

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
30 October 2008 (30.10.2008)

PCT

(10) International Publication Number
WO 2008/129529 A2

(51) International Patent Classification:

A61K 31/352 (2006.01) A61P 3/10 (2006.01)
A61P 25/00 (2006.01)

(21) International Application Number:

PCT/IL2008/000477

(22) International Filing Date: 6 April 2008 (06.04.2008)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

60/912,720 19 April 2007 (19.04.2007) US
190 531 31 March 2008 (31.03.2008) IL

(71) Applicant (for all designated States except US): **YISSUM RESEARCH DEVELOPMENT COMPANY OF THE HEBREW UNIVERSITY OF JERUSALEM** [IL/IL]; Hi Tech Park, Edmond J. Safra Campus, Givat Ram P.O. Box 39135, 91390 Jerusalem (IL).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **BERRY, Elliot**

[IL/IL]; 17 Gihon St., Jerusalem 93547 (IL). **ME-CHOULAM, Raphael** [IL/IL]; 12 Tchernichovsky Street, Jerusalem, 92581 (IL). **AVRAHAM, Yosefa** [IL/IL]; 19a Nof Harim, 96190 Jerusalem (IL). **DAGON, Yossi** [IL/IL]; 20 David Elhazar, Nes Ziona (IL).

(74) Agent: **WOLFF, BREGMAN & GOLLER**; Patent Attorneys, P.O. Box 1352em, 19B Keren Hayesod St., Jerusalem 91013 (IL).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH,

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(54) Title: CANNABINOID FOR THE TREATMENT OF NEURONAL DAMAGE IN DIABETIC PATIENTS

Fig. 1A

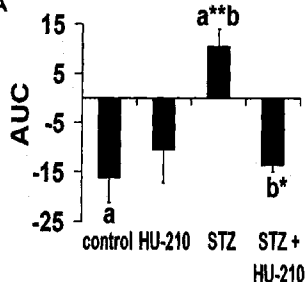


Fig. 1B

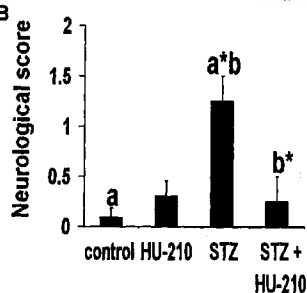


Fig. 1C

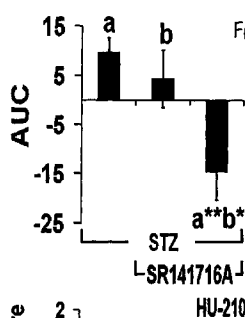
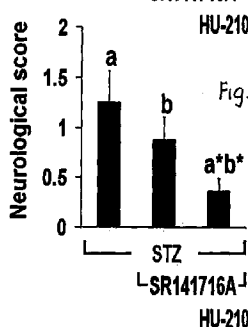
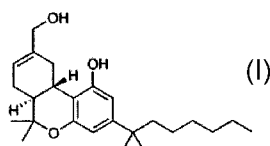


Fig. 1D



(57) Abstract: The invention provides for the use of an effective amount of at least one cannabinoid selected from the group consisting of HU-210 and Tetrahydrocannabinol (THC) for the manufacture of a pharmaceutical composition for alleviating neuronal damage due to hyperglycemia, or for treating neuronal damage in a diabetic subject, wherein HU-210 is the (+)-1,1-dimethylheptyl analog of 7-hydroxy- Δ^6 -tetrahydrocannabinol of the formula (I).





GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report*

CANNABINOID FOR THE TREATMENT OF NEURONAL DAMAGE IN DIABETIC PATIENTS

The invention provides for the use of an effective amount of at least one cannabinoid for the manufacture of a pharmaceutical composition for alleviating neuronal damage due to hyperglycemia, or for treating neuronal damage in a diabetic subject.

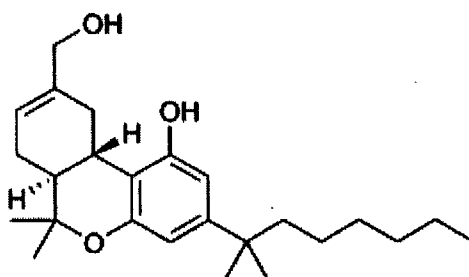
BACKGROUND OF THE INVENTION

Diabetes mellitus is a heterogeneous metabolic disorder characterized by chronic hyperglycemia due to a deficiency (type 1) or resistance (type 2) to insulin (Zimmet et al., 2001). The long-term neurological complications of diabetes which includes peripheral damage (diabetic neuropathy referred to hereinafter as DN), as well as central nervous system neuropathy, is referred to as "diabetic encephalopathy". (Biessels et al., 1994). DN is one of the most common long-term complications of diabetes causing a significant morbidity. Most studies suggest that 50% of patients with a 20 year history of diabetes, whether type 1 or type 2, develop neuropathy (Feldman et al., 1997). Clinical studies in diabetic patients demonstrate changes in cognitive function and decline in memory and mental speed (Strachan et al., 1997; Awad et al., 2004) which is more pronounced in the elderly (Sinclair et al., 2000) and in whom the incidence of dementia appears to be doubled (Ott et al., 1999). Hyperglycemia resulting from uncontrolled diabetes is recognized as the proximal, causal link in the evolution of the neuropathy (Giardino et al., 1996; Brownlee, 2001). Hyperglycemia, in both animals and in vitro models of diabetes, activates a number of pathways of glucose metabolism which are implicated in the development of the neuropathy. These include sorbitol and fructose accumulation, NAD(P)-redox imbalances, protein kinase C (PKC) activation, increased hexosamine pathway, superoxide overproduction and reduced levels of key antioxidative enzymes (Zaltzberg et al., 1999) - all of which result in elevated cellular oxidative stress. Clinical evidence shows that hyperglycemia-induced oxidative stress predisposes to complications in diabetic patients, and its inhibition may block the initiation and progression of the neuropathy (Greene et al., 1992; Osen et al., 2001). Oxidative stress is generated in the neural tissue when the production of free radical moieties exceeds its antioxidant capacity. Insufficient antioxidant capacity leads to free

radical attack which damages proteins, lipids, and nucleic acids. This cellular damage triggers apoptosis in neurons and supporting glial cells, which contributes to the neuropathology associated with diabetes (Greene et al., 1999). To date, antioxidant therapy is the most promising approach to the prevention of diabetic neuropathy. However, efficacy of this kind of DN treatment has yet to be demonstrated in clinical trials (Vincent et al., 2004). Synthetic and endogenous cannabinoids exert neuroprotective effects in animals as well as in vitro models of various forms of acute neuronal injury, such as cerebral ischaemia, traumatic brain injury, neurodegenerative diseases, and Huntington's disease (Grundy et al., 2001; Baker et al., 2003). Recently, the neuroprotective actions of cannabidiol and other cannabinoids were shown in cultured cortical neurons to be mediated by a potent antioxidative effect which was receptor independent (Hampson et al., 1998). Studies in animal models of focal cerebral ischemia suggest that the antioxidative effect of cannabinoids was at least as effective in vivo as found in vitro (Hampson et al., 2000).

SUMMARY OF THE INVENTION

The present invention is based on the finding of neuroprotective effects of cannabinoids in experimental models of diabetes – in vivo and in vitro. The synthetic cannabinoid agonist HU-210 which is the (+)-1,1-dimethylheptyl analog of 7-hydroxy-*delta*-6-tetrahydrocannabinol of the formula:



was found to be much more potent than other widely used cannabinoid compounds such as anandamide and 2-AG (Pop et al., 1999). It has now been found that HU-210 attenuates neural oxidative stress, brain function impairment, neural apoptosis, proliferation arrest and differentiation *without* affecting glycemic control. It has further

been found that isolated tetrahydrocannabinol (THC) was able to improve brain function in diabetic mice.

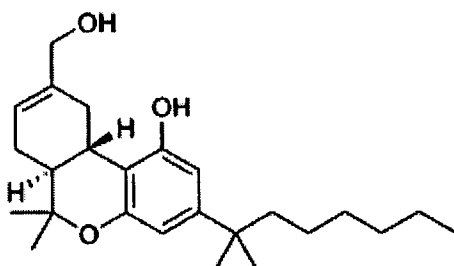
SUMMARY OF THE INVENTION

The present invention is based on the finding that the synthetic cannabinoid, HU-210 was capable of alleviating the oxidative damage and cognitive impairment, in streptozotocin (STZ)-induced diabetic mice, without affecting glycemic parameters.

