DRUG COUNSELING INFORMATION

Date: 10/30/2014 **Page:** 1

Patient: KELLY PATTON

Rx Number: 41-1979730

Doctor Name: VIVIAN ILLERA, MD

Directions: INJECT CONTENTS OF 2 SYRINGES (400 MG) SUBCUTANEOUSLY EVERY FOUR WEEKS. SINGLE-US SYRINGE. STORE REFRIGERATED. DO NOT FREEZE.

Drug Name: CIMZIA

GENERIC NAME: CERTOLIZUMAB (SER-toe-LIZ-oo-mab)

COMMON USES: This medicine is TNF (tumor necrosis factor)-blocker used to reduce signs and symptoms of Crohn disease in certain patients. This medicine is also used to treat active rheumatoid arthritis or psoriatic arthritis. It may be used alone or in combination with other medicine.

HOW TO USE THIS MEDICINE: Follow the directions for using this medicine provided by your doctor. This medicine has a MEDICATION GUIDE approved by the U.S. Food and Drug Administration. Read it carefully. Ask your doctor, nurse, or pharmacist any questions that you may have about this medicine. This medicine is usually administered as an injection under the skin at your doctor's office, hospital, or clinic. If you will be using it at home, a health care provider will teach you how to use it. Be sure you understand how to use this medicine. Follow the procedures you are taught when you use a dose. Contact your health care provider if you have any questions. THIS MEDICINE SHOULD BE A CLEAR COLORLESS TO YELLOW LIQUID. Do not use this medicine if it contains particles, is cloudy or discolored, or if the vial is cracked or damaged. Let this medicine come to room temperature before you inject it, as directed by your health care provider. USE THE PROPER TECHNIQUE taught to you by your doctor. Inject deep under the skin, NOT into muscle. This medicine may be injected in your stomach or thigh area. IF YOUR DOSE REQUIRES 2 INJECTIONS, be sure to use separate injection sites for each injection. BE SURE TO ROTATE INJECTION SITES. Do not inject into areas of the skin that are sore, bruised, red, or hard. DISCARD ANY UNUSED MEDICINE remaining in the syringe or vial after use. DO NOT RECEIVE A LIVE VACCINE (eg, measles, mumps) or treatment with a weakened bacteria (eg, BCG for bladder cancer) while you are taking this medicine. Talk with your doctor before you receive any vaccine. STORE THIS MEDICINE in the refrigerator, between 36 and 46 degrees F (2 and 8 degrees C), away from heat, moisture, and light. Do not freeze. Do not use this medicine past the expiration date on the label. KEEP THIS PRODUCT, as well as syringes and needles, out of the reach of children and pets. Do not reuse needles, syringes, or other materials. Ask your health care provider how to dispose of these materials after use. Follow all local rules for disposal. IF YOU MISS A DOSE OF THIS MEDICINE, use it as soon as possible. Then go back to your regular dosing schedule. DO NOT USE 2 doses at once.

CAUTIONS: DO NOT USE THIS MEDICINE if you have had an allergic reaction to it or if you are allergic to any ingredient in this product. Tell your doctor if you have had a severe allergic reaction (eg, severe rash, hives, difficulty breathing, dizziness) to another TNF blocker (eg, infliximab). DO NOT EXCEED THE RECOMMENDED DOSE or use more often than prescribed without checking with your doctor. LABORATORY AND/OR MEDICAL TESTS, including TB tests, complete blood cell counts, liver function, and skin examinations, may be performed to monitor your progress or to check for side effects. Keep all doctor and laboratory appointments while you are using this medicine.

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THIS MEDICINE MAY AFFECT CERTAIN LAB TEST RESULTS, including blood clotting tests and TB tests. Make sure laboratory personnel and your doctors know you use this medicine. BEFORE YOU HAVE ANY MEDICAL OR DENTAL TREATMENTS, emergency care, or surgery, tell the doctor or dentist that you are using this medicine. THIS MEDICINE MAY LOWER YOUR RESISTANCE TO INFECTION. Prevent infection by avoiding contact with people with colds or other infections. Do not touch your eyes or the inside of your nose unless you have thoroughly washed your hands first. THIS MEDICINE MAY REDUCE THE NUMBER OF BLOOD CELLS THAT ARE NEEDED FOR CLOTTING. To prevent bleeding, avoid situations where bruising or injury may occur. THIS MEDICINE MAY INCREASE THE RISK OF DEVELOPING BLOOD CANCER (eg, leukemia, lymphoma) and other types of cancer. This may be fatal in some cases. Discuss any questions or concerns with your doctor. Tell your doctor if you have ever had cancer. Contact your doctor right away if you develop any unusual symptoms, such as unusual bruising, unusual lumps or swelling (eg, in your neck, armpit, or groin), night sweats, recurring fever, unusual tiredness or weakness, unexplained cough or shortness of breath, persistent unexplained itching, or unexplained weight loss. NEW OR WORSENING NERVOUS SYSTEM PROBLEMS (eg, multiple sclerosis, Guillain-Barre syndrome, seizures, inflammation of the nerves of the eyes) have occurred with TNF blockers. Tell your doctor if you have a condition that affects your nervous system. Discuss any questions or concerns with your doctor. SOME PATIENTS WHO USE THIS MEDICINE HAVE DEVELOPED NEW OR WORSENING PSORIASIS. Tell your doctor right away if you notice any new or worsening skin problems (eg, red, flaky, or itchy skin patches; raised bumps filled with pus). BEFORE YOU BEGIN TAKING ANY NEW MEDICINE, either prescription or over-the-counter, check with your doctor or pharmacist. CAUTION IS ADVISED WHEN USING THIS MEDICINE IN THE ELDERLY because they may be more sensitive to the effects of the medicine, especially infection. FOR WOMEN: IF YOU BECOME PREGNANT, discuss with your doctor the benefits and risks of using this medicine during pregnancy. If you used this medicine during pregnancy, tell your baby's doctor. IT IS UNKNOWN IF THIS MEDICINE IS EXCRETED in breast milk. DO NOT BREAST-FEED while taking this medicine.

POSSIBLE SIDE EFFECTS: SIDE EFFECTS that may occur while taking this medicine include minor pain, redness, or swelling at the injection site. If they continue or are bothersome, check with your doctor. A RARE TYPE OF CANCER called HSTCL has been reported in patients using TNF blockers. These cases have been fatal. Most of these cases occurred in teenagers or young adults. Most of these patients had Crohn disease or ulcerative colitis. Patients who developed this cancer were usually using this medicine along with certain other medicines (azathioprine, 6-mercaptopurine). Tell your doctor if you have or have ever had any type of cancer. Tell your doctor right away if you develop stomach pain or tenderness, fever, night sweats, or unexplained weight loss. CONTACT YOUR DOCTOR IMMEDIATELY if you experience burning, numbness, or tingling; a butterfly-shaped rash on the nose and cheeks; chest pain; confusion; dizziness; fainting; irregular heartbeat; joint pain; mental or mood changes (eg, anxiety, depression, suicidal thoughts or actions); muscle weakness; one-sided weakness; red, swollen, peeling, or blistered skin; seizure; severe or

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persistent headache; severe or persistent pain, swelling, or redness at the injection site; shortness of breath; slurred speech; sudden unusual weight gain; swelling of the hands, ankles, or feet; symptoms of infection (eg, fever, chills, persistent cough or sore throat, white patches in the mouth, painful or frequent urination, unusual vaginal discharge or odor, unusual weight loss, persistent feeling of being unwell); symptoms of liver problems (eg, yellowing of the skin or eyes, dark urine, pale stools, decreased appetite, nausea or stomach pain); unusual bruising or bleeding; unusual lumps (eg, in the armpits, groin, neck); unusual sweating; unusual tiredness or weakness; unusually pale skin; or vision changes. AN ALLERGIC REACTION to this medicine is unlikely, but seek immediate medical attention if it occurs. Symptoms of an allergic reaction include rash; hives; itching; difficulty breathing; tightness in the chest; flushing; swelling of the mouth, face, lips, throat, or tongue; or unusual hoarseness. This is not a complete list of all side effects that may occur. If you have questions about side effects, contact your healthcare provider. Call your doctor for medical advice about side effects. You may report side effects to FDA at 1-800-FDA-1088.

BEFORE USING THIS MEDICINE: WARNING: PATIENTS WHO USE THIS MEDICINE HAVE AN INCREASED RISK OF DEVELOPING SERIOUS AND SOMETIMES FATAL INFECTIONS such as bacterial, viral, or fungal infections; or tuberculosis (TB). Most patients who developed these infections were taking medicines that suppressed their immune systems (eg, methotrexate, corticosteroids). TB may be caused by a new infection or by reactivation of a previous infection. Patients should receive a TB skin test before using this medicine. Patients who test positive for TB should begin treatment for TB before starting this medicine. All patients should also be monitored for signs of TB while using this medicine, even if their TB test is negative. Contact your doctor immediately if you develop signs of TB or any other type of infection (eg, persistent cough; muscle aches or weakness; unexplained weight loss; fever, chills, or persistent sore throat; shortness of breath; unusual tiredness; warm, red, or painful skin or sores; diarrhea or stomach pain; increased or painful urination). THIS MEDICINE IS A TNF BLOCKER. Lymphoma and other types of cancer have been reported in children and teenagers treated with TNF blockers. This has been fatal in some cases. This medicine is not approved for use in children or teenagers. Talk with your doctor for more information. Some medicines or medical conditions may interact with this medicine. INFORM YOUR DOCTOR OR PHARMACIST of all prescription and over-the-counter medicine that you are taking. DO NOT TAKE THIS MEDICINE if you are also using certain biologic medicines (eg, abatacept, adalimumab, anakinra, etanercept, golimumab, infliximab, natalizumab, rituximab, tocilizumab). ADDITIONAL MONITORING OF YOUR DOSE OR CONDITION may be needed if you are taking nonsteroidal anti-inflammatory drugs (NSAIDs) (eg, ibuprofen), or corticosteroids (eg, prednisone), methotrexate, or any other medicine that may weaken the immune system. Tell your doctor if you are using medicine to treat an infection. DO NOT START OR STOP any medicine without doctor or pharmacist approval. Inform your doctor if you have recently received or are scheduled to receive a vaccine. Tell your

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doctor if you have any type of infection, including bacterial, viral, or protozoal infection; fungal infection (eg, histoplasmosis, coccidiomycosis); TB infection; skin infection; flu-like symptoms or other signs of infection (eg, fever; chills; cough; warm, red, or painful skin); open cuts or sores on your body; a history of chronic or recurring infections; a history of positive TB skin test, or if you have recently been around someone who might have TB. Tell your doctor if you live, have lived in, or traveled to an area where TB is common or certain parts of the country (eg, Ohio or Mississippi river valleys) where certain types of fungal infections (eg, histoplasmosis, coccidioidomycosis, blastomycosis) are common. Check with your doctor if you are not sure if you have lived in an area where these infections are common. Tell your doctor if you have a weakened immune system, HIV infection, or any other immune system problems, or if you have any condition that may increase your risk of infection. Inform your doctor of any other medical conditions, including a history of hepatitis B infection or other liver problems; diabetes; heart problems (eg, heart failure); high blood pressure; blood problems (eg, anemia); bone marrow problems; autoimmune problems (eg, lupus); numbness, tingling, or other nervous system problems (eg, seizures, multiple sclerosis (MS), Guillain-Barre syndrome); allergies; pregnancy; or breast-feeding. Tell your doctor if you have a risk for skin cancer or a history of cancer (eg, lymphoma, skin cancer). USE OF THIS MEDICINE IS NOT RECOMMENDED if you have an active infection. Contact your doctor or pharmacist if you have any questions or concerns about taking this medicine.

OVERDOSE: If overdose is suspected, contact your local poison control center or emergency room immediately.

ADDITIONAL INFORMATION: DO NOT SHARE THIS MEDICINE with others for whom it was not prescribed. DO NOT USE THIS MEDICINE for other health conditions. Do not reuse needles, syringes, or other materials. Dispose of properly after use. Ask your doctor, nurse, or pharmacist to explain local regulations for selecting an appropriate container and properly disposing of the container when it is full. CHECK WITH YOUR PHARMACIST about how to dispose of unused medicine.

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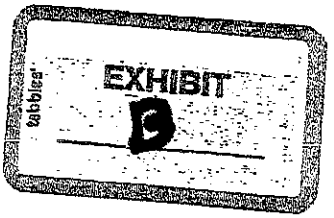

Acetaminophen / hydrocodone Side Effects

Not all side effects for acetaminophen / hydrocodone may be reported. You should always consult a doctor or healthcare professional for medical advice. Side effects can be reported to the FDA here.

For the Consumer

Applies to acetaminophen / hydrocodone: oral capsule, oral elixir, oral liquid, oral solution, oral syrup, oral tablet

In addition to its needed effects, some unwanted effects may be caused by acetaminophen / hydrocodone. In the event that any of these side effects do occur, they may require medical attention.

You should check with your doctor immediately if any of these side effects occur when taking acetaminophen / hydrocodone:

More common

- Dizziness
- lightheadedness

Incidence not known

- Back, leg, or stomach pains
- black, tarry stools
- bleeding gums
- blood in the urine or stools
- blood in vomit
- bluish lips or skin
- chills
- choking
- cough or hoarseness
- dark urine
- decrease in the frequency of urination
- decrease in urine volume
- difficult or troubled breathing

- difficulty in passing urine (dribbling)
- difficulty with breathing
- difficulty with swallowing
- fast heartbeat
- fever
- fever with or without chills
- general body swelling
- general feeling of tiredness or weakness
- headache
- irregular, fast or slow, or shallow breathing
- light-colored stools
- loss of appetite
- lower back or side pain
- nausea or vomiting
- nosebleeds
- not breathing
- painful or difficult urination
- pale or blue lips, fingernails, or skin
- pinpoint red spots on the skin
- puffiness or swelling of the eyelids or around the eyes, face, lips, or tongue
- severe or continuing stomach pain
- shortness of breath or troubled breathing
- skin rash, hives, or itching
- sore throat
- sore tongue
- sores, ulcers, or white spots on the lips or in the mouth
- tightness in the chest
- unable to speak
- unusual bleeding or bruising
- unusual tiredness or weakness
- upper right abdominal or stomach pain
- yellow eyes and skin

If any of the following symptoms of overdose occur while taking acetaminophen / hydrocodone, get emergency help immediately:

Symptoms of overdose

- Bloody or cloudy urine
- change in consciousness
- chest pain or discomfort
- cold and clammy skin
- decreased awareness or responsiveness
- extreme drowsiness
- general feeling of discomfort or illness
- increased sweating
- irregular heartbeat
- lightheadedness, dizziness, or fainting
- loss of consciousness
- no blood pressure or pulse
- no muscle tone or movement
- not breathing
- severe sleepiness
- slow or irregular heartbeat
- stopping of heart
- sudden decrease in the amount of urine
- unconsciousness
- unpleasant breath odor

Some of the side effects that can occur with acetaminophen / hydrocodone may not need medical attention. As your body adjusts to the medicine during treatment these side effects may go away. Your health care professional may also be able to tell you about ways to reduce or prevent some of these side effects. If any of the following side effects continue, are bothersome or if you have any questions about them, check with your health care professional:

More common

- Drowsiness
- relaxed and calm
- sleepiness

Incidence not known

- Belching
- changes in mood
- difficulty having a bowel movement (stool)
- fear or nervousness
- feeling of indigestion
- hearing loss

- impaired hearing
- pain in the chest below the breastbone
- unusual drowsiness, dullness, tiredness, weakness, or feeling of sluggishness

For Healthcare Professionals

Applies to acetaminophen / hydrocodone: oral capsule, oral elixir, oral liquid, oral solution, oral tablet

General

The adverse effects of hydrocodone are generally similar to the adverse effects observed with other narcotic analgesics. Acetaminophen is generally well-tolerated when administered in therapeutic doses.[Ref]

Nervous system

One study has suggested that the respiratory depression caused by hydrocodone may be of benefit in the treatment of dyspnea related to chronic obstructive pulmonary disease and restrictive lung disease. However, the potential for the precipitation of respiratory insufficiency makes such use of hydrocodone hazardous and such use should be undertaken, if at all, only with extreme caution.[Ref]

Nervous system side effects of hydrocodone include mental depression, dizziness, lightheadedness, respiratory depression (which is sometimes fatal), stupor, delirium, somnolence, agitation, and dysphoria.[Ref]

Other

Other side effects have included withdrawal symptoms, after either abrupt cessation or fast tapering of narcotic analgesics. Such symptoms may include agitation, restlessness, anxiety, insomnia, tremor, abdominal cramps, blurred vision, vomiting, and sweating.[Ref]

Hepatic

Alcoholic patients may develop hepatotoxicity after even modest doses of acetaminophen. In healthy patients, approximately 15 grams of acetaminophen is necessary to deplete liver glutathione stores by 70% in a 70 kg person. However, hepatotoxicity has been reported following smaller doses. Glutathione concentrations may be repleted by the antidote N-acetylcysteine. One case report has suggested that hypothermia may also be beneficial in decreasing liver damage during overdose.

In a recent retrospective study of 306 patients admitted for acetaminophen overdose, 6.9% had severe liver injury but all recovered. None of the 306 patients died.

A 19-year-old female developed hepatotoxicity, reactive plasmacytosis and agranulocytosis followed by a leukemoid reaction after acute acetaminophen toxicity.

The adverse effects of hydrocodone may be more likely and more severe in patients with liver disease.[Ref]

Hepatic side effects including severe and sometimes fatal dose dependent hepatitis have been reported in alcoholic patients. Hepatotoxicity has been increased during fasting. Several cases of hepatotoxicity from

chronic acetaminophen therapy at therapeutic doses have also been reported despite a lack of risk factors for toxicity.[Ref]

Gastrointestinal

Gastrointestinal side effects with the use of acetaminophen are rare except in alcoholics and after overdose. Cases of acute pancreatitis have been reported rarely.

Gastrointestinal side effect including nausea, vomiting, constipation, and dry mouth are relatively common effects of narcotic analgesics.[Ref]

One study has suggested that acetaminophen may precipitate acute biliary pain and cholestasis. The mechanism of this effect may be related to inhibition of prostaglandin and alterations in the regulation of the sphincter of Oddi.[Ref]

Genitourinary

Genitourinary side effects including ureteral spasm, spasm of vesicle sphincters, and urinary retention have been reported.[Ref]

Dermatologic

Dermatologic side effects including narcotic-induced rashes have been reported. General erythematous skin rashes associated with acetaminophen have been reported, but are rare. A rare case of bullous erythema associated with acetaminophen has been reported. Acetaminophen has been associated with a risk of rare but potentially fatal serious skin reactions know as Stevens-Johnson syndrome (SJS), toxic epidermal necrolysis (TEN), and acute generalized exanthematous pustulosis (AGEP).[Ref]

Renal

Acute tubular necrosis usually occurs in conjunction with liver failure, but has been observed as an isolated finding in rare cases.

The adverse effects of hydrocodone may be more likely and more severe in patients with renal insufficiency.[Ref]

Renal side effects of acetaminophen are rare and include acute tubular necrosis and interstitial nephritis. Adverse renal effects are most often observed after overdose, from chronic abuse (often with multiple analgesics), or in association with acetaminophen-related hepatotoxicity.[Ref]

Hematologic

Hematologic side effects including rare cases of thrombocytopenia associated with acetaminophen have been reported. Acute thrombocytopenia has also been reported as having been caused by sensitivity to acetaminophen glucuronide, the major metabolite of acetaminophen. Methemoglobinemia with resulting cyanosis has also been observed in the setting of acute overdose.[Ref]

Hypersensitivity

Hypersensitivity side effects to acetaminophen have been reported rarely.[Ref]

Respiratory

Respiratory side effects have included a case of eosinophilic pneumonia which has been associated with acetaminophen.[Ref]

Metabolic

In the case of metabolic acidosis, causality is uncertain as more than one drug was ingested. The case of metabolic acidosis followed the ingestion of 75 grams of acetaminophen, 1.95 grams of aspirin, and a small amount of a liquid household cleaner. The patient also had a history of seizures which the authors reported may have contributed to an increased lactate level indicative of metabolic acidosis.

Metabolic side effects including metabolic acidosis have been reported following a massive overdose of acetaminophen.

