

(19) **DANMARK**

(10) **DK/EP 3509638 T3**



Patent- og
Varemærkestyrelsen

(12) Oversættelse af
europæisk patentskrift

-
- (51) Int.Cl.: **A 61 K 45/00 (2006.01)** **A 61 K 31/05 (2006.01)** **A 61 K 31/192 (2006.01)**
A 61 K 31/337 (2006.01) **A 61 K 31/352 (2006.01)** **A 61 K 31/495 (2006.01)**
A 61 K 36/185 (2006.01) **A 61 P 1/08 (2006.01)** **C 07 C 69/16 (2006.01)**
C 07 C 69/28 (2006.01) **C 07 C 69/94 (2006.01)**
- (45) Oversættelsen bekendtgjort den: **2022-04-11**
- (80) Dato for Den Europæiske Patentmyndigheds bekendtgørelse om meddelelse af patentet: **2022-01-12**
- (86) Europæisk ansøgning nr.: **18739958.9**
- (86) Europæisk indleveringsdag: **2018-06-20**
- (87) Den europæiske ansøgnings publiceringsdag: **2019-07-17**
- (86) International ansøgning nr.: **IL2018050678**
- (87) Internationalt publikationsnr.: **WO2018235079**
- (30) Prioritet: **2017-06-20 US 201762522243 P**
- (84) Designerede stater: **AL AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO RS SE SI SK SM TR**
- (73) Patenthaver: **Yissum Research Development Company of the Hebrew University of Jerusalem Ltd., Hi-Tech Park , Edmond J. Safra Campus , Givat Ram , P.O.B 39135, 9139002 Jerusalem, Israel**
University of Guelph, 50 Stone Road East, Guelph, Ontario N1G 2W1, Canada
- (72) Opfinder: **MECHOULAM, Raphael, 12 Tchernichovsky Street, 9258115 Jerusalem, Israel**
PARKER, Linda, 3621 Idaho Pl., Campbell River, British Columbia V9W 6Y3, Canada
PERTWEE, Roger, 24 Manor Place, Cults, Aberdeen AB15 9QN, Storbritannien
WELLER, Aron, 79c Hamakabim Street, 6083507 Shoham, Israel
TAM, Joseph, 8 Hadishon Street, 9695608 Jerusalem, Israel
HAJ, Christeen, 15/2 Yasmin Street, 17000 Nazareth Ilit, Israel
SMOUM, Reem, P.O.Box 20437, Salah El Din Street, East Jerusalem, Israel
- (74) Fuldmægtig i Danmark: **HØIBERG P/S, Adelgade 12, 1304 København K, Danmark**
- (54) Benævnelse: **Præparater med cannabidiolsyreestere og anvendelser deraf**
- (56) Fremdragne publikationer:
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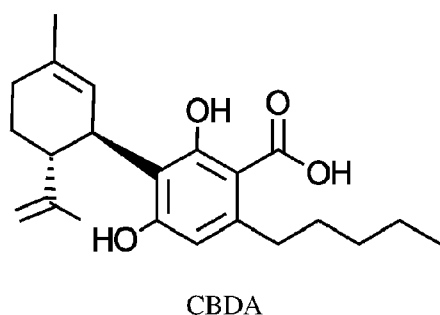
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Description

BACKGROUND OF THE INVENTION

[0001] Cannabidiolic acid (CBDA) is a major constituent of *Cannabis sativa*. It was first isolated in 1955 (Krejčí and Šantavý, 1955) and its structure was elucidated in 1965 by analysis of the physical properties of its methyl ester (Mechoulam and Gaoni, 1965). Its synthesis from cannabidiol was subsequently reported (Mechoulam and Ben-Zvi, 1969).



[0002] Cannabidiolic acid (CBDA) gradually decarboxylates, while still in the plant, to cannabidiol (Mechoulam, 1973), a process that is speeded up by heat. Whereas cannabidiol has been the topic of a large number of publications, and its biological/therapeutic properties have now been reasonably well identified (Mechoulam et al., 2002; Zhornitsky & Potvin, 2012; Cascio and Pertwee, 2014), our knowledge of the pharmacology of cannabidiolic acid is much more limited.

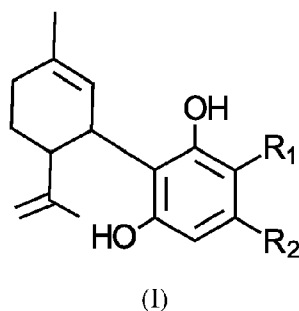
[0003] The limited amount of information on this phyto-cannabinoid that has been published suggests that it may have a wide variety of actions and effects. Accordingly, it has been shown to inhibit breast cancer cell migration (Takeda et al., 2017) and to cause down-regulation of cyclooxygenase-2 (COX-2) (Takeda et al., 2014). Recent evidence suggests that CBDA (at a dose as low as $1 \mu\text{g} \cdot \text{kg}^{-1}$ i.p.) can induce potent 5-HT_{1A} receptor-mediated anti-nausea effects as indicated by its apparent ability to prevent both vomiting in *Suncus murinus* and acute nausea-induced behavior of conditioned gaping in rats (Grill & Norgren, 1978) by enhancing 5-HT_{1A} receptor activation (Bolognini et al., 2013; Rock et al., 2013; 2015b). As well as reducing acute nausea, CBDA has the potential to reduce anticipatory (conditioned) nausea, an effect experienced by chemotherapy patients upon returning to the clinic in which they received

their nauseating treatment (Rock et al., 2014; 2015a; 2016). There are currently no effective selective treatments for anticipatory nausea once it develops in such patients. It is noteworthy, therefore, that Rock et al, (2014; 2015a; 2016) have demonstrated that CBDA reduces contextually-elicited conditioned gaping (a model of anticipatory nau-
 5 sea), also by a 5-HT_{1A} dependent mechanism of action. Finally, like CBD, CBDA has also been shown to produce anxiolytic-like effects under conditions of high stress at doses as low as 0.1 µg·kg⁻¹, i.p (Rock et al., 2017). WO 2015158381 A1 discloses cannabidiol quinone derivatives, and the synthesis of those compounds. US 2010298579 A1 discloses a process for preparing stereoisomers and homologues of cannabinoids.
 10 Yukihiro Shoyama et al. disclose the isolation and structure of four new propyl cannabinoid acids from Thai cannabis 'Meao variant' (Yukihiro Shoyama et al., Chemical & Pharmaceutical Bulletin, vol. 25(9), p. 2306-2311, January 1, 1977). Petrzilka et al. disclose the synthesis of (-)-cannabidiol from (+)-cis- and (+)-trans-p-menthadien-(2,8)-ol-(1) and olivetol (Petrzilka T et al., Helvetica Chimica Acta, vol 54(123,4), p. 1102-1133,
 15 January 1, 1969). WO 2015198071 A1 discloses active pharmaceutical ingredients comprising cannabinoids for use in the treatment of cancer.

[0004] However, CBDA is highly instable, especially when subjected to heat. Hence, there was a growing need to find an analogue of CBDA, having higher stability. It was
 20 surprisingly found by the inventors of the present application that ester derivatives of CBDA provided both the sought-after stability, but also similar or more effective biological profile than CBDA.

SUMMARY OF THE INVENTION

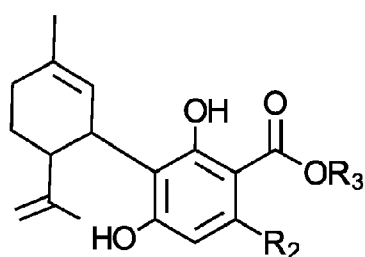
[0005] The invention thus provides a compound having the general formula (I):



for use in the treatment of a condition, disease or symptom associated with 5-HT_{1A} receptor.

[0006] R₁ is selected from -C(=O)OR₃, -OC(=O)R₄; R₂ is selected from a straight or branched C₁-C₁₅ alkyl, straight or branched C₂-C₁₅ alkenyl, straight or branched C₄-C₁₅ alkenynyl and straight or branched C₂-C₁₅ alkynyl; each independently optionally substituted by at least one substituent selected from hydroxy (-OH), halogen, amine and amide or any combinations thereof; R₃ and R₄ are each independently selected from a straight or branched C₁-C₁₅ alkyl, straight or branched C₂-C₁₅ alkenyl, straight or branched C₄-C₁₅ alkenynyl, straight or branched C₂-C₁₅ alkynyl (said alkyl, alkenyl or alkynyl are each optionally substituted with by at least one substituent selected from hydroxy (-OH), halogen, amine and amide or any combinations thereof), halogen, amine and amide. Further, the condition, disease or symptom is selected from anxiety, stress, depression, schizophrenia, panic, withdrawal syndrome, auto-immune disease, inflammation, obesity, metabolic syndrome, retinopathy, nausea, vomiting, kidney dysfunction, renal ischemic/reperfusion injury, neuronal damage, Huntington's disease, Alzheimer's disease, cerebral infarction, hepatic encephalopathy, traumatic brain injury, cerebral ischemia, spinal cord injury, angiogenesis, epilepsy, convulsions, neuropathic pain, airway obstruction, obsessive-compulsive behavior, cognitive impairment, impaired sexual drive and function, sleep disturbance, opioid-related respiratory suppression, addiction and any combinations thereof.

[0007] The invention further provides a compound having the general formula (II):



(II)

for use in the treatment of a condition, disease or symptom as mentioned above.

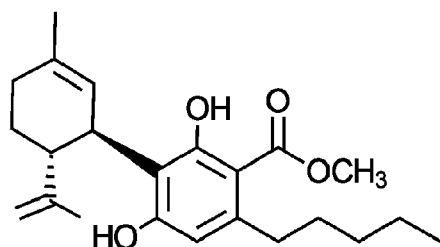
[0008] R₂ is selected from a straight or branched C₁-C₁₅ alkyl, straight or branched C₂-C₁₅ alkenyl, straight or branched C₄-C₁₅ alkenynyl and straight or branched C₂-C₁₅ alkynyl; each independently optionally substituted by at least one substituent selected from hydroxy, halogen, amine and amide or any combinations thereof; R₃ is selected

from a straight or branched C₁-C₁₅ alkyl, straight or branched C₂-C₁₅ alkenyl, straight or branched C₄-C₁₅ alkenynyl, straight or branched C₂-C₁₅ alkynyl (said alkyl, alkenyl or alkynyl are each optionally substituted with by at least one substituent selected from hydroxy (-OH), halogen, amine and amide or any combinations thereof), halogen, amine and amide.

[0009] In some embodiments, R₂ is a straight or branched C₁-C₁₅ alkyl. In other embodiments, R₂ is a straight or branched C₂-C₁₅ alkenyl. In further embodiments, R₂ is a straight or branched C₂-C₁₅ alkynyl or a straight or branched C₄-C₁₅ alkenynyl.

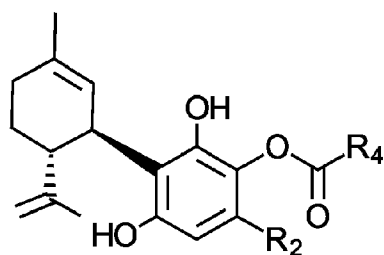
[0010] In some embodiments, R₃ is a straight or branched C₁-C₁₅ alkyl. In other embodiments, R₃ is a straight or branched C₂-C₁₅ alkenyl. In further embodiments, R₃ is a straight or branched C₂-C₁₅ alkynyl or a straight or branched C₄-C₁₅ alkenynyl. In yet further embodiments, R₃ is selected from a halogen, an amine and an amide. In some embodiments, R₃ being an alkyl, alkenyl or alkynyl or alkenynyl is optionally substituted with by at least one substituent selected from hydroxy (-OH), halogen, amine and amide or any combinations thereof.

[0011] A compound of the present invention may be HU-580:



HU-580.

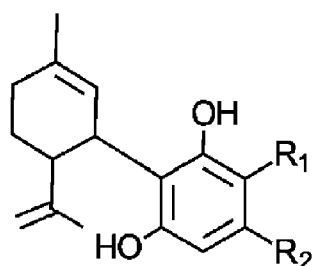
[0012] The disclosure further provides a compound having the general formula (III):



(III)

for use in the treatment of a condition, disease or symptom as mentioned above.

R_2 is selected from a straight or branched C_1 - C_{15} alkyl, straight or branched C_2 - C_{15} alkenyl and straight or branched C_2 - C_{15} alkynyl; each independently optionally substituted by at least one substituent selected from hydroxy, halogen, amine and amide or any combinations thereof; R_4 is selected from a straight or branched C_1 - C_{15} alkyl, straight or branched C_2 - C_{15} alkenyl, straight or branched C_2 - C_{15} alkynyl (said alkyl, alkenyl or alkynyl are each optionally substituted with by at least one substituent selected from hydroxy (-OH), halogen, amine and amide or any combinations thereof), halogen, amine and amide. According to another embodiment the invention also provides a compound having the general formula (I):



(I)

wherein R_1 is selected from $-C(=O)OR_3$, $-OC(=O)R_4$; R_2 is selected from a straight or branched C_2 - C_{15} alkenyl, straight or branched C_4 - C_{15} alkenynyl and straight or branched C_2 - C_{15} alkynyl; each independently optionally substituted by at least one substituent selected from hydroxy, halogen, amine and amide or any combinations thereof; R_3 is selected from a straight or branched C_2 - C_{15} alkynyl, straight or branched C_4 - C_{15} alkenynyl, halogen, amine and amide; and R_4 is selected from a straight or branched C_1 - C_{15} alkyl, straight or branched C_4 - C_{15} alkenynyl, straight or branched C_2 - C_{15} alkenyl and straight or branched C_2 - C_{15} alkynyl, halogen, amine and amide.

[0013] The term "*halogen*" means F, Cl, Br or I.

[0014] The term "*amine*" as used herein refers to an $-NRR'R''$ radical, wherein each of R, R' and R'' is selected from H, straight or branched C_1 - C_{15} alkyl, straight or branched C_2 - C_{15} alkenyl and straight or branched C_2 - C_{15} alkynyl.

[0015] The term "*amide*" as used herein refers to an $-C(=O)NRR'R''$ or $-NRC(=O)R'$ radical, wherein each of R, R' and R'' is selected from H, straight or branched C_1 - C_{15} alkyl, straight or branched C_2 - C_{15} alkenyl and straight or branched C_2 - C_{15} alkynyl.