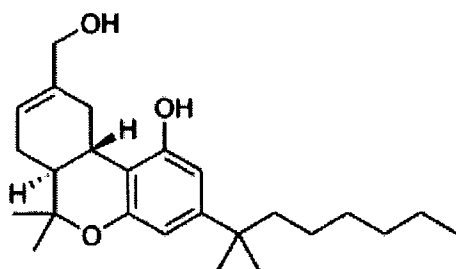
The present invention is further based on the finding that HU-210 was able to alleviate hyperglycemia -induced oxidative stress and cellular injuries in PC12 cells.

The present invention is further based on the finding that isolated THC was able to improve brain function in diabetic mice.

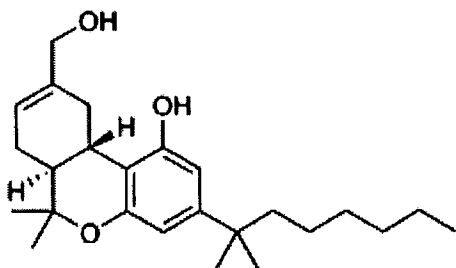
Thus the present invention is directed to the use of an effective amount of at least one cannabinoid selected from the group consisting of isolated Tetrahydrocannabinol (THC) and HU-210 for the manufacture of a pharmaceutical composition for alleviating neuronal damage due to hyperglycemia, wherein HU-210 is the (+)-1,1-dimethylheptyl analog of 7-hydroxy-*delta*-6-tetrahydrocannabinol of the formula:



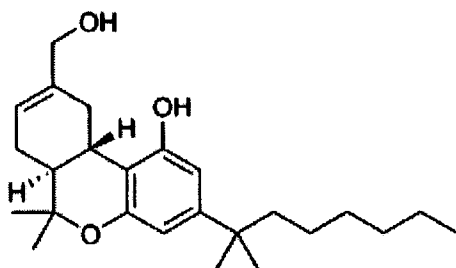
The present invention further concerns the use of at least one cannabinoid selected from the group consisting of isolated Tetrahydrocannabinol (THC) and HU-210 for alleviating neuronal damage due to hyperglycemia, wherein HU-210 is the (+)-1,1-dimethylheptyl analog of 7-hydroxy-*delta*-6-tetrahydrocannabinol of the formula



The present invention is also directed to the use of an effective amount of at least one cannabinoid selected from the group consisting of isolated Tetrahydrocannabinol (THC) and HU-210 for the manufacture of a pharmaceutical composition for treating neuronal damage in a diabetic subject, wherein HU-210 is the (+)-1,1-dimethylheptyl analog of 7-hydroxy-*delta*-6-tetrahydrocannabinol of the formula:

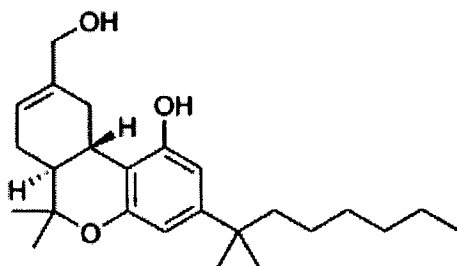


The present invention further concerns the use of at least one cannabinoid selected from the group consisting of isolated Tetrahydrocannabinol (THC) HU-210 for treating neuronal damage in a diabetic subject, wherein HU-210 is the (+)-1,1-dimethylheptyl analog of 7-hydroxy-*delta*-6-tetrahydrocannabinol of the formula

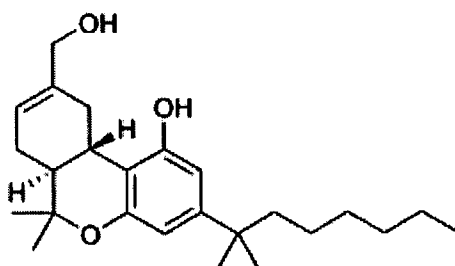


In another aspect of the present invention, there is provided a method for alleviating neuronal damage due to hyperglycemia, the method comprising: administering an effective amount of at least one cannabinoid selected from the group consisting of isolated Tetrahydrocannabinol (THC) and HU-210 wherein HU-210 is

the (+)-1,1-dimethylheptyl analog of 7-hydroxy-*delta*-6-tetrahydrocannabinol of the formula:



The present invention also provides a method for treating neuronal damage in a diabetic subject the method comprising: administering to a subject in need of the treatment a therapeutically effective amount of at least one cannabinoid selected from the group consisting of isolated Tetrahydrocannabinol (THC) and HU-210 and Tetrahydrocannabinol (THC), wherein HU-210 is the (+)-1,1-dimethylheptyl analog of 7-hydroxy-*delta*-6-tetrahydrocannabinol of the formula:



The term "**hyperglycemia**" refers to elevated glucose levels, as compared to the levels neurons are exposed to under normal physiological conditions

In a first aspect of the present invention the hyperglycemia is the result of diabetic mellitus type 1

In a further aspect of the present invention the hyperglycemia is the result of diabetic mellitus type 2.

The "**neuronal damage**" may be central (diabetic encephalopathy) and is mainly measured by electrophysiological and structural changes and limitations in the cognitive functioning of the subject. The neuronal damage may also be peripheral (diabetic neuropathy), affecting the peripheral nerves, and is manifested by numbness, pain, tingling in the feet, double vision or drooping eyelids, or weakness and atrophy of

the thigh muscles. In severe cases diabetic neuropathy may lead to problems in the digestive tract and sexual organs, which can cause indigestion, diarrhea or constipation, dizziness, bladder infections, and impotence. The loss of sensation in the feet may increase the possibility of foot injuries going unnoticed, and which then would develop into ulcers or lesions that become infected. In some embodiments the neuronal damage is for motor weakness which is equivalent to NSS but affecting both sides of the body, and which condition is determined by placement tests to test for proprioception and position. The neuronal damage may also be evident by instability in blood pressure and heart rate.

The term "**treating**" refers to any one of the following: improving at least one undesired symptom of neuronal damage (peripheral or central as defined above); halting the deterioration in the neuronal function as compared to an untreated control; slowing down the rate of deterioration in the neuronal damage as compared to an untreated control; preventing the deterioration of some manifestation of the neuronal damage before it occurs.

The term "**isolated Tetrahydrocannabinol (THC)**", refers to essentially pure THC which is not in combination with one or more of the compounds naturally found in cannabis extract

The pharmaceutical compositions of the present invention can be provided in any form known in the art, for example in a form suitable for oral administration (e.g., a solution, a suspension, a syrup, an emulsion, a dispersion, a suspension, a tablet, a pill, a capsule, a pellet, granules and a powder), for parenteral administration (e.g., intravenous, intramuscular, intraarterial, transdermal, subcutaneous or intraperitoneal), for topical administration (e.g., an ointment, a gel, a cream), for administration by inhalation or for administration via suppository.

In connection with THC the preferred mode of administration is sublingual, preferably by administering 5 mg THC twice a day sublingually in an olive oil solution (5 mg in 0.2 ml oil).

While the invention will now be described in connection with certain preferred embodiments in the following examples and with reference to the accompanying

drawings so that aspects thereof may be more fully understood and appreciated, it is not intended to limit the invention to these particular embodiments. On the contrary, it is intended to cover all alternatives, modifications and equivalents as may be included within the scope of the invention as defined by the appended claims. Thus, the following examples which include preferred embodiments will serve to illustrate the practice of this invention, it being understood that the particulars shown are by way of example and for purposes of illustrative discussion of preferred embodiments of the present invention only and are presented in the cause of providing what is believed to be the most useful and readily understood description of formulation procedures as well as of the principles and conceptual aspects of the invention

DESCRIPTION OF THE DRAWINGS

Figure 1. Effect of HU-210 on brain function of STZ treated mice.

Mice were injected i.p. with 200 mg/kg STZ. 8 weeks later, 0.1 mg/kg HU-210 was administered daily for 5 days. (A) Performance in an eight arm maze was recorded every day after the HU-210 treatment. (B) Neurological score was evaluated after the HU-210 treatment. (C) Performance in an eight arm maze after co-administration with 0.1 mg/kg HU-210 and 5 mg/kg SR141716A. (D) Neurological score evaluation after co-administration with 0.1 mg/kg HU-210 and 5 mg/kg SR141716A. The figures represent 3 independent experiments. Values were calculated as described in *Materials and Methods*.

Figure 2. Effect of HU-210 on blood glucose levels and body weight of STZ-treated mice.

Mice were injected i.p. with 200 mg/kg STZ. 8 weeks later, 0.1 mg/kg HU-210 was administered daily for 5 days. (A) Blood glucose levels (mg/dl) were measured by Glucometer Elite XL and presented as mg per dl. (B) Weights were recorded.

Figure 3. Effect of HU-210 on cerebral oxidative stress following STZ treatment.

Mice were injected I.P with 200 mg/kg STZ. 8 weeks later, 0.1 mg/kg HU-210 was administered daily for 5 days. Cerebral oxidative stress was measured by TBARS and calculated as nmol TBARS per mg protein.