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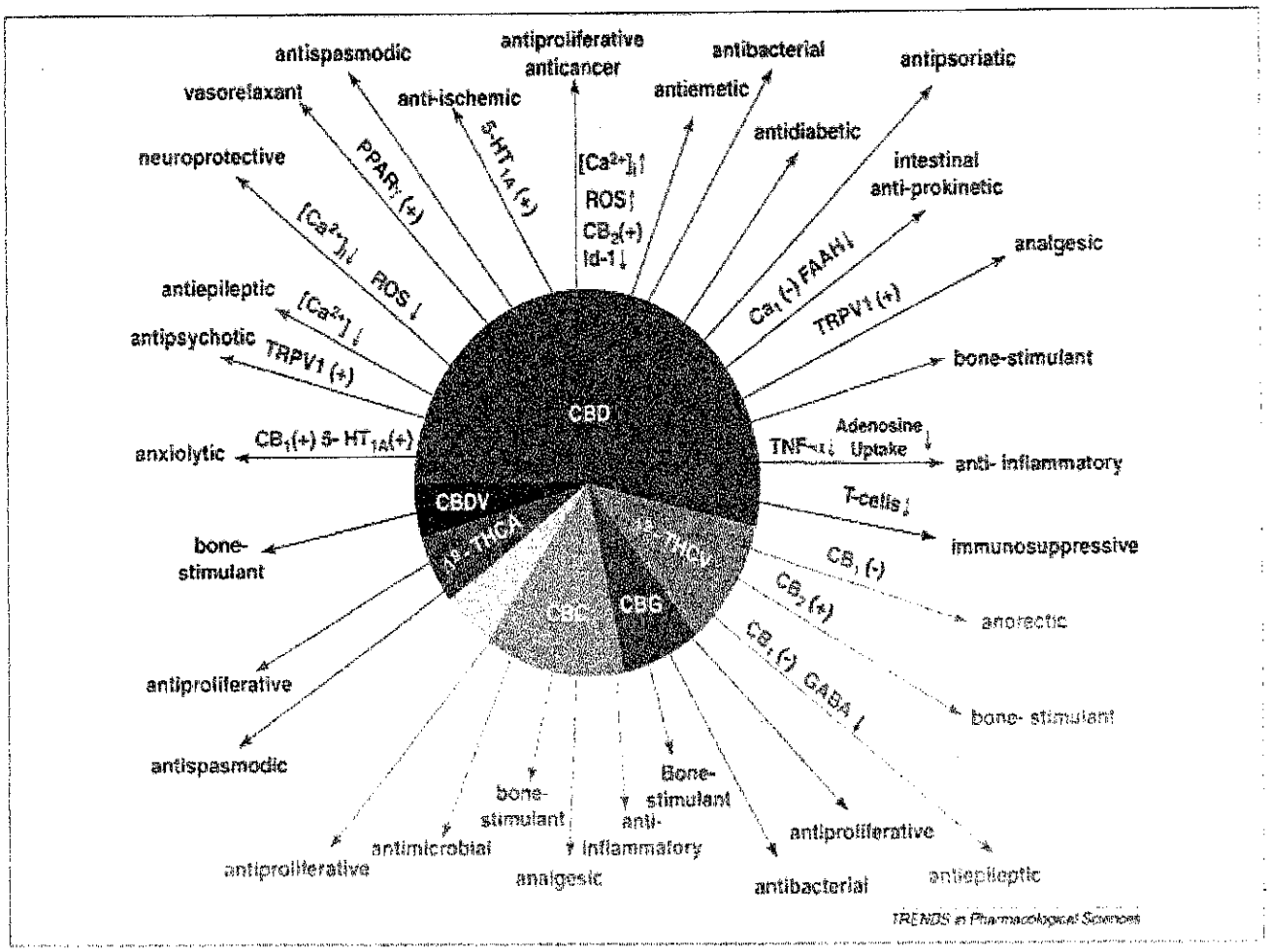

Figure 1. Pharmacological actions of non-psychotropic cannabinoids (with the indication of the proposed mechanisms of action).
Abbreviations: Δ^9-THC, Δ^9-tetrahydrocannabinol; Δ^8-THC, Δ^8-tetrahydrocannabinol; CBN, cannabinol; CBD, cannabidiol; Δ^9-THCV, Δ^9-tetrahydrocannabivarin; CBC, cannabichromene; CBG, cannabigerol; Δ^9-THCA, Δ^9-tetrahydrocannabinolic acid; CBDA, cannabidiolic acid; TRPV1, transient receptor potential vanilloid type 1; PPARγ, peroxisome proliferator-activated receptor γ; ROS, reactive oxygen species; 5-HT_{1A}, 5-hydroxytryptamine receptor subtype 1A; FAAH, fatty acid amide hydrolase.
(+), direct or indirect activation; ↑, increase; ↓, decrease.

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CB_1 cannabinoid receptors are involved in neuroprotection via NF-κB inhibition

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We reported earlier that closed head injury (CHI) in mice causes a sharp elevation of brain 2-arachidonoylglycerol (2-AG) levels, and that exogenous 2-AG reduces brain edema, infarct volume and hippocampal death and improved clinical recovery after CHI. The beneficial effect of 2-AG was attenuated by SR141716A, a CB_1 cannabinoid receptor antagonist, albeit at relatively high doses. In the present study, we further explored the role of CB_1 receptors in mediating 2-AG neuroprotection. CB_1 receptor knockout mice ($CB_1(-/-)$) showed minor spontaneous recovery at 24 h after CHI, in contrast to the significant improvement in neurobehavioral function seen in wild-type (WT) mice. Moreover, administration of 2-AG did not improve neurological performance and edema formation in the $CB_1(-/-)$ mice. In addition, 2-AG abolished the three- to four-fold increase of nuclear factor κB (NF-κB) transactivation, at 24 h after CHI in the WT mice, while it had no effect on NF-κB in the CB_1 $(-/-)$ mice, which was as high as in the WT vehicle-treated mice. We thus propose that 2-AG exerts its neuroprotection after CHI, at least in part, via CB_1 receptor-mediated mechanisms that involve inhibition of intracellular inflammatory signaling pathways.
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Published online 23 February 2005

Keywords: cannabinoid receptors; endocannabinoids; inflammation; neuroprotection; NF-κB; traumatic brain injury

Introduction

Traumatic brain injury leads to secondary damage that includes the release of harmful mediators (e.g. glutamate, reactive oxygen species (ROS), inflammatory cytokines). Protective mechanisms are also set in motion, and recently the endocannabinoid system was proposed to be neuroprotective (van der Stelt *et al*, 2002; Mechoulam, 2002; Mechoulam *et al*, 2002; Grundy *et al*, 2001). Cultured rat hippocampal neurons and cerebral cortical neurons are protected from excitotoxicity or ischemia by cannabinoid receptor agonists (Shen and Thayer, 1998; Sinor *et al*, 2000). In addition, N-methyl-D-aspartate-induced (NMDA) Ca^{2+} flux is reduced by anandamide, while SR141716A, a cannabinoid receptor (CB_1) antagonist, counteracted this activity (Hampson *et al*, 1998). *In vivo* models support these observations. Thus, WIN 55212 (a synthetic cannabinoid) reduced ischemic damage in rat brain (Nagayama *et al*, 1999) and Δ^9-tetrahydrocannabinol (THC), the main psychoactive marijuana constituent, reduced neuronal injury in neonatal rats injected with the Na^+/K^+-ATPase inhibitor ouabain (van der Stelt *et al*, 2001*a*). Hansen *et al* (2002) proposed that *N*-acylethanolamines, particularly anandamide, are neuroprotective and, indeed, anandamide protected rat brain against ouabain-induced neuronal injury (van der Stelt *et al*, 2001*b*). Yet, in contrast, a recent report describes the neurotoxic effects of anandamide in rats, through mechanisms independent of the CB_1 receptor and probably mediated, at least in part, via the vanilloid VR1 receptor (Cernak *et al*, 2004). We have reported that the levels of the endocannabinoid 2-arachidonoylglycerol (2-AG) increased 10-fold within 4 h after closed head injury (CHI) in mice, and that synthetic 2-AG injected after CHI improved outcome (Panikashvili *et al*, 2001). The neuroprotective effect of 2-AG was attenuated by the CB_1 receptor antagonist SR141716A, albeit at relatively high doses. These findings suggest that the neuroprotective effect of 2-AG is apparently cannabinoid receptor mediated.

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The mechanisms of neuroprotection or neurotoxicity by cannabinoids are not yet clear. Some cannabinoids, for example, plant derived (THC), synthetic (WIN 55212) or endogenous (2-AG, anandamide), bind to the CB_1 receptor; others, such as cannabidiol (CBD), a marijuana constituent (Hampson *et al*, 1998), or the synthetic dexanabinol (Shohami and Mechoulam, 2000; Knoller *et al*, 2002) do not. Hence, the effects of cannabinoids could be derived from numerous mechanisms.

Nuclear factor κB (NF-κB), a key regulator of inflammatory response (Perkins, 2000; Karin and Ben-Neriah, 2000) is composed of homo- and heterodimers including p65 and p50. The p65 subunit contains a translocation domain in its C-terminal end. Inactive NF-κB is retained in the cytosol, where its activity is tightly regulated by members of the IκB family. Activation of the IκB kinase by different proinflammatory signals (e.g. endotoxin, tumor necrosis factor-α (TNF-α), IL-1β, oxidative stress; Zingarelli *et al*, 2003) leads to phosphorylation, ubiquitination, and degradation of IκB (Malek *et al*, 2001; Tam and Sen, 2001). Nuclear factor κB, thus released, translocates into the nucleus and activates various genes. Studies from our laboratory recently showed robust transactivation of NF-κB at 24 h up to 8 days after CHI. Inhibition of this activation by melatonin was associated with significant improved outcome (Beni *et al*, 2004).

The present study explores the effect of 2-AG on NF-κB transactivation after CHI in mice. To confirm the role of the CB_1 receptor in neuroprotection, the spontaneous and 2-AG-mediated neurobehavioral recovery and NF-κB activation in CB_1 receptor knockout ($CB_1(-/-)$) mice were compared with those observed in wild-type (WT) mice.

Materials and methods

Animals

The study was performed according to the guidelines of the Institutional Animal Care Committee. (1) Male C57BL/6J mice (Harlan, Israel) weighing 32 to 35 g were used in this study. The animals were divided into groups, treated with 2-AG or vehicle as described further, and killed at different times after CHI. (2) $CB_1(-/-)$ mice were kindly provided by Professor A Zimmer (Bonn, Germany). They were developed as described previously (Zimmer *et al*, 1999). To transfer the CB_1 tm1zim mutation from the MPI2 embryonic stem cell genetic background (129/sv) to a C57BL/6J genetic background, congenic mice were generated by breeding the mutant allele for 10 generations with C57BL/6J mice. Heterozygous mice were bred to yield WT, heterozygous and null mice.

Trauma Model

Mice were subjected to CHI under ether anesthesia, confirmed by loss of pupillary reflex, using a weight-drop device that falls over the left hemisphere, as described elsewhere (Chen *et al*, 1996) and modified by Yatsiv *et al* (2002). In brief, after a sagittal scalp incision, mice were immobilized under a cylindrical calibrated weight drop device. A tipped teflon cone was placed 3 mm lateral to the midline and 1 mm caudal to the left coronal suture, and a metal rod (94 g) was dropped on the cone from a height of 11 to 14 cm (adjusted to body weight) to cause CHI. Sham-treated mice were anesthetized with ether, their scalps were incised, but trauma was not induced.

Evaluation of Functional Outcome

At 1 h after CHI, the functional status of the mice was evaluated according to a set of 10 neurobehavioral tasks (neurological severity score (NSS)) that test reflexes, alertness coordination, and motor abilities (Table 1; Beni-Adani *et al*, 2001). One point was awarded for absence of reflex or failure to perform a particular task. Hence, a score of 10 reflects maximal neurological impairment. Only mice with NSS >4 at 1 h after injury (NSS 1 h) were included in the study. Immediately after evaluation of NSS 1 h, mice were randomly assigned to vehicle or drug treatment (see below), and NSS was evaluated again at 24 h. The extent of spontaneous recovery was calculated as the difference between NSS 1 h and that at 24 h: ΔNSS = NSS (1 h) − NSS (24 h) and compared with that induced by 2-AG treatment.

Cerebral Edema

Cerebral edema was evaluated at 24 h after CHI (time for maximal edema, Chen *et al*, 1996) by determining the tissue water content in the injured brain, as previously described (Chen *et al*, 1996). The percentage of tissue water was calculated as

$$\%H_2O = [(\text{wet weight} - \text{dry weight})/\text{wet weight}] \times 100.$$

Infarct Volume

At 24 h after CHI brains of WT and $CB_1(-/-)$ mice were sliced to 2-mm thick slices using a brain mold. The slices

Table 1 Neurological severity score for head-injured mice

TASK	*NSS*
Presence of mono- or hemiparesis	1
Inability to walk on a 3-cm wide beam	1
Inability to walk on a 2-cm wide beam	1
Inability to walk on a 1-cm wide beam	1
Inability to balance on a 1-cm wide beam	1
Inability to balance on a round stick (0.5-cm wide)	1
Failure to exit a 30-cm diameter circle (for 2 mins)	1
Inability to walk straight	1
Loss of startle behavior	1
Loss of seeking behavior	1
Maximum total	10

One point is awarded for failure to perform a task. NSS at 1 h in the range of 8 to 10: severe CHI.

were placed in a 2% solution of 2,3,5-triphenyltetrazolium chloride (TTC) in PBS and photographed using Stereoscope Stemi SV11 (Zeiss, Germany) and digital photocamera Coolpix E990 (Nikon, Japan). Scion Image-Release Beta 4.0.2 program was used to quantify the infarct volume, to examine whether lacking CB_1 receptor will affect infarct volume.

Electrophoretic Mobility Shift Assay for Nuclear Factor κB DNA Binding

Mice were decapitated 8 and 24 h after CHI or sham operation. Nuclear extracts were prepared as described previously (Beni *et al*, 2004). The injured tissue weighing approximately 100 mg was dissected on ice, transferred briefly into ice-cold 0.32 mol/L sucrose and incubated for 5 to 10 mins. Tissues were homogenized in ice-cold 1:4 (w:v) buffer A (0.5 mol/L sucrose; 10 mmol/L HEPES, pH 7.9; 1.5 mmol/L MgCl2; 10% glycerol; 1 mmol/L EDTA) into which 1 mmol/L DDT, 1 mmol/L PMSF and protease inhibitor cocktail (1:25; Roche Diagnostics; Mannheim, Germany) were added before use. After 15-min incubation on ice, centrifugation at 10,000g for 5 mins at +4°C was performed. Supernatants were discarded and pellets were rewashed with 1:1 (v:v) buffer A and centrifuged at 10,000g for 5 min at +4°C. Supernatants were discarded and nuclear pellets were re-suspended in 1:1 (v:v) ice-cold buffer C (20 mmol/L HEPES, pH 7.9; 25% glycerol ; 0.42 M NaCl; 0.2 mmol/L EDTA), to which 1 mmol/L DDT, 0.5 mmol/L PMSF were added freshly. After 30-min incubation on ice with frequent vortexing, the samples were centrifuged for 20 mins at 15,000g at +4°C, and the supernatants were stored at −80°C until used as nuclear extracts in the EMSA. The consensus sequence for NF-κB was a double-stranded oligonucleotide (5′-AGT TGA GGG GAC TTT CCC AGG C-3′; 3′-TCA ACT CCC CTG AAA GGG TCC G-5′; Promega, E3291; Madison, WI). Oligonucleotides contained 5′-OH blunt ends that were labeled with [γ-^{32}P] (Perkin-Elmer Life Sciences) using T_4 polynucleotide kinase (Promega, M4101) according to the instructions of the manufacturer. The binding reaction mix, containing 10 mmol/L HEPES (pH 7.9), 60 mmol/L KCl, 10% glycerol, 2 μg bovine serum albumin, 0.4 mmol/L DDT, 2 μg poly(dI-dC), 10 μg nuclear protein, and γ-^{32}P-labeled NF-κB (30,000 cpm), was incubated in ice for 1 h. Specificity of the protein-DNA complexes was confirmed by incubation (30 mins) of nuclear extracts with 100-fold excess of unlabeled NF-κB oligonucleotide before the respective γ-^{32}P-labeled probe was added. Supershift assay for NF-κB entailed incubation (30 mins) of nuclear extracts with 3 μl of anti-p65 and anti-p50 monoclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) before addition of the γ-^{32}P-labeled probe. DNA–protein complexes were resolved on a 5% polyacrylamide gel made up in 1 × TGE (50 mmol/L Tris, 400 mmol/L glycine, 2 mmol/L EDTA) at 100 V for 95 mins. The gels were vacuum-dried and exposed to Kodak X-ray films at −80°C. Quantitative data were obtained using Bio-Rad Multi-Analyst (PC Version 1.1). Levels of NF-κB were expressed as the relative optical densities against background within a gel. Each experiment was repeated three to four times, and the data represent the mean of all measurements.

Drugs

2-Arachidonoylglycerol was synthesized in our laboratory according to published procedures (Mechoulam *et al*, 1995). Emulphor was obtained from Sigma Israel. 2-Arachidonoylglycerol was dissolved in anhydrous ethanol:emulphor: saline (1:1:18) and injected intraperitoneally at 100 μl per 10 g body weight at a dose of 5 mg/kg.

Statistical Analysis

Values represent the mean ± s.d. Statistical significance of differences between means was evaluated by the nonparametric Mann–Whitney test for NSS assessment, and by Student's *t*-test for brain water content, and OD measurement. Probability values (P) smaller than 0.05 were considered to be statistically significant.

Results

Spontaneous Recovery after Closed Head Injury in Wild-Type and CB_1 Receptor Deficient Mice

To assess the basal motor activity of $CB_1(-/-)$ mice, the NSS was recorded for naive $CB_1(-/-)$ and WT mice ($n=7$ and 9, respectively). Neurological severity score was higher in the $CB_1(-/-)$ than in the WT mice (1.79 ± 0.58 versus 0.56 ± 0.88, $P=0.008$), indicating some basal deficits, as reported earlier (Zimmer *et al*, 1999). $CB_1(-/-)$ and WT mice were then subjected to CHI and their neurobehavioral outcome was evaluated 1 and 24 h later. Owing to the basal difference in NSS, it is hard to claim that similar values of NSS at 1 h indicates similar severity of injury in both groups, rather, similar NSS 1 h may bias towards more severe injury in the WT. Yet, over the next 24 h the WT mice recovered significantly better than the $CB_1(-/-)$ mice as depicted by their higher ΔNSS (1.89 ± 0.78 versus 0.71 ± 0.76, respectively, $P=0.0164$) (Figure 1A). By expressing the recovery as a difference between the NSS at 1 h and that at 24 h, each mouse serves as it own control, and ΔNSS for the individual mice indeed reflects their post CHI recovery, which is independent of the pre-CHI deficits. Thus, it appears that the $CB_1(-/-)$ mice are more susceptible to the secondary brain damage after CHI than WT mice, and their spontaneous recovery is slower.