[0016] The term " *C_1 - C_{15} alkyl*" as used herein represents a saturated, branched or

straight hydrocarbon group having 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 carbon atoms all connected via sigma bonds.

5 [0017] The term " C_2-C_{15} alkenyl" as used herein represents a branched or straight hydrocarbon group having 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 carbon atoms, wherein at least one of the bonds connecting said carbon atoms is a double bond, all other bonds may be of any other type (single and/or double).

10 [0018] The term " C_2-C_{15} alkynyl" as used herein represents a branched or straight hydrocarbon group having 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 carbon atoms, wherein at least one of the bonds connecting said carbon atoms is a triple bond, all other bonds may be of any other type (single and/or triple). The term " C_4-C_{15} alkenynyl" as used herein represents a branched or straight hydrocarbon group having 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 carbon atoms, wherein at least one of the bonds connect-
15 ing two adjacent carbon atoms is a double bond and at least one of the bonds connecting two adjacent carbon atoms is a triple bond.

[0019] As used herein, the term "*optional substituent*" denotes that the corresponding substituent may be present or may be absent. Accordingly, a compound of the invention may have 1, 2, 3 or more optional substituents at any point of the radical that is defined as having this optional substitution.
20

[0020] It is to be understood that the compounds provided herein may contain one or more chiral centers. Such chiral centers may each be of either of the (*R*) or (*S*) configuration. In case a compound of the invention contains more than one chiral center, each one of those chiral centers may be of the (*R*) or (*S*) configuration, independently. Thus, the compounds provided herein may be enantiomerically pure, or be stereoisomeric or diastereomeric mixtures.
25

30 [0021] The invention further provides a composition comprising at least one compound as defined herein above and below, of general formula (I) and (II) to be used in the treatment of the conditions, diseases or symptoms as disclosed herein above.

[0022] In a further aspect, the invention provides a compound as defined herein above

and below, of general formula (I) and (II), for use in the treatment of a condition, disease or symptom associated with 5-HT_{1A} receptor.

5 **[0023]** In a aspect, the invention provides a compound as defined herein above and below, of general formula (I) and (II), for use in the treatment of a condition, disease or symptom selected from nausea, vomiting, convulsions and any combinations thereof.

10 **[0024]** In a further aspect, the invention provides a compound as defined herein above and below, of general formula (I) and (II), for use in the treatment of a condition, disease or symptom associated with depression.

15 **[0025]** The invention thus provides a compound as defined herein above and below, of general formula (I) and (II), for use in the treatment of a condition, disease or symptom selected from anxiety, stress, depression, schizophrenia, panic, withdrawal syndrome, auto-immune disease, inflammation, reduction of infarct size, increase blood flow in stroke, obesity, metabolic syndrome, retinopathy, nausea, myocardia, liver, renal ischemic/reperfusion injury, neuronal damage, Huntington's disease, Alzheimer's disease, cerebral infarction, hepatic encephalopathy, traumatic brain injury, cerebral ischemia, spinal cord injury, memory rescuing effects, cancer, angiogenesis, epilepsy, 20 convulsions, neuropathic pain, airway obstruction, obsessive-compulsive behavior, cognitive impairment, impaired sexual drive and function, sleep disturbance, opioid-related respiratory suppression, addiction and any combinations thereof.

25 **[0026]** By another aspect the present disclosure concerns a method which may be useful in the treating of a disease, the disease characterized by being improved by cannabidiolic acid (CBDA), comprising: administering to a subject in need of such treatment a therapeutically effective amount of a compound as defined herein above and below, of general formula (I) and (II).

30 **[0027]** Non-limiting examples of disease, conditions and symptoms are: Anxiety and stress, depression, schizophrenia, panic and anxiety, withdrawal symptoms in cannabis and tobacco addiction, reward-facilitating effect of morphine and cocaine, auto-immune diseases of any type (diabetes type 1, GVHD being specific non-limiting examples), Inflammation (Crohn's disease, colitis, pancreatitis, rheumatoid arthritis), reduction of in-

farct size and increase blood flow in stroke, obesity (treated by reduced food consumption; or by lowering appetite), metabolic syndrome, retinopathy associated with diabetes, nausea, myocardial, liver, renal ischemic/reperfusion injury, neuronal damage (due to neurological diseases or injury, Parkinson's disease, Huntington's disease, Alzheimer's disease, cerebral infarction, hepatic encephalopathy, traumatic brain injury, cerebral ischemia, spinal cord injury, memory rescuing effects, cancer and resistance to cancer chemotherapy, cancer cell migration (metastasis), angiogenesis, epilepsy and convulsions, chronic inflammatory and neuropathic pain, airway obstruction, Obsessive-compulsive behavior and any combinations thereof.

[0028] In some embodiments, the diseases conditions and symptoms are selected from nausea (both anticipatory and acute), vomiting, anxiety and affective disorders of any type, such as depression (including major depression, mild depression and bipolar disease).

[0029] The present disclosure concerns a method that may be useful in the treatment of a disease wherein a clinical beneficial effect is evident by the activation of 5HT_{1A} receptors, comprising administering to a subject in need of such treatment an effective amount of at least one compound of general formula (I) or (II).

[0030] Non-limiting examples of such diseases and conditions are: hypertension, anxiety, vomiting and nausea, pain, Schizophrenia, Parkinson's disease, cognitive impairment, impaired sexual drive and function, obesity (the effect is suppressed food consumption), sleep disturbance (in particular short rem duration), opioid-related respiratory suppression, addiction and any combinations thereof.

[0031] In particular, the diseases that show an improved clinical outcome, due to 5HT_{1A} activation are nausea (both anticipatory and acute) and vomiting (anti emetic and anti-nausea), anxiety and affective disorder of any type, mainly depression (including major depression, mild depression and bipolar disease) and any combinations thereof.

[0032] Thus, when referring herein to a "*condition, symptom or disease associated with 5HT_{1A} receptor*" it should be understood to include hypertension, anxiety, vomiting and

nausea, pain, Schizophrenia, Parkinson's disease, cognitive impairment, impaired sexual drive and function, obesity (the effect is suppressed food consumption), sleep disturbance (in particular short rem duration), opioid-related respiratory suppression, addiction, nausea (both anticipatory and acute) and vomiting (anti emetic and anti-nausea), anxiety and affective disorder of any type, mainly depression (including major depression, mild depression and bipolar disease) and any combinations thereof.

[0033] By another aspect the present disclosure concerns a method which may be useful in the treatment of a disease selected from: a nausea (both anticipatory and acute), anxiety and affective disorder of any type, mainly depression (including major depression, mild depression and bipolar disease); comprising: administering to a subject in need of such treatment an effective amount of CBDA-ME.

[0034] In another one of its aspects the invention provides a compound as disclosed herein above and below for use in the treatment of at least one disease, condition, symptom or disorder associated with kidney dysfunction. The disclosure further provides a method which may be useful in treating at least one disease, condition, symptom or disorder associated with kidney dysfunction in a patient in need thereof; comprising administering to said patient at least one compound of the present invention.

[0035] When referring to "*kidney dysfunction*" in the context of the present application, it should be understood to include any type (qualitative or quantitative) of reduction or failure of kidney function and may be acute or chronic. Such kidney dysfunction may be caused by any reason including injury, disease, genetic disposition and so forth.

Causes of acute kidney dysfunction include, low blood pressure, blockage of the urinary tract, medications, muscle breakdown, hemolytic uremic syndrome, and any combinations thereof. Additional causes of chronic kidney dysfunction include, to diabetes, high blood pressure, nephrotic syndrome, polycystic kidney disease and any combinations thereof.

[0036] In some embodiments, such diseases, conditions, symptoms and disorders that are associated with kidney dysfunction include, diabetic nephropathy, chronic and acute kidney injuries, chronic and acute kidney diseases, acute-on-chronic kidney failure, obesity-associated kidney damage, and any combinations thereof.

5 [0037] Conditions and symptoms associated with kidney dysfunction include: high levels of urea in the blood, vomiting, diarrhea, nausea, weight loss, nocturnal urination, changes in urination frequency and quantity, blood in the urine, pressure, or difficulty
10 urinating, buildup of phosphates in the blood, itching, bone damage, nonunion in broken bones, muscle cramps, buildup of potassium in the blood, abnormal heart rhythms, muscle paralysis, failure of kidneys to remove excess fluid, swelling of the legs, ankles, feet, face, or hands, shortness of breath, polycystic kidney disease, large fluid-filled cysts on the kidneys, pain in the back or side, lowering production of erythropoietin, decreased production of red blood cells, anemia, foamy or bubbly urine, swelling in the
15 hands, feet, abdomen, or face, appetite loss, excess protein in the blood and urine, seizures when administered with high doses of penicillin and any combinations thereof.

15 [0038] The present disclosure also relates to pharmaceutical compositions comprising a compound of the subject invention in admixture with pharmaceutically acceptable auxiliaries, and optionally other therapeutic agents. The auxiliaries must be "acceptable" in the sense of being compatible with the other ingredients of the composition and not deleterious to the recipients thereof.

20 [0039] Pharmaceutical compositions include those suitable for oral, rectal, nasal, topical (including transdermal, buccal and sublingual), vaginal or parenteral (including subcutaneous, intramuscular, intravenous and intradermal) administration or administration via an implant. The compositions may be prepared by any method well known in the art of pharmacy.

25 [0040] Such methods include the step of bringing in association compounds used in the invention or combinations thereof with any auxiliary agent. The auxiliary agent(s), also named accessory ingredient(s), include those conventional in the art, such as carriers, fillers, binders, diluents, disintegrants, lubricants, colorants, flavoring agents, anti-oxidants, and wetting agents.
30

[0041] Pharmaceutical compositions suitable for oral administration may be presented as discrete dosage units such as pills, tablets, dragées or capsules, or as a powder or granules, or as a solution or suspension. The active ingredient may also be presented

as a bolus or paste. The compositions can further be processed into a suppository or enema for rectal administration.

5 [0042] The disclosure further relates to a pharmaceutical composition, as hereinbefore described, in combination with packaging material, including instructions for the use of the composition for a use as hereinbefore described.

10 [0043] For parenteral administration, suitable compositions include aqueous and nonaqueous sterile injection. The compositions may be presented in unit-dose or multi-dose containers, for example sealed vials and ampoules, and may be stored in a freeze-dried (lyophilised) condition requiring only the addition of sterile liquid carrier, for example water, prior to use. For transdermal administration, e.g. gels, patches or sprays can be contemplated. Compositions or formulations suitable for pulmonary administration e.g. by nasal inhalation include fine dusts or mists which may be generated
15 by means of metered dose pressurized aerosols, nebulisers or insufflators.

[0044] The exact dose and regimen of administration of the composition will necessarily be dependent upon the therapeutic or nutritional effect to be achieved and may vary with the particular formula, the route of administration, and the age and condition
20 of the individual subject to whom the composition is to be administered.

[0045] The invention relates to a compound for use in the treatment of a condition, disease or symptom associated with 5-HT_{1A} receptor; comprising administering to a patient in need thereof at least one compound disclosed herein or above (compounds of
25 formula (I), (II) and so forth).

[0046] The disclosure further relates to a compound for use in the treatment of a condition, disease or symptom selected from nausea, vomiting, convulsions and any combinations thereof; comprising administering to a patient in need thereof at least one compound disclosed herein or above (compounds of formula (I), (II) and so forth).
30

[0047] The disclosure further relates to a compound for use in the treatment of a condition, disease or symptom associated with depression; comprising administering to a patient in need thereof at least one compound disclosed herein or above (compounds
35 of formula (I), (II) and so forth).

- 5 [0048] The disclosure relates to a compound for use in the treatment of a condition, disease or symptom selected from anxiety, stress, depression, schizophrenia, panic, withdrawal syndrome, auto-immune disease, inflammation, reduction of infarct size, increase blood flow in stroke, obesity, metabolic syndrome, retinopathy, nausea, myocardia, liver, renal ischemic/reperfusion injury, neuronal damage, Huntington's disease, Alzheimer's disease, cerebral infarction, hepatic encephalopathy, traumatic brain injury, cerebral ischemia, spinal cord injury, memory rescuing effects, cancer, angiogenesis, epilepsy, convulsions, neuropathic pain, airway obstruction, obsessive-compulsive behavior, cognitive impairment, impaired sexual drive and function, sleep disturbance, 10 opioid-related respiratory suppression, addiction and any combinations thereof; comprising administering to a patient in need thereof at least one compound disclosed herein or above (compounds of formula (I), (II) and so forth).
- 15 [0049] The disclosure further relates to a compound for use in the treatment of at least one disease, condition, symptom or disorder associated with kidney dysfunction; comprising administering to a patient in need thereof at least one compound disclosed herein or above (compounds of formula (I), (II) and so forth).
- 20 [0050] As used herein, the term "*treating a disease, disorder, condition or symptom*" refers to a slowing of, or a reversal of, the progress of the disease, disorder or symptom thereof. Treating a disease or disorder includes treating a symptom and/or reducing the symptoms of the disease.

BRIEF DESCRIPTION OF THE DRAWINGS

25

- [0051] The subject matter regarded as the invention is particularly pointed out and distinctly claimed in the concluding portion of the specification. The invention, however, both as to organization and method of operation, together with objects, features, and advantages thereof, may best be understood by reference to the following detailed description when read with the accompanying drawings in which:
- 30

Figures 1A-1E show the effect of CBDA (0.01, 0.1, 1.0, 10 or 100 nM) on 8-OH-DPAT-induced stimulation of [³⁵S]-GTPγS binding to membranes obtained

from CHO cells stably transfected with human 5-HT_{1A} receptors. Symbols represent mean values \pm SEM ($n = 6$). Mean E_{\max} and EC_{50} values for 8-OH-DPAT determined in the presence of CBDA or just of its vehicle (VEH), DMSO, together with the 95% confidence limits of these values, are listed in Table 1.

5 **Figures 2A-2F** show the effect of HU-580 (0.001, 0.01, 0.1, 1.0, 10 or 100 nM) on 8-OH-DPAT-induced stimulation of [³⁵S]-GTP γ S binding to membranes obtained from CHO cells stably transfected with human 5-HT_{1A} receptors. Symbols represent mean values \pm SEM ($n = 6$). Mean E_{\max} and EC_{50} values for 8-OH-DPAT determined in the presence of HU-580 or just of its vehicle (VEH),
10 DMSO, together with the 95% confidence limits of these values, are listed in Table 2.

