold increase in lung cancer risk. Cigarette smokers, too, developed COPD more often in the study, and researchers found that marijuana did not impair lung function.

Tashkin, supported by other research, concluded that the active ingredient tetrahydrocannabinol, or THC, has an "anti-tumoral effect" in which "cells die earlier before they age enough to develop mutations that might lead to lung cancer."

However, the smoke from marijuana did swell the airways and lead to a greater risk of chronic bronchitis.

"Early on, when our research appeared as if there would be a negative impact on lung health, I was opposed to legalization because I thought it would lead to increased use and that would lead to increased health effects," Tashkin says. "But at this point, I'd be in favor of legalization. I wouldn't encourage anybody to smoke any substances, because of the potential for harm. But I don't think it should be stigmatized as an illegal substance.

"Tobacco smoking causes far more harm. And in terms of an intoxicant, alcohol causes far more harm."

Cognitive function

A 2006 study in the journal Neurology found that speed of thinking, attention and verbal fluency were affected as much as 70 percent by long-term heavy use (four or more joints per week).

But a 2003 review of literature in the Journal of the International Neuropsychological Society found that marijuana smoking had a "small effect" on memory in longtime users. However, users had no lasting effects in reaction time, attention or verbal function.

"Surprisingly, we saw very little evidence of deleterious effects," Dr. Igor Grant, researcher at the University of California, San Diego, School of Medicine, said in a statement.

Other studies: A 2002 study in the Journal of the American Medical Association found that heavy users did worse on recall memory tests. A 2006 study in Greece showed users had slower mental-processing speed than the control group.

Then again, a 2007 study at the University of Lausanne in Switzerland, published in Archives of Pediatrics & Adolescent Medicine, found that students who smoked marijuana had better grades than those who used only tobacco or those who did not smoke any substance. In terms of brain development, a 2000 study in the Journal of Addictive Diseases found changes in brain structure in those who started using marijuana before age 17 but not in those who started at an older age.

A 2009 Children's Hospital of Philadelphia study used brain imaging to show that heavy adolescent users are more likely to have disrupted brain development in regions involving memory, attention, decision making and language.

But a 2008 Ohio State University study found that marijuana can reduce brain inflammation and perhaps reduce memory impairment that could delay Alzheimer's disease.

Psychosis

Yes, there is an increased risk in psychotic behavior and long-term risk of mental illness from marijuana use, according to a 2007 review of literature commissioned by Great Britain's Department of Health and published in the Lancet.

But the risk is small, because the risk of developing psychosis in the general population is 3 percent over a lifetime and rises to 5 percent for marijuana users, lead researcher Stanley Zammit told the
Los Angeles Times. "So 95 percent of the people are not going to get psychotic, even if they smoke on a daily basis," he told the paper.

In 2005, New Zealand researchers studied a group of people with a gene variant the researchers believe predisposes that group to developing psychosis. Those in the group who smoked marijuana as teens had a tenfold increase in risk of psychosis than those who abstained.

**Depression**

A study published in 2001 in the American Journal of Psychiatry followed nearly 2,000 adults over 15 years. It found that marijuana users who had no symptoms of depression at the start were four times more likely than non-users of developing symptoms during that time frame.

In 2008, the U.S. Office of National Drug Control Policy stated that early marijuana use could increase the likelihood of mental illness by as much as 40 percent later in life.

However, researchers at McGill University in Montreal in 2007 reported in the Journal of Neuroscience that THC in low doses actually serves as an antidepressant similar to Prozac, producing serotonin. At higher doses, however, they found it could lead not only to depression but also to psychotic episodes.

**Summary**

“For first time we now have clear message from fed government saying they will not stand in way of states that wish to implement alternative regulatory schemes in lieu of federal prohibition,” Many have predicted that within the next one to three years, five or six other states may join Colorado and Washington in legalizing the drug, setting the stage for the rest of the country to follow. The Age of Deception is Ending In 2003, the U.S. Government as represented by the Department of Health and Human Services filed for, and was awarded a patent on cannabinoids. The reason? Because research into cannabinoids allowed pharmaceutical companies to acquire practical knowledge on one of the most powerful antioxidants and neuroprotectants known to the natural world. The U.S. Patent 6630507 was specifically initiated when researchers found that cannabinoids had specific antioxidant properties making them useful in the treatment and prophylaxis of wide variety of oxidation associated diseases, such as ischemic, age-related, inflammatory and autoimmune diseases.

The cannabinoids are found to have particular application as neuroprotectants, for example in limiting neurological damage following ischemic insults, such as stroke and trauma, or in the treatment of neurodegenerative diseases, such as Alzheimer’s disease, Parkinson’s disease and HIV dementia. Nonpsychoactive cannabinoids, such as cannabidoil, are particularly advantageous to use because they avoid toxicity that is encountered with psychoactive cannabinoids at high doses useful in the method of the present invention. Besides the top 10 health benefits below, findings published in the journal PLoS ONE, researchers have now discovered that marijuana-like chemicals trigger receptors on human immune cells that can directly inhibit a type of human immuno-deficiency virus (HIV) found in late-stage AIDS.