Infarct Volume and Edema after Closed Head Injury in CB_1 Receptor Deficient Mice

Using TTC staining 24 h after CHI infarct volume was measured in WT and $CB_1(-/-)$ mice. In spite of the difference in functional recovery, there was no difference in the infarct volumes between the groups

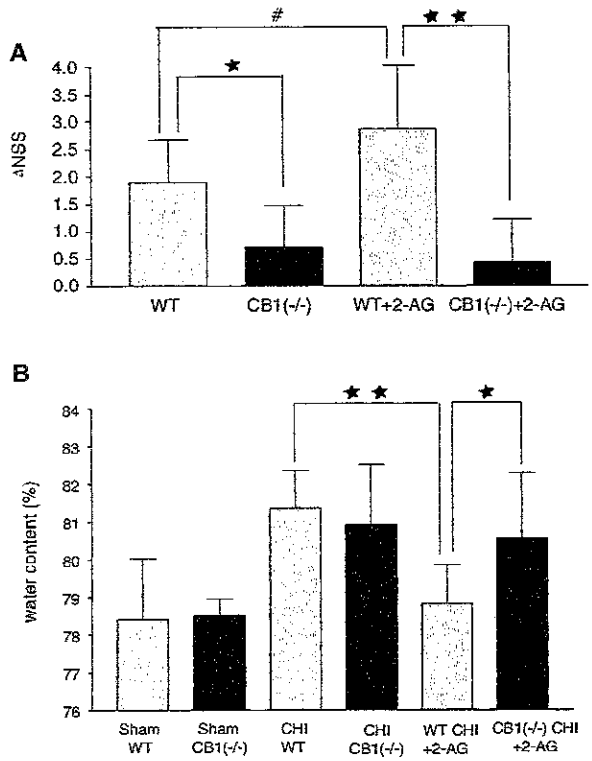

Figure 1 $CB_1(-/-)$ mice display smaller spontaneous recovery after CHI as compared with WT mice, and do not respond to 2-AG therapy. (**A**) Motor function was assessed using the NSS; a neurobehavioral score that awards one point for failure to perform a task. The spontaneous recovery of the $CB_1(-/-)$ ($n=7$; filled bars) was significantly smaller than that of the WT ($n=9$; gray bars). 2-Arachidonoylglycerol treatment, which increased ΔNSS in the WT, was not effective in the $CB_1(-/-)$ ($n=7$/group). $^*P<0.05$ $CB_1(-/-)$ versus WT. $^{**}P<0.05$ $CB_1(-/-)$ versus WT after 2-AG treatment. $^{\#}P<0.05$ effect of 2-AG on WT. (**B**) Basal water content (sham mice) was similar in WT (gray bars) and $CB_1(-/-)$ (filled bars) mice, and a similar increase occurred after CHI. However, treatment with 2-AG effectively reduced edema in the WT, but not in the $CB_1(-/-)$ mice. $^*P<0.05$ WT treated with 2-AG versus $CB_1(-/-)$ treated with 2-AG. $^{**}P<0.05$ WT versus WT treated with 2-AG.

12.3 ± 7.4 versus 15 ± 10.6 respectively, $P=0.51$. Similarly, basal water content in the cortex of nontraumatized $CB_1(-/-)$ mice was similar to that of the WT. After CHI, significant water accumulation was found in both groups (Figure 1B), but edema was not statistically different between these groups (81.37 ± 0.99 versus 80.92 ± 1.59 %).

Effect of Exogenous 2-Arachidonoylglycerol on Neurological Severity Score and Edema in Wild-Type and CB1(−/−) Mice

We next addressed the question whether the protective effects of exogenous 2-AG, which were reported earlier, are mediated via the CB_1 receptor. Wild-type and $CB_1(-/-)$ mice were treated with 2-AG 1 h after CHI. 2-Arachidonoylglycerol significantly improved the neurobehavioral status of the WT, but not of the $CB_1(-/-)$ mice ($\Delta NSS=2.86\pm1.46$ versus 0.43 ± 0.79, respectively; $P=0.0041$, Figure 1A). Moreover, treatment with 2-AG effectively reduced edema only in the WT mice (Figure 1B, 81.37 ± 0.99, where water content was down to normal (78.81 ± 1.046), but not in the $CB_1(-/-)$, where it remained as high as in the nontreated mice (80.57 ± 1.71, $P=0.0308$ versus WT treated with 2-AG). It should be noted that basal water content, namely in sham-operated mice, in $CB_1(-/-)$ mice was similar to that in the WT (78.52 ± 0.43 versus 78.41 ± 1.61).

Effect of 2-Arachidonoylglycerol on Nuclear Factor κB Translocation

Since inflammation and oxidative stress are major components of the post-CHI responses, we decided to investigate the effect of 2-AG on a key transcription factor of these pathways. The pattern of NF-κB–DNA binding of nuclear extract prepared 24 h after CHI is described in Figure 2A. Three bands were obtained and their specificity was proved by adding excess of unlabeled (cold) oligonucleotide NF-κB and specific antibodies for the p65 and p50 subunits. Supershift assays show that the lower band is mostly composed of p50 and the upper band is p50–p65 heterodimer. Closed head injury in control mice (lanes 5 and 9) induced significant increase in NF-κB–DNA binding as compared with sham (lane 10) (optical density 0.84 ± 0.29 versus 0.27 ± 0.15; Figure 2B). Treatment of WT mice with 2-AG (lanes 1, 3, 6, 8) completely abolished this increase (0.37 ± 0.27; $P<0.001$). We next investigated NF-κB transactivation after CHI in the $CB_1(-/-)$ mice, with and without 2-AG treatment. Figure 3 depicts the results of this study showing that, like in the WT, there is approximately three-fold increase of NF-κB activation 24 h after CHI in $CB_1(-/-)$ mice (lanes 2 and 9 versus 6). However, in contrast to WT mice, treatment with 2-AG in $CB_1(-/-)$ was not effective (lanes 3, 5, 8 and 10), and did not reduce activity of this transcription factor (optical density 1.37 ± 0.8 versus 1.53 ± 0.62; Figure 3B).

Discussion

The present study extends our findings on the beneficial effect of 2-AG after CHI in mice, shows the role of the CB_1 receptor in mediating these effects and provides some mechanistic clue to its action. We reported earlier (Panikashvili *et al*, 2001) that 2-AG treatment decreased edema formation, hippocampal cell death, infarct volume and neurological dysfunction. The CB_1 antagonist SR141716A partly inhibited 2-AG protection, albeit at a relatively high dose

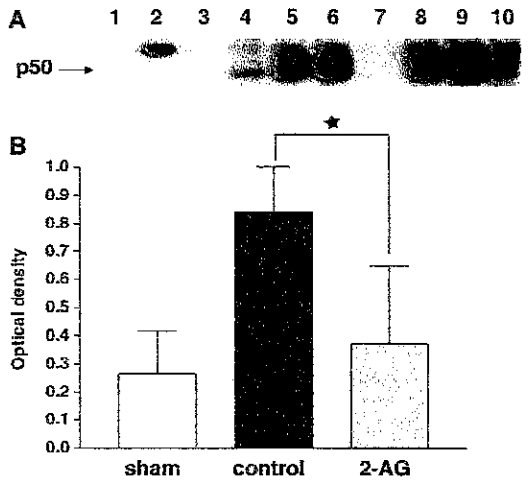

Figure 2 2-Arachidonoylglycerol inhibits NF-κB nuclear translocation 24 h after CHI. **(A)** Nuclear factor κB DNA binding was determined in brain nuclear extracts, using EMSA. Samples were isolated 24 h after CHI from control (vehicle-treated mice; lanes 5,9), from 2-AG-treated mice (lanes 1,3,6,8) and from sham-operated mice (lane 10); lane 7: competition with access of unlabeled NF-κB oligonucleotide. Supershift assay of a vehicle-treated sample pre-incubated with anti-p65 (lane 4) or anti-p50 (lane 2) antibodies (see text). **(B)** Quantitative data, expressed as relative optical densities, were obtained from the gel shown in **(A)**, using the Bio-Rad Multi-Analyst. Each experiment was repeated four to five times, and the data represent the mean ± s.d. of all the measurements. $^*P<0.05$ versus control.

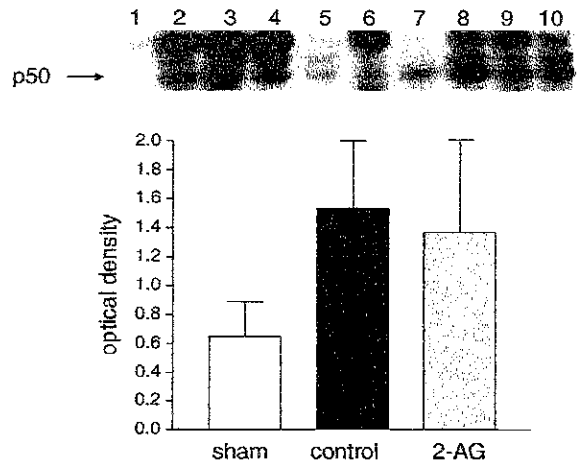

Figure 3 2-Arachidonoylglycerol does not abolish the increase in NF-κB transactivation 24 h after CHI in $CB_1(-/-)$ mice. **(A)** A pattern of EMSA similar to that shown in Figure 2 for the WT mice was found for the $CB_1(-/-)$ control CHI mice (lanes 2,4,9). Treatment with 2-AG (lanes 3,5,8,10) had no effect on NF-κB nuclear translocation. Anti p65 was added to mouse in lane 7, competition with protein excess is shown in lane 1, and sham mouse is shown in lane 6. **(B)** Quantitative data of Figure 3A, expressed as relative optical densities, calculated as described in Figure 2. Sham mice—empty bar ($n=2$); control CHI-filled bar ($n=4$) and 2-AG treated mice—gray bar ($n=5$).

(20 mg/kg). Since the major brain cannabinoid receptor is CB_1, and the specificity of SR141716A as a pure CB_1 antagonist is controversial, we decided to use the CB_1 knockout mice to study the role of CB_1 receptor in neuroprotection. As reported by Zimmer *et al* (1999), we also found that the naive $CB_1(-/-)$ mice display some motor deficits, unrelated to the trauma. As expected, after injury, their spontaneous recovery was extremely slow, as compared with the WT mice, suggesting that the endogenous cannabinoids play a role in the spontaneous recovery after CHI. In another experiment (preliminary data, not shown), NSS was evaluated at 3 and 7 days, and the same pattern was found, namely, ΔNSS of the CB1(−/−) mice remained below 1 during the whole period, while that of the WT slowly, but consistently, increased. These findings agree with those of Parmentier-Batteur *et al* (2002), who showed increased severity of stroke in $CB_1(-/-)$ mice. However, the present findings do not show greater infarct volume or edema in the $CB_1(-/-)$, indicating that other endogenous mechanisms are probably involved. To further confirm the role of the CB_1 receptor in 2-AG neuroprotection, WT and $CB_1(-/-)$ mice were treated with exogenous 2-AG. As expected, no beneficial effects, neither on neurobehavior nor on edema formation, were noted in the $CB_1(-/-)$ mice, in contrast to a significant effect on the WT, confirming our earlier reports (Panikashvili *et al*, 2001).

Proinflammatory cytokines play a crucial role in traumatic brain injury. Tumor necrosis factor-α is released early (within 1 to 4 h) after CHI (Shohami *et al*, 1997; Stover *et al*, 2000) and acts on specific receptors. On binding, the cytosolic portions of both TNF receptors recruit multiple intracellular adapter proteins that activate the transcription factor NF-κB, starting with hydrolysis of the inhibitory protein IκB that allows the p65/p50 complex to translocate to the nucleus and to regulate the expression of various (Baeuerle and Henkel, 1994). We have recently reported that inhibition of NF-κB transactivation after CHI (at 1 to 8 days) is associated with better functional outcome (Beni *et al*, 2004), and therefore decided to investigate the effect of 2-AG on NF-κB activation after CHI. Indeed, treatment with 2-AG completely abolished the robust activation of this transcription factor, which is a key event in the proinflammatory signaling after CHI.

Closed head injury-induced activation of NF-κB in the $CB_1(-/-)$ mice was three to four fold higher than in the respective noninjured (sham) $CB_1(-/-)$ mice, similar to that observed in the WT. This suggests that the endogenous 2-AG does not affect the inflammatory signaling. However, in response to exogenous 2-AG, WT and $CB_1(-/-)$ mice differed dramatically, and the latter did not respond at all to 2-AG treatment. In addition to their antiinflammatory activities, cannabinoids act also as antioxidants. Cannabidiol is a potent antioxidative agent (Hampson *et al*, 1998) and the nonpsychotropic

synthetic cannabinoid, dexanabinol (HU-211), has also antioxidative properties (Shohami and Mechoulam, 2000). 2-Arachidonoylglycerol was also shown to suppress formation of ROS and TNF-α by murine macrophages *in vitro* after stimulation with lipopolysaccharide (Gallily *et al*, 2000). The antioxidant properties of 2-AG may well add to its profile as neuroprotectant and inhibitor of NF-κB transactivation. Oxidative stress is one of the major components in the pathophysiology of traumatic brain injury (Lewen *et al*, 2000), and ample evidence suggests that ROS also regulate signal transduction pathways such as the NF-κB and AP-1 (Vollgraf *et al*, 1999). Thus, taken together, anti-inflammatory and antioxidant properties of 2-AG may either add or synergize to enhance its activity as a neuroprotective agent.

Cannabinoids produce a variety of neurobehavioral effects, and a major focus of cannabinoid research has been the substantiation of the assumption that the pharmacological actions of cannabinoids are receptor-mediated. Indeed, the $CB_1(-/-)$ mice showed spontaneous phenotypes, including hypoactivity, reduced locomotion and rearing, supraspinal hypoalgesia, increased mortality (Zimmer *et al*, 1999), spontaneous reduction in feeding behavior (Di Marzo *et al*, 2001), changes in male hormone balance (Paria *et al*, 2001), and suckling behavior within 1 day of birth Fride *et al* (2001). For the most part, results observed in mice treated with selective CB_1 receptor antagonists mimic the findings observed in the transgenic animals. However, developmental changes may occur to compensate for the lack of CB_1 receptors, as has been suggested from studies of neuropeptide expression (Steiner *et al*, 1999). Our current findings fit into this body of evidence regarding the importance of the endocannabinoid system, acting via the CB_1 receptor, in brain function under physiological and pathological conditions. Recently, van der Stelt *et al* (2002) and Mechoulam (2002) discussed the role of the endocannabinoid system as a general endogenous protection system. The pharmacological picture is further complicated by the fact that there seems to be species differences. While we found 2-AG production to be enhanced in mice after CHI, Panikashvili *et al* (2001) and Sugiura *et al* (2000) saw elevation in 2-AG level in picrotoxin-administered rat brain; Hansen *et al* (2001), in contrast, found anandamide and not 2-AG enhancement in rats after TBI.

Several pharmacological agents have been described to inhibit NF-κB at one or multiple activation steps of the signaling pathway. These agents include proteasome inhibitors, glucocorticoids (such as dexamethasone), nonsteroidal anti-inflammatory drugs and anti-inflammatory cytokines (Zingarelli *et al*, 2003). Cannabinoids were found to be very effective in different models of inflammation. In the 1970s, Sofia *et al* (1973*a*,*b*, 1974) showed robust anti-inflammatory effects of crude marijuana extract, of the active marijuana constituent THC, as well as of the marijuana nonpsychoactive constituents CBD and cannabinol (CBN) in paw edema inflammation model in rats. Some of these effects were later shown to be CB_1 or CB_2 receptors mediated (Clayton *et al*, 2002; Hanus *et al*, 1999). Cannabinol and 2-AG were shown to inhibit IL-2 expression in activated thymocytes through inhibition of NF-κB (Herring and Kaminski, 1999; Ouyang *et al*, 1998). Yet, the molecular mechanisms of all these beneficial effects remained unclear. The complexity and controversy in the field of endocannabinoids is further shown in a recent report on the 'dark side' of anandamide that describes its toxic effects, both *in vitro* and *in vivo*, in rats (Cernak *et al*, 2004). This study suggests that mechanisms independent of the CB_1 receptor, probably vanilloid receptor VR1-mediated, are involved in anandamide's neurotoxicity.

In conclusion, we report for the first time that the endocannabinoid 2-AG exerts neuroprotection after traumatic brain injury, at least in part, by inhibition of NF-κB transactivation through CB_1 receptors. We suggest to further study drugs of similar pharmacological profile as novel candidates for treatment of traumatic brain injury.

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US006630507B1

(12) **United States Patent**
Hampson et al.

(10) **Patent No.: US 6,630,507 B1**
(45) **Date of Patent: Oct. 7, 2003**

(54) **CANNABINOIDS AS ANTIOXIDANTS AND NEUROPROTECTANTS**

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(73) Assignee: **The United States of America as represented by the Department of Health and Human Services**, Washington, DC (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

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(86) PCT No.: **PCT/US99/08769**

§ 371 (c)(1),
(2), (4) Date: **Feb. 2, 2001**

(87) PCT Pub. No.: **WO99/53917**

PCT Pub. Date: **Oct. 28, 1999**

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(51) **Int. Cl.**[7] **A61K 31/35**
(52) **U.S. Cl.** **514/454**
(58) **Field of Search** 514/454

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Primary Examiner—Kevin E. Weddington
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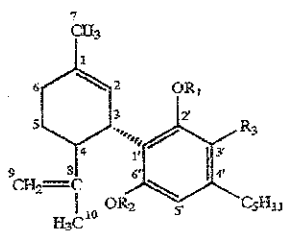
(57) **ABSTRACT**

Cannabinoids have been found to have antioxidant properties, unrelated to NMDA receptor antagonism. This new found property makes cannabinoids 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. A particular disclosed class of cannabinoids useful as neuroprotective antioxidants is formula (I) wherein the R group is independently selected from the group consisting of H, CH_3, and $COCH_3$.

(I)



26 Claims, 7 Drawing Sheets

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FIG. 1

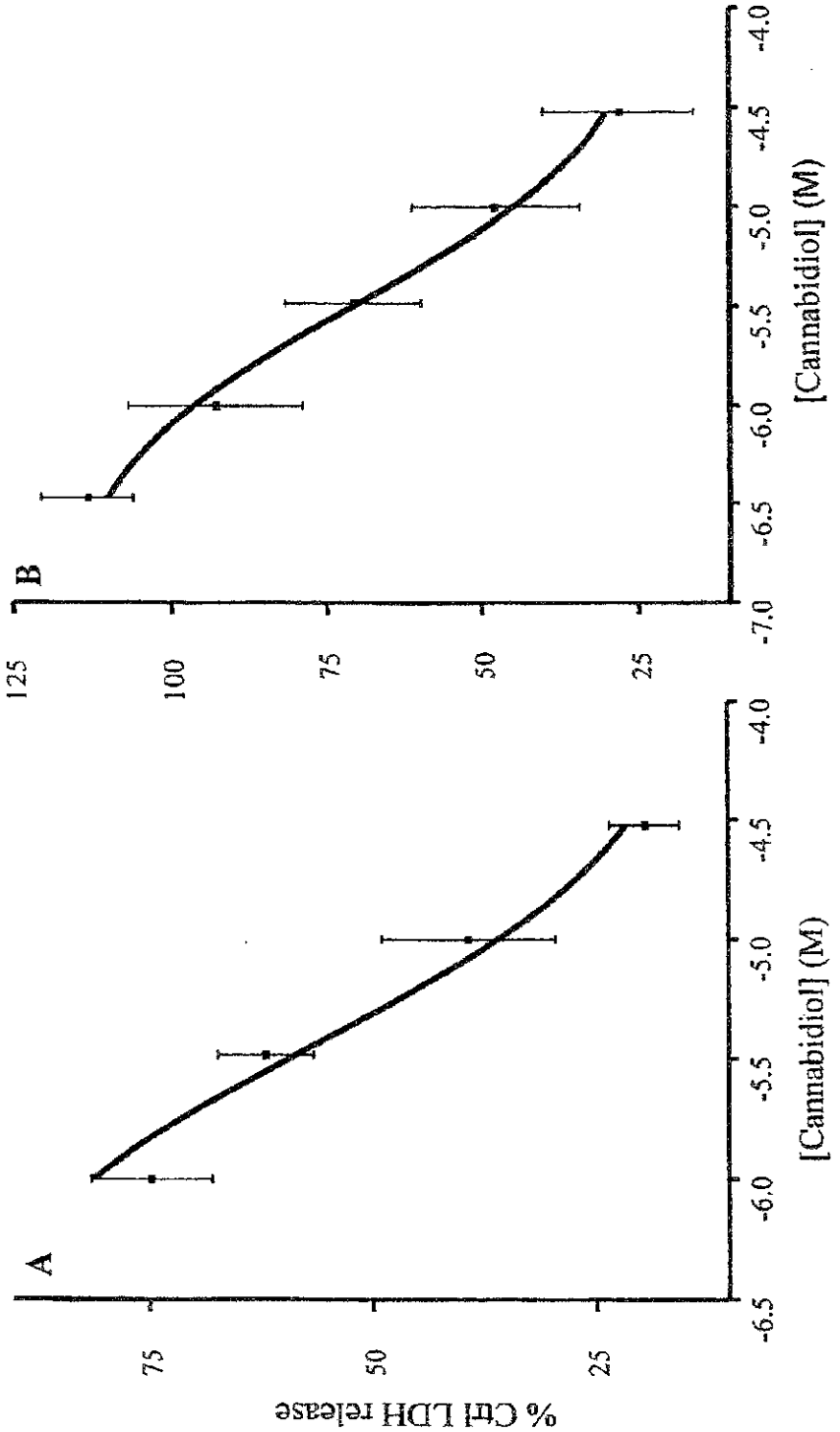

A
B
% Ctrl LDH release
[Cannabidiol] (M)
[Cannabidiol] (M)
25
50
75
100
125
-6.5
-6.0
-5.5
-5.0
-4.5
-4.0
-7.0