Figure 3 shows the mean number of conditioned gapes elicited by a LiCl-paired saccharin solution among the rats pretreated with various doses of CBDA ($n = 8$ per group) or HU-580 ($n = 8$ per group) or just with vehicle (VEH; $n = 8$). Additional groups were administered a pretreatment of WAY100635 (0.1 mg·kg⁻¹)
15 15 min prior to 0.1 mg·kg⁻¹ HU-580 ($n = 6$) or VEH ($n = 8$). Results are presented as mean \pm SEM and * $P < 0.05$, depicts mean responses to CBDA or HU-580, which differed significantly from mean responses to VEH.

Figures 4A shows the effect of CBDA or HU-580 (0.01, 0.1 μ g·kg⁻¹) or vehicle (VEH) administered i.p. 45 min prior to the anticipatory nausea test ($n = 6$ per group). Additional groups were administered a pretreatment of WAY100635 (0.1 mg·kg⁻¹) 15 min prior to 0.1 mg·kg⁻¹ HU-580 ($n = 8$) or VEH ($n = 8$). The mean number of conditioned gaping responses was measured during the anticipatory nausea test trial. Each bar represents the mean \pm SEM. * $P < 0.05$, significant difference from the VEH-treated control animals.
25

Figure 4B shows the mean distance (cm) travelled was measured in an activity test performed after the anticipatory nausea test. Each bar represents the mean \pm SEM.

Figure 5 shows the mean time spent by rats in the light box, 24 h following exposure to no foot shocks (No-FS) or to FSs. All rats were injected i.p. with vehicle (VEH; $n = 9$ or 12), 0.01 μ g·kg⁻¹, CBDA ($n = 8$) or 0.01 μ g·kg⁻¹ HU-580 ($n = 8$), 45 min prior to a 5 min light-dark box emergence test. Additional groups were injected with 0.1 mg·kg⁻¹ WAY100635 15 min prior to VEH ($n = 7$ or 8) or 0.01 μ g·kg⁻¹ HU-580 ($n = 8$). Each bar represents the mean \pm SEM. * $P < 0.05$,
30 indicates a significant difference between FS and No FS stress groups.
35

[0052] It will be appreciated that for simplicity and clarity of illustration, elements shown in the figures have not necessarily been drawn to scale. For example, the dimensions of some of the elements may be exaggerated relative to other elements for clarity. Further, where considered appropriate, reference numerals may be repeated among the figures to indicate corresponding or analogous elements.

DETAILED DESCRIPTION OF THE PRESENT INVENTION

[0053] In the following detailed description, numerous specific details are set forth in order to provide a thorough understanding of the invention. However, it will be understood by those skilled in the art that the present invention may be practiced without these specific details. In other instances, well-known methods, procedures, and components have not been described in detail so as not to obscure the present invention.

SYNTHESIS

Synthesis of Cannabidiolic Acid (CBDA):

[0054] A mixture of Cannabidiol (CBD, 314 mg, 1 mmol) and 2 molar solution of Magnesium Methyl Carbonate (MMC/2M, 1.5 ml, 3 mmol) in dimethylformamide (DMF) is heated at 130°C for 3 hours. Then the reaction is cooled to 0°C, acidified with 10% hydrochloric acid and extracted with ether. The organic layer is washed with saline, dried over the drying agent magnesium sulfate (MgSO_4) and then evaporated. The crude compound is then cleaned by column chromatography (20% ether-petroleum ether).

Synthesis of Cannabidiolic Acid Methyl Ester (HU-580):

[0055] To a solution of Cannabidiolic Acid (CBDA) (175 mg, 0.488 mmol) in 2.5 ml dichloromethane (CH_2Cl_2), add 0.02 ml of methanol (CH_3OH , 0.488 mmol) and 7.2 mg of 4-Pyrrolidinopyridine (0.048 mmol). The reaction is stirred for 5 minutes at room temperature followed by the addition of the coupling agent, N,N'-Dicyclohexylcarbodiimide (DCC) (121 mg, 0.585 mmol) and stirred overnight. Then the solvent was evaporated and the crude mixture acidified with 5% hydrochloric acid and extracted with dichloromethane (CH_2Cl_2). The organic layer is washed with saturated aqueous sodium bicarbonate (NaHCO_3), dried over the drying agent magnesium sulfate (MgSO_4) and then

evaporated. The crude compound is then cleaned by column chromatography (2% ether-petroleum ether).

[0056] ¹H-NMR spectra were obtained using a Bruker AMX 300 MHz apparatus using the deuterated DMSO. Thin-layer chromatography (TLC) was run on silica gel 60F₂₅₄ plates (Merck). Column chromatography was performed on silica gel 60 Å (Merck). Compounds were located using a UV lamp at 254 nm. GCMS analyses were performed on an HP GCMS instrument (Model GCD PLUS) with an EI detector and 30 m methyl silicone column.

[0057] ¹H NMR (300 MHz, ((CD₃)₂SO)) δ 6.18 (1H, s, Ar), 5.07 (1H, s), 4.44 (1H, s), 4.41 (1H, s), 3.82 (3H, s), 3.35 (1H, m), 2.66 (1H, m), 2.49 (2H, t), 2.09 (1H, b), 1.95 (3H, s), 1.71-1.05 (12, ms), 0.86 (3H, t). GC MS = 314 m/z.

BIOLOGICAL METHODS

In vitro procedures

[0058] *CHO cells*. CHO cells stably transfected with cDNA encoding human 5-HT_{1A} receptors (a generous gift from Dr Keith Parker) were maintained at 37°C and 5% CO₂ in Gibco™ Ham's F-12 Nutrient Mix supplied by Fisher Scientific UK Ltd that was supplemented both with 2 mM L-glutamine, 10% FBS and 0.6% penicillin-streptomycin, all also supplied by Fisher Scientific UK Ltd, and with the disulphate salt of G418 [(2R,3S,4R,5R,6S)-5-amino-6-[[[(1R,2S,3S,4R,6S)-4,6-diamino-3-[[[(2R,3R,4R,5R)-3,5-dihydroxy-5-methyl-4-(methylamino)oxan-2-yl]oxy]2-hydroxy cyclohexyl]oxy]-2-[(1R)-1-hydroxyethyl]oxane-3,4-diol; 600 mg·mL⁻¹] supplied by Sigma-Aldrich UK.

[0059] [³⁵S]-GTPγS binding assay. Each assay was carried out with human 5-HT_{1A} CHO cell membranes (50μg protein per well), GTPγS-binding buffer (50 mM Tris-HCl; 50 mM Tris- Base; 5 mM MgCl₂; 1 mM EDTA; 100 mM NaCl; 1 mM DTT and 0.1% BSA), 0.1nM [³⁵S]-GTPγS and 30μM GDP, in a final volume of 500μL (Cascio *et al.*, 2010). Binding was initiated by the addition of [³⁵S]-GTPγS to the wells. Non-specific binding was measured in the presence of 30μM GTPγS. Assays were performed at 30°C for 60 min (Cascio *et al.*, 2010). The reaction was terminated by a rapid vacuum filtration method using Tris-binding buffer as described previously by Cascio *et al.* (2010), and the radioactivity was quantified by liquid scintillation spectrometry. In all

the [³⁵S]-GTPγS-binding assays, 0.1 nM [³⁵S]-GTPγS was used, 30 mM GDP and a protein concentration of 5 µg per well. CBDA, HU-580, 8-OH-DPAT and WAY100635 were stored at -20°C as 10 mM stock solutions dissolved in DMSO.

***In vivo* procedures**

5 **[0060] *Animals.*** Animal procedures complied with the Canadian Council on Animal Care, and the protocols were approved by the Institutional Animal Care Committee at University of Guelph. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath and Lilley, 2015). A total of 200 naïve male Sprague-Dawley rats, obtained from Charles River Laboratories (St Constant, Que-
10 bec), were used for all *in vivo* studies. Rats were individually housed (for acute nausea studies) or pair- housed [for anticipatory nausea and light-dark emergence studies] in home cages made of opaque white plastic (48 × 26 × 20 cm), containing bed-o-cob bedding from Harlan Laboratories, Inc. (Mississauga, Ontario), a brown paper towel, and Crinkl'Nest™ from The Andersons, Inc. (Maumee, Ohio). Additionally, in the home
15 cage, rats were provided with a soft white paper container that was 14 cm long and 12 cm in diameter. All rats were subjected to an ambient temperature of 21°C and a 12/12 h light-dark schedule (lights off at 07:00 h) and maintained on food (Highland Rat Chow [8640]) and water *ad libitum*. For the acute and anticipatory nausea studies, their body weights ranged from 263 to 329 g on the day of conditioning. For the light-dark emer-
20 gence studies, their body weights ranged from 320 to 387 g on the day of test.

[0061] *Apparatus.* For the studies of acute nausea (*in vivo* experiment 1), rats were placed in taste reactivity (Grill and Norgren, 1978) chambers with their cannula at-
25 tached to an infusion pump (Model KDS100, KD Scientific, Holliston, MA, USA) for fluid delivery. The taste reactivity chambers were made of clear Plexiglas (22.5 × 26 × 20 cm) that sat on a table with a clear glass top. A mirror beneath the chamber at a 45° angle facilitated viewing of the ventral surface of the rat to observe orofacial responses. The conditioning chamber was in a dark room next to a 25 W light source. A video
30 camera (Sony DCR-HC48, Henry's Cameras, Waterloo, ON, Canada) fire-wired into a computer was focused on the mirror and used to record each rat's orofacial reactions during the 2 min taste reactivity test. The video tapes were later scored using 'The Observer' (Noldus Information Technology Inc., Leesburg, VA, USA) software.

[0062] For *in vivo* experiment 2, contextually elicited conditioned gaping (a model of

anticipatory nausea) was measured using a distinctive conditioning chamber made of opaque black Plexiglass (22.5 × 26 × 20 cm) with an opaque lid that sat on a table with a clear glass top. A mirror beneath the chamber at a 45° angle facilitated viewing of the ventral surface of the rat to observe orofacial responses. The conditioning chamber was in a dark room next to a 25 W light source. A video camera that was fire-wired into a computer was focused on the mirror to record each rat's orofacial reactions during the 5 min test trial. The video tapes were later scored using 'The Observer' software. To assess activity, an activity chamber made of white Plexiglas (60 × 25 × 25 cm) was used, illuminated by a red light found in a different room than the contextual chamber was used to create a different context from the AN chamber. The activity of each rat was captured by video camera and sent to the Ethovision software program (Noldus, Inc., NL) to measure distance (cm) travelled.

[0063] For the *in vivo* experiment 3, anxiolytic-like responding was evaluated using the light-dark emergence apparatus, which consisted of an opaque white plastic rectangular box that was divided into two compartments: a small (25 cm wide × 20.5 cm long × 20.5 cm high) enclosed dark box built of opaque black plastic with a door (8 cm wide × 10 cm high) leading to a larger (39.5 cm long × 25 cm wide) open lit box. The open lit box was illuminated by one lamp (with a 60 W bulb, 180 lux in the light chamber) positioned 115 cm above the center of the lit box. A video camera was mounted over the top of the light-dark box, and the video tapes were analyzed by the Ethovision software (Noldus Information Technology, Leesburg, VA, USA) for the duration of time spent in the light box for the 5 min test. For the foot shock (FS) session, the rats were placed in sound attenuating MED Associates fear conditioning chambers (St. Albans, VT, USA). The 6 min FS session consisted of six 0.8 mA foot shocks delivered 1 min apart. Each 0.5 s shock was preceded by a 30s auditory tone (90 Db, 5000 Hz) as described by Bluett *et al.* (2014).

***In vivo* procedures**

[0064] *In vivo* experiment 1: dose-related effects of CBDA and HU-580 on acute nausea and 5-HT_{1A} receptor mediation of HU-580 effects.

[0065] All rats were surgically implanted with an intraoral cannula according to the procedures described by Limebeer *et al.* (2010). On the day of surgery, the rats were injected with an antibiotic (Derapin: 0.0 mg·kg⁻¹ s.c.; Pfizer Animal Health, Pfizer Canada

Inc, Kirkland, Quebec, Canada) 30 min prior to being anaesthetized with isoflurane (4-5% induction, 1.5% maintenance in O₂). Surgical plane anesthesia, as indicated by absence of the hind limb withdrawal reflex and defined by the Canadian Council of Animal Care, was induced before any surgery began, and was adjusted as necessary. Once sufficient anesthesia had been induced, a 2 cm² section of skin was shaved at the back of the neck at the level of the scapula. The skin was prepared by cleaning with soap (Bactistat; Ecolab, St. Paul, MN, USA) and wiping with 70% isopropyl alcohol followed by 7% Betadine solution (Purdue Products L.P., Stamford, CT, USA). Each rat was then administered a 5 mg·kg⁻¹ injection (i.p.) of the antiinflammatory/analgesic drug carprofen (Rimadyl; Pfizer Canada Inc., Kirkland, Quebec, Canada). A thin-walled 15-gauge stainless steel needle was inserted into the shaved area on the neck, directed subcutaneously around the ear and brought out behind the first molar inside the mouth. A 10 cm length of Intra Medic PE90 tubing (Clay Adams Brand; Becton Dickinson and Co., Sparks, MD, USA) with an inner diameter of 0.86 mm and an outer diameter of 1.27 mm was then inserted through the needle after which the needle was removed. Betadine (10%) was applied to the puncture site and three elastic discs (2 cm²) were placed over the exposed end of the tubing and drawn to the skin at the back of the neck for the purpose of stabilizing the cannula. The cannula was held secure in the oral cavity by a 6 mm disc of polypropylene mesh (297 micron; Small Parts Inc., Miramar, FL, USA) secured behind the heat flanged intraoral opening. The rats were then returned to their home cage and monitored daily for 3 days. For 3 days following surgery the rats were weighed and their cannulae were flushed with an antiseptic mouth wash. During this time, the rats were also monitored for activity, vocalization, dehydration, rigidity, and presence of porphyrin staining around the eyes. On the first post-surgical day, the rats were also given an analgesic/anti-inflammatory injection of Rimadyl (5 mg·kg⁻¹ i.p.).