Figure 4. PC12 cells viability in response to hyperglycemic condition and HU-210 treatment.

PC12 cells (2×10^4) were seeded in a medium containing 8% FBS+ 8% HS. The cells were exposed to 27 g/L glucose and treated with vehicle or 5-20 μ M HU-210 for up to 72 h. WST-1 was supplemented to the medium for 2 h. Absorbance was determined at the respective wavelength with an ELISA reader. The figures are averages of five independent experiments.

Figure 5. Effect of HU-210 on the hyperglycemia pro-apoptotic activity.

PC12 cells (1×10^5) were seeded in a medium containing 8% FBS and 8% HS, exposed to 27 g/L glucose and treated with the indicated concentrations of HU-210. After 48 h, the cells were co-stained with annexin V antibodies and propidium iodide and were then analyzed by flow-cytometry. (A). FACS analysis of PC12 cells exposed to 27 g/l glucose and treated with vehicle or 20 μ M HU210. Apoptotic cells are represented by the two right hand rectangles in each panel and calculated as such. (B) Apoptotic rates of PC12 cells exposed to 27 g/L glucose and treated with the indicated dosage of HU-210. The figures are averages of three independent experiments.

Figure 6. The cell cycle profile of PC12 is modulated by hyperglycemia and HU-210.

PC12 cells (1×10^5) were seeded in a medium containing 8% FBS and 8% HS, exposed to 27 g/L glucose and treated with the indicated concentrations of HU-210. At 24 h, the cells were stained with propidium iodide and subjected to cell cycle profiling by flow-cytometry. The figures are averages of five independent experiments.

Figure 7. Effect of HU-210 and hyperglycemia on NGF-mediated neurite outgrowth.

PC12 cells (2×10^4) were maintained in a medium containing 1% FBS, 1% HS and 50 ng/ml NGF. (A) Representative phase-contrast images showing neurite outgrowth in PC12 cells exposed to 27 g/L glucose and treated with vehicle or 20 μ M HU210. Panels were randomly selected and are representative fields. (B) Graph comparing neurite length in PC12 cells treated with the indicated dosage of HU-210

under normal or 27 g/L medium glucose. The figures are averages of three independent experiments.

Figure 8. Effect of HU-210 on hyperglycemia-induced hyper dopamine levels in PC12 cells.

PC12 cells (1×10^6) were seeded in a medium containing 8% FBS and 8% HS, exposed to 27 g/L glucose and treated with vehicle or 20 μ M HU-210 for 6 h. Cells were harvested and analyzed by HPLC. The figures represent 5 independent experiments.

Figure 9. PC12 intracellular ROS formation in response hyperglycemic conditions and HU-210 treatment.

PC12 cells were exposed 27 g/l glucose for 72h. Intracellular ROS levels were measured by fluorescent probe DCFH-DA. The values represent 5 independent experiments.

Fig 10 Effect of THC on brain function of STZ treated mice.

Mice were injected i.p. with 200 mg/kg STZ. 8 saline, THC (0.1mg/kg) or THC+SR144528 (0.1mg/kg) were administered intraperitoneally for 1 day, 1 h before the maze testing was carried out and results are shown in the graph .

DETAILED DESCRIPTION OF THE INVENTION

Materials and Methods

Cells and reagents

PC12 cells were kindly provided by O. Meyuhas, Dept. of Biochem., Faculty of Medicine, Jerusalem. These cells were grown either in Dulbecco's modified Eagle's medium (DMEM) supplemented with 8% horse serum (HS), 8% fetal bovine serum (FBS), glutamine, and gentamicin or with 1% HS, in the presence of nerve growth factor (NGF) (50 ng/ml), which causes the cells to differentiate. HU-210 was provided by Prof. Raphael Mechoulam.

Mice

Eight- to 10-week old female Sabra mice (29-32g) were assigned at random to different groups of 10 mice per cage and were used in all experiments. All cages contained wood-chip bedding and were placed in a temperature-controlled room at

22 °C, on a 12 h light/dark cycle (lights on at 07.00 a.m.). The mice had free access to water 24 h a day. The food provided was Purina chow and the animals were maintained in the animal facility (SPF unit) of the Hebrew University Hadassah Medical School, Jerusalem.

Mice were sacrificed after treatment by decapitation between 10.00-12.00 a.m. Brains were rapidly removed, dissected and kept at -70°C.

In-vivo experimental protocol

Mice were made diabetic by intraperitoneal (i.p.) injection of 200 mg/kg of body weight STZ (Sigma) in 0.05M citrate buffer (pH 4.5). Mice receiving an injection of citrate buffer were used as controls. Hyperglycemia was determined 4 days after injection by blood glucose analysis. Mice with blood glucose levels >300 mg/dl were considered to have diabetes. Mice were divided into four groups: control mice treated with saline, control mice treated with HU-210, diabetic mice treated with saline, and diabetic mice treated with HU-210. HU-210 or saline were administered i.p. daily.

Glucose assay

Blood glucose levels were measured by Glucometer Elite XL according to the manufacturer's instruction.

Thiobarbituric acid-reactive substances (TBA-RS) measurement

TBA-RS was determined according to the method of Esterbauer and Cheeseman (Esterbauer and Cheeseman., 1990). Briefly, 300 uL of cold 10% trichloroacetic acid were added to 150 uL of brain supernatant and centrifuged at 300 g for 10 min. Three hundred microlitres of the supernatant were transferred to a Pyrex tube and incubated with 300 uL of 0.67% thiobarbituric acid (TBA) in 7.1% sodium sulphate in boiling water bath for 25 min. The mixture was allowed to cool on running tap water for 5 min. The resulting pink-stained TBA-RS was determined in a spectrophotometer at 532 nm. A calibration curve was performed using 1,1,3,3-tetramethoxypropane. Each curve point was subjected to the same treatment as supernatants. TBARS was calculated as nmol TBARS/mg protein.

Eight-arm maze

The animals were placed in an eight-arm maze as described previously (Dagon, et al, 2005). A water deprivation method was used and performed by limiting water consumption overnight and presenting a reward of 50 μ l of water at the end of each arm. The mice were tested until they made entries into all eight arms or until they completed 24 entries, whichever came first. Hence, the lower the score is, the better the performance. Maze performance was calculated each day for five consecutive days. Results were presented as area under the curve (AUC) utilizing the formula: (day 2 + day 3 + day 4 + day 5) - 4*(day 1).

Neurological function

Neurological function was assessed by a 10 point scale based on reflexes and task performance (Chen et al., 1996): exit from a circle 1 meter in diameter in less than 1 minute; seeking; walking a straight line; startle reflex; grasping reflex; righting reflex; placing reflex; corneal reflex; maintaining balance on a beam 3, 2 and 1 cm in width; and climbing onto a square and a round pole. For each task failed or abnormal reflex reaction, a score of 1 was assigned. Thus, a higher score indicates poorer neurological function. The neurological score was assessed one day after TAA induction (day 2). The mice were then divided between treatment groups so that each group had a similar baseline neurological score before STZ administration.

2',7'-dihydrodichlorofluorescein (DCFH) oxidation.

Reactive species production was assessed according to LeBel et al.,1992 by using 2',7'-dihydrodichlorofluorescein diacetate (DCF-DA). DCF-DA prepared in 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl, was incubated with pre-treated cerebral cortex supernatants during 30 min at 37 °C. DCF-DA is enzymatically hydrolyzed by intracellular esterases to form non-fluorescent DCFH, which is then rapidly oxidized to form highly fluorescent 2',7'-dichlorofluorescein (DCF) in the presence of reactive species (RS). The DCF fluorescence intensity parallels the amount of RS formed. Fluorescence was measured using excitation and emission wavelengths of 480 nm and 535 nm, respectively. Calibration curve was performed with standard DCF (0.25–10 mM) and the levels of RS were calculated as pmol DCF formed/mg protein.

Cell viability

Cell viability was evaluated in control and hyperglycemic conditions treated with HU-210 for 24-72h. The assay was performed using the Cell proliferation Reagent WST-1 kit, based on the cleavage of the tetrazolium salt WST-1 (Berridge M.V., 1996), according to the manufacturer's instruction (Roche Applied Science, Germany). Values are presented as percent of control.

Cell cycle analysis and annexin V staining

Cells were grown in 60 mm plate, exposed to 27 g/L glucose and treated with HU-210 as before. Both attached and floating cells were collected after 24 h and centrifuged at 5000xg for 5 min. The cell pellet was fixed with 1 ml 70% ethanol (1 ml), centrifuged and re-suspended in 1 ml PBS containing RNase A and propidium iodide (50 mg/ml each). Stained cells were analyzed for relative DNA content by a Coulter FACSsort flow cytometer (Becton Dickinson).