FIG. 2

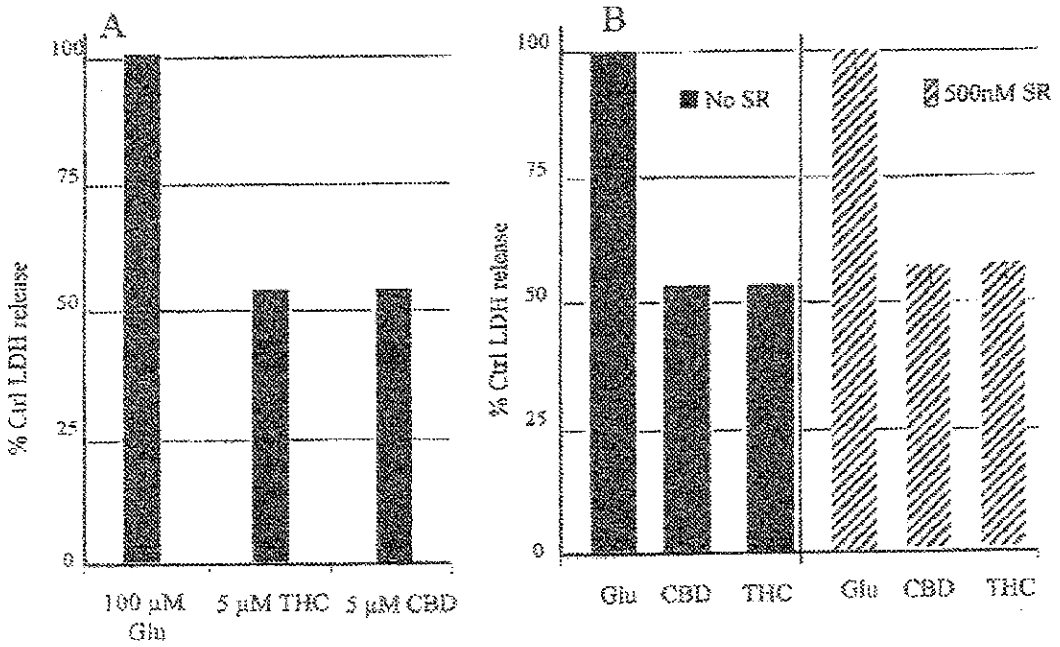

A
100
75
50
25
0
% Ctrl LDH release
100 μM Glu
5 μM THC
5 μM CBD
B
100
75
50
25
0
% Ctrl LDH release
No SR
500nM SR
Glu
CBD
THC
Glu
CBD
THC

FIG. 3

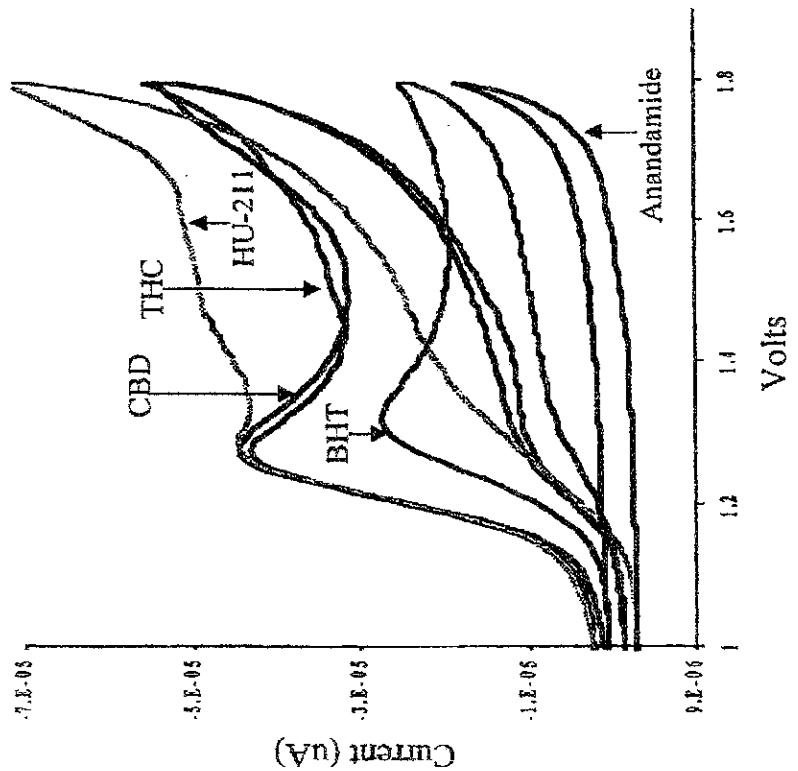

Current (uA)
Volts
CBD
THC
HU-211
BHT
Anandamide

FIG. 4

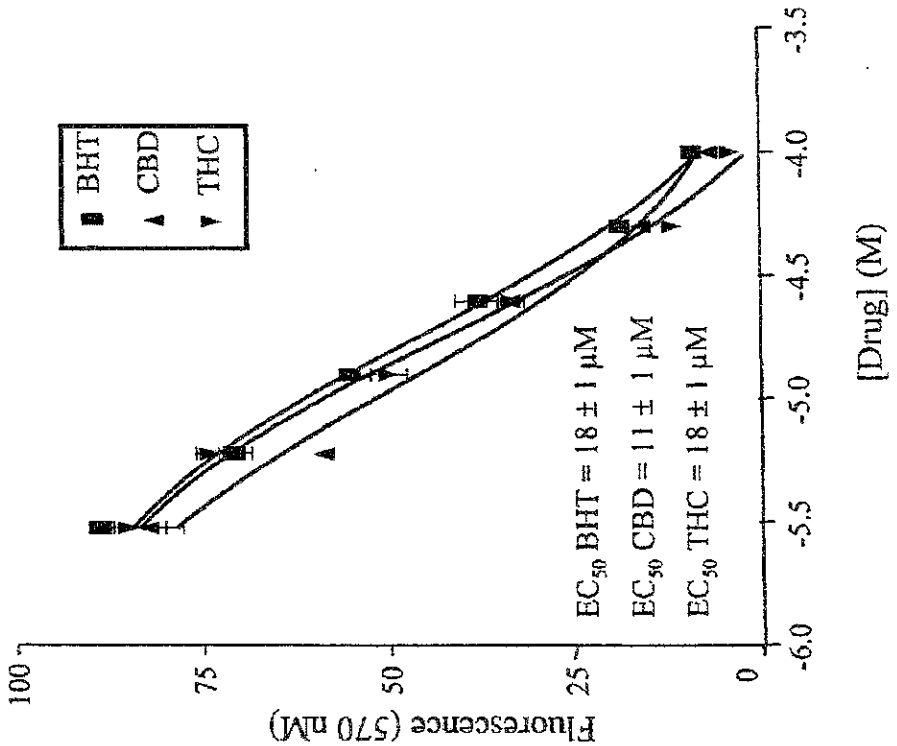

Fluorescence (570 nM)
[Drug] (M)
BHT
CBD
THC
EC50 BHT = 18 ± 1 µM
EC50 CBD = 11 ± 1 µM
EC50 THC = 18 ± 1 µM

FIG. 5

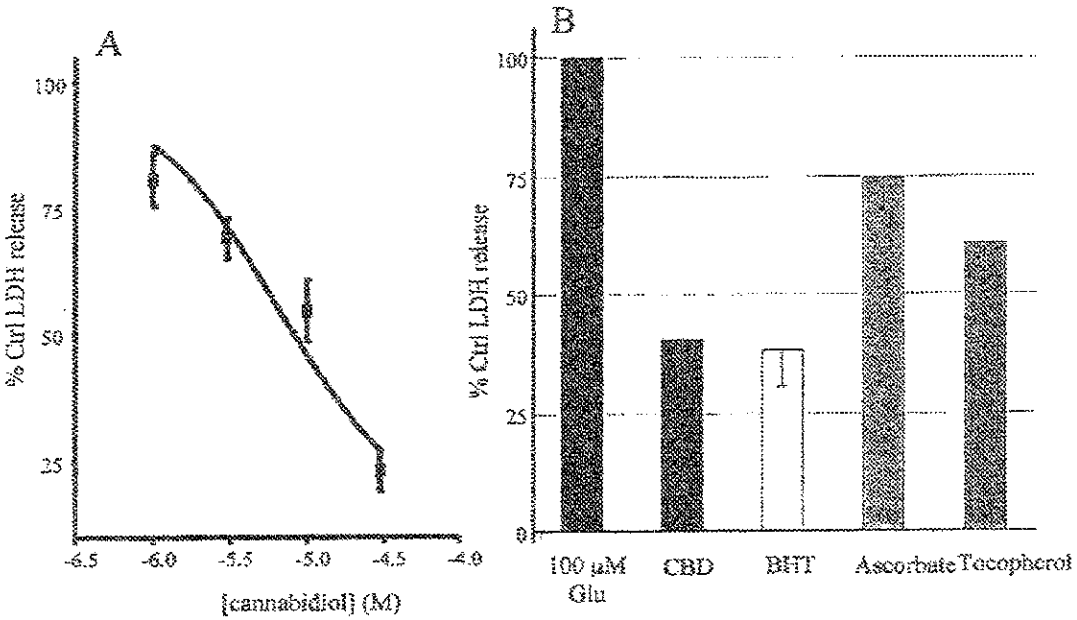

A
100
75
50
25
% Ctrl LDH release
-6.5
-6.0
-5.5
-5.0
-4.5
-4.0
[cannabidiol] (M)
B
100
75
50
25
0
% Ctrl LDH release
100 μM Glu
CBD
BHT
Ascorbate
Tocopherol

FIG. 6

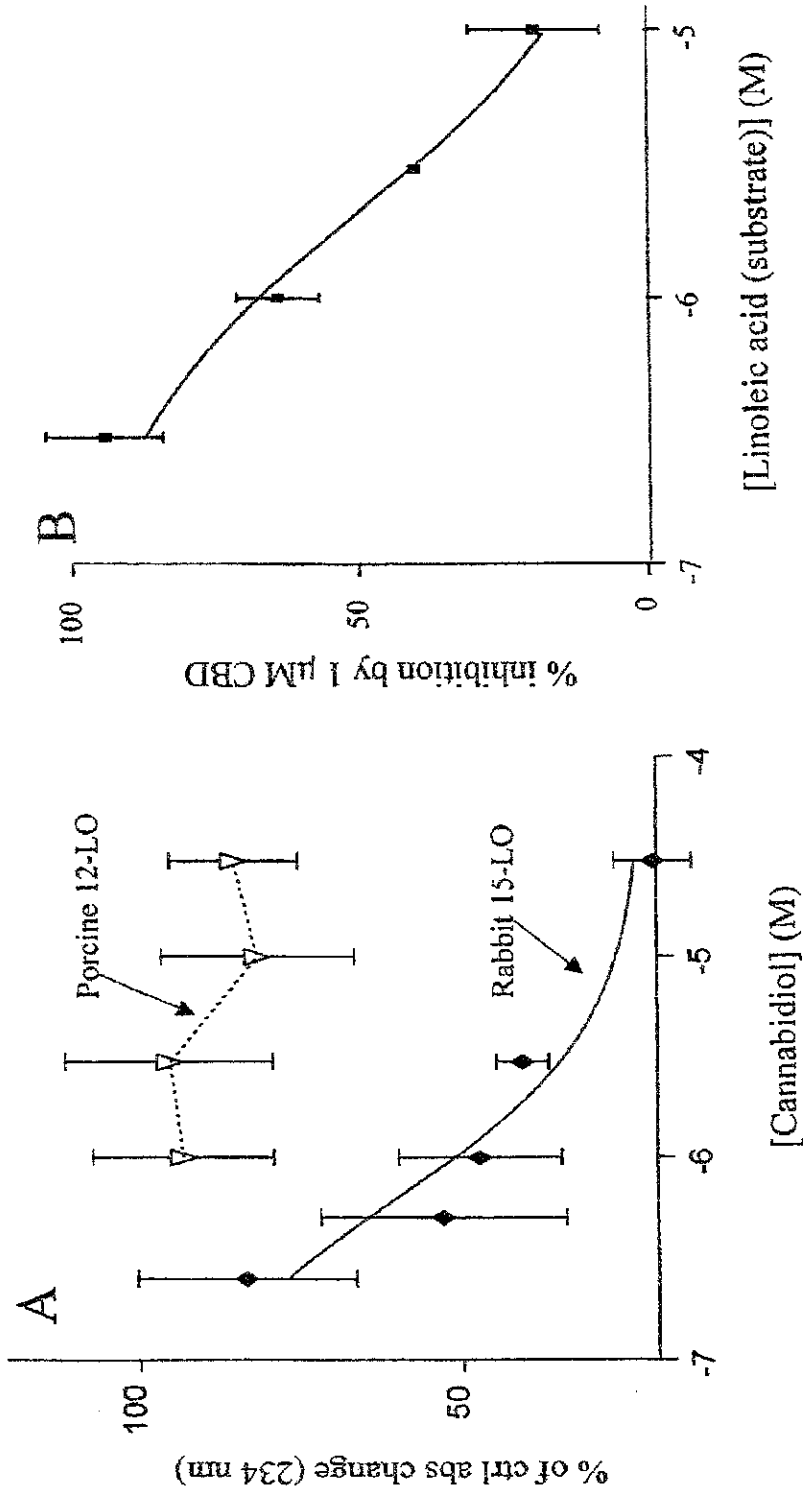

A
% of ctrl abs change (234 nm)
100
50
Porcine 12-LO
Rabbit 15-LO
-7
-6
-5
-4
[Cannabidiol] (M)
B
% inhibition by 1 μM CBD
100
50
0
-7
-6
-5
[Linoleic acid (substrate)] (M)

FIG. 7

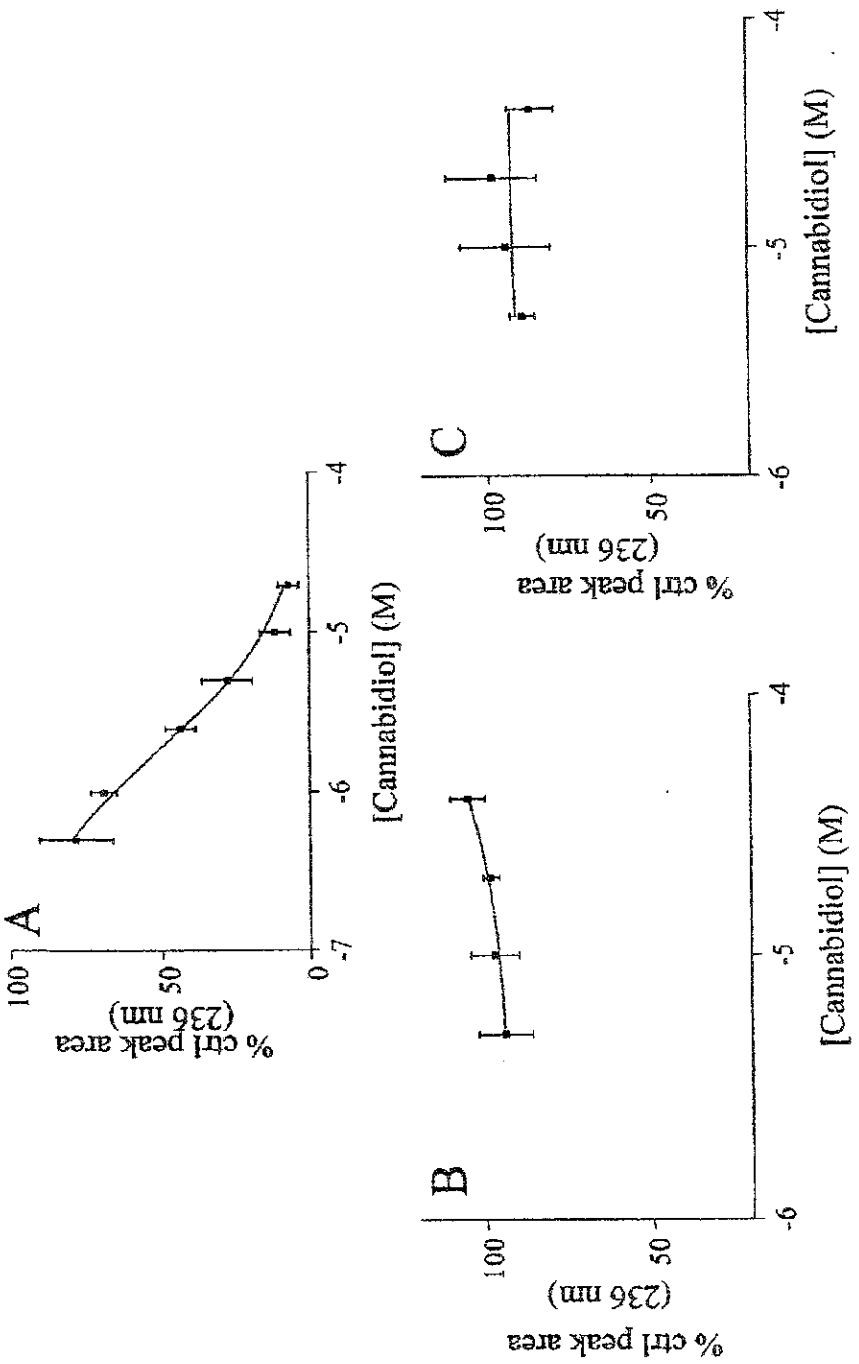
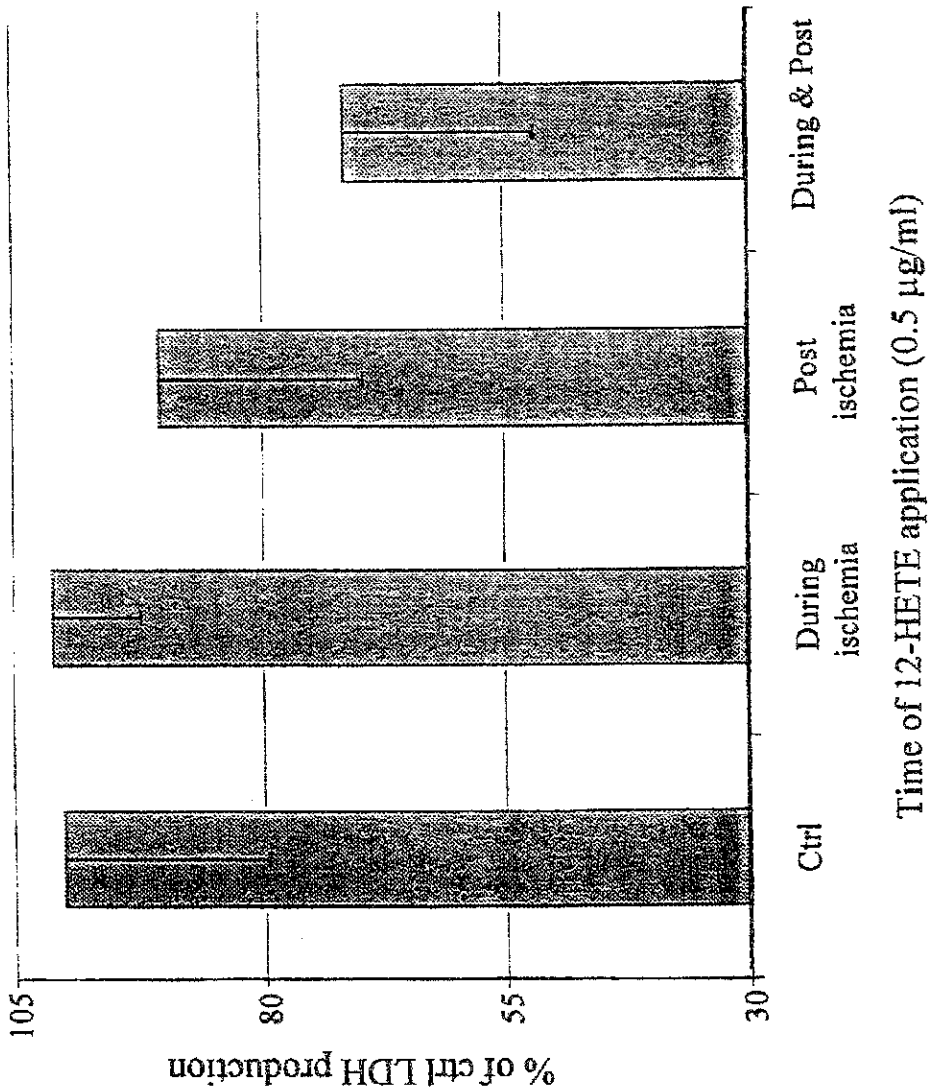


FIG. 8


105
80
55
30
% of ctrl LDH production
Ctrl
During ischemia
Post ischemia
During & Post
Time of 12-HETE application (0.5 µg/ml)

CANNABINOIDS AS ANTIOXIDANTS AND NEUROPROTECTANTS

This application is a 371 of PCT/US99/08769 filed Apr. 21, 1999, which claims benefit of No. 60/082,589 filed Apr. 21, 1998, which claims benefit of No. 60/095,993 filed Aug. 10, 1998.