[0066] Following post-surgical monitoring, the rats received an adaptation trial in which they were placed in the taste reactivity chamber with each rat's cannula attached to the infusion pump. During adaptation, water was infused into their intraoral cannulae for 2 min at a rate of 1 mL·min⁻¹. On the day following the adaptation trial, the rats received a conditioning trial in which they were administered a pretreatment injection of vehicle (VEH) (*n* = 8), CBDA (0.01, 0.1, 1 µg·kg⁻¹; *n* = 8 per group) or HU-580 (0.01, 0.1, 1 µg·kg⁻¹; *n* = 8 per group). Forty-five minutes after the pretreatment injection, the rats were individually placed in the chamber and infused, p.o., with 0.1% saccharin solution

for 2 min at the rate of $1 \text{ mL} \cdot \text{min}^{-1}$. Immediately after the saccharin infusion, all rats were injected with $20 \text{ mL} \cdot \text{kg}^{-1}$ of 0.15 M LiCl and returned to their home cage. Seventy-two hours later, rats were tested drug-free. Rats were again infused p.o. with 0.1% saccharin solution for 2 min at the rate of $1 \text{ mL} \cdot \text{min}^{-1}$ while the orofacial responses were video recorded from a mirror at a 45° angle beneath the chambers. Rats were then returned to their home cages. Two additional groups were added to determine the mechanism of action. These rats were injected with WAY100635 ($0.1 \text{ mg} \cdot \text{kg}^{-1}$) 15 min prior to an injection of either vehicle ($n = 8$) or $0.1 \text{ } \mu\text{g} \cdot \text{kg}^{-1}$ HU-580 ($n = 6$). The video tapes were later scored by an observer blind to the experimental conditions using 'The Observer' for the behaviors of gaping (large openings of the mouth and jaw, with lower incisors exposed).

[0067] *In vivo experiment 2: effect of CBDA and HU-580 on anticipatory nausea and 5-HT_{1A} receptor mediation of HU-580 effects.*

[0068] To compare the potential of HU-580 and CBDA to reduce anticipatory nausea, the contextually elicited conditioned gaping paradigm was used (e.g. Limebeer *et al.*, 2010; Rock *et al.*, 2014). Rats underwent four conditioning trials during which the distinctive context was paired with $127 \text{ mg} \cdot \text{kg}^{-1}$ LiCl. On each trial, rats were injected with LiCl and then immediately placed in the conditioning chamber for 30 min. This procedure was repeated four times with a 48h interval between conditioning trials. For the test trial, rats were randomly assigned to one of five treatment groups ($n = 6$ per group): VEH, $0.1 \text{ } \mu\text{g} \cdot \text{kg}^{-1}$ CBDA, $0.1 \text{ } \mu\text{g} \cdot \text{kg}^{-1}$ HU-580, $0.01 \text{ } \mu\text{g} \cdot \text{kg}^{-1}$ CBDA, $0.01 \text{ } \mu\text{g} \cdot \text{kg}^{-1}$ HU-580. Pretreatments were injected 45 min before the rats were given an saline injection ($20 \text{ mL} \cdot \text{kg}^{-1}$ i.p.) and individually placed in the conditioning (contextual) chamber for 5 min, and orofacial responses were video recorded. To investigate the mechanism of action of HU-580, two additional groups of rats were administered $0.1 \text{ mg} \cdot \text{kg}^{-1}$ WAY-VEH ($n = 8$), $0.1 \text{ mg} \cdot \text{kg}^{-1}$ WAY- $0.1 \text{ } \mu\text{g} \cdot \text{kg}^{-1}$ HU-580 ($n = 8$). VEH or WAY100635 were administered 15 min before HU-308 or VEH. The video tapes from the test trial were scored by an observer blind to the experimental conditions using 'The Observer' for the behaviors of gaping (large openings of the mouth and jaw, with lower incisors exposed). Immediately following the test trial, rats were put in the activity chamber (white Plexiglas, $60 \times 25 \times 25 \text{ cm}$, illuminated by a red light) for 15 min, and locomotor activity was captured by a video camera and sent to a computer using EthoVision software (Noldus, Inc, NL) to measure distance (cm) travelled.

[0069] *In vivo experiment 3: effect of CBDA and HU-580 on anxiety-like responding and 5-HT_{1A} receptor mediation of HU-580 effects.*

5 **[0070]** The effect of CBDA and HU-580 on anxiety-like responding was evaluated using the light-dark box emergence test following either foot shock stress or no foot shock (No FS) stress. Bluett *et al.* (2014) have demonstrated that anxiety-like responding in this test is greatly enhanced 24 h following foot shock stress. Also, Rock *et al.* (2017) have shown that CBDA (at doses as low as 0.1 $\mu\text{g}\cdot\text{kg}^{-1}$ i.p.) prevents the enhanced
10 anxiety-like responding following foot shock stress, by a 5-HT_{1A}-dependent mechanism of action. Therefore, the relative effectiveness of an even lower dose (0.01 $\mu\text{g}\cdot\text{kg}^{-1}$, i.p.) of CBDA and HU-580 was compared. Since it was found that HU-580 was anxiolytic at this low dose, it was subsequently evaluated the ability of the 5HT_{1A} receptor antagonist, WAY100635, to reverse the suppression of anxiety-like responding by HU-580.

15 **[0071]** All rats were acclimatized to the facility for 13 days prior to experimental manipulations, with weighing and handling occurring for eight of these days. After this acclimatization, the rats received a single FS stress session or No FS stress session 24 h before the light-dark emergence test (Bluett *et al.*, 2014). For the FS group, the rats
20 were placed in sound-attenuating MED Associates fear conditioning chambers (St. Albans, VT, USA). The 6 min FS session consisted of six 0.8 mA FSs delivered 1 min apart. Each 0.5 s shock was preceded by a 30 s auditory tone (90 Db, 5000 Hz) as described by Bluett *et al.* (2014). The No FS stress group remained in their home cage during this session.

25 **[0072]** Twenty-four hours later, the rats were subjected to the light-dark emergence test. Rats in the FS group and the No FS group were pretreated with VEH, 0.01 $\mu\text{g}\cdot\text{kg}^{-1}$ CBDA or 0.01 $\mu\text{g}\cdot\text{kg}^{-1}$ HU-580. Forty-five minutes later, they were placed in the dark chamber of the light-dark box, and their movement was tracked for a 5 min test. To investigate the possibility that the effect of HU-580 was 5-HT_{1A} receptor-mediated, additional groups were injected with WAY100635, 15 min prior to VEH or 0.01 $\mu\text{g}\cdot\text{kg}^{-1}$ HU-
30 580. The number of seconds spent in the light box was measured. Groups were as follows: No FS-VEH ($n = 9$), FS-VEH ($n = 12$), No FS-0.01 $\mu\text{g}\cdot\text{kg}^{-1}$ CBDA ($n = 8$), FS-0.01 $\mu\text{g}\cdot\text{kg}^{-1}$ CBDA ($n = 8$), No FS- 0.01 HU-580 ($n = 8$), FS-0.01 HU-580 ($n = 8$), No FS-0.1

$\mu\text{g}\cdot\text{kg}^{-1}$ WAY-VEH ($n = 8$), FS-0.1 $\mu\text{g}\cdot\text{kg}^{-1}$ WAY-VEH ($n = 7$), No FS-0.1 $\mu\text{g}\cdot\text{kg}^{-1}$ WAY-0.01 $\mu\text{g}\cdot\text{kg}^{-1}$ HU580 ($n = 8$), FS-0.1 $\mu\text{g}\cdot\text{kg}^{-1}$ WAY-0.01 $\mu\text{g}\cdot\text{kg}^{-1}$ HU-580 ($n = 8$).

In vitro and in vivo data analysis

5 [0073] Net agonist-stimulated [^{35}S]-GTP γ S binding values were calculated by subtracting basal binding values (obtained in the absence of agonist) from agonist-stimulated values (obtained in the presence of agonist) (Cascio *et al.*, 2010). Values are expressed as means and variability as SEM or as 95% confidence limits. Mean EC₅₀ and mean maximal effect (E_{max}) values, and SEM or 95% confidence limits of these values, have been calculated by nonlinear regression analysis using the equation for a sigmoid concentration-response curve (GraphPad Prism). P values <0.05 were considered significant. The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015).

15 [0074] For analysis of data from the acute nausea experiment (*In vivo* experiment 1), a single factor ANOVA was conducted for the mean number of gapes in the 2 min test, and subsequent pairwise comparisons were assessed with least significant difference (LSD) *post hoc* tests. For analysis of data from the anticipatory nausea (AN) experiment (*In vivo* experiment 2), a single factor ANOVA was conducted for the number of gapes in the 5 min AN test and for the total distance travelled in the activity test, and subsequent pairwise comparisons were assessed with LSD *post hoc* tests. For analysis of data from the anxiety-like responding experiment (*In vivo* experiment 3), the amount of time spent in the light box during the light-dark emergence test was entered into a 2×5 between factors ANOVA with the factors of FS stress/No FS stress and each pretreatment and $\mu\text{g}\cdot\text{kg}^{-1}$ i.p. dose condition (VEH, 0.01 $\mu\text{g}\cdot\text{kg}^{-1}$ CBDA, 0.01 $\mu\text{g}\cdot\text{kg}^{-1}$ HU-580, WAY-VEH or WAY-HU-580). Subsequent independent t -tests were conducted to explore the interaction. Significance levels were set at $P < 0.05$.

30 [0075] *Drugs and materials used in vitro.* 8-OH-DPAT and WAY100635 were supplied by Bio-Techne (Abingdon, UK). [^{35}S]-GTP γ S (1250 Ci mmol $^{-1}$) was purchased from PerkinElmer Life Sciences, Inc. (Boston, MA, USA), and GTP γ S, GDP and DMSO from Sigma-Aldrich UK. CBDA and its methyl ester (HU-580) were provided by Raphael Mechoulam.

[0076] *Drugs used in vivo.* Lithium chloride (LiCl; Sigma Aldrich) was prepared in a

0.15 M solution with sterile water and was administered i.p. at a volume of 20 mL·kg⁻¹ (127.2 mg·kg⁻¹ dose). CBDA and its methyl ester (HU-580), both provided by Raphael Mechoulam, were dissolved in a glass graduated tube in 1 mL ethanol with 1 mL Tween80 (Sigma) added to the solution, and the ethanol was evaporated off with a nitrogen stream, after which 9 mL of saline was added (final Tween80: saline ratio = 1:9). CBDA or HU-580 were administered to rats i.p. at a dose of 0.01, 0.1 or 1.0 µg·kg⁻¹, in a volume of 1 mL·kg⁻¹, using a stock solution containing one or other of these compounds at a concentration of 0.01, 0.1 or 1.0 µg·mL⁻¹ respectively. WAY100635 (Sigma, St Louis, MO, USA) was dissolved in saline at a concentration of 0.1 mg·mL⁻¹ and administered to rats i.p. at a dose of 0.1 mg·kg⁻¹ (1 mL·kg⁻¹).

RESULTS

[0077] *CBDA and HU-580 enhance the ability of a 5-HT_{1A} receptor agonist to stimulate [³⁵S] GTPγS binding to human 5-HT_{1A} receptors in vitro*

[0078] As found previously in [³⁵S]GTPγS binding experiments performed with rat brainstem membranes (Bolognini *et al.*, 2013), CBDA enhanced the stimulation of [³⁵S]-GTPγS binding induced by the selective 5-HT_{1A} receptor agonist, 8-OH-DPAT, to membranes obtained from CHO cells stably transfected with human 5-HT_{1A} receptors (Figure 1A-1E and Table 1). Concentrations of CBDA in the sub-micromolar range, producing significant increases in the mean E_{\max} of 8-OH-DPAT at 0.1, 1.0 and 10 nM, but not at 0.01 or 100 nM. None of these increases in mean E_{\max} was accompanied by any significant change in the mean EC_{50} of 8-OH-DPAT ($P > 0.05$; Table 1). The methyl ester of CBDA, HU-580, was even more potent than CBDA at enhancing 8-OH-DPAT-induced stimulation of [³⁵S]-GTPγS binding to human 5-HT_{1A} receptor-expressing CHO cell membranes (Figure 2A-2F and Table 2). Thus, it produced a significant increase in the mean E_{\max} of 8-OH-DPAT not only at 0.1, 1.0 and 10 nM (like CBDA) but also at 0.01 nM (unlike CBDA). HU-580 did not increase the mean E_{\max} of 8-OH-DPAT either at 100 nM (like CBDA) or at 0.001 nM and did not significantly affect the mean EC_{50} of 8-OH-DPAT at any of the concentrations investigated (Table 2). When administered by itself, at concentrations of 0.01, 0.1, 1, 10 or 100 nM, HU-580 did not behave as a 5-HT_{1A} receptor agonist or inverse agonist as indicated by the lack of a detectable effect of any of these concentrations on [³⁵S]-GTPγS binding to membranes obtained from human 5-HT_{1A} receptor-transfected CHO cells ($n = 6$; data not shown).

Table 1: Effects of various concentrations of CBDA on the mean EC_{50} and E_{max} values of 8-OH-DPAT for its stimulation of [35 S]GTP γ S binding to membranes obtained from CHO cells stably transfected with human 5-HT $_{1A}$ receptors (See also Figures 1A-1E)

| Pretreat- ment | Mean EC_{50} (nM) | 95% con- fidence limits (nM) | Mean E_{max} (%) | 95% con- fidence limits (%) | n |
|----------------------------------|------------------------|------------------------------------|--------------------|-----------------------------------|---|
| Vehicle | 17.48 | 10.14 & 30.12 | 97.69 | 88.57 & 106.8 | 6 |
| 0.01 nM CBDA (Fi- gure 1A) | 22.66 | 10.32 & 49.46 | 95.64 | 83.23 & 108.1 | 6 |
| Vehicle | 10.77 | 5.74 & 20.21 | 98.67 | 88.26 & 109.1 | 6 |
| 0.1 nM CBDA (Fi- gure 1B) | 11.19 | 8.18 & 15.29 | 115.3* | 109.6 & 120.9 | 6 |
| Vehicle | 15.52 | 7.51 & 32.08 | 96.56 | 84.51 & 108.6 | 6 |
| 1.0 nM CBDA (Fi- gure 1C) | 72.81 | 31.86 & 166.4 | 167.1* | 138.3 & 195.9 | 6 |
| Vehicle | 16.77 | 8.58 & 32.80 | 101.7 | 89.55 & 113.9 | 6 |
| 10 nM CBDA (Fi- gure 1D) | 57.44 | 23.55 & 140.1 | 143.9* | 118.4 & 169.4 | 6 |
| Vehicle | 20.00 | 11.46 & 34.88 | 108.6 | 98.03 & 119.1 | 6 |
| 100 nM CBDA (Fi- gure 1E) | 22.86 | 10.73 & 48.70 | 109.0 | 96.14 & 121.9 | 6 |

5 [0079] Each asterisk indicates a significant difference ($*P < 0.05$) between a mean E_{max} value of 8-OH-DPAT determined in the presence of a particular concentration of CBDA, and the mean E_{max} value of 8-OH-DPAT displayed in the previous row,

that was determined in the same experiment in the presence of vehicle (DMSO) instead of CBDA. Significant differences are indicated by non-overlapping 95% confidence limits.