Apoptosis was evaluated in cells exposed to 27 g/l glucose and treated with HU-210 for 48 h using the Annexin V FITC Detection Kit according to the manufacturer's instruction (Oncogene Research Products, Cambridge MA). Briefly, both attached and floating cells were collected, washed with cold PBS, re-suspended at a density of 5×10^5 /ml in 0.5 ml DMEM, stained for annexin V and analyzed by flow cytometry.

Measurement of neurite extension

To assess the effect of HU-210 and hyperglycemia on NFG-mediated neurite outgrowth, PC12 cells were grown in the presence of NGF (50 ng/ml) and assayed by measurement of the number of cells bearing at least one extended neurite (>2 mm). Neurites were counted in 100 cells per field in four separated fields per well. Triplicate wells were used routinely for each experimental condition or treatment.

Catecholamine measurements

Catecholamines were measured as described previously (Dagon et al., 2005). 12×10^6 cells were collected, washed with cold PBS and re-suspended for each analysis. The assay for dopamine was performed by HPLC separation and detection using HPLC-ECD. Values are presented as a concentration of ng per 1 g of re-suspended cells.

Statistics

Data from 3 to 5 independent experiments was used for calculating average values. The statistical difference between sets of values was calculated by Student's T-test of unpaired data.

RESULTS

HU-210 ameliorates brain function impairment of diabetic mice.

Cannabinoids have been shown to protect the brain from various insults and to improve several neurodegenerative diseases (Grundy et al., 2001). However, their direct effect on diabetic encephalopathy has not been demonstrated. The effect of the synthetic cannabinoid HU-210 was tested on STZ-induced brain dysfunction. Mice were assigned randomly to saline or STZ treatment groups. After 8 weeks of diabetes, control and STZ administered mice were treated daily with HU-210 0.1 mg/kg and tested in the eight arm maze. Diabetic mice demonstrated a progressive decline in performances throughout the 5 days of testing. HU-210 treatment did not significantly affect performance in the control group. However, performance of the diabetic mice was significantly improved by HU-210 treatment (**Fig.1A**). Diabetic mice also exhibited reduced neurological score. This effect was overcome by HU-210 treatment (**Fig. 1B**). Recent studies have claimed that much of the cannabinoid neuroprotective effect is mediated through the cerebral CB-1 receptor signaling (Van der Stelt et al, 2005). To study this pathway, STZ administered mice were co-treated daily with the CB-1 antagonist SR141716A alone and together with HU-210 0.1 mg/kg. Mice were subjected to the eight arm maze as before. SR141716A treatment did not significantly affect the progressive decline in performance demonstrated by the diabetic mice or the improvement following HU-210 treatment (**Fig.1C**). In addition, no decrease in the beneficial effect of HU-210 on the neurological index was recorded in response to the SR141716A administration (**Fig.1D**). These results demonstrated a potential benefit of HU-210 treatment for the brain function impairment in diabetes through a pathway that is not mediated via CB1 receptors.

HU-210 treatment does not alter STZ induced hyperglycemia.

Clinical evidence suggests that the cognitive disturbances characterizing diabetic neuropathy are associated with chronic hyperglycemia (Ryan et al., 1992). At the onset of the study all animals had similar blood glucose levels (data not shown). To assess the effect of HU-210 on the STZ induced systemic hyperglycemia, mice were assigned randomly to saline or STZ treatment for 8 weeks as before. Blood glucose levels were recorded in control and STZ administrated STZ mice daily treated with 0.1 mg/kg HU-210 for 5 days. HU-210 treatment had no significant effect on the hyperglycemic index in diabetic animals or on normal levels in the treated controls (Fig. 2A). HU-210 did not affect the animal's weights (Fig. 2B).

HU-210 attenuates cerebral oxidative stress in diabetic mice.

One of the mechanisms underlying hyperglycemia-induced neural degeneration is increased oxidative stress (Greene et al., 1999). The effect of HU-210 on cerebral ROS formation in the diabetic animals was investigated. STZ treated mice (which exhibited brain function impairment as described previously) demonstrated a significant increase of cerebral ROS levels when compared to the control group. Elevated ROS levels were normalized by treatment with 0.1 mg/kg HU-210 (Fig. 3). This result implied that the mechanism by which HU-210 improved cognitive function may also involve attenuation of ROS production

HU-210 protects PC12 cells cultured in hyperglycemic conditions.

To elucidate the neural actions of HU-210 in vivo, the effect of HU-210 on cultured PC12 cells under hyperglycemic conditions was studied. Since the optimal glucose concentration for PC12 cell-cultures is 4.5 g/l, in vivo hyperglycemia was stimulated by increasing the medium glucose level to up to 27 g/l (6 fold of the optimal glucose concentration). PC12 cells exposed to increasing levels of glucose for 24h exhibited a dose dependent toxicity, reaching a maximal effect in response to 27 g/l medium glucose. This concentration was selected for further study as representative of hyperglycemic conditions (data not shown). Prolonged exposure to hyperglycemia (72h) caused a time-dependent cell loss. HU-210 treatment increased cell viability during the incubation periods in a dose dependent manner (Fig. 4). These results

established a direct effect of HU-210 on neuronal targets and implied a neuroprotective effect of HU-210 on hyperglycemic PC12 cells.

HU-210 counteracts hyperglycemia-induced apoptotic cell death.

To study the mechanism underlying the neuroprotective effects of HU-210, The apoptotic index of hyperglycemic cells in response to treatment was studied. PC12 cells were exposed to hyperglycemic conditions and treated with 5-20 μ M HU-210. Annexin V staining showed a sharp increase of apoptotic cell number in response to the hyperglycemic condition. This effect was almost completely abolished following 20 μ M HU-210 administration (**Fig. 5**). Treatment of control cells with 5-20 μ M HU-210 had no significant effects (data not shown).

HU-210 overcomes hyperglycemia-induced cell cycle arrest.

To study further the cellular outcome of HU-210 treatment, the cell cycle profile of PC12 cells under hyperglycemic conditions was investigated. Exposure of the cells to hyperglycemia led to inhibition of cell cycle progress from G1 to S, as determined by FACS. While 5-20 μ M HU-210 treatment of control cells produced no significant changes in the cell cycle profile (data not shown), HU-210 treatment abolished the cell cycle arrest and restored the percent of cells in G1 and S phases to control values (**Fig. 6**).

HU-210 improves hyperglycemia-induced impaired neuritogenesis.

Treatment of PC12 pheochromocytoma cells with NGF triggers differentiation from a chromaffin-like to a sympathetic neuron-like phenotype, making these cells a suitable model for studies of neural differentiation (Greene et al., 1976). Following 50 ng/ml NGF treatments, the cells produce neurite out-growth processes which are strongly inhibited by exposure to hyperglycemic conditions (27 g/l). As shown in **Fig. 7**, this inhibition by the hyperglycemic environment was completely abolished by 5-20 μ M HU-210 treatment in a dose-dependent manner. One of the interesting observations from these experiments was that HU-210 by itself appeared to trigger a differentiation response with a bell-shaped curve which reached a peak at 10 μ M. This result clearly demonstrates the rehabilitative effect of HU-210 on impaired neural differentiation in vitro.

HU-210 withholds hyper catecholamine levels in PC12 cells following hyperglycemia.

PC12 cells synthesize and store the catecholamine neurotransmitters dopamine and norepinephrine (Greene et al., 1976). The effect of hyperglycemia on catecholamines levels was studied. Exposure of the cells to hyperglycemia promptly enhanced dopamine levels after 3h (and longer). Treatment of these cells with 20 μ M HU-210 restored elevated dopamine levels to control levels (Fig. 8).

HU-210 blocks cellular oxidative stress in PC12 exposed to hyperglycemic conditions.

Hyperglycemia has been demonstrated to increase intracellular ROS production in PC12 cells exposed to hyperglycemic conditions (Lelkes et al., 2001). To study the effect of HU-210 on ROS formation in PC12 cells, the fluorescent probes DSF-DA were used. As shown in Fig. 9, DSF-DA associated fluorescence significantly increased after 48 and 72h exposure to hyperglycemic conditions. 5-20 μ M HU-210 treatment had no effects on the ROS levels in control cells. However, it blocked elevated ROS formation in cells exposed to hyperglycemia in a dose-dependent manner.