FIELD OF THE INVENTION

The present invention concerns pharmaceutical compounds and compositions that are useful as tissue protectants, such as neuroprotectants and cardioprotectants. The compounds and compositions may be used, for example, in the treatment of acute ischemic neurological insults or chronic neurodegenerative diseases.

BACKGROUND OF THE INVENTION

Permanent injury to the central nervous system (CNS) occurs in a variety of medical conditions, and has been the subject of intense scientific scrutiny in recent years. It is known that the brain has high metabolic requirements, and that it can suffer permanent neurologic damage if deprived of sufficient oxygen (hypoxia) for even a few minutes. In the absence of oxygen (anoxia), mitochondrial production of ATP cannot meet the metabolic requirements of the brain, and tissue damage occurs. This process is exacerbated by neuronal release of the neurotransmitter glutamate, which stimulates NMDA (N-methyl-D-aspartate), AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionate) and kainate receptors. Activation of these receptors initiates calcium influx into the neurons, and production of reactive oxygen species, which are potent toxins that damage important cellular structures such as membranes, DNA and enzymes.

The brain has many redundant blood supplies, which means that its tissue is seldom completely deprived of oxygen, even during acute ischemic events caused by thromboembolic events or trauma. A combination of the injury of hypoxia with the added insult of glutamate toxicity is therefore believed to be ultimately responsible for cellular death. Hence if the additive insult of glutamate toxicity can be alleviated, neurological damage could also be lessened. Anti-oxidants and anti-inflammatory agents have been proposed to reduce damage, but they often have poor access to structures such as the brain (which are protected by the blood brain barrier).

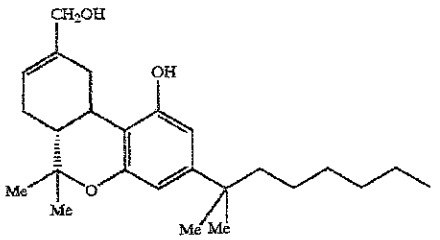
Given the importance of the NMDA, AMPA and kainate receptors in the mechanism of injury, research efforts have focused on using antagonists to these receptors to interfere with the receptor mediated calcium influx that ultimately leads to cellular death and tissue necrosis. In vitro studies using cultured neurons have demonstrated that glutamate receptor antagonists reduce neurotoxicity, but NMDA and AMPA/kainate receptor antagonists have different effects. Antagonists to NMDAr prevent neurotoxicity if present during the glutamate exposure period, but are less effective if added after glutamate is removed. In contrast, AMPA/kainate receptor antagonists are not as effective as NMDA antagonists during the glutamate exposure period, but are more effective following glutamate exposure.

Some of the research on these antagonists has focused on cannabinoids, a subset of which have been found to be NMDA receptor antagonists. U.S. Pat. No. 5,538,993 (3S, 4S-delta-6-tetrahydrocannabinol-7-oic acids), U.S. Pat. No. 5,521,215 (sterospecific (+) THC enantiomers), and U.S. Pat. No. 5,284,867 (dimethylheptyl benzopyrans) have reported that these cannabinoids are effective NMDA receptor blockers. U.S. Pat. No. 5,434,295 discloses that the 1,1 dimethylheptyl (DMH) homolog of [3R,4R]-7-hydroxy-

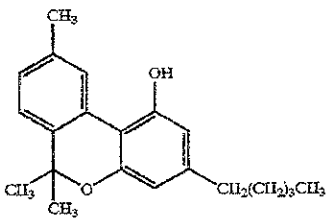
Δ^6THC (known as HU-210) is a superpotent cannabinoid receptor agonist with cannabinomimetic activity two orders of magnitude greater than the natural Δ^9 THC. The HU-210 dimethylheptyl cannabinoid, has severe side effects, including fatigue, thirst, headache, and hypotension. *J. Pharmacol. Sci.* 60:1433–1457 (1971). Subjects who received this synthetic cannabinoid with a dimethylheptyl group experienced marked psychomotor retardation, and were unwilling or incapable of assuming an erect position.

In contrast to HU-210, the (−)(3R,4R) THC-DMH enantiomer (known as HU-211) displays low affinity to the cannabinoid receptors, but retains NMDA receptor antagonist neuroprotective activity.

HU-211

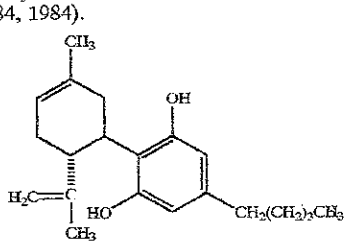


THC (tetrahydrocannabinol) is another of the cannabinoids that has been shown to be neuroprotective in cell cultures, but this protection was believed to be mediated by interaction at the cannabinoid receptor, and so would be accompanied by undesired psychotropic side effects.



THC

Although it has been unclear whether cannabimimetic activity plays a role in neuroprotection against glutamate induced neurological injury, the teaching in this field has clearly been that a cannabinoid must at least be an antagonist at the NMDA receptor to have neuroprotective effect. Hence cannabidiol (2-[3-methyl-6-(1-methylethenyl)-2-cyclohexen-1-yl]-5-pentyl-1,3-benzenediol or CBD), a cannabinoid devoid of psychoactive effect (*Pharm. Rev.* 38:21–43, 1986), has not been considered useful as a neuroprotectant. Cannabidiol has been studied as an antiepileptic (Carlini et al., *J. Clin. Pharmacol.* 21:417S–427S, 1981; Karler et al., *J. Clin. Pharmacol.* 21:437S–448S, 1981, Consroe et al., *J. Clin Phannacol.* 21:428S–436S, 1981), and has been found to lower intraocular pressure (Colasanti et al, *Exp. Eye Res.* 39:251–259, 1984 and *Gen. Pharmac.* 15:479–484, 1984).



Cannabidiol (CBD)

No signs of toxicity or serious side effects have been observed following chronic administration of cannabidiol to healthy volunteers (Cunha et al., *Pharmacology* 21:175–185, 1980), even in large acute doses of 700 mg/day (Consroe et al., *Pharmacol. Biochem. Behav.* 40:701–708, 1991) but cannabidiol is inactive at the NMDA receptor. Hence in spite of its potential use in treating glaucoma and seizures, cannabidiol has not been considered a neuroprotective agent that could be used to prevent glutamate induced damage in the central nervous system.

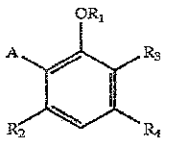
SUMMARY OF THE INVENTION

It is an object of this invention to provide a new class of antioxidant drugs, that have particular application as neuroprotectants, although they are generally useful in the treatment of many oxidation associated diseases.

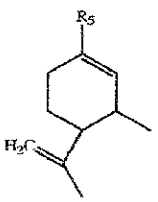
Yet another object of the invention is to provide a subset of such drugs that can be substantially free of psychoactive or psychotoxic effects, are substantially non-toxic even at very high doses, and have good tissue penetration, for example crossing the blood brain barrier.

It has surprisingly been found that cannabidiol and other cannabinoids can function as neuroprotectants, even though they lack NMDA receptor antagonist activity. This discovery was made possible because of the inventor's recognition of a previously unanticipated antioxidant property of the cannabinoids in general (and cannabidiol in particular) that functions completely independently of antagonism at the NMDA, AMPA and kainate receptors. Hence the present invention includes methods of preventing or treating diseases caused by oxidative stress, such as neuronal hypoxia, by administering a prophylactic or therapeutically effective amount of a cannabinoid to a subject who has a disease caused by oxidative stress.

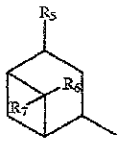
The cannabinoid may be a cannabinoid other than THC, HU-210, or other potent cannabinoid receptor agonists. The cannabinoid may also be other than HU-211 or any other NMDA receptor antagonist that has previously been reported. A potent cannabinoid receptor agonist is one that has an EC_{50} at the cannabinoid receptor of 50 nM or less, but in more particular embodiments 190 nM or 250 nM or less. In disclosed embodiments the cannabinoid is not psychoactive, and is not psychotoxic even at high doses. In some particularly disclosed embodiments, the cannabinoid is selected from the group:



where A is aryl, and particularly

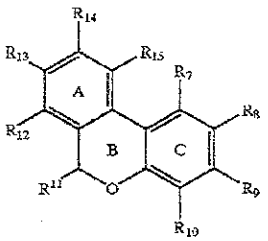


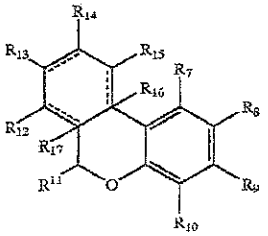
but not a pinene such as:

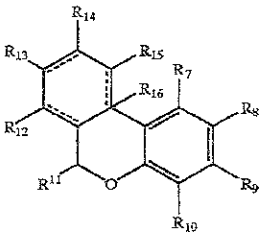


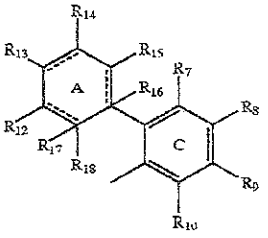
and the R_1–R_5 groups are each independently selected from the groups of hydrogen, lower substituted or unsubstituted alkyl, substituted or unsubstituted carboxyl, substituted or unsubstituted alkoxy, substituted or unsubstituted alcohol, and substituted or unsubstituted ethers, and R_6–R_7 are H or methyl. In particular embodiments, there are no nitrogens in the rings, and/or no amino substitutions on the rings.

In other embodiments, the cannabinoid is one of the following:









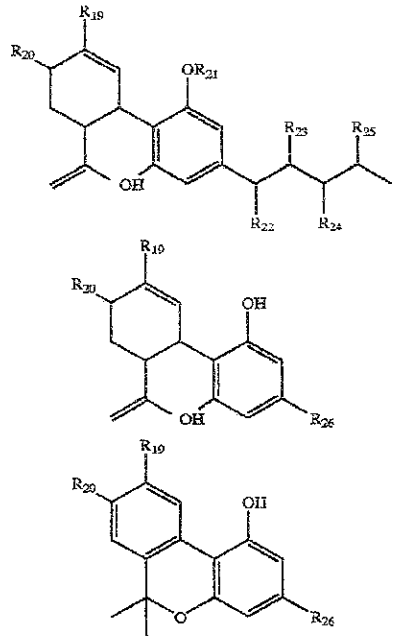
where there can be 0 to 3 double bonds on the A ring, as indicated by the optional double bonds indicated by dashed lines on the A ring. The C ring is aromatic, and the B ring can be a pyran. Particular embodiments are dibenzo pyrans and cyclohexenyl benzenediols. Particular embodiments of the cannabinoids of the present invention may also be highly lipid soluble, and in particular embodiments can be dis-

solved in an aqueous solution only sparingly (for example 10 mg/ml or less). The octanol/water partition ratio at neutral pH in useful embodiments is 5000 or greater, for example 6000 or greater. This high lipid solubility enhances penetration of the drug into the CNS, as reflected by its volume of distribution (V_d) of 1.5 L/kg or more, for example 3.5 L/kg, 7 L/kg, or ideally 10 L/kg or more, for example at least 20 L/kg. Particular embodiments may also be highly water soluble derivatives that are able to penetrate the CNS, for example carboxyl derivatives.

R_{7-18} are independently selected from the group of H, substituted or unsubstituted alkyl, especially lower alkyl, for example unsubstituted C_1–C_3 alkyl, hydroxyl, alkoxy, especially lower alkoxy such as methoxy or ethoxy, substituted or unsubstituted alcohol, and unsubstituted or substituted carboxyl, for example COOH or $COCH_3$. In other embodiments R_{7-18} can also be substituted or unsubstituted amino, and halogen.

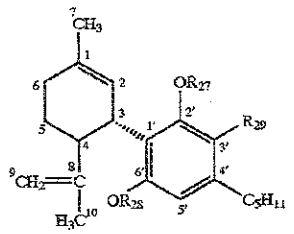
The cannabinoid has substantially no binding to the NMDAr (for example an IC_{50} greater than or equal to 5 μM or 10 μM), has substantially no psychoactive activity mediated by the cannabinoid receptor (for example an IC_{50} at the cannabinoid receptor of greater than or equal to 300 nM, for example greater than 1 μM and a K_i greater than 250 nM, especially 500–1000 nM, for example greater than 1000 nM), and antioxidant activity, as demonstratable by the Fenton reaction or cyclic voltametry.

In other particular embodiments, the cannabinoids are one of the following:

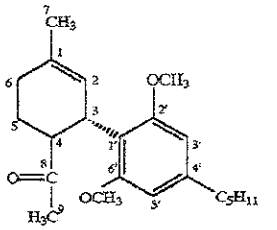


where R_{19} is substituted or unsubstituted alkyl, such as lower alkyl (for example methyl), lower alcohol (such as methyl alcohol) or carboxyl (such as carboxylic acid) and oxygen (as in =O); R_{20} is hydrogen or hydroxy; R_{21} is hydrogen, hydroxy, or methoxy; R_{22} is hydrogen or hydroxy; R_{23} is hydrogen or hydroxy; R_{24} is hydrogen or hydroxy; R_{25} is hydrogen or hydroxy; and R_{26} is substituted or unsubstituted alkyl (for example n-methyl alkyl), substituted or unsubstituted alcohol, or substituted or unsubstituted carboxy.

In yet other embodiments of the invention, the cannabinoids are

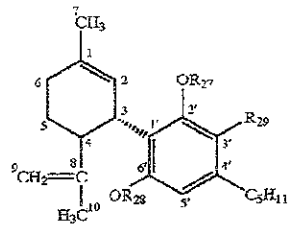


wherein numbering conventions for each of the ring positions are shown, and R_{27}, R_{28} and R_{29} are independently selected from the group consisting of H, unsubstituted lower alkyl such as CH_3, and carboxyl such as $COCH_3$. Particular examples of nonpsychoactive cannabinoids that fall within this definition are cannabidiol and



and other structural analogs of cannabidiol.

In more particular embodiments, the cannabinoid is used to prevent or treat an ischemic or neurodegenerative disease in the central nervous system of a subject, by administering to the subject a therapeutically effective amount of a cannabinoid to protect against oxidative injury to the central nervous system. The cannabinoid may be any of the compounds set forth above, or more specifically



wherein R_{27}, R_{28} and R_{29} are independently selected from the group consisting of H, lower alkyl such as CH_3, and carboxyl such as $COCH_3$, and particularly wherein

a) R_{27}=R_{28}=R_{29}=H

b) R_{27}=R_{29}=H; R_{28}=CH_3

c) R_{27}=R_{28}=CH_3; R_{29}=H

d) R_{27}=R_{28}=$COCH_3$; R_{29}=H

e) R_{27}=H; R_{28}=R_{29}=$COCH_3$

When R_{27}=R_{28}=R_{29}=H, then the compound is cannabidiol. When R_{27}=R_{29}=H and R_{28}=CH_3, the compound is CBD monomethyl ether. When R_{27}=R_{28}=CH_3 and R_{29}=H, the compound is CBD dimethyl ether. When R_{27}=R_{28}=$COCH_3$ and R_{29}=H, the compound is CBD diacetate. When R_{27}=H and R_{28}=R_{29}=$COCH_3$, the compound is CBD monoacetate. The ischemic or neurodegenerative disease may be, for

example, an ischemic infarct, Alzheimer's disease, Parkinson's disease, Down's syndrome, human immunodeficiency virus (HIV) dementia, myocardial infarction, or treatment and prevention of intraoperative or perioperative hypoxic insults that can leave persistent neurological deficits following open heart surgery requiring heart/lung bypass machines, such as coronary artery bypass grafts (CABG).

The invention also includes an assay for selecting a cannabinoid to use in treating a neurological disease by determining whether the cannabinoid is an antioxidant. Once it has been determined that the cannabinoid is an antioxidant, an antioxidant effective amount of the cannabinoid is administered to treat the neurological disease, such as a vascular ischemic event in the central nervous system, for example the type caused by a neurovascular thromboembolism. Similarly, the method of the present invention includes determining whether a disease is caused by oxidative stress, and if the disease is caused by oxidative stress, administering the cannabinoid in a therapeutically effective antioxidant amount.

The invention also includes identifying and administering antioxidant and neuroprotective compounds (such as cannabidiol) which selectively inhibit the enzyme activity of both 5- and 15-lipoxygenase more than the enzyme activity of 12-lipoxygenase. In addition, such compounds posses low NMDA antagonist activity and low cannabinoid receptor activity. Assays for selecting compounds with the desired effect on lipoxygenase enzymes, and methods for using identified compounds to treat neurological or ischemic diseases are also provided. Such diseases may include a vascular ischemic event in the central nervous system, for example a thromboembolism in the brain, or a vascular ischemic event in the myocardium. Useful administration of the compounds involves administration both during and after an ischemic injury.

These and other objects of the invention will be understood more clearly by reference to the following detailed description and drawings.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1A is a graph showing NMDA induced cellular damage in a neuron (as measured by LDH release) in cells that were exposed to glutamate for 10 minutes, which demonstrates that increasing concentrations of cannabidiol in the cell culture protects against cellular damage.

FIG. 1B is a graph similar to FIG. 1A, but showing that AMPA/kainate receptor mediated damage (induced by glutamate and the AMPA/kainate receptor potentiating agents cyclothiazide or concanavalin A) is also reduced in a concentration dependent manner by the presence of cannabidiol in the culture medium.

FIG. 2A is a bar graph showing cellular damage (as measured by LDH release) in the presence of glutamate alone (100 μM Glu), and in the presence of glutamate and 5 μM cannabidiol (CBD) or 5 μM THC, and demonstrates that CBD and THC were similarly protective.

FIG. 2B is a bar graph similar to FIG. 2A, but showing the cellular damage assessed in the presence of the cannabinoid receptor antagonist SR 141716A (SR), which was not found to alter the neuroprotective effect of CBD (5 μM) or THC (5 μM), indicating the effect is not a typical cannabinoid effect mediated by the cannabinoid receptor.

FIG. 3 is a graph showing the reduction oxidation potentials determined by cyclic voltametry for some natural and synthetic cannabinoids, the antioxidant BHT, and the non-cannabinoid anandamide (arachidonyl ethanolamide) which is a ligand for the cannabinoid receptor. The voltage at which initial peaks occur is an indication of antioxidant activity.

FIG. 4 is a graph that demonstrates the antioxidant properties of BHT, CBD and THC, by plotting the fluorescence of a fluorescent dye against concentrations of these substances, where declining fluorescence is an indication of greater antioxidant activity.

FIG. 5A is a graph illustrating decreased t-butyl peroxide induced toxicity (as measured by LDH release) in the presence of increasing concentrations of cannabidiol, demonstrating that cannabidiol is an effective antioxidant in living cells.

FIG. 5B is a bar graph comparing the antioxidant activity of several antioxidants against glutamate induced toxicity in neurons, showing that CBD has superior antioxidant activity.

FIG. 6A is a graph showing the effect of CBD (as measured by the change in absorbance at 234 nm) on the enzymatic activity of two lipoxygenase enzymes, rabbit 15-LO and porcine 12-LO, which demonstrates that CBD inhibits 15-LO, but not 12-LO enzyme.

