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DEP: 2020 DIRECCION DE NUEVAS CREACIONES	EVE: 379 FASE NACIONAL INCLUIDO CAPITULO II
TRA: 11 PCT-PATENTE DE INVENCION CAPITULOS I Y II	FOLIOS: 6
ACT: 519 PRESENTACION	

Abogado(a)/Señor(a)
SILVANA MARIA LÓPEZ DÍAZ

Asunto:	Radicación:	12-19374- -6
	Trámite:	11
	Evento:	379
	Actuación:	519
	Oficio:	241
	Folios:	6

Patente de Invención	X			Patente de Modelo de Utilidad	
Vía Nacional					
Vía PCT (Fase Nacional)	X	Capítulo I		Capítulo II	X
No. Solicitud Internacional	PCT/GB2010/051066				
Fecha de Presentación Internacional	29/06/2010				

Descripción:	Radicación 12-19374-00000	03/Febrero/2012
Reivindicaciones:	Radicación 12-19374-00000	03/Febrero/2012
Prioridad:	GB0911580	03/Julio/2009

Se acepta la reivindicación de prioridad porque su contenido técnico corresponde con el de la prioridad reivindicada (Art. 9 D 486) y fue presentada dentro del plazo establecido por la D. 486.

De acuerdo con lo establecido en el numeral 1.2.2.2 del Título X de la Circular Única para efectos del examen de patentabilidad, se tendrá en cuenta la tasa pagada por el solicitante al momento de comenzar el examen para las reivindicaciones adicionales; esto es que cuando la solicitud contenga más de diez (10) reivindicaciones y no se haya pagado la tasa complementaria, sólo se estudiarán las cubiertas por la tasa pagada. En el presente caso se estudiarán las reivindicaciones 1 a 14 del capítulo reivindicatorio.

1er Art 45.

canabinoides en el tratamiento de epilepsia”.

El problema planteado en esta solicitud consiste en diseñar un tratamiento alternativo para la epilepsia.

Para resolver este problema técnico la solicitud en estudio proporcionar fito-canabinoides o combinaciones de fito-canabinoides que tienen uso en el tratamiento de formas específicas de convulsiones asociadas con epilepsia.

El objeto de la presente solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): A61K31/352 // A61P25/08.

4. Excepción a la Patentabilidad (Art 20 D 486 literal d))

El objeto de las reivindicación 14 no es patentable de acuerdo con el Art citado.

5. Reivindicaciones relativas a un uso o a un segundo uso (Art 14 y 21 D 486)

La referencia al uso presente en las reivindicaciones 1 a 13 no es patentable, por lo tanto se sugiere eliminarlo. La materia reclamada si bien se encabeza en los términos del compuesto Cannabidiol (CBD) se define en términos del uso terapéutico asociado a la actividad farmacológica del compuesto no psicomimético, para el que se ha reportado actividad anticonvulsivante destinada al tratamiento de enfermedades como epilepsia, Alzheimer y de enfermedades cardiovasculares.

6. De la solicitud de patente

Titulo

El título no corresponde con el objeto de la solicitud porque la referencia al uso terapéutico. Se sugiere a la solicitante asignar un nuevo título a la solicitud.

7. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación internacional: julio 03 de 2009, y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud.

Ítem	Documento	Título	Fecha de Publicación
D1	Noriyuki Usami. et al.	“Synthesis and Pharmacological Evaluation in Mice of Halogenated Cannabidiol Derivatives”	Noviembre/1999
D2	Iuvone Teresa. et al.	“Neuroprotective effect of cannabidiol, a non-psychoactive component from Cannabis sativa, on β -amyloid-induced toxicity in PC12 cells”	Abril/2004
D3	Resstel, Leonardo B.M. et al.	“5-HT _{1A} receptors are involved in the cannabidiol-induced attenuation of behavioural and cardiovascular responses to acute restraint stress in rats”	Enero/2009
D4	Scuderi Caterina, et al.	“Cannabidiol in Medicine: A review of its	09/Octubre/2008

Ítem	Documento	Título	Fecha de Publicación
		therapeutic potential in CNS disorders"	En línea Mayo/2009 En físico

El documento D1¹ divulga que los cannabinoides son los principales agentes bioactivos de la marihuana, específicamente los compuestos cannabinal (CBN) y cannabidiol (CBD, 1) (Pág. 1641). El documento D1 también señala que el cannabidiol es conocido por su potente capacidad barbitúrica, inductor de sueño y efecto anticonvulsivante. Además indica que algunos estudios sugieren su capacidad como antiepiléptica.

El documento D2² divulga que el Cannabidiol ejerce un efecto combinado neuroprotector, antioxidante, antiapoptótico frente al efecto citotóxico del péptido β -amiloide en células PC12 (células de médula adrenal diferenciadas a neuronas por factores de crecimiento) y que inhibe la caspasa 3 y su precursor pro-caspasa 3 (Resumen).