The beneficial effect of THC on mice administered with STZ is mediated by the CB2 receptors

Cognitive function was evaluated by spontaneous alternation (T-maze). T-maze is a 'two-trial' phenomena in which an animal is said to alternate if its choice on the second trial of testing is opposite from that of the first trial. The method was based on that described by Henderson (1970) with modifications (Zahalka et al., 1995). The apparatus consisted of a T-shaped maze made of opaque Plexiglas® and a transparent cover. The width and the height of the T-maze were 4×4 cm. Female Sabra mice administered with STZ as described hereinbefore, were randomized into each treatment group. Saline, THC (0.1mg/kg) or THC+SR144528 (0.1mg/kg) were administered intraperitoneally for 1 day, 1 h before the maze testing was carried out. The mouse was placed in the entrance arm and the dividing door was open. Immediately after the mouse entered one of the horizontal arms, it was returned to the entrance arm for the next trial. Each mouse was allowed a maximum of four

alternations (five entrances) The number of alternations was measured (the higher the score the better the performance). STZ administration decreased significantly the performance in the maze ($p < 0.001$) while THC reversed its performance ($p < 0.001$). Administration of SR144528 (CB2 antagonist) impaired the performance. Thus it seems that the effect of THC is mediated by the CB2 receptor

DISCUSSION

Much research on neural and cognitive function in diabetes has been derived from a variety of experimental models of the disease. These demonstrate impairment in both cognition and synaptic plasticity. While experimental diabetes caused by insulin deficiency, exhibits reproducibly an impaired cognitive phenotype, other models caused by insulin resistance demonstrate much more varied behavior. This most probably reflects the neuromodulatory effects of the genetic alterations required to generate these models (Biessels et al., 2005). Therefore, the STZ agent, which induces insulin deficiency was used, as an experimental model to evaluate the neuroprotective potential of HU210. Additionally, HU-210 was administered systemically since the intracerebral route may be toxic (Blokland et al., 1994). The compound improved the cognitive dysfunction associated with the experimental diabetes. Although the exact pathogenetic mechanisms are under debate, hyperglycemia is recognized as the major proximal cause, since cognitive impairment can be ameliorated by improving glycemic control (Mooradian., 1997). Recognizing that cannabinoids are involved in both local and systemic energy balance (Di Marzo et al., 2005), there originally was concern that HU-210 might aggravate hyperglycemia by stimulating appetite and weight gain. Nevertheless, the doses used of HU-210 had no such effects and it was concluded that its protective actions were at the tissue level. Furthermore, this same dosage of HU-210 did not improve cognitive dysfunction induced by severe diet restriction - a stress which is associated with the converse situation of hypoglycemia (Dagon et al; unpublished results). This observation reinforces the conclusion that the specificity of the neuroprotective effect was against hyperglycemic stress rather than through other metabolic / nutritional actions of endocannabinoids. Since the therapeutic implications of cannabinoids treatment were initially recognized, much attention was given to the

mechanism of action. One of the leading possibilities is that cannabinoids may act as CB-1 agonists which activate signaling pathway(s) responsible for their neuroprotective effects. However, the present results clearly show that CB-1 antagonism does not block the neuroprotective actions of HU-210 treatment. Therefore it is believed that HU-210 acts through another mechanism not yet elucidated.

According to the present invention THC was found also to act through a mechanism other than CB1 Receptor.

Previous studies have shown that hyperglycemia leads to apoptosis in both PC12 cells (Lelkes et al., 2001; Okouchi et al., 2005) and in cultured neurons (Russell et al., 1999, ; Russell et al., 2002). It has now been demonstrated that HU-210 was able to counteract in a time-, and dose- dependent manner apoptotic cell death induced by hyperglycemia. The focus of the former studies was upon the loss of neuronal function and decreased survival as a cause of DN. However, diabetes-induced neuronal damage is not only derived from apoptotic cell loss, but also from impaired regeneration processes (Olydefkis et al., 2003 ; Apfel., 1999). In patients with diabetic neuropathy, the balance between degeneration and regeneration shifts towards the former, while therapeutic efforts attempt to promote regeneration (Apfel., 1999). Thus, the finding that HU-210 overcomes hyperglycemia-induced proliferation arrest and inhibition of differentiation, suggests that in addition to preventing neural cell loss, cognitive improvement resulted from stimulating cell regeneration (As seen in Fig 7A).

The origin of PC12 cells from a transplantable rat adrenal pheochromocytoma suggests a peripheral effect for HU-210 treatment alongside the central one. Thus, the potential therapeutic benefits of HU-210 could partly be derived from attenuation of peripheral nerve damage which is responsible for a major part of DN pathology.

In summary, DN is one of the most common complications of diabetes, severely interfering with the quality of life of patients. Currently, improvement of glycemic control remains the principal therapeutic strategy to prevent or delay its onset or progression (Zochodne., 1999). A vast effort is invested in searching for therapies based on superior antioxidative properties for the prevention of neuropathy in diabetic patients. HU-210 administration ameliorated the neural insults of diabetes, both in vivo and in vitro.

These results suggest the potential therapeutic use of cannabinoid-like compounds in the management of diabetic neurological complications.

It will be evident to those skilled in the art that the invention is not limited to the details of the foregoing illustrative examples and that the present invention may be embodied in other specific forms without departing from the essential attributes thereof, and it is therefore desired that the present embodiments and examples be considered in all respects as illustrative and not restrictive, reference being made to the appended claims, rather than to the foregoing description, and all changes which come within the meaning and range of equivalency of the claims are therefore intended to be embraced therein.

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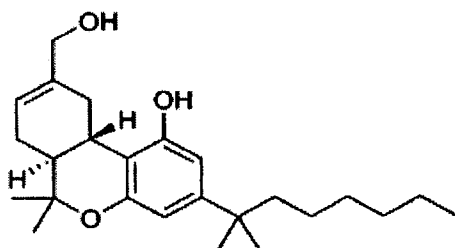
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WHAT IS CLAIMED IS:

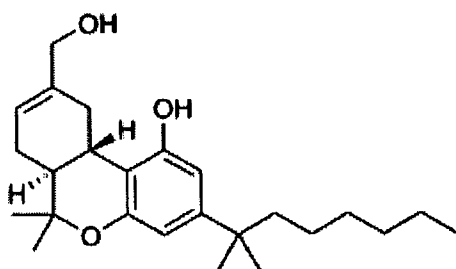
1. The use of an effective amount of at least one cannabinoid selected from the group consisting of isolated Tetrahydrocannabinol (THC) and HU-210 for the manufacture of a pharmaceutical composition for alleviating neuronal damage due to hyperglycemia, wherein HU-210 is the (+)-1,1-dimethylheptyl analog of 7-hydroxy-*delta*-6-tetrahydrocannabinol of the formula:



2. The use of claim 1 wherein the hyperglycemia is the result of diabetic mellitus type 1.

3. The use of claim 1 wherein the hyperglycemia is the result of diabetic mellitus type 2.

4. The use of an effective amount of at least one cannabinoid selected from the group consisting of isolated Tetrahydrocannabinol (THC) and HU-210 for the manufacture of a pharmaceutical composition for treating neuronal damage in a diabetic subject, wherein HU-210 is the (+)-1,1-dimethylheptyl analog of 7-hydroxy-*delta*-6-tetrahydrocannabinol of the formula:

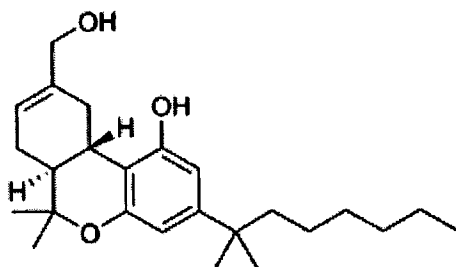


5. The use of claim 4 wherein the diabetes is diabetic mellitus type 1.

6. The use of claim 4 wherein the diabetes is diabetic mellitus type 2.

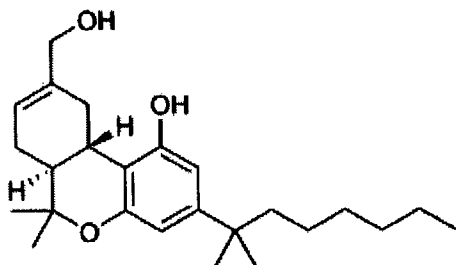
7. The use of claim 4 wherein the neuronal damage is diabetic encephalopathy.