FIG. 6B is a graph demonstrating that inhibitory effect of CBD on 15-LO is competitive.

FIG. 7A is a graph similar to FIG. 6A, but was performed in whole cells rather than purified enzyme preparations, and shows the effect of CBD (as measured by the change in absorbance at 236 nm) on the enzymatic activity of 5-LO from cultured rat basophillic leukemia cells (RBL-2H3), which demonstrates that CBD inhibits 5-LO.

FIG. 7B is a graph showing the effect of CBD (as measured by the change in absorbance at 236 nm) on the formation of 12-HETE (the product of 12-LO) by human leukocytes (12-LO type 1).

FIG. 7C is a graph similar to FIG. 7B, showing the effect of CBD (as measured by the change in absorbance at 236 nm) on the formation of 12-HETE by human platelets (12-LO type 2).

FIG. 8 is a bar graph demonstrating that 12-HETE can protect cortical neurons from NMDAr toxicity most effectively when administered during and post ischemia.

DETAILED DESCRIPTION OF SOME SPECIFIC EMBODIMENTS

This invention provides antioxidant compounds and compositions, such as pharmaceutical compositions, that include cannabinoids that act as free radical scavengers for use in prophylaxis and treatment of disease. The invention also includes methods for using the antioxidants in prevention and treatment of pathological conditions such as ischemia (tissue hypoxia), and in subjects who have been exposed to oxidant inducing agents such as cancer chemotherapy, toxins, radiation, or other sources of oxidative stress. The compositions and methods described herein are also used for preventing oxidative damage in transplanted organs, for inhibiting reoxygenation injury following reperfusion of ischemic tissues (for example in heart disease), and for any other condition that is mediated by oxidative or free radical mechanisms of injury. In particular embodiments of the invention, the compounds and compositions are used in the treatment of ischemic cardiovascular and neurovascular conditions, and neurodegenerative diseases. However the present invention can also be used as an antioxidant treatment in non-neurological diseases.

Molecular oxygen is essential for aerobic organisms, where it participates in many biochemical reactions, includ-

ing its role as the terminal electron acceptor in oxidative phosphorylation. However excessive concentrations of various forms of reactive oxygen species and other free radicals can have serious adverse biological consequences, including the peroxidation of membrane lipids, hydroxylation of nucleic acid bases, and the oxidation of sulfhydryl groups and other protein moieties. Biological antioxidants include tocopherols and tocotrieneols, carotenoids, quinones, bilirubin, ascorbic acid, uric acid, and metal binding proteins. However these endogenous antioxidant systems are often overwhelmed by pathological processes that allow permanent oxidative damage to occur to tissue.

Free radicals are atoms, ions or molecules that contain an unpaired electron, are usually unstable, and exhibit short half-lives. Reactive oxygen species (ROS) is a collective term, designating the oxygen radicals (e.g. $.O_2^-$ superoxide radical), which by sequential univalent reduction produces hydrogen peroxide (H_2O_2) and hydroxyl radical (.OH). The hydroxyl radical sets off chain reactions and can interact with nucleic acids. Other ROS include nitric oxide (NO.) and peroxy nitrite (NOO.), and other peroxyl (RO_2.) and alkoxyl (RO.) radicals. Increased production of these poisonous metabolites in certain pathological conditions is believed to cause cellular damage through the action of the highly reactive molecules on proteins, lipids and DNA. In particular, ROS are believed to accumulate when tissues are subjected to ischemia, particularly when followed by reperfusion.

The pharmaceutical compositions of the present invention have potent antioxidant and/or free radical scavenging properties, that prevent or reduce oxidative damage in biological systems, such as occurs in ischemic/reperfusion injury, or in chronic neurodegenerative diseases such as Alzheimer's disease, HIV dementia, and many other oxidation associated diseases.

DEFINITIONS

"Oxidative associated diseases" refers to pathological conditions that result at least in part from the production of or exposure to free radicals, particularly oxyradicals, or reactive oxygen species. It is evident to those of skill in the art that most pathological conditions are multifactorial, and that assigning or identifying the predominant causal factors for any particular condition is frequently difficult. For these reasons, the term "free radical associated disease" encompasses pathological states that are recognized as conditions in which free radicals or ROS contribute to the pathology of the disease, or wherein administration of a free radical inhibitor (e.g. desferroxamine), scavenger (e.g. tocopherol, glutathione) or catalyst (e.g. superoxide dismutase, catalase) is shown to produce detectable benefit by decreasing symptoms, increasing survival, or providing other detectable clinical benefits in treating or preventing the pathological state.

Oxidative associated diseases include, without limitation, free radical associated diseases, such as ischemia, ischemic reperfusion injury, inflammatory diseases, systemic lupus erythematosis, myocardial ischemia or infarction, cerebrovascular accidents (such as a thromboembolic or hemorrhagic stroke) that can lead to ischemia or an infarct in the brain, operative ischemia, traumatic hemorrhage (for example a hypovolemic stroke that can lead to CNS hypoxia or anoxia), spinal cord trauma, Down's syndrome, Crohn's disease, autoimmune diseases (e.g. rheumatoid arthritis or diabetes), cataract formation, uveitis, emphysema, gastric ulcers, oxygen toxicity, neoplasia, undesired cellular apoptosis, radiation sickness, and others. The present invention is believed to be particularly beneficial in the treatment of oxidative associated diseases of the CNS, because of the ability of the cannabinoids to cross the blood brain barrier and exert their antioxidant effects in the brain. In particular embodiments, the pharmaceutical composition of the present invention is used for preventing, arresting, or treating neurological damage in Parkinson's disease, Alzheimer's disease and HIV dementia; autoimmune neurodegeneration of the type that can occur in encephalitis, and hypoxic or anoxic neuronal damage that can result from apnea, respiratory arrest or cardiac arrest, and anoxia caused by drowning, brain surgery or trauma (such as concussion or spinal cord shock).

As used herein, an "antioxidant" is a substance that, when present in a mixture containing an oxidizable substrate biological molecule, significantly delays or prevents oxidation of the substrate biological molecule. Antioxidants can act by scavenging biologically important reactive free radicals or other reactive oxygen species ($.O_2^-$, H_2O_2, .OH, HOCl, ferryl, peroxyl, peroxynitrite, and alkoxyl), or by preventing their formation, or by catalytically converting the free radical or other reactive oxygen species to a less reactive species. Relative antioxidant activity can be measured by cyclic voltametry studies of the type disclosed in Example 5 (and FIG. 3), where the voltage (x-axis) is an index of relative antioxidant activity. The voltage at which the first peak occurs is an indication of the voltage at which an electron is donated, which in turn is an index of antioxidant activity.

"Therapeutically effective antioxidant doses" can be determined by various methods, including generating an empirical dose-response curve, predicting potency and efficacy of a congener by using quantitative structure activity relationships (QSAR) methods or molecular modeling, and other methods used in the pharmaceutical sciences. Since oxidative damage is generally cumulative, there is no minimum threshold level (or dose) with respect to efficacy. However, minimum doses for producing a detectable therapeutic or prophylactic effect for particular disease states can be established.

As used herein, a "cannabinoid" is a chemical compound (such as cannabinol, THC or cannabidiol) that is found in the plant species *Cannabis sativa* (marijuana), and metabolites and synthetic analogues thereof that may or may not have psychoactive properties. Cannabinoids therefore include (without limitation) compounds (such as THC) that have high affinity for the cannabinoid receptor (for example K_i<250 nM), and compounds that do not have significant affinity for the cannabinoid receptor (such as cannabidiol, CBD). Cannabinoids also include compounds that have a characteristic dibenzopyran ring structure (of the type seen in THC) and cannabinoids which do not possess a pyran ring (such as cannabidiol). Hence a partial list of cannabinoids includes THC, CBD, dimethyl heptylpentyl cannabidiol (DMHP-CBD), 6,12-dihydro-6-hydroxy-cannabidiol (described in U.S. Pat. No. 5,227,537, incorporated by reference); (3S,4R)-7-hydroxy-Δ^6-tetrahydrocannabinol homologs and derivatives described in U.S. Pat. No. 4,876,276, incorporated by reference; (+)-4-[4-DMH-2,6-diacetoxy-phenyl]-2-carboxy-6,6-dimethylbicyclo[3.1.1]hept-2-en, and other 4-phenylpinene derivatives disclosed in U.S. Pat. No. 5,434,295, which is incorporated by reference; and cannabidiol (−)(CBD) analogs such as (−)CBD-monomethylether, (−)CBD dimethyl ether; (−)CBD diacetate; (−)3'-acetyl-CBD monoacetate; and ±AF11, all of which are disclosed in Consroe et al., *J. Clin. Pharmacol.*

21:428S–436S, 1981, which is also incorporated by reference. Many other cannabinoids are similarly disclosed in Agurell et al., *Pharmacol. Rev.* 38:31–43, 1986, which is also incorporated by reference.

As referred to herein, the term "psychoactivity" means "cannabinoid receptor mediated psychoactivity." Such effects include, euphoria, lightheadedness, reduced motor coordination, and memory impairment. Psychoactivity is not meant to include non-cannabinoid receptor mediated effects such as the anxiolytic effect of CBD.

The "lipoxygenase enzyme activity" refers to the relative level of lipoxygenase enzyme activity for a particular lipoxgenase, such as 5-, 15- or 12-lipoxygenase, as measured in Example 8. A compound would be said to "selectively inhibit a lipoxgenase enzyme" if the concentration of inhibitor required to reduce enzyme activity by 50% was at least about 5 times less than the amount required to reduce activity of a second lipoxgenase enzyme by the same degree (under the same conditions, i.e. temperature, substrate concentration, etc.)

An "antagonist" is a compound that binds and occupies a receptor without activating it. In the presence of a sufficient concentration of antagonist, an agonist cannot activate its receptor. Therefore, antagonists may decrease the neurotoxicity mediated by NMDA (as described in Example 3) or AMPA and Kainate (as described in Example 4).

An "agonist" is a compound that activates a receptor. When the receptor is activated for a longer than normal period of time, this may cause neurotoxicity, as in the case of NMDA, AMPA and kainate receptors (see Examples 3 and 4).

The term "alkyl" refers to a cyclic, branched, or straight chain alkyl group containing only carbon and hydrogen, and unless otherwise mentioned contains one to twelve carbon atoms. This term is further exemplified by groups such as methyl, ethyl, n-propyl, isobutyl, t-butyl, pentyl, pivalyl, heptyl, adamantyl, and cyclopentyl. Alkyl groups can either be unsubstituted or substituted with one or more substituents, e.g. halogen, alkyl, alkoxy, alkylthio, trifluoromethyl, acyloxy, hydroxy, mercapto, carboxy, aryloxy, aryloxy, aryl, arylalkyl, heteroaryl, amino, alkylamino, dialkylamino, morpholino, piperidino, pyrrolidin-1-yl, piperazin-1-yl, or other functionality.

The term "lower alkyl" refers to a cyclic, branched or straight chain monovalent alkyl radical of one to seven carbon atoms. This term is further exemplified by such radicals as methyl, ethyl, n-propyl, i-propyl, n-butyl, t-butyl, i-butyl (or 2-methylpropyl), cyclopropylmethyl, i-amyl, n-amyl, hexyl and heptyl. Lower alkyl groups can also be unsubstituted or substituted, where a specific example of a substituted alkyl is 1,1-dimethyl heptyl.

"Hydroxyl" refers to —OH.

"Alcohol" refers to R—OH, wherein R is alkyl, especially lower alkyl (for example in methyl, ethyl or propyl alcohol). An alcohol may be either linear or branched, such as isopropyl alcohol.

"Carboxyl" refers to the radical —COOH, and substituted carboxyl refers to —COR where R is alkyl, lower alkyl or a carboxylic acid or ester.

The term "aryl" or "Ar" refers to a monovalent unsaturated aromatic carbocyclic group having a single ring (e.g. phenyl) or multiple condensed rings (e.g. naphthyl or anthryl), which can optionally be unsubstituted or substituted with, e.g., halogen, alkyl, alkoxy, alkylthio, trifluoromethyl, acyloxy, hydroxy, mercapto, carboxy, aryloxy, aryl, arylalkyl, heteroaryl, amino, alkylamino, dialkylamino, morpholino, piperidino, pyrrolidin-1-yl, piperazin-1-yl, or other functionality.

The term "alkoxy" refers to a substituted or unsubstituted alkoxy, where an alkoxy has the structure —O—R, where R is substituted or unsubstituted alkyl. In an unsubstituted alkoxy, the R is an unsubstituted alkyl. The term "substituted alkoxy" refers to a group having the structure —O—R, where R is alkyl which is substituted with a non-interfering substituent. The term "arylalkoxy" refers to a group having the structure —O—R—Ar, where R is alkyl and Ar is an aromatic substituent. Arylalkoxys are a subset of substituted alkoxys. Examples of useful substituted alkoxy groups are: benzyloxy, naphthyloxy, and chlorobenzyloxy.

The term "aryloxy" refers to a group having the structure —O—Ar, where Ar is an aromatic group. A particular aryloxy group is phenoxy.

The term "heterocycle" refers to a monovalent saturated, unsaturated, or aromatic carbocyclic group having a single ring (e.g. morpholino, pyridyl or faryl) or multiple condensed rings (e.g. indolizinyl or benzo[b]thienyl) and having at least one heteroatom, defined as N, O, P, or S, within the ring, which can optionally be unsubstituted or substituted with, e.g. halogen, alkyl, alkoxy, alkylthio, trifluoromethyl, acyloxy, hydroxy, mercapto, carboxy, aryloxy, aryl, arylakyl, heteroaryl, amino, alkylamino, dialkylamino, morpholino, piperidino, pyrrolidin-1-yl, piperazin-1-yl, or other functionality.

"Arylalkyl" refers to the groups —R—Ar and —R—HetAr, where Ar is an aryl group. HetAr is a heteroaryl group, and R is a straight-chain or branched chain aliphatic group. Example of arylaklyl groups include benzyl and furfuryl. Arylalkyl groups can optionally be unsubstituted or substituted with, e.g., halogen, alkyl, alkoxy, alkylthio, trifluoromethyl, acyloxy, hydroxy, mercapto, carboxy, aryloxy, aryl, arylalkyl, heteroaryl, amino, alkylamino, dialkylamino, morpholino, peperidino, pyrrolidin-1-yl, piperazin-1-yl, or other functionality.

The term "halo" or "halide" refers to fluoro, bromo, chloro and iodo substituents.

The term "amino" refers to a chemical functionality —NR'R" where R' and R" are independently hydrogen, alkyl, or aryl. The term "quaternary amine" refers to the positively charged group —N^+R'R", where R'R" and R" are independently selected and are alkyl or aryl. A particular amino group is —NH_2.

A "pharmaceutical agent" or "drug" refers to a chemical compound or composition capable of inducing a desired therapeutic or prophylactic effect when properly administered to a subject.

All chemical compounds include both the (+) and (−) stereoisomers, as well as either the (+) or (−) stereoisomer.

Other chemistry terms herein are used according to conventional usage in the art, as exemplified by *The McGraw-Hill Dictionary of Chemical Terms* (1985) and *The Condensed Chemical Dictionary* (1981).

The following examples show that both nonpsychoactive cannabidiol, and psychoactive cannabinoids such as THC, can protect neurons from glutamate induced death, by a mechanism independent of cannabinoid receptors. Cannabinoids are also be shown to be potent antioxidants capable of preventing ROS toxicity in neurons.

EXAMPLE 1

Preparation of Cannabinoids and Neuronal Cultures

Cannabidiol, THC and reactants other than those specifically listed below were purchased from Sigma Chemical,

Co. (St. Louis, Mo.). Cyclothiazide, glutamatergic ligands and MK-801 were obtained from Tocris Cookson (UK). Dihydrorhodamine was supplied by Molecular Probes (Eugene, Oreg.). T-butyl hydroperoxide, tetraethylammonium chloride, ferric citrate and sodium dithionite were all purchased from Aldrich (WI). All culture media were Gibco/BRL (MD) products.

Solutions of cannabinoids, cyclothiazide and other lipophiles were prepared by evaporating a 10 mM ethanolic solution (under a stream of nitrogen) in a siliconized microcentrifuge tube. Dimethyl sulfoxide (DMSO, less than 0.05% of final volume) was added to ethanol to prevent the lipophile completely drying onto the tube wall. After evaporation, 1 ml of culture media was added and the drug was dispersed using a high power sonic probe. Special attention was used to ensure the solution did not overheat or generate foam. Following dispersal, all solutions were made up to their final volume in siliconized glass tubes by mixing with an appropriate quantity of culture media.

Primary neuronal cultures were prepared according to the method of Ventra et al. (J. Neurochem. 66:1752–1761, 1996). Fetuses were extracted by Cesarian section from a 17 day pregnant Wistar rat, and the feral brains were placed into phosphate buffered saline. The cortices were then dissected out, cut into small pieces and incubated with papain for nine minutes at 37° C. After this time the tissue was dissociated by passage through a fire polished Pasteur pipette, and the resultant cell suspension separated by centrifugation over a gradient consisting of 10 mg/ml bovine serum albumin and 10 mg/ml ovomucoid (a trypsin inhibitor) in Earls buffered salt solution. The pellet was then re-suspended in high glucose, phenol red free Dulbecco's modified Eagles medium containing 10% fetal bovine serum, 2 mM glutamine, 100 IU penicillin, and 100 μg/ml streptomycin (DMEM). Cells were counted, tested for vitality using the trypan blue exclusion test and seeded onto poly-D-lysine coated 24 multiwell plates. After 96 hours, 10 μM fluoro-deoxyuridine and 10 μM uridine were added to block glial cell growth. This protocol resulted in a highly neuron-enriched culture.

EXAMPLE 2

Preparation of Astrocytes and Conditioned Media

Astrocyte conditioned DMEM was used throughout the AMPA/kainate toxicity procedure and following glutamate exposure in the NMDAr mediated toxicity protocol. Media was conditioned by 24 hour treatment over a confluent layer of type I astrocytes, prepared from two day old Wistar rat pups. Cortices were dissected, cut into small pieces, and enzymatically digested with 0.25% trypsin. Tissue was then dissociated by passage through a fire polished Pasteur pipette and the cell suspension plated into untreated 75 cm^2 T-flasks. After 24 hours the media was replaced and unattached cells removed. Once astrocytes achieved confluence, cells were divided into four flasks. Media for experiments was conditioned by a 24 hour exposure to these astrocytes, after which time it was frozen at −20° C. until use. Astrocyte cultures were used to condition DMEM for no longer than two months.

EXAMPLE 3

NMDA Mediated Toxicity Studies

Glutamate neurotoxicity can be mediated by NMDA, AMPA or kainate receptors. To examine NMDAr mediated toxicity, cultured neurons (cultured for 14–18 days) were exposed to 250 μM glutamate for 10 minutes in a magnesium free saline solution. The saline was composed of 125 mM NaCl, 25 mM glucose, 10 mM HEPES (pH 7.4), 5 mM KCl, 1.8 mM calcium chloride and 5% bovine serum albumin. Following exposure, cells were washed twice with saline, and incubated for 18 hours in conditioned DMEM. The level of lactate dehydrogenase (LDH) in the media was used as an index of cell injury.

Toxicity was completely prevented by addition of the NMDAr antagonist, MK-801 (500 nM, data not shown). However, FIG. 1A shows that cannabidiol also prevented neurotoxicity (maximum protection 88±9%) with an EC_{50} of 2–4 μM (specifically about 3.5 μM).

EXAMPLE 4

AMPA and Kainate Receptor Mediated Toxicity Studies

Unlike NMDA receptors, which are regulated by magnesium ions, AMPA/kainate receptors rapidly desensitize following ligand binding. To examine AMPA and kainate receptor mediated toxicity, neurons were cultured for 7–13 days, then exposed to 100 μM glutamate and 50 μM cyclothiazide (used to prevent AMPA receptor desensitization). Cells were incubated with glutamate in the presence of 500 nM MK-801 (an NMDAr antagonist) for 18–20 hours prior to analysis. Specific AMPA and kainate receptor ligands were also used to separately examine the effects of cannabinoids on AMPA and kainate receptor mediated events. Fluorowillardiine (1.5 μM) was the AMPA agonist and 4-methyl glutamate (10 μM) was the kainate agonist used to investigate receptor mediated toxicity. When specifically examining kainate receptor activity, cyclothiazide was replaced with 0.15 mg/ml Concanavalin-A.