El documento D3³ divulga que el Cannabidiol (CBD) es un compuesto no-psicomimético derivado de la *Cannabis sativa* que induce efecto ansiolítico y antipsicótico en roedores por efecto de la activación del receptor 5-HT_{1A} (Resumen).

El documento D4⁴ divulga que el Cannabidiol (CBD) es el principal componente no psicotrópico de las partes áreas de la planta *Cannabis sativa* y señala que dentro de los efectos farmacológicos atribuidos se encuentran el efecto anticonvulsivante, sedante, hipnótico, antipsicótico, antiinflamatorio y neuroprotector (Resumen). El documento señala además el perfil farmacológico y una revisión de estudios preclínicos y clínicos de CBD para el tratamiento de una variedad de desórdenes del sistema nervioso central (SNC). En particular el documento incorpora por referencia los hallazgos de *Consroe y colaboradores* en 1986 en un estudio en pacientes con enfermedad de Parkinson en el año 1986 a quienes se les administraron dosis entre 100-600mg/día por un periodo de seis semanas (Pág. 600, Col. 2).

Examen de Patentabilidad (Art 14 D486)

1 Noriyuki Usami. et al. "Synthesis and Pharmacological Evaluation in Mice of Halogenated Cannabidiol Derivatives". Chem. Pharm. Bull. 47(11) 1641-1645. November 1999. Pages 1641 – 1645. Disponible en: http://cpb.pharm.or.jp/cpb/199911/C11_1641.pdf

2 Iuvone Teresa. et al. "Neuroprotective effect of cannabidiol, a non-psychoactive component from Cannabis sativa, on β -amyloid-induced toxicity in PC12 cells". Journal of Neurochemistry, 2004, 89. Pages 134-141. Disponible en: <http://onlinelibrary.wiley.com/doi/10.1111/j.1471-4159.2003.02327.x/abstract>

3 Resstel, Leonardo B.M. et al. "5-HT_{1A} receptors are involved in the cannabidiol-induced attenuation of behavioural and cardiovascular responses to acute restraint stress in rats". British Journal of Pharmacology (2009), 156, 181-188. Disponible en: <http://onlinelibrary.wiley.com/doi/10.1111/j.1476-5381.2008.00046.x/pdf>

4 Scuderi Caterina, et al. "Cannabidiol in Medicine: A review of its therapeutic potential in CNS disorders". Phytotherapy research. 23, 597-602 (2009). Published online 9 October 2008 in Wiley InterScience. Disponible en: <http://onlinelibrary.wiley.com/doi/10.1002/ptr.2625/abstract>

Novedad (Art 16 D 486)

La reivindicación 1 consiste en Cannabidiol (CBD) para uso en el tratamiento de convulsiones parciales en donde el CBD está presente en una cantidad que proporciona una dosis diaria de por lo menos 400mg.

Características esenciales	D1	D2	D3	D4
Cannabidiol (CBD)	Cannabidiol (1)	Cannabidiol	Cannabidiol	Cannabidiol
i) Dosis diaria de por lo menos 400mg	Do 10mg/kg en ratas	Concentración 10 ⁻⁵ M en células PC-12	Do administrada 1, 10 o 20mg/kg de peso del roedor (Pág. 182, Métodos)	Do de 100-600mg/día

En conclusión, las características esenciales de la reivindicación 1 se encuentran anticipadas por cualquiera de los documentos D1, D2, D3 o D4 que divulgan el compuesto Cannabidiol (CBD). Por lo anterior se considera que la citada reivindicación no cumple con el requisito de novedad (Art. 16).

Por su parte, las reivindicaciones adicionales 2 a 13 no presentan elementos esenciales adicionales por lo cual se considera que tampoco cumplen con el citado requisito.

La reivindicación 12 consiste en una composición que comprende Cannabidiol (CBD) para tratar convulsiones parciales.

Características esenciales	D1	D2	D3	D4
Composiciones	Composiciones	Composiciones	Composiciones	Composiciones
Cannabidiol (CBD)	Cannabidiol	Cannabidiol	Cannabidiol	Cannabidiol

Teniendo en cuenta que las composiciones se caracterizan exclusivamente por el hecho de incluir el principio activo cannabidiol y que en el estado de la técnica se divulgan sistemas para administrarlo a diferentes modelos biológicos se considera que dicha reivindicación no es nueva.

En conclusión, las características esenciales de la reivindicación 12 se encuentran anticipadas por cualquiera de los documentos D1, D2, D3 o D4 que divulgan sistemas para administrar el compuesto Cannabidiol (CBD). Por lo anterior se considera que la citada reivindicación no cumple con el requisito de novedad (Art. 16).

8. Conclusión

Los requisitos de patentabilidad de la presente solicitud están afectados así:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	Noriyuki Usami. et al.	1 a 13	Novedad
D2	Iuvone Teresa. et al.	1 a 13	Novedad

D3	Resstel, Leonardo B.M. et al.	1 a 13	Novedad
D4	Scuderi Caterina, et al.	1 a 13	Novedad

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir del día siguiente de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante de sesenta (60) días para dar respuesta a este requerimiento y presentarla por escrito.