8. The use of claim 4 wherein the neuronal damage is diabetic neuropathy.
9. The use of claim 4 wherein the neuronal damage is selected from the group consisting of motor weakness and instability in blood pressure and heart rate.
10. The use as defined in any one of the preceding claims wherein the cannabinoid is HU-210 which is the (+)-1,1-dimethylheptyl analog of 7-hydroxy-*delta*-6-tetrahydrocannabinol of the formula:



11. A method according to any one of claims 1 to 9 wherein the cannabinoid is isolated Tetrahydrocannabinol(THC).

12. A method for alleviating neuronal damage due to hyperglycemia, the method comprising: administering an effective amount of at least one cannabinoid selected from the group consisting of isolated Tetrahydrocannabinol (THC) and HU-210, wherein HU-210 is the (+)-1,1-dimethylheptyl analog of 7-hydroxy-*delta*-6-tetrahydrocannabinol of the formula:



13. A method for treating neuronal damage in a diabetic subject, the method comprising administering to a subject in need of the treatment a therapeutically effective amount of at least one cannabinoid selected from the group consisting of

isolated Tetrahydrocannabinol (THC) and HU-210 wherein HU-210 is the (+)-1,1-dimethylheptyl analog of 7-hydroxy-*delta*-6-tetrahydrocannabinol of the formula:

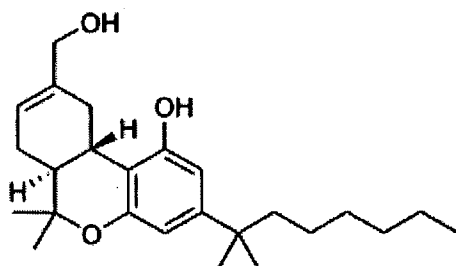


Fig. 1A

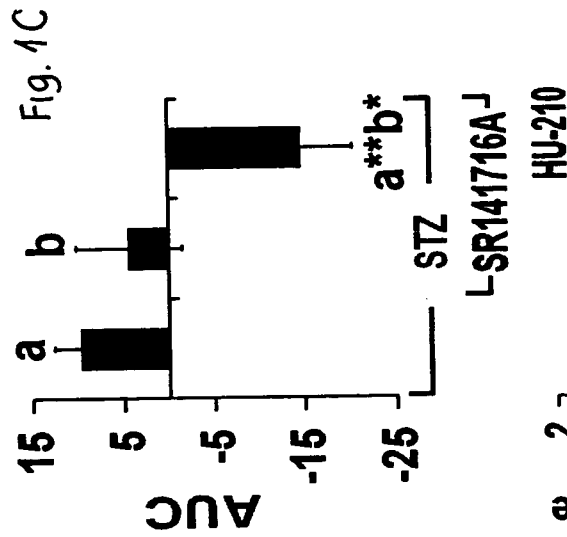
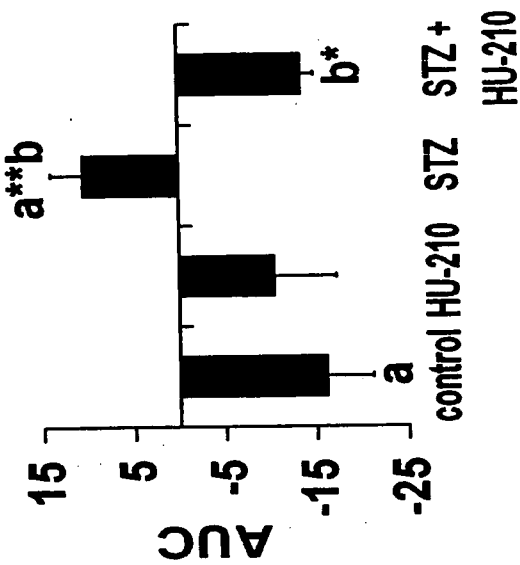


Fig. 1 B

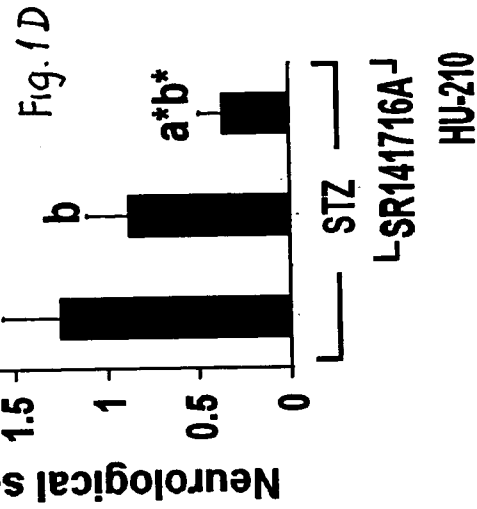
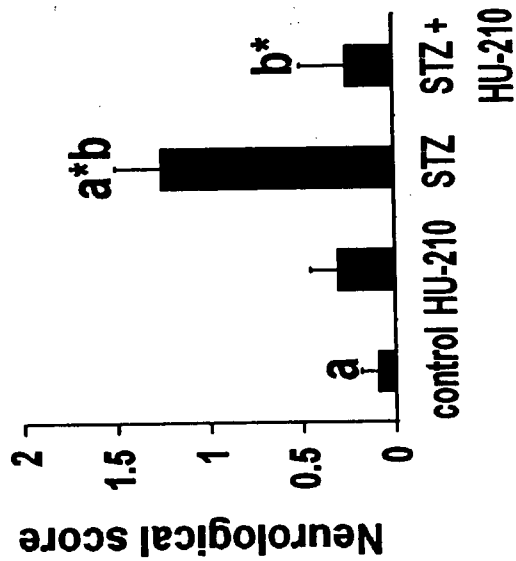


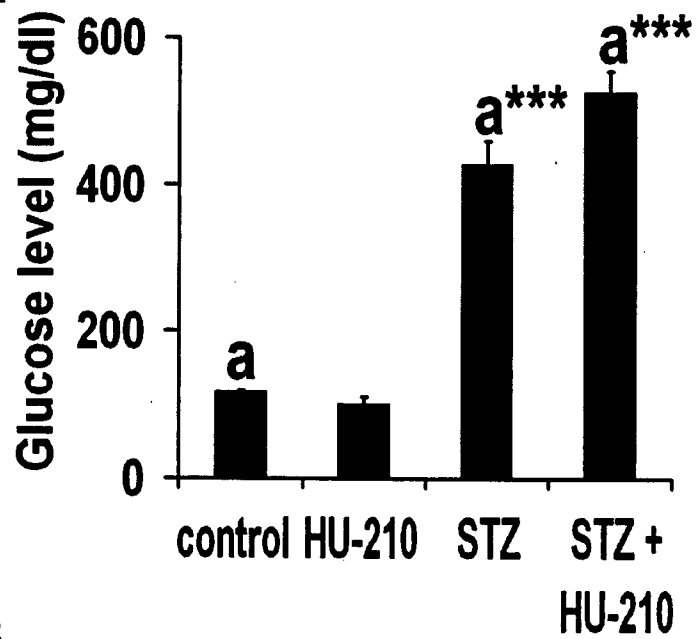
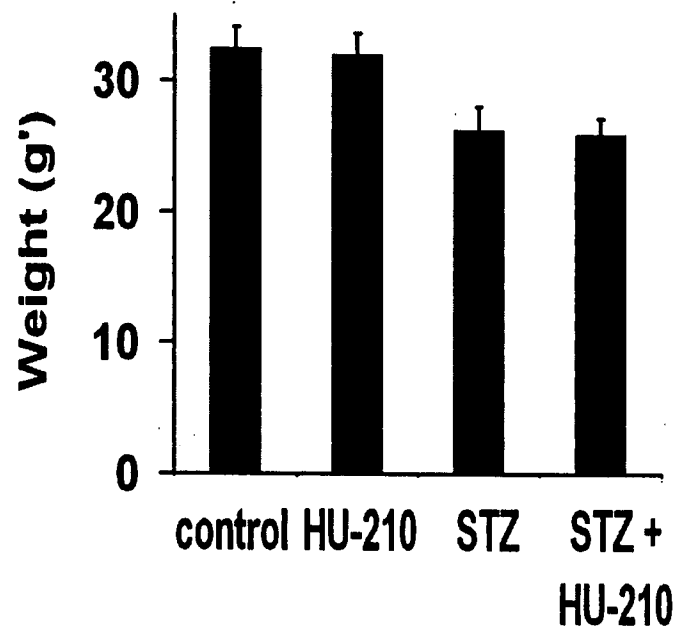
Fig. 2A**Fig.2 B**