5 **Table 2:** Effects of various concentrations of HU-580 on the mean EC_{50} and E_{max} values of 8-OH-DPAT for its stimulation of [35 S]GTP γ S binding to membranes obtained from CHO cells stably transfected with human 5-HT $_{1A}$ receptors (see also Figures 2A-2F)

| Pretreatment | Mean EC_{50} (nM) | 95% confidence limits (nM) | Mean E_{max} (%) | 95% confidence limits (%) | n |
|----------------------------|---------------------|----------------------------|--------------------|---------------------------|---|
| Vehicle | 18.05 | 10.73 & 30.38 | 102.4 | 93.21 & 111.7 | 6 |
| 0.001nM HU-580 (Figure 2A) | 14.44 | 9.37 & 22.25 | 99.80 | 98.33 & 106.3 | 6 |
| Vehicle | 15.49 | 7.84 & 30.59 | 96.25 | 85.34 & 107.2 | 6 |
| 0.01nM HU-580 (Figure 2B) | 34.15 | 18.58 & 62.77 | 126.9* | 113.5 & 140.3 | 6 |
| Vehicle | 18.69 | 9.74 & 35.83 | 97.62 | 86.22 & 109.0 | 6 |
| 0.1nM HU-580 (Figure 2C) | 12.94 | 6.88 & 24.34 | 125.3* | 113.0 & 137.6 | 6 |
| Vehicle | 19.75 | 11.44 & 34.12 | 108.4 | 98.0 & 118.7 | 6 |
| 1.0nM HU-580 (Figure 2D) | 48.22 | 31.31 & 74.26 | 140.5* | 128.7 & 152.4 | 6 |
| Vehicle | 14.58 | 6.83 & 31.15 | 99.36 | 86.58 & 112.1 | 6 |
| 10 nM HU-580 (Figure 2E) | 23.94 | 8.82 & 64.97 | 138.8* | 115.7 & 161.9 | 6 |
| Vehicle | 19.89 | 11.78 & 33.58 | 101.6 | 92.04 & 111.1 | 6 |

| Pretreatment | Mean EC ₅₀ (nM) | 95% con- fidence limits (nM) | Mean E_{\max} (%) | 95% con- fidence limits (%) | n |
|---------------------------|-------------------------------|------------------------------------|---------------------|-----------------------------------|---|
| 100 nM HU-580 (Figure 2F) | 8.49 | 1.87 & 38.59 | 91.96 | 71.6 & 112.3 | 6 |

[0080] Each asterisk indicates a significant difference ($*P < 0.05$) between a mean E_{\max} value of 8-OH-DPAT determined in the presence of a particular concentration of HU-580, and the mean E_{\max} value of 8-OH-DPAT displayed in the previous row, that was determined in the same experiment in the presence of vehicle (DMSO) instead of HU-580. Significant differences are indicated by non-overlapping 95% confidence limits.

[0081] *In vivo experiment 1: dose-related effects of CBDA and HU-580 on acute nausea and 5-HT_{1A} receptor mediation of HU-580 effects*

[0082] At a dose of 0.1 $\mu\text{g}\cdot\text{kg}^{-1}$, but not at 0.01 or 1 $\mu\text{g}\cdot\text{kg}^{-1}$, HU-580 was more effective than CBDA in reducing acute nausea as assessed by the rat gaping model. HU-580's suppressive effect on acute nausea (0.1 $\mu\text{g}\cdot\text{kg}^{-1}$) was blocked by WAY100635. A single factor ANOVA revealed a significant group effect $F(8, 61) = 3.9$; $P < 0.05$. Figure 3 presents the mean number of gapes displayed by the various pretreatment groups. Subsequent LSD *post hoc* comparison tests revealed that both compounds reduced LiCl-induced gaping responses relative to vehicle at a dose of 1 $\mu\text{g}\cdot\text{kg}^{-1}$ ($P < 0.05$), replicating our previous findings (Limebeer *et al.*, 2010; Rock and Parker, 2013). However, at the even lower dose of 0.1- $\mu\text{g}\cdot\text{kg}^{-1}$, that is, subthreshold for a CBDA-induced reduction of nausea-like behavior, HU-580 reduced LiCl-induced conditioned gaping behavior relative to vehicle ($P < 0.05$). Rats pretreated with HU-580 (0.1 $\mu\text{g}\cdot\text{kg}^{-1}$) also gaped significantly less than group WAY-0.1 $\mu\text{g}\cdot\text{kg}^{-1}$ HU-580 ($P < 0.05$), indicating a 5-HT_{1A} receptor-mediated effect.

[0083] *In vivo experiment 2: effect of CBDA and HU-580 on anticipatory nausea and 5-HT_{1A} receptor mediation of HU-580 effects*

[0084] At an extremely low dose of 0.01 $\mu\text{g}\cdot\text{kg}^{-1}$, but not at 0.1 $\mu\text{g}\cdot\text{kg}^{-1}$, HU-580 was more effective than CBDA in reducing anticipatory nausea as assessed by the contextually elicited conditioned gaping model. The suppressive effect of HU-580 (0.1 $\mu\text{g}\cdot\text{kg}^{-1}$) was blocked by pretreatment with WAY100635. A single factor ANOVA revealed a significant group effect $F(6, 39) = 8.7$; $P < 0.05$. Figure 4A presents the mean number of gapes displayed. Subsequent LSD *post hoc* comparisons revealed that compared to VEH controls, at a dose of 0.1 $\mu\text{g}\cdot\text{kg}^{-1}$, both CBDA and HU-580 reduced conditioned gaping (P values < 0.05); however, the groups did differ at a dose of 0.01 $\mu\text{g}\cdot\text{kg}^{-1}$, with group HU-580 gaping significantly less than VEH controls ($P < 0.05$) and group 0.01 CBDA ($P = 0.05$). Rats pretreated with HU-580 (0.1 $\mu\text{g}\cdot\text{kg}^{-1}$) also gaped significantly less than group WAY-0.1 $\mu\text{g}\cdot\text{kg}^{-1}$ HU-580 ($P < 0.05$), indicating a 5-HT_{1A} receptor-mediated effect. A single factor ANOVA for the locomotor activity test (Figure 4B) revealed no significant effect on distance moved, $F(6, 39) = 0.9$, $P > 0.05$.

In vivo experiment 3: anxiolytic effects of CBDA and HU-580

[0085] Figure 5 presents the mean number of seconds spent by rats in the light box for each of the various pretreatment groups that received FS or No FS 24 h prior the light-dark test. As can be seen, FS stress greatly enhanced the anxiety-like responding of decreased time spent in the light box. At a low dose of 0.01 $\mu\text{g}\cdot\text{kg}^{-1}$, HU-580, but not CBDA, reversed the effect of FS on the anxiety-like responding of decreased time spent in the light box. The 2 \times 5 ANOVA for the number of seconds spent in the light box revealed a significant main effect of FS stress, $F(1, 84) = 25.6$; $P < 0.05$, and a FS stress \times pretreatment interaction, $F(4, 84) = 3.2$; $P, 0.05$). To analyze the interaction, subsequent independent *t*-tests revealed that rats pretreated with VEH ($P < 0.05$), 0.01 $\mu\text{g}\cdot\text{kg}^{-1}$ CBDA ($P < 0.05$), WAY-VEH ($P < 0.05$) or WAY-0.01 $\mu\text{g}\cdot\text{kg}^{-1}$ HU-580 ($P = 0.05$) spent less time in the light box following FS stress than following No FS stress, but rats pretreated with 0.01 $\mu\text{g}\cdot\text{kg}^{-1}$ HU-580 did not display this anxiogenic-like response. Furthermore, subsequent single factor ANOVAs of the time spent in the light box revealed a significant pretreatment effect among the FS groups, $F(4, 38) = 4.6$; $P < 0.05$, but not among the No FS groups. Among the FS groups subsequent Bonferroni tests revealed that only group 0.01 $\mu\text{g}\cdot\text{kg}^{-1}$ HU-580 spent significantly more time in the light box than group VEH ($P < 0.05$).

Discussion

[0086] The results obtained confirmed that CBDA displays significant potency both at producing an apparent enhancement of the activation of 5-HT_{1A} receptors, by the direct 5-HT_{1A} receptor agonist, 8-OH-DPAT, *in vitro*, and at producing a 5-HT_{1A} receptor-mediated reduction of both acute and anticipatory nausea in rats, *in vivo*.

[0087] The new *in vitro* data shows, first, that CBDA can enhance the activation not only of rat brain stem 5-HT_{1A} receptors (Bolognini et al., 2013), but also of human 5-HT_{1A} receptors and, second, that at both rat brain stem and human 5-HT_{1A} receptors, CBDA induces such enhancement with a bell-shaped concentration-response curve in the sub-micromolar range. The *in vitro* data described herein also reveals an important similarity between the pharmacological effects of CBDA and its methyl ester, HU-580. More specifically, these data have provided convincing evidence that HU-580 shares the ability of CBDA to produce an apparent enhancement of the activation of human 5-HT_{1A} receptors by 8-OH-DPAT in the [³⁵S]-GTPγS binding assay. Importantly, HU-580 produced such enhancement both with greater potency and with an even broader bell-shaped concentration-response curve than CBDA. Thus, significant enhancement was induced by HU-580 at concentrations of 0.01 to 10 nM (Table 2) and by CBDA at concentrations of 0.1 to 10 nM (Table 1). Whereas, at concentrations of 1, 10 and 100 nM, HU-580 produced slightly less enhancement of 8-OH-DPAT-induced 5-HT_{1A} receptor activation than CBDA, HU-580 produced slightly greater enhancement of this activation than CBDA, at concentrations of 0.01 and 0.1 nM (Tables 1 and 2).

[0088] It is noteworthy that none of the concentrations of CBDA and HU-580 that significantly increased E_{max} values of 8-OH-DPAT for its stimulation of [³⁵S]-GTPγS binding to 5-HT_{1A} receptors produced any significant change in the EC₅₀ of 8-OH-DPAT (Tables 1 and 2). This finding shows that CBDA and HU-580 may have been acting as positive allosteric modulators of the activation of these receptors by 8-OH-DPAT, there being evidence that some positive allosteric modulators do indeed increase the E_{max} values but not the potencies of agonists at certain receptors (Christopoulos *et al.*, 2014). There is a possibility that CBDA and HU-580 target an allosteric site on the 5-HT_{1A} receptor, as positive allosteric modulators. It is also noteworthy that the positive *in vitro* data for CBDA and HU-580 that was obtained herein all came from experiments performed with CHO cells transfected with human 5-HT_{1A} receptors.

[0089] The *in vivo* data reveals similarities between the pharmacological effects of HU-580 and CBDA. Thus, these data show that the ability of CBDA to reduce acute and anticipatory nausea in rats extends to HU-580. Importantly, as also found in our *in vitro* experiments, HU-580 displayed even greater potency than CBDA. More specifically, effective suppression of acute nausea-induced conditioned gaping was induced by HU-580 at a dose as low as $0.1 \mu\text{g}\cdot\text{kg}^{-1}$ i.p., whereas the lowest effective dose of CBDA for the production of such suppression was $1 \mu\text{g}\cdot\text{kg}^{-1}$ i.p. (Figure 3). Indeed, it was found that at a dose as low as $0.01 \mu\text{g}\cdot\text{kg}^{-1}$ i.p., HU-580, but not CBDA, suppressed contextually elicited conditioned gaping. It was also shown that suppression of LiCl-induced gaping and contextually elicited conditioned gaping by HU-580 can be completely prevented by the 5-HT_{1A} receptor-selective antagonist, WAY100635. Finally, although CBDA has recently been found to reduce FS enhancement of anxiogenic-like behavior in the light-dark box emergence test at doses of 0.1, 1 and $100 \mu\text{g}\cdot\text{kg}^{-1}$ i.p. (Rock *et al.*, 2017), it was found herein that it did not share the ability of HU-580 to reduce FS enhancement of anxiogenic-like behavior in the light-dark emergence test at the lower dose of $0.01 \mu\text{g}\cdot\text{kg}^{-1}$ i.p., suggesting that HU-580 may be even more potent than CBDA in reducing stress-induced anxiety. Furthermore, it was also shown that HU-580 has the ability to reduce FS enhancement of anxiogenic-like behavior is 5-HT_{1A} receptor-mediated. The results show that HU-580 is both more stable than CBDA, and more potent than CBDA (acute and anticipatory nausea).

[0090] Ideally, drugs used as medicines should, when stored, display stability over a reasonable period of time. Hence, since stored CBDA undergoes significant decomposition, even at 4°C, a major aim of this project was to develop a compound that produces no less potency than CBDA in the assays described in this paper but displays much greater stability over a reasonable length of time when stored at this temperature. It is noteworthy, therefore, that the inventors have found that HU-580 is, indeed, more stable than CBDA when stored at 4°C for 21 days. In addition, the finding showing that HU-580 is more potent than CBDA both *in vitro* and *in vivo* supports the hypothesis that the pharmacological effects produced by HU-580 in our experiments did not depend on its decomposition or metabolism to CBDA.

[0091] In conclusion, the evidence shows that HU-580, displays greater potency than

CBDA at suppressing signs both of acute and anticipatory nausea, and of stress-induced anxiety in rats, and that it produces these effects in a 5-HT_{1A} receptor-dependent manner.