Cannabidiol protection against AMPA/kainate mediated neurotoxicity is illustrated in FIG. 1B, where LDH in the media was used as an index of cell injury. The neuroprotective effect of cannabidiol was similar to that observed in the NMDA mediated toxicity model (FIG. 1A). Cannabidiol prevented neurotoxicity (maximum protection 80±17%) with an EC_{50} of 2–4 μM (specifically about 3.3 μM). Comparable results were obtained with either the AMPA receptor ligand, fluorowillardiine or the kainate receptor specific ligand, 4-methyl-glutamate (data not shown). Hence cannabidiol protects similarly against toxicity mediated by NMDA, AMPA or kainate receptors.

Unlike cannabidiol, THC is a ligand (and agonist) for the brain cannabinoid receptor. The action of THC at the cannabinoid receptor has been proposed to explain the ability of THC to protect neurons from NMDAr toxicity in vitro. However in AMPA/kainate receptor toxicity assays, THC and cannabidiol were similarly protective (FIG. 2A), indicating that cannabinoid neuroprotection is independent of cannabinoid receptor activation. This was confirmed by inclusion of cannabinoid receptor antagonist SR-141716A in the culture media (SR in FIG. 2B). See Mansbach et al., *Psychopharmacology* 124:315–22, 1996, for a description of SR-141716A. Neither THC nor cannabidiol neuroprotection was affected by cannabinoid receptor antagonist (FIG. 2B).

EXAMPLE 5

Cyclic Voltametery Studies or ReDox Potentials

To investigate whether cannabinoids protect neurons against glutamate damage by reacting with ROS, the anti-

oxidant properties of cannabidiol and other cannabinoids were assessed. Cyclic voltametry, a procedure that measures the ability of a compound to accept or donate electrons under a variable voltage potential, was used to measure the oxidation potentials of several natural and synthetic cannabinoids. These studies were performed with an EG&G Princeton Applied Research potentiostat/galvanostat (Model 273/PAR 270 software, NJ). The working electrode was a glassy carbon disk with a platinum counter electrode and silver/silver chloride reference. Tetraethylammonium chloride in acetonitrile (0.1 M) was used as an electrolyte. Cyclic voltametry scans were done from +0 to 1.8 V at scan rate of 100 mV per second. The reducing ability of cannabidiol (CBD), THC, HU-211, and BHT were measured in this fashion. Anandamide, a cannabinoid receptor ligand without a cannabinoid like structure, was used as a non-responsive control. Each experiment was repeated twice with essentially the same results.

Cannabidiol, THC and the synthetic cannabinoid HU-211 all donated electrons at a similar potential as the antioxidant BHT. Anandamide (arachidonyl ethanolamide) did not undergo oxidation at these potentials (FIG. 3). Several other natural and synthetic cannabinoids, including cannabidiol, nabilone, and levanantrodol were also tested, and they too exhibited oxidation profiles similar to cannabidiol and THC (data not shown).

EXAMPLE 6

Iron Catalyzed Dihydrorhodamine Oxidation (Fenton Reaction)

The ability of cannabinoids to be readily oxidized, as illustrated in Example 5, indicated they possess antioxidant properties comparable to BHT. The antioxidant activity of BHT was examined in a Fenton reaction, in which iron is catalyzed to produce ROS. Cannabidiol (CBD) and tetrahydrocannabinol (THC) were evaluated for their ability to prevent oxidation of dihydrorhodamine to the fluorescent compound rhodamine. Oxidant was generated by ferrous catalysis (diothionite reduced ferric citrate) of t-butyl hydroperoxide in a 50:50 water:acetonitrile (v/v) solution. Dihydrorhodamine (50 μM) was incubated with 300 μM t-butyl hydroperoxide and 0.5 μM iron for 5 minutes. After this time, oxidation was assessed by spectrofluorimetry (Excit=500 nm, Emiss=570 nm). Various concentrations of cannabinoids and BHT were included to examine their ability to prevent dihydrorhodiamine oxidation.

Cannabidiol, THC and BHT all prevented dihydrorhodamine oxidation in a similar, concentration dependent manner (FIG. 4), indicating that cannabinoids have antioxidant potency comparable to BHT.

To confirm that cannabinoids act as antioxidants in the intact cell, neurons were also incubated with the oxidant t-butyl hydroperoxide and varying concentrations of cannabidiol (FIG. 5A). The t-butyl hydroperoxide oxidant was chosen for its solubility in both aqueous and organic solvents, which facilitates oxidation in both cytosolic and membrane cell compartments. Cell toxicity was assessed 18–20 hours after insult by measuring lactate dehydrogenase (LDH) release into the culture media. All experiments were conducted with triple or quadruple values at each point and all plates contained positive (glutamate alone) and baseline controls. The assay was validated by comparison with an XTT based metabolic activity assay. As shown in FIG. 5A, cannabidiol protected neurons against ROS toxicity in a dose related manner, with an EC_{50} of about 6 μM. The maximum protection observed was 88±9%.

Cannabidiol was also compared with known antioxidants in an AMPA/kainate toxicity protocol. Neurons were exposed to 100 μM glutamate and equimolar (5 μM) cannabidiol, α-tocopherol, BHT or ascorbate (FIG. 5B). Although all of the antioxidants attenuated glutamate toxicity, cannabidiol was significantly more protective than either α-tocopherol or ascorbate. The similar antioxidant abilities of cannabidiol and BHT in this chemical system (FIG. 4), and their comparable protection in neuronal cultures (FIG. 5B), implies that cannabidiol neuroprotection is due to an antioxidant effect.

EXAMPLE 7

In vivo Rat Studies

The middle cerebral artery of chloral hydrate anesthetized rats was occluded by insertion of suture thread into it. The animals were allowed to recover from the anesthetic and move freely for a period of two hours. After this time the suture was removed under mild anesthetic and the animals allowed to recover for 48 hours. Then the animals were tested for neurological deficits, sacrificed, and the infarct volume calculated. To examine the infarct volume, animals were anesthetized, ex-sanguinated, and a metabolically active dye (3-phenyl tetrazolium chloride) was pumped throughout the body. All living tissues were stained pink by the dye, while morbid regions of infarcted tissue remained white. Brains were then fixed for 24 hours in formaldehyde, sliced and the infarct volumes measured.

One hour prior to induction of ischemia 20 mg/kg of cannabidiol was administered by intra-peritoneal injection (ip) in a 90% saline:5% emulphor 620 (emulsifier):5% ethanol vehicle. A second ip 10 mg/kg dose of cannabidiol was administered 8 hours later using the same vehicle. Control animals received injections of vehicle without drug. IV doses would be expected to be 3–5 times less because of reduction of first pass metabolism.

The infarct size and neurological assessment of the test animals is shown Table 1.

TABLE 1

Cannabidiol protects rat brains from ischemia damage

	Volume of Infarct (mm3)		Behavioral Deficit Score	
Animal	Drug	Control	Drug	Control
1	108.2	110.5	3	2
2	83.85	119.6	4	4
3	8.41	118.9	3	4
4	75.5	177.7	1	4
5	60.53	33.89	1	3
6	27.52	255.5	1	5
7	23.16	143	1	4
Mean	55.3	137.0	2.0	3.7
SEM	13.8	25.7	0.5	0.4
	p = 0.016 significant		p = 0.015 significant	

*Neurological scoring is performed on a subjective 1–5 scale of impairment. 0 = no impairment, 5 = severe (paralysis)

This data shows that infarct size was approximately halved in the animals treated with cannabidiol, which was also accompanied by a substantial improvement in the neurological status of the animal.

These studies with the nonpsychotropic marijuana constituent, cannabidiol, demonstrate that protection can be achieved against both glutamate neurotoxicity and free radical induced cell death. THC, the psychoactive principle

of cannabis, also blocked glutamate neurotoxicity with a potency similar to cannabidiol. In both cases, neuroprotection is unaffected by the presence of a cannabinoid receptor antagonist. These results therefore surprisingly demonstrate that cannabinoids can have useful therapeutic effects that are not mediated by cannabinoid receptors, and therefore are not necessarily accompanied by psychoactive side effects. Cannabidiol also acts as an anti-epileptic and anxiolytic, which makes it particularly useful in the treatment of neurological diseases in which neuroanatomic defects can predispose to seizures (e.g. subarachnoid hemorrhage).

A particular advantage of the cannabinoid compounds of the present invention is that they are highly lipophilic, and have good penetration into the central nervous system. The volume of distribution of some of these compounds is at least 100 L in a 70 kg person (1.4 L/kg), more particularly at least 250 L, and most particularly 500 L or even 700 L in a 70 kg person (10 L/kg). The lipophilicity of particular compounds is also about as great as that of THC, cannabidiol or other compounds that have excellent penetration into the brain and other portions of the CNS.

Cannabinoids that lack psychoactivity or psychotoxicity are particularly useful embodiments of the present invention, because the absence of such side effects allows very high doses of the drug to be used without encountering unpleasant side effects (such as dysphoria) or dangerous complications (such as obtundation in a patient who may already have an altered mental status). For example, therapeutic antioxidant blood levels of cannabidiol can be 5–20 mg/kg, without significant toxicity, while blood levels of psychoactive cannabinoids at this level would produce obtundation, headache, conjunctival irritation, and other problems. Particular examples of the compounds of the present invention have low affinity to the cannabinoid receptor, for example a K_i of greater than 250 nM, for example $K_i \geqq 500$–1000 nM. A compound with a $K_i \geqq 1000$ nM is particularly useful, which compound has essentially no psychoactivity mediated by the cannabinoid receptor.

Cannabidiol blocks glutamate toxicity with equal potency regardless of whether the insult is mediated by NMDA, AMPA or kainate receptors. Cannabidiol and THC have been shown to be comparable to the antioxidant BHT, both in their ability to prevent dihydrorhodamine oxidation and in their cyclic voltametric profiles. Several synthetic cannabinoids also exhibited profiles similar to the BHT, although anandamide, which is not structurally related to cannabinoids, did not. These findings indicate that cannabinoids act as antioxidants in a non-biological situation, which was confirmed in living cells by showing that cannabidiol attenuates hydroperoxide induced neurotoxicity. The potency of cannabidiol as an antioxidant was examined by comparing it on an equimolar basis with three other commonly used compounds.

In the AMPA/kainate receptor dependent neurotoxicity model, cannabidiol neuroprotection was comparable to the potent antioxidant, BHT, but significantly greater than that observed with either α-tocopherol or ascorbate. This unexpected superior antioxidant activity (in the absence of BHT tumor promoting activity) shows for the first time that cannabidiol, and other cannabinoids, can be used as antioxidant drugs in the treatment (including prophylaxis) of oxidation associated diseases, and is particularly useful as a neuroprotectant. The therapeutic potential of nonpsychoactive cannabinoids is particularly promising, because of the absence of psychotoxicity, and the ability to administer higher doses than with psychotropic cannabinoids, such as THC. Previous studies have also indicated that cannabidiol is not toxic, even when chronically administered to humans or given in large acute doses (700 mg/day).

EXAMPLE 8

Effect of Cannabidiol on Lipoxygenase Enzymes

This example describes in vitro and in vivo assays to examine the effect of cannabidiol (CBD) on three lipoxygenase (LO) enzymes: 5-LO, 12-LO and 15-LO.

In vitro Enzyme Assay

The ability of CBD to inhibit lipoxygenase was examined by measuring the time dependent change in absorption at 234 nM following addition of 5 U of each lipoxygenase (rabbit 15-LO purchased from Biomol (PA), porcine 12-LO purchased from Cayman chemicals (MI)) to a solution containing 10 μM (final concentration) linoleic acid.

Enzyme studies were performed using a u.v. spectrophotometer and a 3 ml quartz cuvette containing 2.5 ml of a stirred solution of 12.5 μM sodium linoleic acid (sodium salt) in solution A (25 mM Tris (pH 8.1), 1 mM EDTA 0.1% methyl cellulose). The reaction was initiated by addition of 0.5 ml enzyme solution (10 U/ml enzyme in solution A) and recorded for 60 seconds. Lipoxygenase exhibits non-Michaelis-Menten kinetics, an initial "lag" (priming) phase followed by a linear phase which is terminated by product inhibition. These complications were reduced by assessing enzyme activity (change in absorption) over the "steepest" 20 second period in a 60 second run time. Recordings examined the absorption at 234 nm minus the value at a reference wavelength of 280 nm. Linoleic acid was used as the substrate rather than arachidonic acid, because the products are less inhibitory to the enzyme, thereby providing a longer "linear phase".

Cell Purification and Separation

Human platelets and leukocytes were purified from buffy coat preparations (NIH Blood Bank) using a standard Ficoll based centrifugation method used in blood banks. Prior to use, cells were washed three times to eliminate contaminating cell types. Cultured rat basophillic leukemia cells (RBL-2H3) were used as a source of 5-lipoxygenase.

In vivo Determination of Lipoxygenase Activity

Cells were incubated with arachidonic acid and stimulated with the calcium ionophore A23187. Lipids were extracted and separated by reverse phase HPLC. Product formation was assessed as the area of a peak that co-eluted with an authentic standard, had a greater absorbance at 236 nm than at either 210 or 280 nm, and the formation of which was inhibited by a lipoxygenase inhibitor.

Cell pellets were triturated in DMEM culture media, aliquoted and pre-incubated for 15 minutes with 20 μM arachidonic acid and varying concentrations of cannabidiol and/or 40 μM nordihydroguaiaretic acid (a lipxygenase inhibitor). Platelets and leukocytes were also pre-incubated with 80 μM manoalide (Biomol) to prevent phospholipase A2 activation. Product formation was initiated by addition of 5 μM A23187 and incubation for 10 minutes at 37° C. At the end of the incubation, the reaction was stopped by addition of 15% 1M HCl and 10 ng/ml prostaglandin B2 (internal standard). Lipids were extracted with 1 volume of ethyl ether, which was dried under a stream of nitrogen. Samples were reconstituted in 50% acetonitrile:50% H_2O and separated by reverse phase HPLC using a gradient running from 63% acetonitrile: 37% H_2O:0.2% acetic acid to 90% acetonitrile (0.2% acetic acid) over 13 minutes.

Measurement of NMDAr Toxicity

The ability of 12-HETE (12-(s)-hydroxy-eicosatetraenoic acid, the product of the action of 12-lipoxygenase on arachi-

donic (eicosatetraenoic) acid) to protect cortical neurons from NMDAr toxicity was measured as described in Example 3. The 12-HETE (0.5 μg/ml) was added either during ischemia (co-incubated with the glutamate), during post-ischemia (co-incubated with the DMEM after washing the cells), or during both ischemia and post-ischemia.

Results

Using semi-purified enzyme preparations, the effect of CBD on rabbit 15-LO and porcine 12-LO was compared. As shown in FIGS. 6A and B, CBD is a potent competitive inhibitor of 15-LO with an EC_{50} of 598 nM. However, CBD had no effect on the 12-LO enzyme.

Using whole cell preparations, the effect of CBD on 5- and 12-LO enzymes was investigated. As shown in FIG. 7A, CBD inhibited 5-LO in cultured rat basophillic leukemia cells (RBL-2H3) with an EC_{50} of 1.92 μM. However, CBD had no effect on 12-LO, as monitored by the production of 12-HETE (the product of 12-LO), in either human leukocytes or platelets (FIGS. 7B and C). The leukocyte 12-LO is similar, while the platelet 12-LO is structurally and functionally different, from the porcine 12-LO used in the in vitro enzyme study.

The ability of 12-HETE to protect cortical neurons from NMDAr toxicity is shown in FIG. 8. To achieve best protection from NMDAr toxicity, 12-HETE was administered both during and post ischemia.

Therefore, CBD serves as a selective inhibitor of at least two lipoxygenase enzymes, 5-LO and 15-LO, but had no effect on 12-LO. Importantly, this is the first demonstration (FIG. 8) that the 12-LO product 12-HETE can play a significant role in protecting neurons from NMDAr mediated toxicity. Although the mechanism of this protection is unknown at the present time, 12-HETE is known to be an important neuromodulator, due to its ability to influence potassium channel activity.

EXAMPLE 9

Methods of Treatment

The present invention includes a treatment that inhibits oxidation associated diseases in a subject such as an animal, for example a rat or human. The method includes administering the antioxidant drugs of the present invention, or a combination of the antioxidant drug and one or more other pharmaceutical agents, to the subject in a pharmaceutically compatible carrier and in an effective amount to inhibit the development or progression of oxidation associated diseases. Although the treatment can be used prophylactically in any patient in a demographic group at significant risk for such diseases, subjects can also be selected using more specific criteria, such as a definitive diagnosis of the condition. The administration of any exogenous antioxidant cannabinoid would inhibit the progression of the oxidation associated disease as compared to a subject to whom the cannabinoid was not administered. The antioxidant effect, however, increases with the dose of the cannabinoid.

The vehicle in which the drug is delivered can include pharmaceutically acceptable compositions of the drugs of the present invention using methods well known to those with skill in the art. Any of the common carriers, such as sterile saline or glucose solution, can be utilized with the drugs provided by the invention. Routes of administration include but are not limited to oral, intracranial ventricular (icv), intrathecal (it), intravenous (iv), parenteral, rectal, topical ophthalmic, subconjunctival, nasal, aural, sublingual (under the tongue) and transdermal. The antioxidant drugs of the invention may be administered intravenously in any conventional medium for intravenous injection such as an aqueous saline medium, or in blood plasma medium. Such medium may also contain conventional pharmaceutical adjunct materials such as, for example, pharmaceutically acceptable salts to adjust the osmotic pressure, lipid carriers such as cyclodextrins, proteins such as serum albumin, hydrophilic agents such as methyl cellulose, detergents, buffers, preservatives and the like. Given the low solubility of many cannabinoids, they may be suspended in sesame oil.

Given the excellent absorption of the compounds of the present invention via an inhaled route, the compounds may also be administered as inhalants, for example in pharmaceutical aerosols utilizing solutions, suspensions, emulsions, powders and semisolid preparations of the type more fully described in *Remington: The Science and Practice of Pharmacy* (19th Edition, 1995) in chapter 95. A particular inhalant form is a metered dose inhalant containing the active ingredient, in a suspension or a dispersing agent (such as sorbitan trioleate, oleyl alcohol, oleic acid, or lecithin, and a propellant such as 12/11 or 12/114).

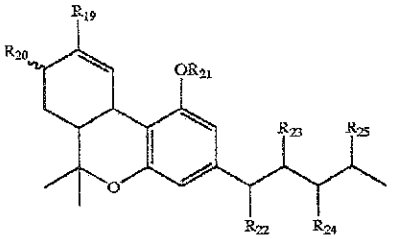
Embodiments of the invention comprising pharmaceutical compositions can be prepared with conventional pharmaceutically acceptable carriers, adjuvants and counterions as would be known to those of skill in the art. The compositions are preferably in the form of a unit dose in solid, semi-solid and liquid dosage forms such as tablets, pills, powders, liquid solutions or suspensions, injectable and infusible solutions, for example a unit dose vial, or a metered dose inhaler. Effective oral human dosage ranges for cannabidiol are contemplated to vary from about 1–40 mg/kg, for example 5–20 mg/kg, and in particular a dose of about 20 mg/kg of body weight.

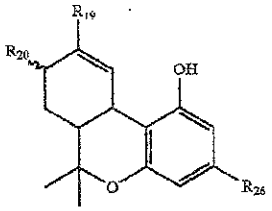
If the antioxidant drugs are to be used in the prevention of cataracts, they may be administered in the form of eye drops formulated in a pharmaceutically inert, biologically acceptable carrier, such as isotonic saline or an ointment. Conventional preservatives, such as benzalkonium chloride, can also be added to the formulation. In ophthalmic ointments, the active ingredient is admixed with a suitable base, such as white petrolatum and mineral oil, along with antimicrobial preservatives. Specific methods of compounding these dosage forms, as well as appropriate pharmaceutical carriers, are known in the art. *Remington: The Science and Practice of Pharmacy*, 19th Ed., Mack Publishing Co. (1995), particularly Part 7.