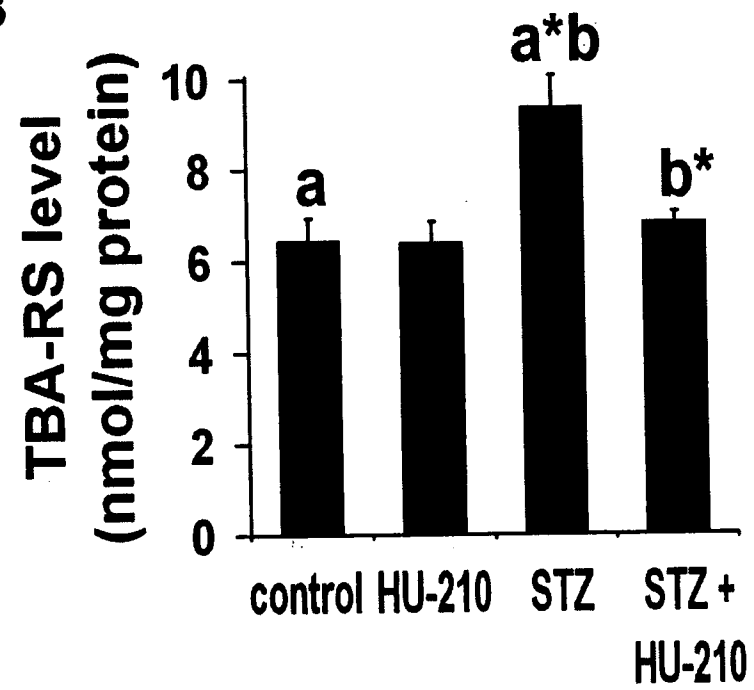
Fig. 3

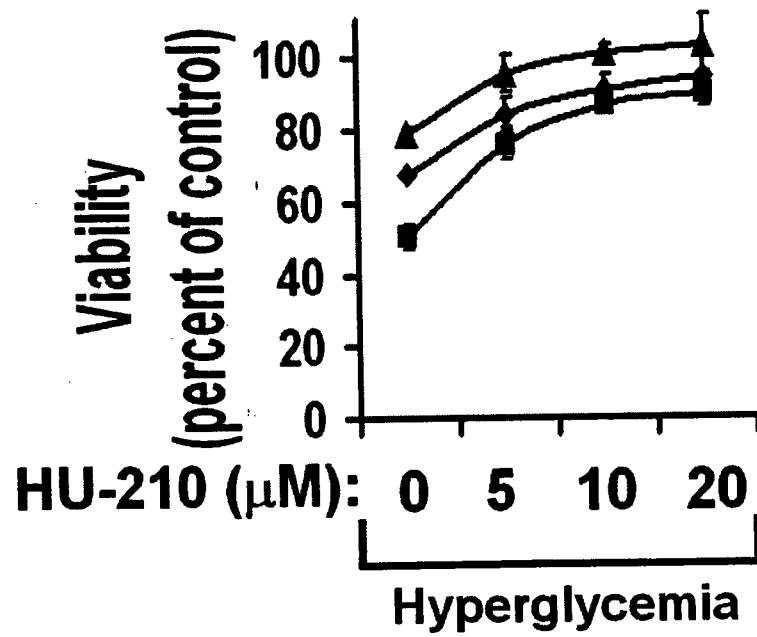
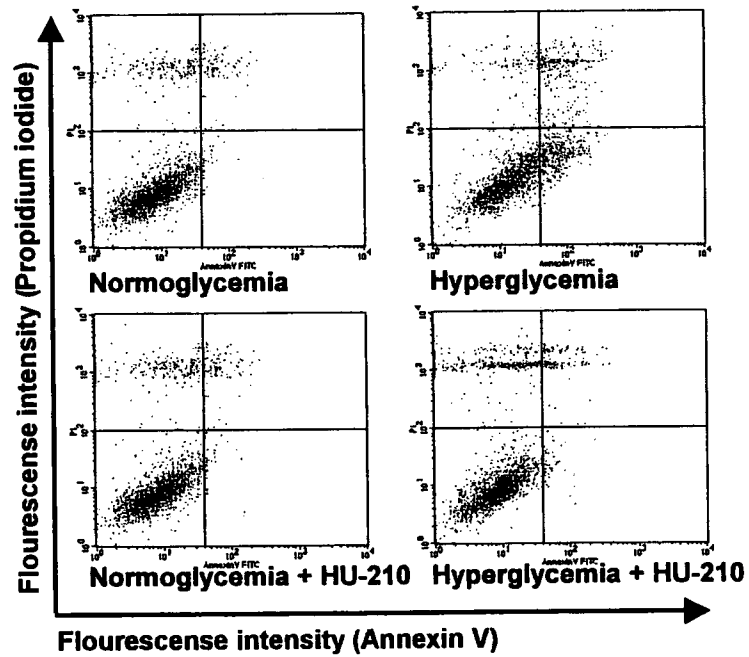
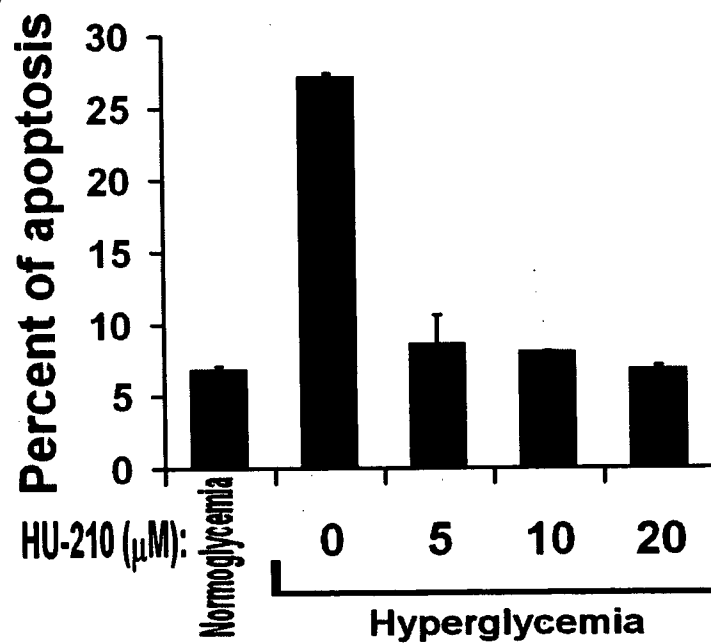
Fig. 4