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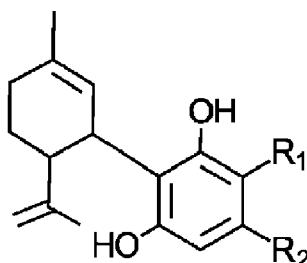
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PATENTKRAV

1. Forbindelse til anvendelse i behandlingen af en tilstand, en sygdom eller et symptom forbundet med 5-HT_{1A}-receptor, hvilken forbindelse har den almene formel (I):

5



(I)

hvor

R₁ er valgt blandt -C(=O)OR₃, -OC(=O)R₄;

10 R₂ er valgt blandt en ligekædet eller forgrenet C₁-C₁₅ alkyl, ligekædet eller forgrenet C₂-C₁₅ alkenyl, ligekædet eller forgrenet C₄-C₁₅ alkenynyl og ligekædet eller forgrenet C₂-C₁₅ alkynyl; hver uafhængigt eventuelt substitueret med mindst én substituent valgt blandt hydroxy, halogen, amin, amid og en hvilken som helst kombination deraf;

15 R₃ og R₄ hver uafhængigt er valgt blandt en ligekædet eller forgrenet C₁-C₁₅ alkyl, ligekædet eller forgrenet C₂-C₁₅ alkenyl, ligekædet eller forgrenet C₄-C₁₅ alkenynyl, ligekædet eller forgrenet C₂-C₁₅ alkynyl, halogen, amin og amid, hvor tilstanden, sygdommen eller symptomet er valgt blandt angst, stress, depression, skizofreni, panik, abstinenssyndrom, autoimmun sygdom, inflammation, fedme, metabolisk syndrom, retinopati, kvalme, opkastning, nyredysfunktion, nyreiskæmisk/reperfusionsskade, neuronal skade, Huntingtons sygdom, Alzheimers sygdom, hjerneinfarkt, hepatisk encefalopati, traumatisk hjerne-
 20 skade, cerebral iskæmi, rygmarvsskade, angiogenese, epilepsi, kramper, neuropatisk smerte, luftvejsobstruktion, obsessiv-kompulsiv adfærd, kognitiv svækkelse, nedsat seksualdrift og -funktion, søvnforstyrrelser, opioidrelateret respirationsundertrykkelse, afhængighed og en hvilken som helst kombination deraf.

25

2. Forbindelse til anvendelse ifølge krav 1, hvor tilstanden, sygdommen eller symptomet er valgt blandt kvalme, opkastning, kramper og en hvilken som helst kombination

deraf.

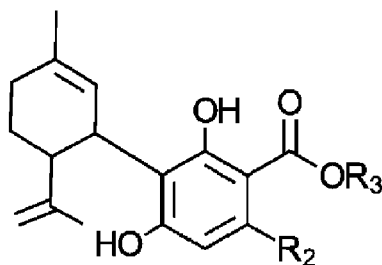
3. Forbindelse til anvendelse ifølge krav 1, hvor tilstanden, sygdommen eller symptomet er forbundet med depression.

5

4. Forbindelse til anvendelse ifølge krav 1, hvor sygdommen, tilstanden, symptomet eller lidelsen er forbundet med nyredysfunktion.

5. Forbindelse til anvendelse ifølge et hvilket som helst af kravene 1 til 4, hvor forbindelsen er en forbindelse med den almene formel (II):

10



(II)

hvor

15

R_2 er valgt blandt en ligekædet eller forgrenet C_1 - C_{15} alkyl, ligekædet eller forgrenet C_2 - C_{15} alkenyl, ligekædet eller forgrenet C_4 - C_{15} alkenynyl og ligekædet eller forgrenet C_2 - C_{15} alkynyl; hver uafhængigt eventuelt substitueret med mindst én substituent valgt blandt hydroxy, halogen, amin og amid, og en hvilken som helst kombination deraf;

20

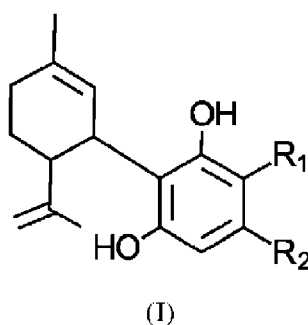
R_3 er valgt blandt en ligekædet eller forgrenet C_1 - C_{15} alkyl, ligekædet eller forgrenet C_2 - C_{15} alkenyl, ligekædet eller forgrenet C_4 - C_{15} alkenynyl, ligekædet eller forgrenet C_2 - C_{15} alkynyl, halogen, amin og amid.

6. Forbindelse til anvendelse ifølge et hvilket som helst af kravene 1 til 5, hvor R_2 er en ligekædet eller forgrenet C_1 - C_{15} alkyl.

25

7. Forbindelse til anvendelse ifølge et hvilket som helst af kravene 1 til 5, hvor R_2 er en ligekædet eller forgrenet C_2 - C_{15} alkenyl.

8. Forbindelse til anvendelse ifølge et hvilket som helst af kravene 1 til 5, hvor R_2 er en ligekædet eller forgrenet C_2 - C_{15} alkynyl eller en ligekædet eller forgrenet C_4 - C_{15} alkenynyl.
- 5 9. Forbindelse til anvendelse ifølge et hvilket som helst af kravene 1 til 5, hvor R_3 er en ligekædet eller forgrenet C_1 - C_{15} alkyl.
- 10 10. Forbindelse til anvendelse ifølge et hvilket som helst af kravene 1 til 5, hvor R_3 er en ligekædet eller forgrenet C_2 - C_{15} alkenyl.
- 11 11. Forbindelse til anvendelse ifølge et hvilket som helst af kravene 1 til 5, hvor R_3 er en ligekædet eller forgrenet C_2 - C_{15} alkynyl eller en ligekædet eller forgrenet C_4 - C_{15} alkenynyl.
- 15 12. Forbindelse til anvendelse ifølge et hvilket som helst af kravene 1 til 5, hvor R_3 er valgt blandt et halogen, en amin og et amid.
- 20 13. Præparat omfattende mindst én forbindelse ifølge et hvilket som helst af kravene 1 til 12 til anvendelse i behandlingen af en tilstand, en sygdom eller et symptom forbundet med 5-HT_{1A}-receptor, hvor tilstanden, sygdommen eller symptomet er valgt blandt angst, stress, depression, skizofreni, panik, abstinenssyndrom, autoimmun sygdom, inflammation, fedme, metabolisk syndrom, retinopati, kvalme, opkastning, nyredysfunktion, nyreiskæmi/reperfusionsskade, neuronal skade, Huntingtons sygdom, Alzheimers sygdom, hjerneinfarkt, hepatisk encefalopati, traumatisk hjerneskade, cerebral iskæmi, rygmarvsskade, angiogenese, epilepsi, kramper, neuropatisk smerte, luftvejsobstruktion, obsessiv-kompulsiv adfærd, kognitiv svækkelse, nedsat seksualdrift og -funktion, søvnforstyrrelser, opioidrelateret respirationsundertrykkelse, afhængighed og en hvilken som helst kombination deraf.
- 25 30 14. Forbindelse med den almene formel (I):



hvor

- 5 R_1 er valgt blandt $-C(=O)OR_3$, $-OC(=O)R_4$;
 R_2 er valgt blandt en ligekædet eller forgrenet C_2-C_{15} alkenyl, ligekædet eller
 forgrenet C_4-C_{15} alkenynyl og ligekædet eller forgrenet C_2-C_{15} alkynyl; hver uaf-
 hængigt eventuelt substitueret med mindst én substituent valgt blandt hydroxy,
 halogen, amin og amid eller en hvilken som helst kombination deraf;
- 10 R_3 er valgt blandt en ligekædet eller forgrenet C_2-C_{15} alkynyl, ligekædet eller for-
 grenet C_4-C_{15} alkenynyl, halogen, amin og amid; og
 R_4 er valgt blandt en ligekædet eller forgrenet C_1-C_{15} alkyl, ligekædet eller for-
 grenet C_4-C_{15} alkenynyl, ligekædet eller forgrenet C_2-C_{15} alkenyl og ligekædet
 eller forgrenet C_2-C_{15} alkynyl, halogen, amin og amid.