The compounds of the present invention are ideally administered as soon as a diagnosis is made of an ischemic event, or other oxidative insult. For example, once a myocardial infarction has been confirmed by electrocardiograph, or an elevation in enzymes characteristic of cardiac injury (e.g. CKMB), a therapeutically effective amount of the cannabinoid drug is administered. A dose can also be given following symptoms characteristic of a stroke (motor or sensory abnormalities), or radiographic confirmation of a cerebral infarct in a distribution characteristic of a neurovascular thromboembolic event. The dose can be given by frequent bolus administration, or as a continuous IV dose. In the case of cannabidiol, for example, the drug could be given in a dose of 5 mg/kg active ingredient as a continuous intravenous infusion; or hourly intramuscular injections of that dose.

EXAMPLE 10

The following table lists examples of some dibenzopyran cannabinoids that may be useful as antioxidants in the method of the present invention.

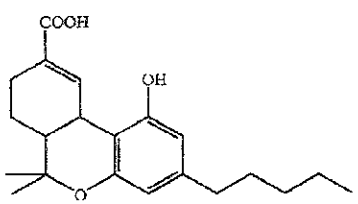




	Compound		R_{19}	R_{20}	R_{21}	R_{22}	R_{23}	R_{24}	R_{25}	R_{26}
H	5	7-OH-Δ^1-THC	CH_2OH	H	H	H	H	H	H	C_5H_{11}
H	6	6α-OH-Δ^1-THC	CH_3	α-OH						
H	7	6β-OH-Δ^1-THC	CH_3	β-OH						
	8	1"-OH-Δ^1-THC	CH_3			OH				
H	9	2"-OH-Δ^1-THC	CH_3				OH			
	10	3"-OH-Δ^1-THC	CH_3					OH		
	11	4"-OH-Δ^1-THC	CH_3						OH	
H	12	6α,7-diOH-Δ^1-THC	CH_2OH	α-OH						
H	13	6β,7-diOH-Δ^1-THC	CH_2OH	β-OH						
	14	1",7-diOH-Δ^1-THC	CH_2OH			OH				
H	15	2",7-diOH-Δ^1-THC	CH_2OH				OH			
H	16	3",7-diOH-Δ^1-THC	CH_2OH					OH		
H	17	4",7-diOH-Δ^1-THC	CH_2OH						OH	
	18	1",6β-diOH-Δ^1-THC	CH_3	β-OH		OH				
	19	1",3"-diOH-Δ^1-THC	CH_3			OH		OH		
	20	1",6α,7-triOH-Δ^1-THC	CH_2OH	α-OH		OH				
H	21	Δ^1-THC-6-one	CH_3	=O						
	22	Epoxyhexahydrocannabinol (EHHC)*	CH_3							
	23	7-oxo-Δ^1-THC	CHO							
H	24	Δ^1-THC-7"-oic acid	COOH							
H	25	Δ^1-THC-3"-oic acid	CH_2							C_2H_4COOH
H	26	1"-OH-Δ^1-THC-7"-oic acid	COOH			OH				
H	27	2"-OH-Δ^1-THC-7"-oic acid	COOH				OH			
H	28	3"-OH-Δ^1-THC-7"-oic acid	COOH					OH		
H	29	4"-OH-Δ^1-THC-7"-oic acid	COOH						OH	
H	30	3",4",5"-trisnor-2"-OH-Δ^1-THC-7-oic acid	COOH							C_2H_4OH
H	31	7-OH-Δ^1-THC-2"-oic acid	CH_2OH							CH_2COOH
H	32	6β-OH-Δ^1-THC-2"-oic acid	CH_3	β-OH						CH_2COOH
H	33	7-OH-Δ^1-THC-3"-oic acid	CH_2OH							C_2H_4COOH
H	34	6β-OH-Δ^1-THC-3"-oic acid	CH_3	β-OH						C_2H_4COOH
H	35	6α-OH-Δ^1-THC-4"-oic acid	CH_3	α-OH						C_3H_6COOH
H	36	2",3"-dehydro-6U-OH-Δ^1-THC-4"-oic acid	CH_3	α-OH						C_3H_4COOH
H	37	Δ^1-THC-1",7-dioic acid	COOH							COOH
H	38	Δ^1-THC-2",7-dioic acid	COOH							CH_2COOH
H	39	Δ^1-THC-3",7-dioic acid	COOH							C_2H_4COOH
H	40	Δ^1-THC-4",7-dioic acid	COOH							C_3H_6COOH
H	41	1",2"-dehydro-Δ^1-THC-3",7-dioic acid	COOH							C_2H_2COOH
H	42	Δ^1-THC-glucuronic acid	CH_2		gluc†					
H	43	Δ^1-THC-7-oic acid glucuronide	COO	gluc†						

*Epoxy group in C-1 and C-2 positions
†Glucuronide
Note: R-group substituents are H if not indicated otherwise.

Chemical structures of some of the dibenzopyran cannabinoids are shown below.



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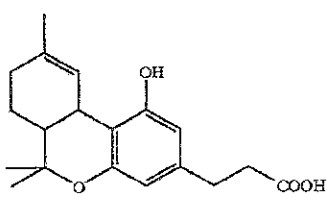

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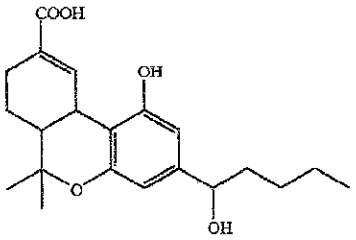

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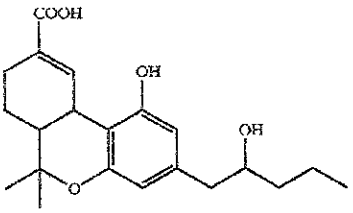

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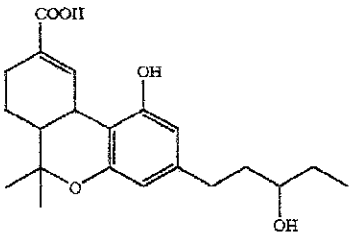

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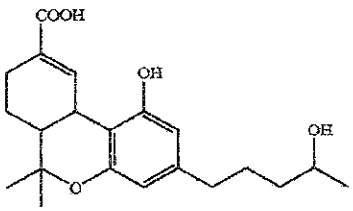

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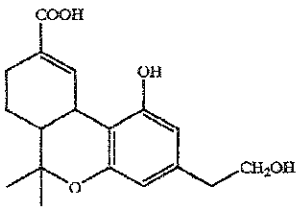

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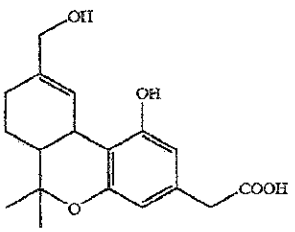

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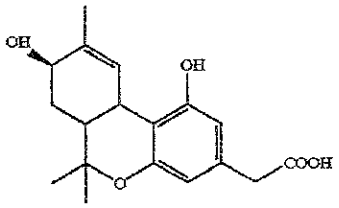

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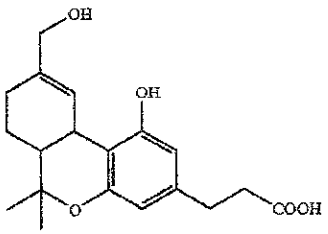

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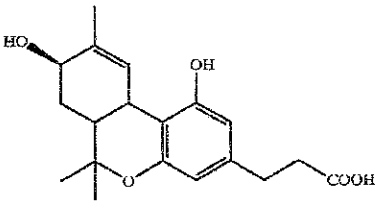

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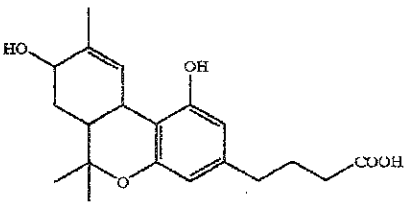

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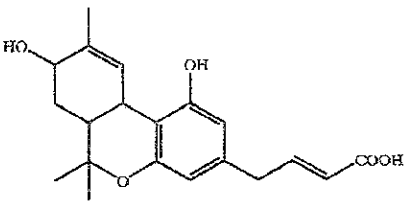

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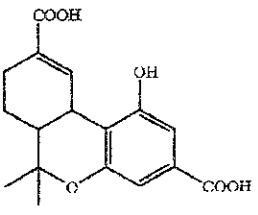

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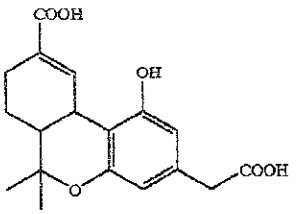

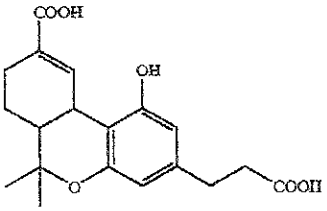

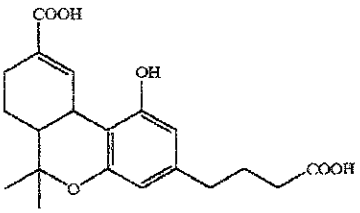

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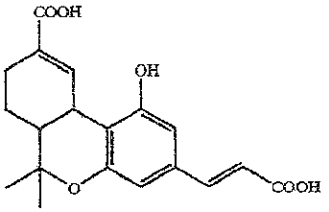
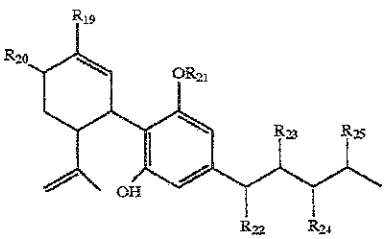
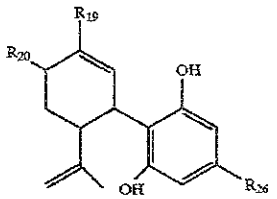


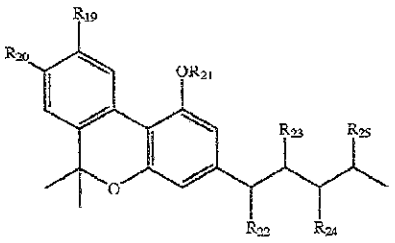
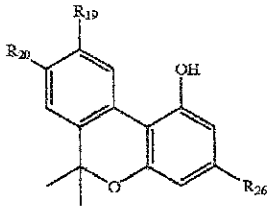
EXAMPLE 11

Examples of Structural Analogs of Cannabidiol

The following table lists examples of some cannabinoids which are structural analogs of cannabidiol and that may be useful as antioxidants in the method of the present invention. A particularly useful example is compound CBD, cannabidiol.

Compound	R_{19}	R_{20}	R_{21}	R_{22}	R_{23}	R_{24}	R_{25}	R_{26}
								
44 CBD	CH_3	H	H	H	H	H	H	C_5H_{11}
45 7-OH—CBD	CH_2OH							
46 6α-	CH_3	α-OH						
47 6β-	CH_3	β-OH						
48 1"-	CH_3			OH				
49 2"-	CH_3				OH			
50 3"-	CH_3					OH		
51 4"-	CH_3						OH	
52 5"-	CH_3							$C_4H_8CH_2OH$
53 6,7-diOH—CBD	CH_2OH	OH						
54 3",7-diOH—CBD	CH_2OH					OH		
55 4",7-diOH—CBD	CH_2OH						OH	
56 CBD-7-oic acid	COOH							
57 CBD-3"-oic acid	CH_3							C_2H_4COOH

-continued

Compound	R_{19}	R_{20}	R_{21}	R_{22}	R_{23}	R_{24}	R_{25}	R_{26}
								
58 CBN	CH_3	H	H	H	H	H	H	C_5H_{11}
59 7-OH—CBN	CH_2OH							
60 1"-OH—CBN	CH_3			OH				
61 2"-OH—CBN	CH_3				OH			
62 3"-OH—CBN	CH_3					OH		
63 4"-OH—CBN	CH_3						OH	
64 5"-OH—CBN	CH_3							$C_4H_8CH_2OH$
65 2"-7-diOH—CBN	CH_2OH				OH			
66 CBN-7-oic acid	COOH							
67 CBN-1"-oic acid	CH_3							COOH
68 CBN-3"-oic acid	CH_3							C_2H_4COOH

Note: R-group substituents are H if not indicated otherwise.

The invention being thus described, variation in the materials and methods for practicing the invention will be apparent to one of ordinary skill in the art. Such variations are to be considered within the scope of the invention, which is set forth in the claims below.

We claim:

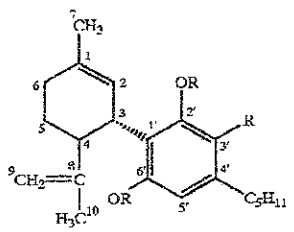
1. A method of treating diseases caused by oxidative stress, comprising administering a therapeutically effective amount of a cannabinoid that has substantially no binding to the NMDA receptor to a subject who has a disease caused by oxidative stress.

2. The method of claim 1, wherein the cannabinoid is nonpsychoactive.

3. The method of claim 2, wherein the cannabinoid has a volume of distribution of 10 L/kg or more.

4. The method of claim 1, wherein the cannabinoid is not an antagonist at the NMDA receptor.

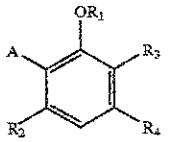
5. The method of claim 1, wherein the cannabinoid is:



where R is H, substituted or unsubstituted alkyl, carboxyl, alkoxy, aryl, aryloxy, arylalkyl, halo or amino.

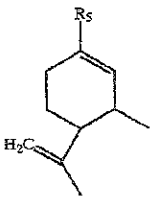
6. The method of claim 5, wherein R is H, substituted or unsubstituted alkyl, carboxyl or alkoxy.

7. The method of claim 2, wherein the cannabinoid is:



where

A is cyclohexyl, substituted or unsubstituted aryl, or



but not a pinene;

R_1 is H, substituted or unsubstituted alkyl, or substituted or unsubstituted carboxyl;

R_2 is H, lower substituted or unsubstituted alkyl, or alkoxy;

R_3 is of H, lower substituted or unsubstituted alkyl, or substituted or unsubstituted carboxyl;

R_4 is H, hydroxyl, or lower substituted or unsubstituted alkyl; and

R_5 is H, hydroxyl, or lower substituted or unsubstituted alkyl.

8. The method of claim 7, wherein

R_1 is lower alkyl, COOH or $COCH_3$;

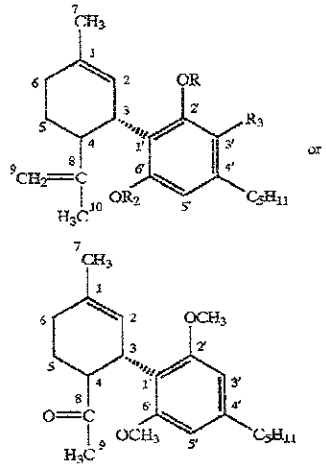
R_2 is unsubstituted C_1–C_5 alkyl, hydroxyl, methoxy or ethoxy;

R_3 is H, unsubstituted C_1–C_3 alkyl, or $COCH_3$;

R_4 is hydroxyl, pentyl, heptyl, or diemthylheptyl; and

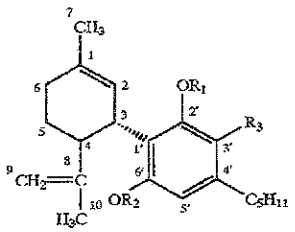
R_5 is hydroxyl or methyl.

9. The method of claim 1, wherein the cannabinoid is:



where R_1, R_2 and R_3 are independently H, CH_3, or $COCH_3$.

10. The method of claim 9, wherein the cannabinoid is:



where:

a) R_1=R_2=R_3=H;

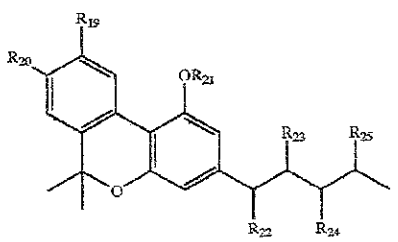
b) R_1=R_3=H, R_2=CH_3;

c) R_1=R_2=CH_3, R_3=H;

d) R_1=R_2=$COCH_3$, R_3=H; or

e) R_1=H, R_2=R_3=$COCH_3$.

11. The method of claim 2, wherein the cannabinoid is:



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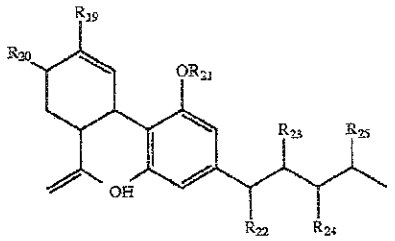
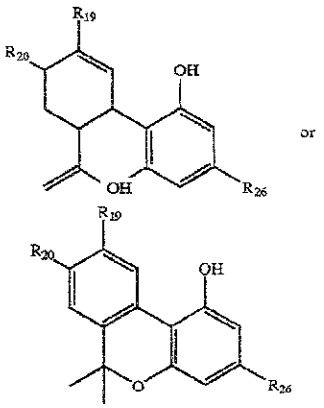


where R_{19} is H, lower alkyl, lower alcohol, or carboxyl; R_{20} is H or OH; and R_{21}–R_{25} are independently H or OH.

12. The method of claim 11, wherein R_{19} is H, CH_3, CH_2OH, or COOH, and R_{20}–R_{24} are independently H or OH.

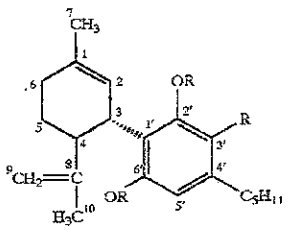
13. The method of claim 2, wherein the cannabinoid is:



where R_{19} and R_{20} are H, and R_{26} is alkyl.

14. The method of claim 10, wherein the cannabinoid is cannabidiol.

15. A method of treating an ischemic or neurodegenerative disease in the central nervous system of a subject, comprising administering to the subject a therapeutically effective amount of a cannabinoid, where the cannabinoid is



where R is H, substituted or unsubstituted alkyl, carboxyl, alkoxy, aryl, aryloxy, arylalkyl, halo or amino.

16. The method of claim 15, wherein the cannabinoid is not a psychoactive cannabinoid.

17. The method of claim 15 where the ischemic or neurodegenerative disease is an ischemic infarct, Alzheimer's disease, Parkinson's disease, and human immunodeficiency virus dementia, Down's syndrome, or heart disease.

18. A method of treating a disease with a cannabinoid that has substantially no binding to the NMDA receptor, comprising determining whether the disease is caused by oxi-

dative stress, and if the disease is caused by oxidative stress, administering the cannabinoid in a therapeutically effective antioxidant amount.

19. The method of claim **18**, wherein the cannabinoid has a volume of distribution of at least 1.5 L/kg and substantially no activity at the cannabinoid receptor.

20. The method of claim **19**, wherein the cannabinoid has a volume of distribution of at least 10 L/kg.

21. The method of claim **1**, wherein the cannabinoid selectively inhibits an enzyme activity of 5- and 15-lipoxygenase more than an enzyme activity of 12-lipoxygenase.

22. A method of treating a neurodegenerative or ischemic disease in the central nervous system of a subject, comprising administering to the subject a therapeutically effective amount of a compound selected from any of the compounds of claims **9** through **13**.

23. The method of claim **22** where the compound is cannabidiol.

24. The method of claim **22**, wherein the ischemic or neurodegenerative disease is an ischemic infarct, Alzheimer's disease, Parkinson's disease, and human immunodeficiency virus dementia, Down's syndrome, or heart disease.

25. The method of claim **24** wherein the disease is an ischemic infarct.

26. The method of claim **1**, wherein the cannabinoid is not an antagonist at the AMPA receptor.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,630,507 B1
DATED : October 7, 2003
INVENTOR(S) : Hampson et al.

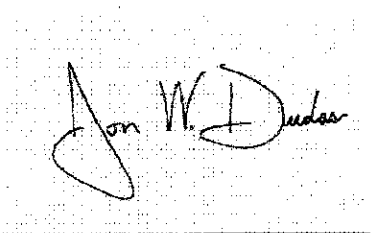
It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 13,
Line 23, "feral" should read -- fetal --.

Column 30,
Line 16, reads "R_{20}-R_{24}" should read -- R_{20}-R_{25} --.

Signed and Sealed this

Fifteenth Day of June, 2004



JON W. DUDAS
Acting Director of the United States Patent and Trademark Office