Fig. 5A**Fig. 5 B**

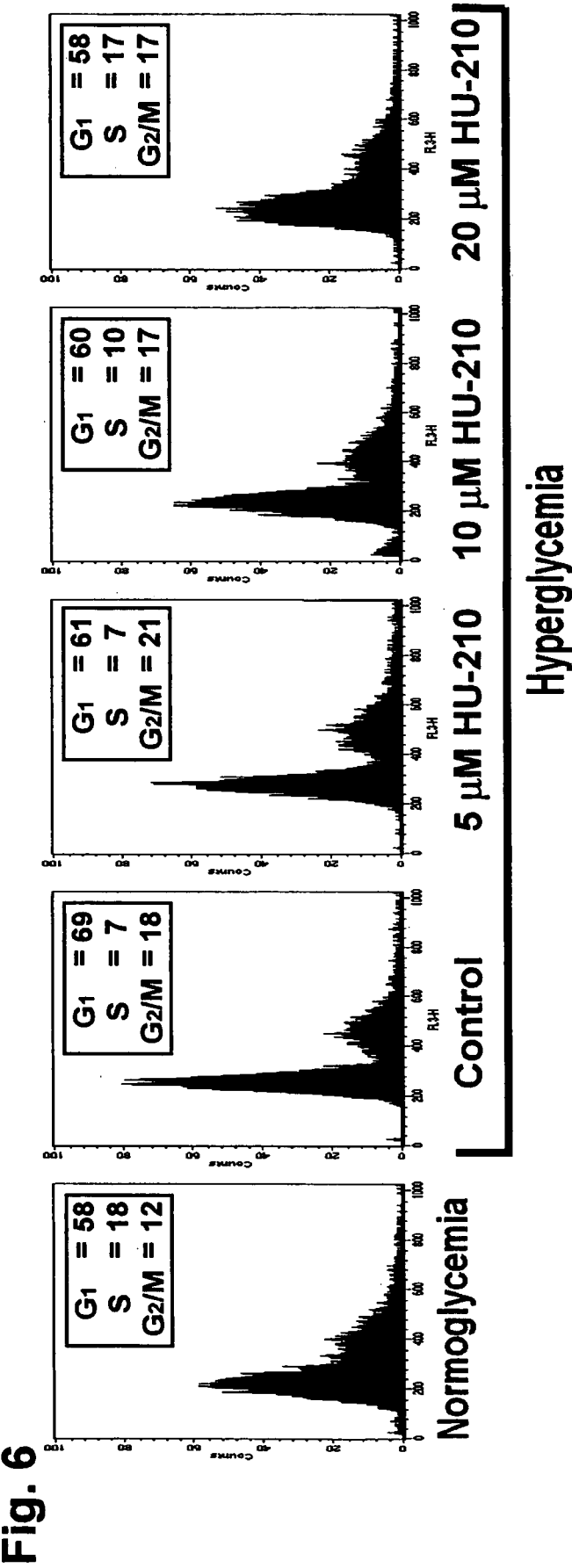


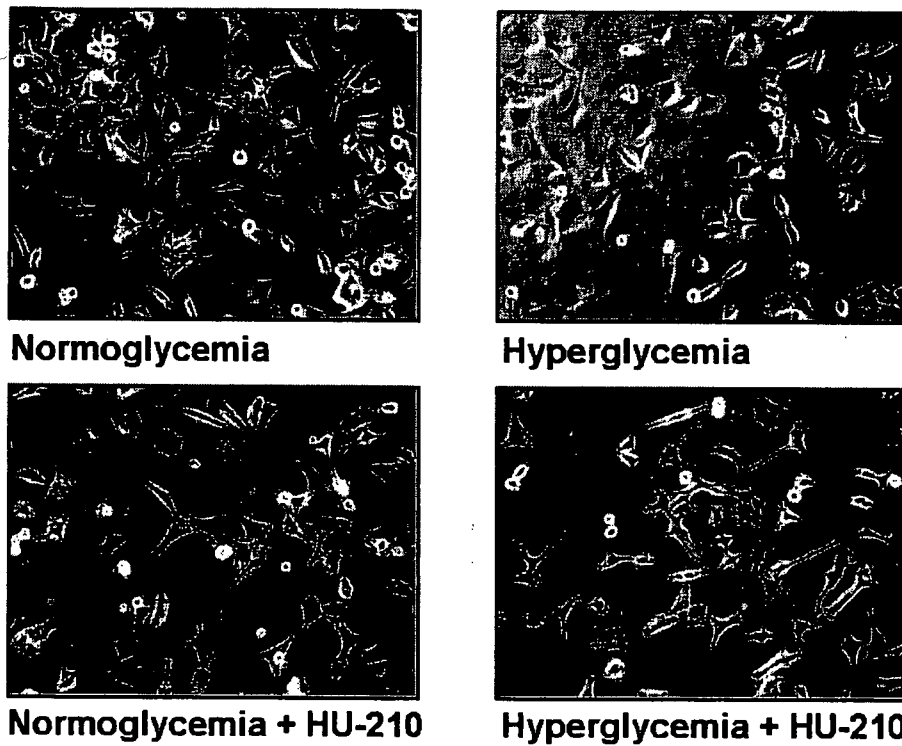
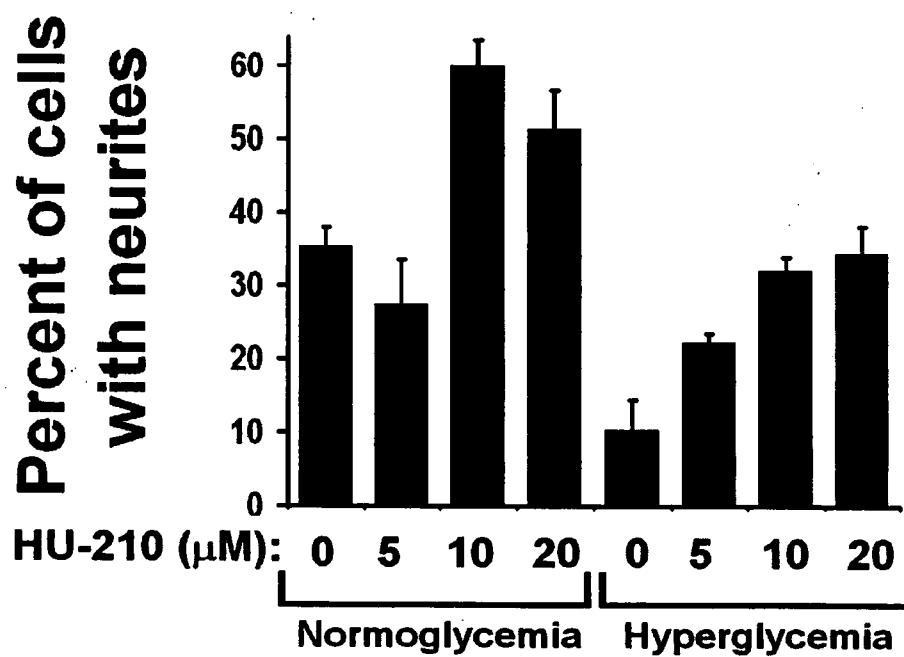
Fig. 7A**Fig. 7 B**

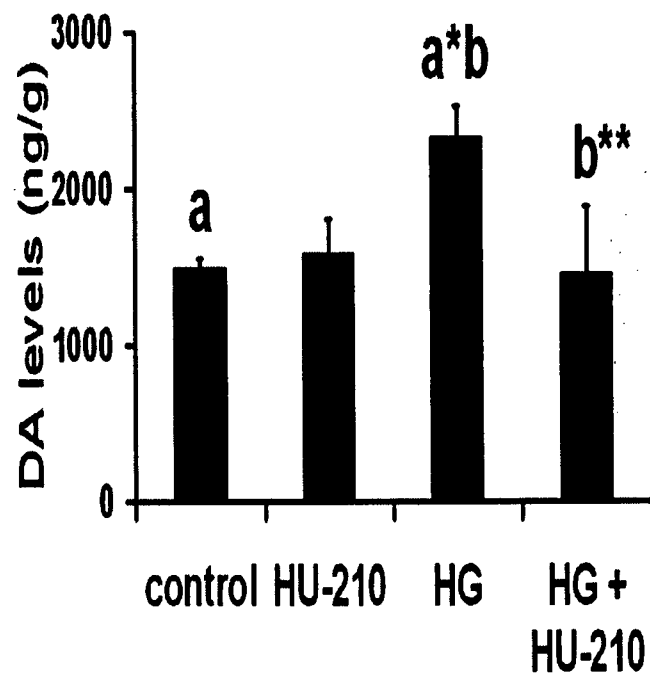
Fig. 8

Fig. 9

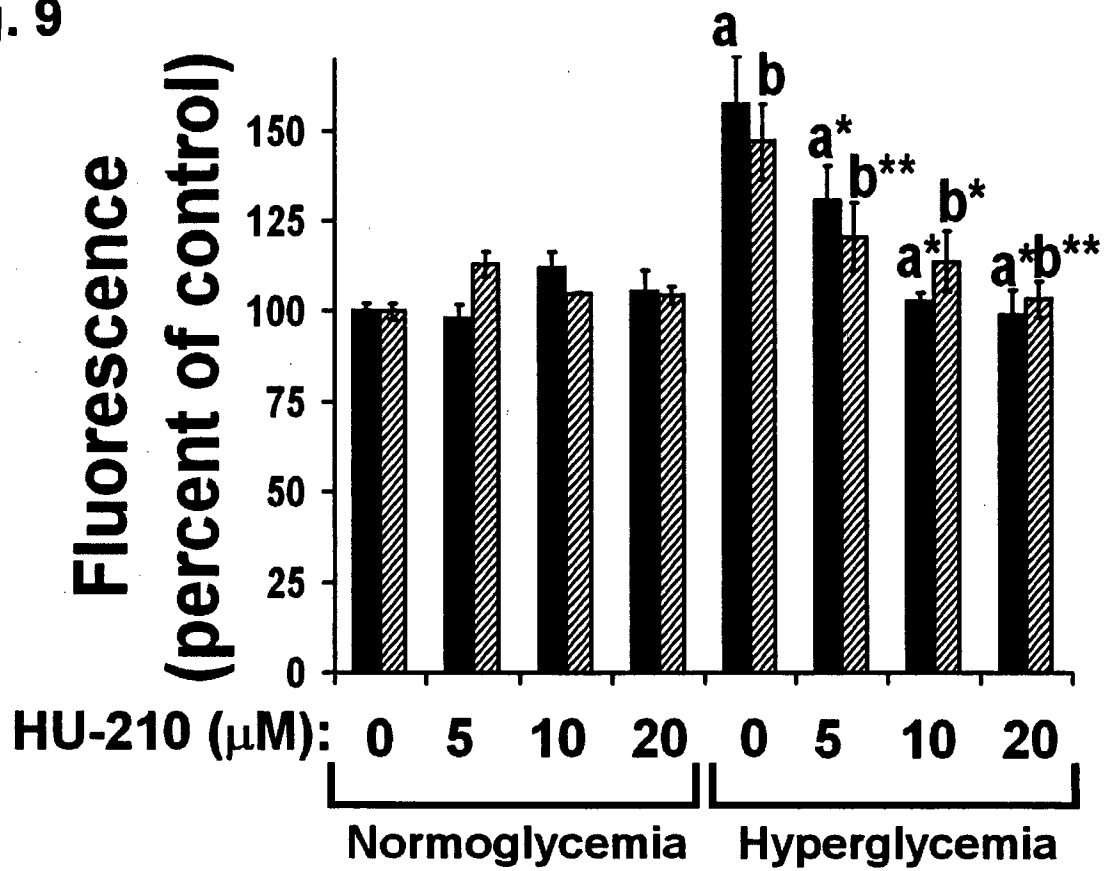


FIG. 10