15

DRAWINGS

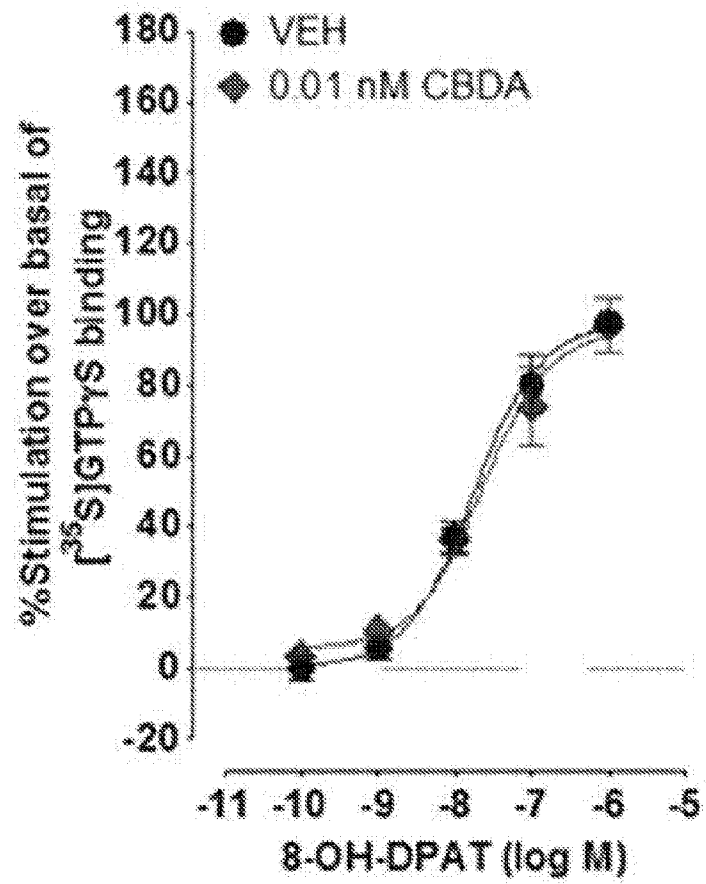


Figure 1A

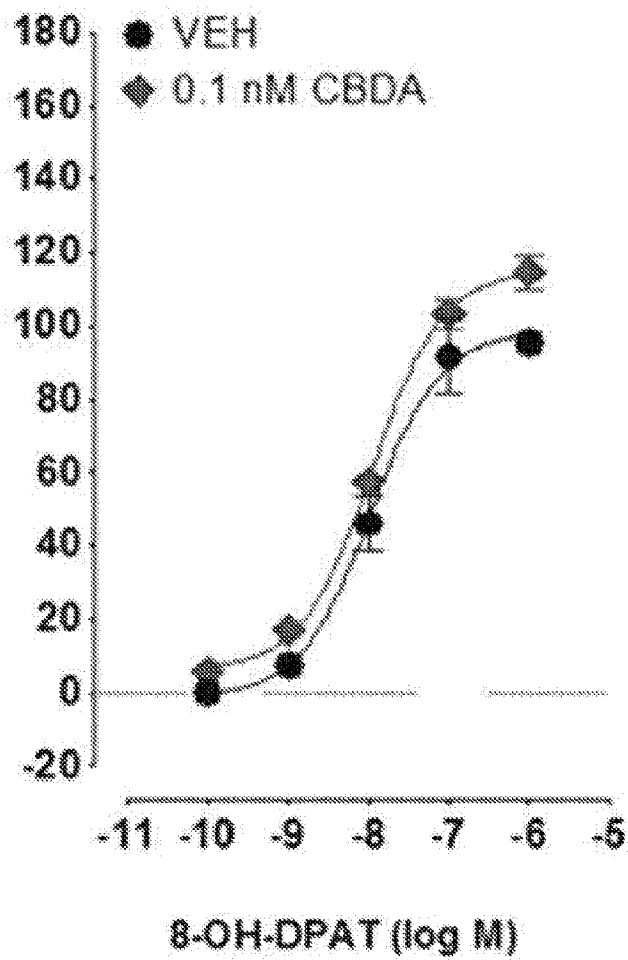


Figure 1B

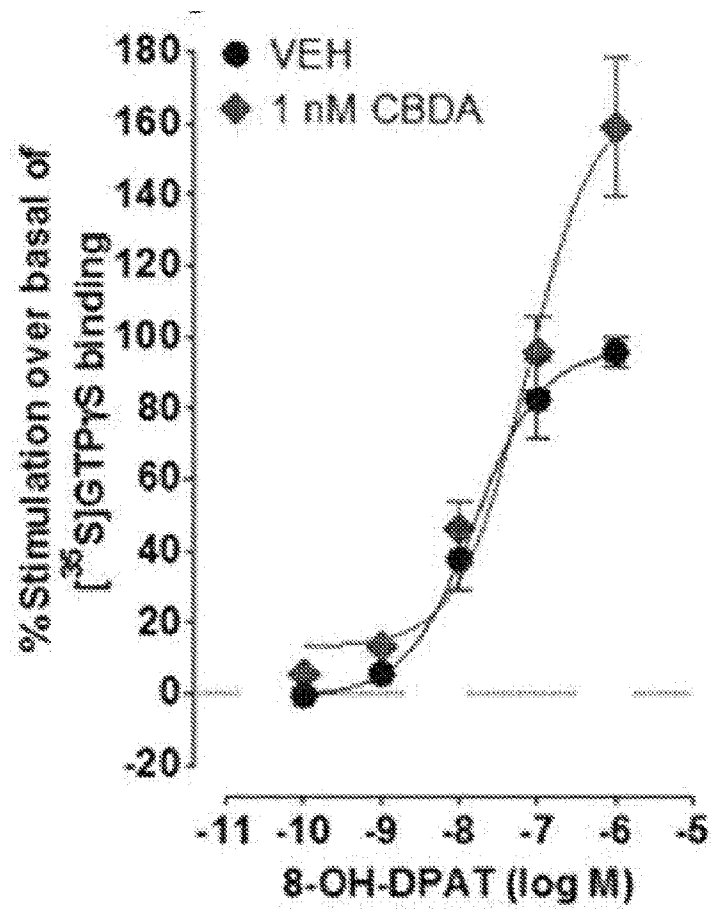


Figure 1C

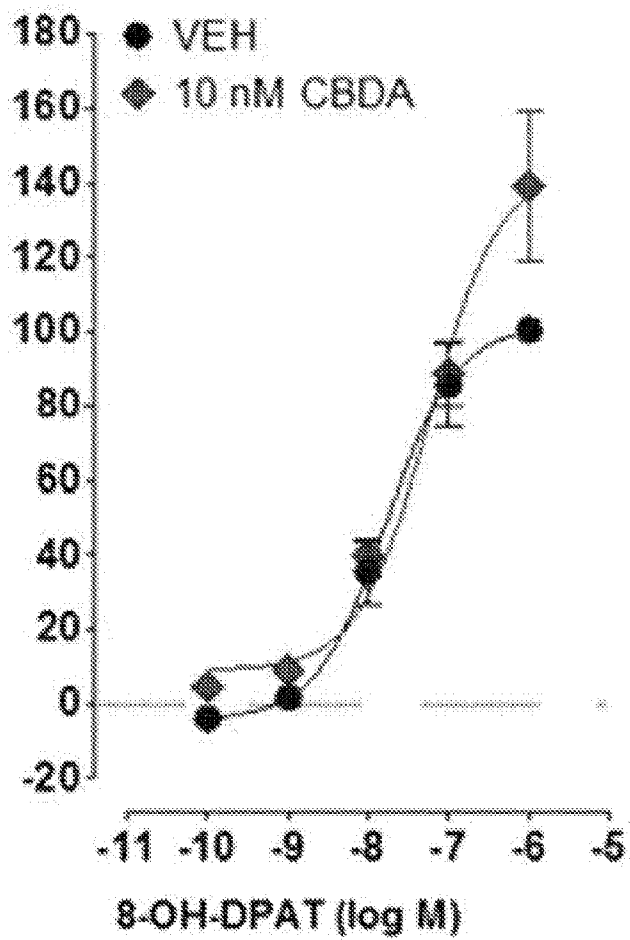


Figure 1D

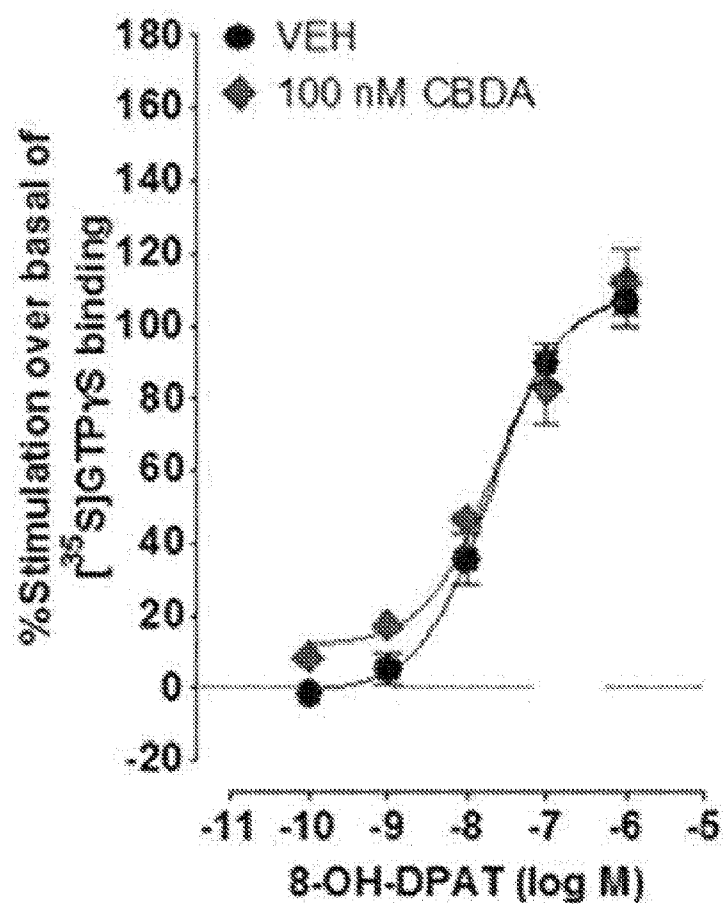


Figure 1E

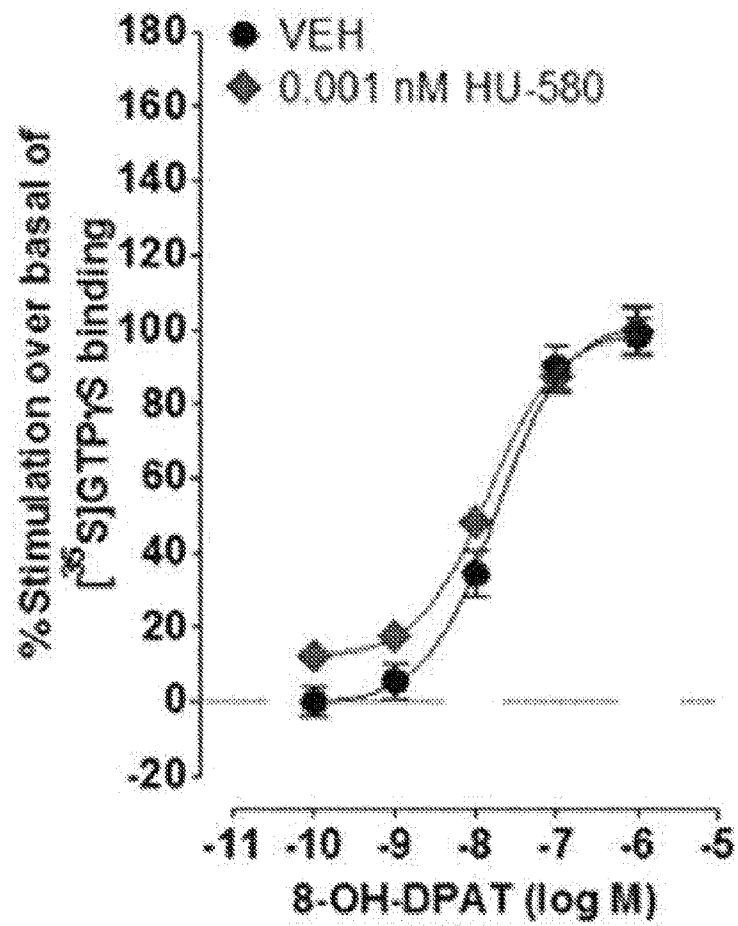


Figure 2A

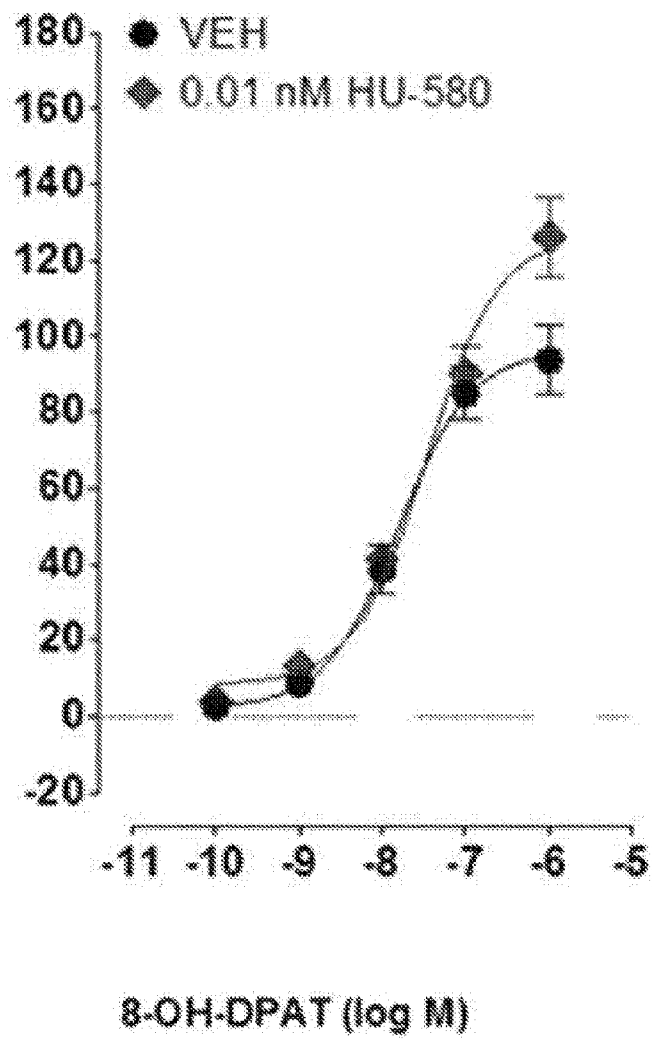


Figure 2B