Recent studies have even shown it to be an effective atypical anti-psychotic in treating schizophrenia a disease many other studies have inconsistently found it causing. Top 10 Health
Benefits of Marijuana 1. Cancer Cannabinoids, the active components of marijuana, inhibit tumor growth in laboratory animals and also kill cancer cells. Western governments have known this for a long time yet they continued to suppress the information so that cannabis prohibition and the profits generated by the drug industry proliferated. THC that targets cannabinoid receptors CB1 and CB2 is similar in function to endocannabinoids, which are cannabinoids that are naturally produced in the body and activate these receptors. The researchers suggest that THC or other designer agents that activate these receptors might be used in a targeted fashion to treat lung cancer. 2. Tourette’s Syndrome Tourette’s syndrome is a neurological condition characterized by uncontrollable facial grimaces, tics, and involuntary grunts, snorts and shouts. Dr. Kirsten Mueller-Vahl of the Hanover Medical College in Germany led a team that investigated the effects of chemicals called cannabinoids in 12 adult Tourette’s patients. A single dose of the cannabinol produced a significant reduction in symptoms for several hours compared to placebo, the researchers reported. 3. Seizures Marijuana is a muscle relaxant and has “antispasmodic” qualities that have proven to be a very effective treatment for seizures. There are actually countless cases of people suffering from seizures that have only been able to function better through the use of marijuana. 4. Migraines Since medicinal marijuana was legalized in California, doctors have reported that they have been able to treat more than 300,000 cases of migraines that conventional medicine couldn’t through marijuana. 5. Glaucoma Marijuana’s treatment of glaucoma has been one of the best documented. There isn’t a single valid study that exists that disproves marijuana’s very powerful and popular effects on glaucoma patients. 6. Multiple Sclerosis Marijuana’s effects on multiple sclerosis patients became better documented when former talk-show host, Montel Williams began to use pot to treat his MS. Marijuana works to stop the neurological effects and muscle spasms that come from the fatal disease. 7. ADD and ADHD A well documented USC study done about a year ago showed that marijuana is not only a perfect alternative for Ritalin but treats the disorder without any of the negative side effects of the pharmaceutical. 8. IBS and Crohn’s Marijuana has shown that it can help with symptoms of the chronic diseases as it stops nausea, abdominal pain, and diarrhea. 9. Alzheimer’s Despite what you may have heard about marijuana’s effects on the brain, the Scripps Institute, in 2006, proved that the THC found in marijuana works to prevent Alzheimer’s by blocking the deposits in the brain that cause the disease. 10. Premenstrual Syndrome Just like marijuana is used to treat IBS, it can be used to treat the cramps and discomfort that causes PMS symptoms. Using marijuana for PMS actually goes all the way back to Queen Victoria. Mounting Evidence Suggests Raw Cannabis is Best Cannabinoids can prevent cancer, reduce heart attacks by 66% and insulin dependent diabetes by 58%. Cannabis clinician Dr. William Courtney recommends drinking 4 – 8 ounces of raw flower and leaf juice from any Hemp plant, 5 mg of Cannabidiol (CBD) per kg of body weight, a salad of Hemp seed sprouts and 50 mg of THC taken in 5 daily doses. Why raw? Heat destroys certain enzymes and nutrients in plants. Incorporating raw cannabis allows for a greater
availability of those elements. Those who require large amounts of cannabinoids without the psychoactive effects need to look no further than raw cannabis. In this capacity, it can be used at 60 times more tolerance than if it were heated. Raw cannabis is considered by many experts as a dietary essential. As a powerful anti-inflammatory and antioxidant, raw cannabis may be right there with garlic and turmeric.

---

**Pot Smoking in U.S. Tops the Charts**

An estimated 42 percent of Americans and New Zealanders have smoked marijuana at least once, significantly more than most nations and more than twice the percentage of European countries.

### Estimated Cannabis Use Among Population
(at least one usage)

<table>
<thead>
<tr>
<th>Country</th>
<th>Use Rate</th>
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<tbody>
<tr>
<td>Colombia</td>
<td>10.8%</td>
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<tr>
<td>Mexico</td>
<td>7.8%</td>
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<tr>
<td>United States</td>
<td>42.4%</td>
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<tr>
<td>Belgium</td>
<td>10.4%</td>
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<td>France</td>
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<td>Netherlands</td>
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<td>Spain</td>
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<td>Ukraine</td>
<td>6.4%</td>
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<tr>
<td>Israel</td>
<td>11.5%</td>
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<tr>
<td>Lebanon</td>
<td>4.6%</td>
</tr>
<tr>
<td>Nigeria</td>
<td>2.7%</td>
</tr>
<tr>
<td>South Africa</td>
<td>8.4%</td>
</tr>
<tr>
<td>Japan</td>
<td>1.5%</td>
</tr>
<tr>
<td>China</td>
<td>0.3%</td>
</tr>
<tr>
<td>New Zealand</td>
<td>41.9%</td>
</tr>
</tbody>
</table>

Marijuana Science: Why Pot Heads Are Slackers

By Denise Chow, Staff Writer  |  July 01, 2013 04:53pm ET

The stereotype of pot smokers as lackadaisical loafers is supported by new research: People who smoke marijuana regularly over long periods of time tend to produce less of a chemical in the brain that is linked to motivation, a new study finds.

Researchers in the United Kingdom scanned the brains of 19 regular marijuana users, and 19 nonusers of the same sex and age, using positron emission tomography (PET), which helps measure the distribution of chemicals throughout the brain.

They found that the long-term cannabis users tended to produce less dopamine, a "feel good" chemical in the brain that plays an important role in motivation and reward-driven behavior.