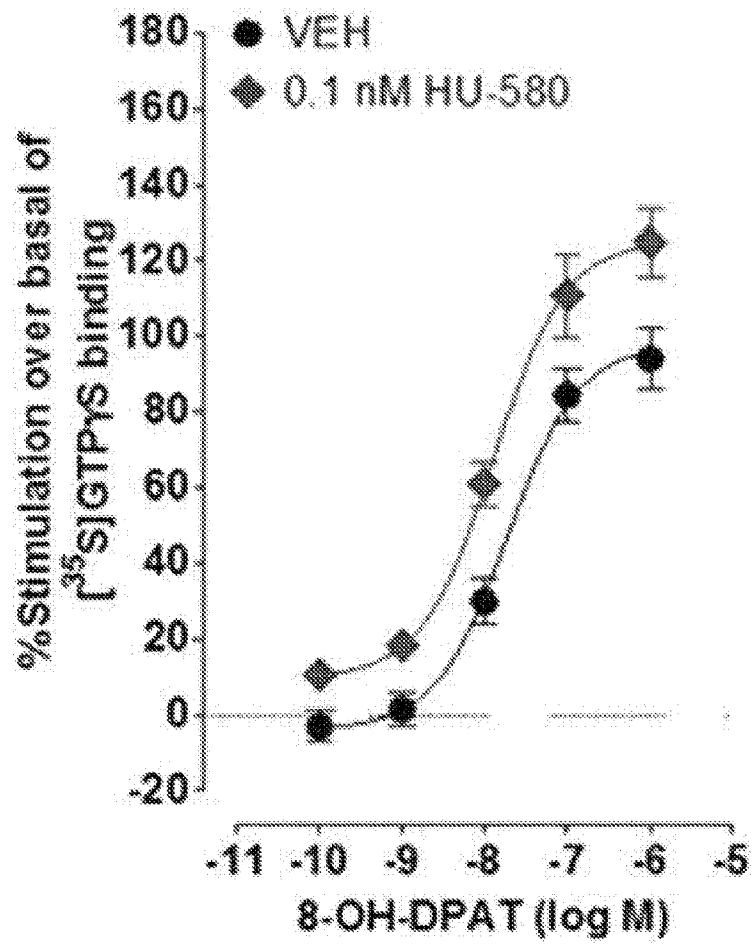


Figure 2C

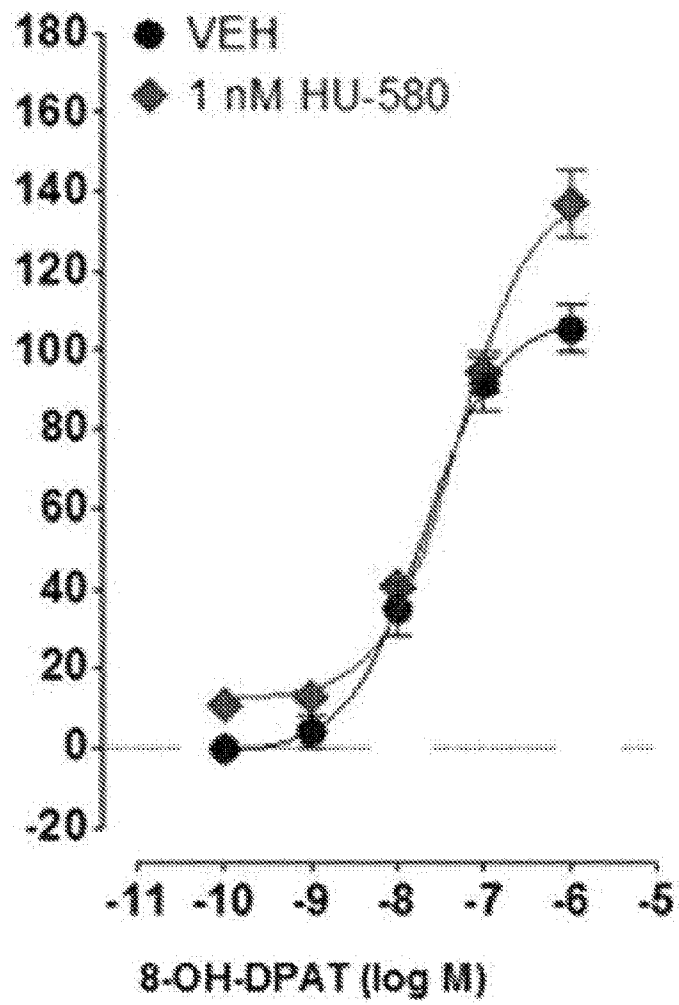


Figure 2D

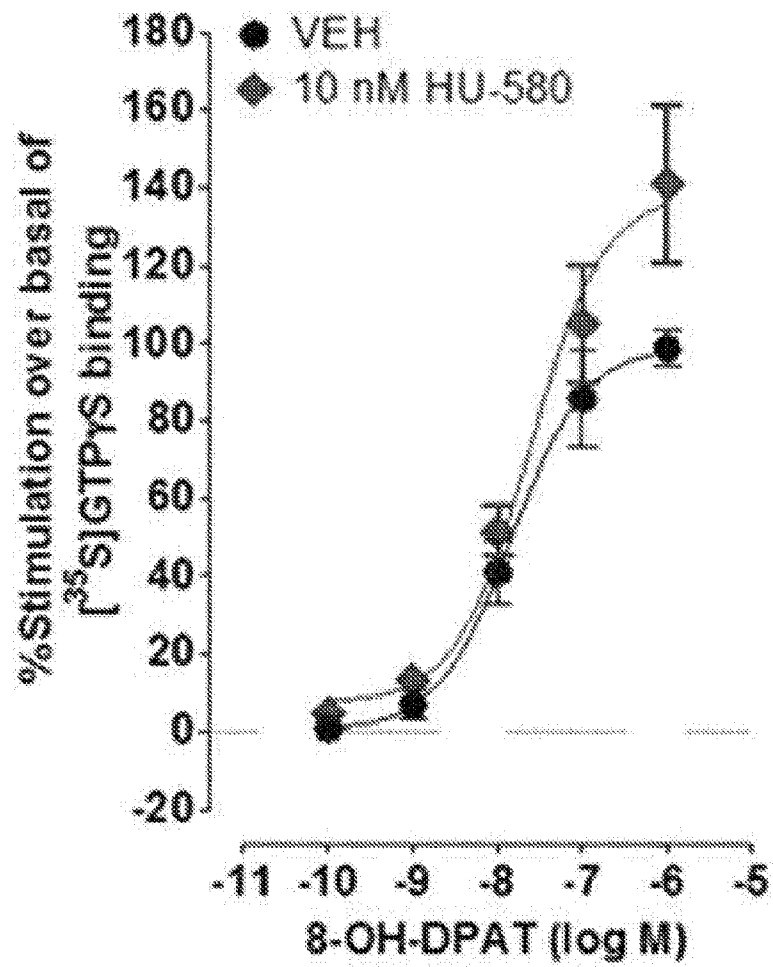


Figure 2E

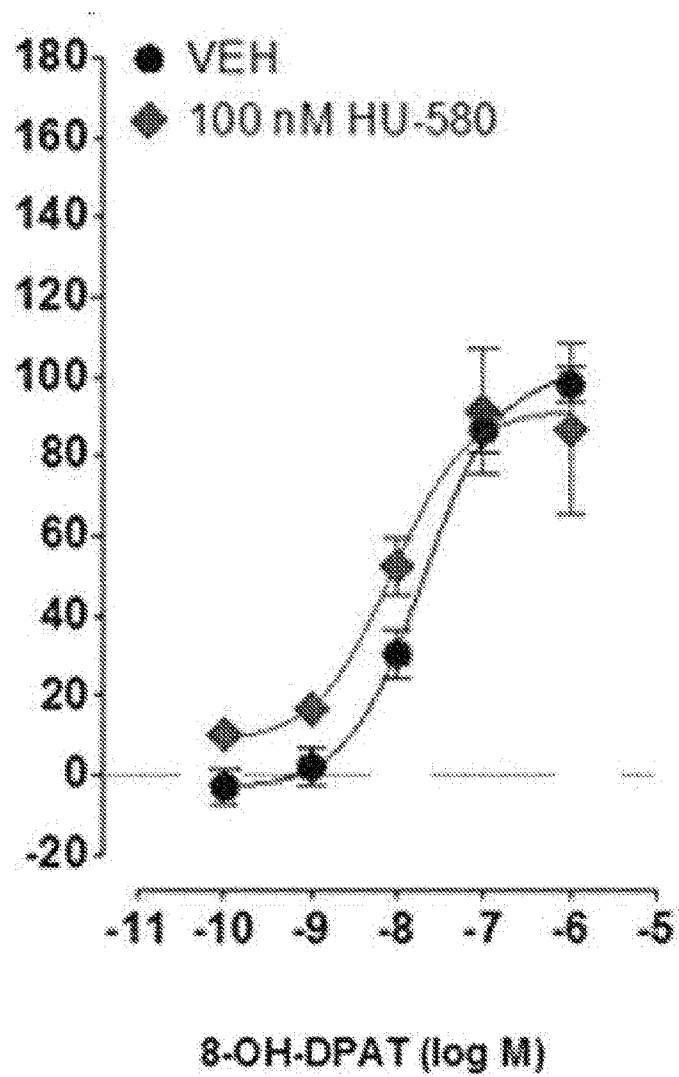


Figure 2F

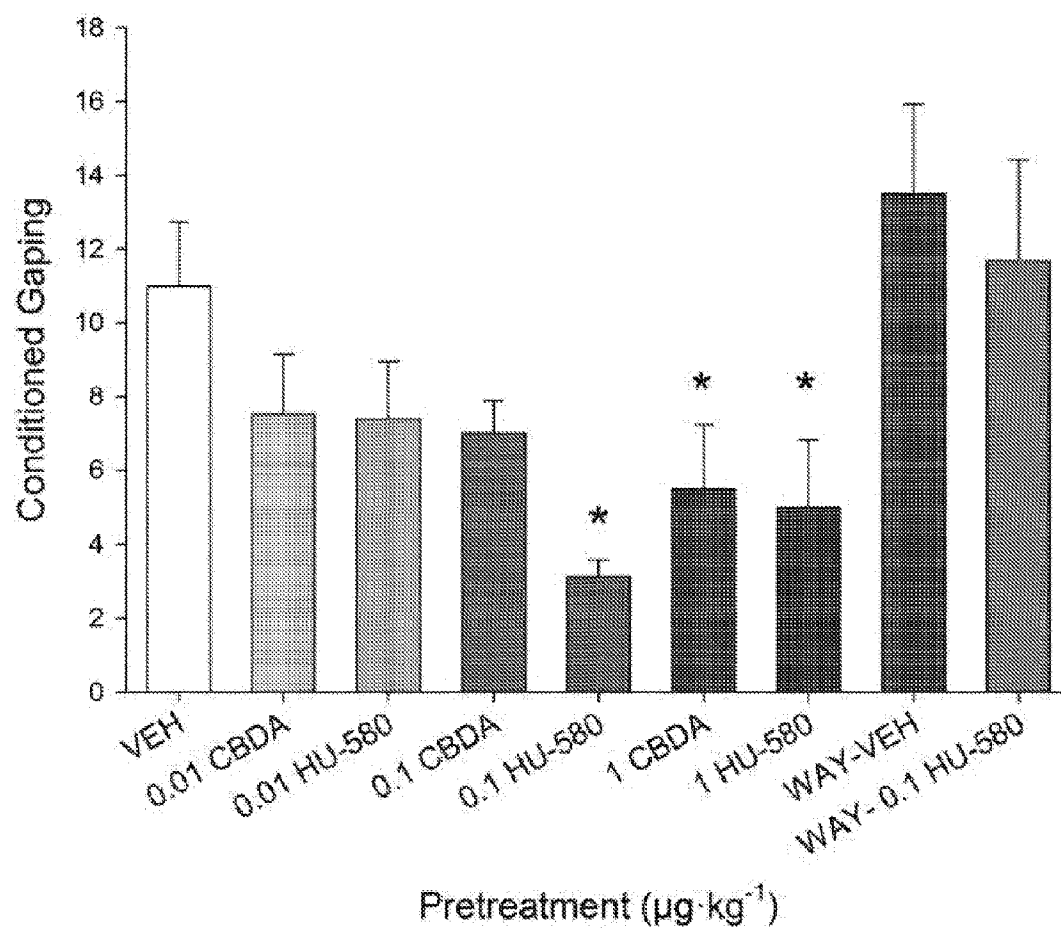
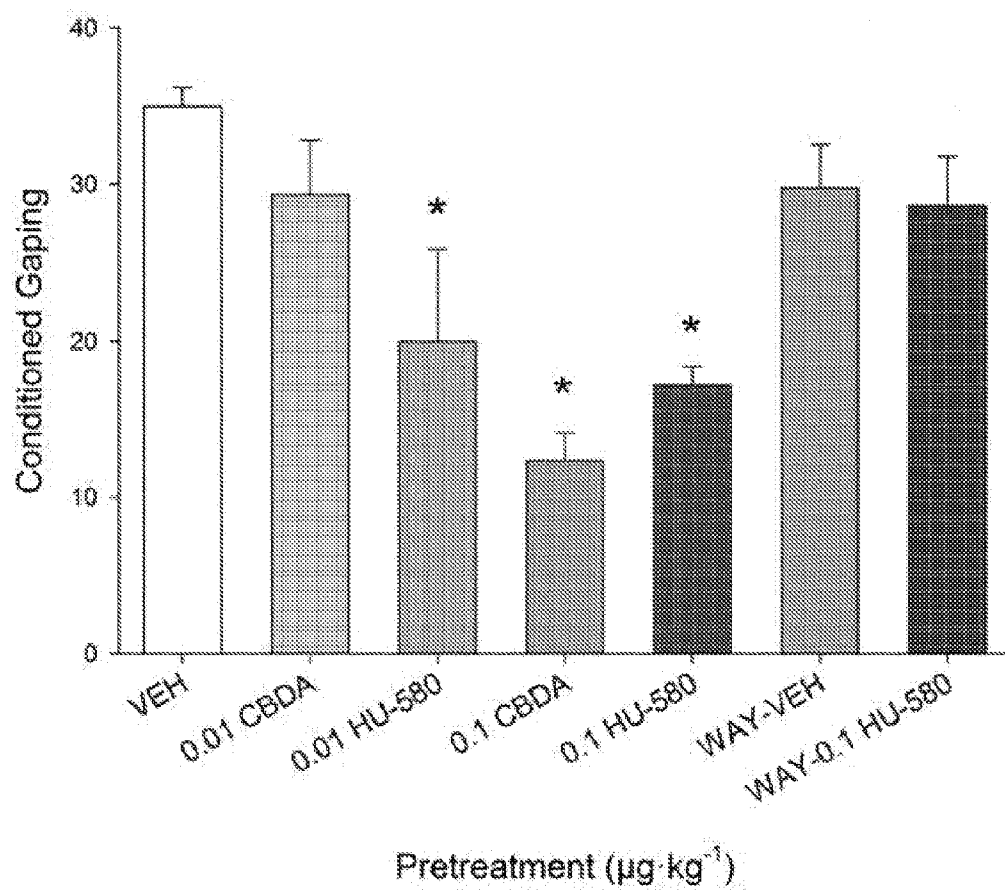


Figure 3

**Figure 4A**

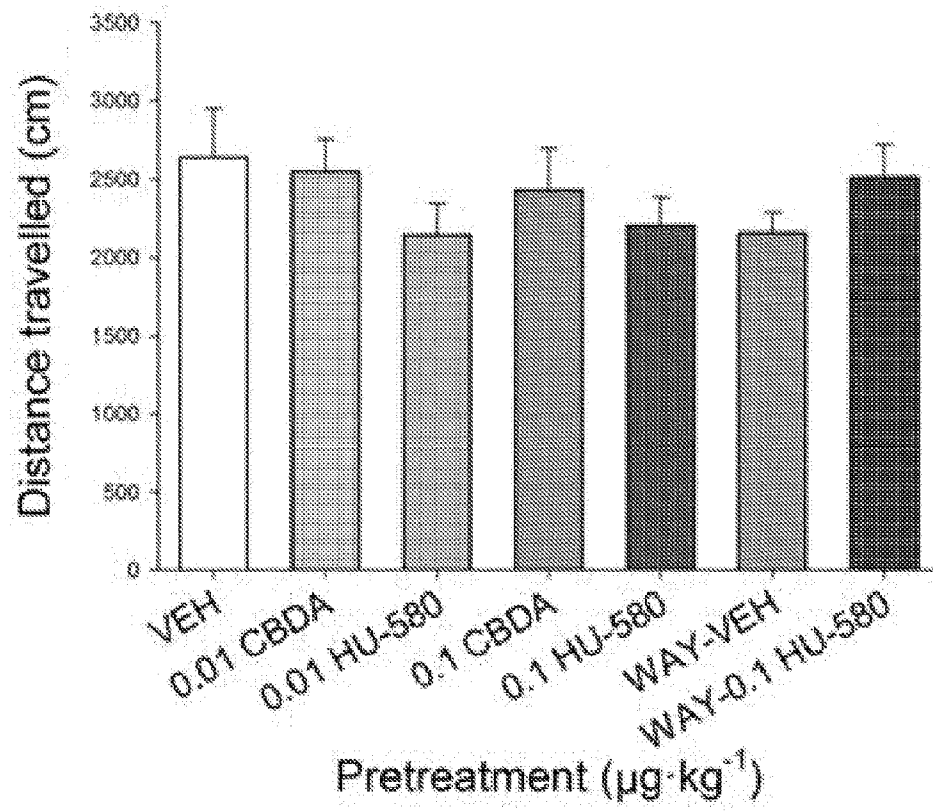


Figure 4B

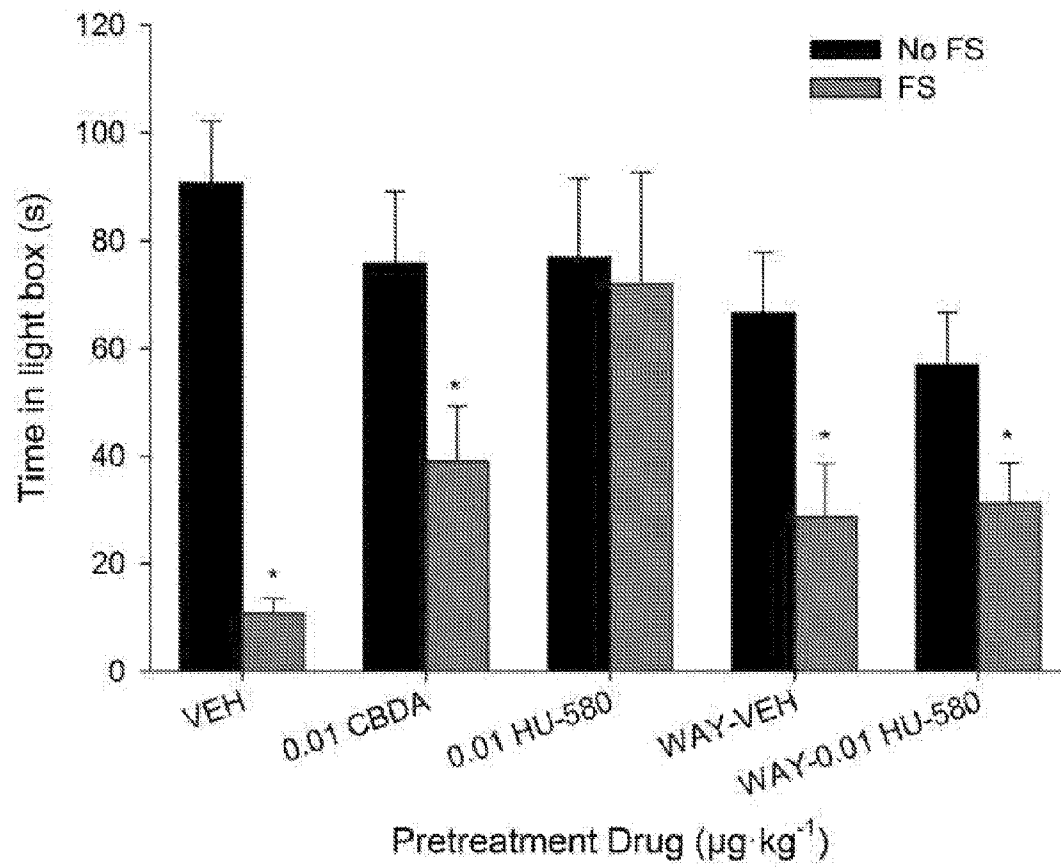


Figure 5