Finalmente, se le recuerda al solicitante que si como respuesta a este requerimiento llegara a modificar el capítulo reivindicatorio y éste contenga más de diez (10) reivindicaciones, no pagará la tasa de modificación voluntaria pero sí la correspondiente al pago por reivindicación adicional.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.



JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (c)

El anterior requerimiento fue notificado por fijación en lista, de fecha 2014-01-21

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RESEARCH PAPER

5-HT_{1A} receptors are involved in the cannabidiol-induced attenuation of behavioural and cardiovascular responses to acute restraint stress in rats

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Background and purpose: Cannabidiol (CBD) is a non-psychotomimetic compound from *Cannabis sativa* which induces anxiolytic- and antipsychotic-like effects in rodents. These effects could be mediated by facilitation of the endocannabinoid system or by the activation of 5-HT_{1A} receptors. As either of these mechanisms could promote adaptation to inescapable stress, the aim of the present work was to test the hypothesis that CBD would attenuate the autonomic and behavioural consequences of restraint stress (RS). We also investigated if the responses to CBD depended on activation of 5-HT_{1A} receptors.

Experimental approach: Male Wistar rats received i.p. injections of vehicle or CBD (1, 10 or 20 mg kg⁻¹) and 30 min later were submitted to 60 min of restraint where their cardiovascular responses were recorded. The protocol of the second experiment was similar to the first one except that animals received i.p. injections of the 5-HT_{1A} receptor antagonist WAY100635 (0.1 mg kg⁻¹) before CBD treatment and exposure to restraint. 24 h later they were also tested in the elevated plus-maze (EPM), an animal model of anxiety.

Key results: Exposure to RS increased blood pressure and heart rate and induced an anxiogenic response in the EPM 24 h later. These effects were attenuated by CBD. WAY100635 by itself did not change the cardiovascular and anxiogenic response to RS, but blocked the effects of CBD.

Conclusion and implications: The results suggest that CBD can attenuate acute autonomic responses to stress and its delayed emotional consequences by facilitating 5-HT_{1A} receptor-mediated neurotransmission.

British Journal of Pharmacology (2009) **156**, 181–188; doi:10.1111/j.1476-5381.2008.00046.x

Keywords: cannabinoids; cardiovascular system; elevated plus-maze; 5-HT_{1A} receptor

Abbreviations: CBD, cannabidiol; EPM, elevated plus-maze; HR, heart rate; MAP, mean arterial pressure; THC, Δ^9 -tetrahydrocannabinol

Introduction

Marijuana (from *Cannabis sativa*) is one of the most widely abused drugs in the world. In humans, it elicits subjective changes that include euphoria, heightened sensitivity to external stimuli and relaxation (Martin *et al.*, 1991; Compton *et al.*, 1992; Johns, 2001). The major constituent of cannabis

is Δ^9 -tetrahydrocannabinol (THC) and this is thought to be the main ingredient responsible for its psychoactive properties (Mechoulam, 1970; Mechoulam *et al.*, 1970; Ilan *et al.*, 2005). The discovery of specific binding sites for THC led to the discovery of the cannabinoid receptors (Devane *et al.*, 1988; Matsuda *et al.*, 1990; Munro *et al.*, 1993) and, so far, two sub-types of the cannabinoid receptor have been identified, CB₁ and CB₂ (Pertwee, 2005; nomenclature follows Alexander *et al.*, 2008). The activation of CB₁ receptors by THC is thought to account for most of the central effects of cannabis (Huestis *et al.*, 2001). Anandamide and 2-arachidonoyl glycerol, referred to as endocannabinoids, are the major endogenous agonists of the CB₁ receptor (Di Marzo *et al.*, 1998; Piomelli, 2003).

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Cannabidiol (CBD), another cannabinoid generally found in relatively high concentrations in cannabis, exhibits a somewhat different pharmacology compared with THC (Mechoulam *et al.*, 2002). CBD attenuates the psychotomimetic and anxiogenic effects of THC in humans (Karniol *et al.*, 1974; Zuardi *et al.*, 1982). Moreover, systemic administration of CBD induced antipsychotic (Zuardi *et al.*, 1991; Zuardi *et al.*, 2006) and anxiolytic-like effects (Guimaraes *et al.*, 1990; Resstel *et al.*, 2006).

The mechanism of action of CBD is not fully understood. It has a low affinity for cannabinoid receptors (Petitet *et al.*, 1998; Thomas *et al.*, 1998) but can block the reuptake of anandamide (Bisogno *et al.*, 2001) and its metabolism by the enzyme, fatty acid amide hydrolase (FAAH), (Watanabe *et al.*, 1998; Di Marzo *et al.*, 1999; Mechoulam and Hanus, 2002; Mechoulam *et al.*, 2002). Moreover, CBD may possess agonistic properties at 5-HT_{1A} receptors (Russo *et al.*, 2005). Although there are contradictory results, several studies indicate that activation of these receptors can induce anxiolytic-like effects and mediate adaptation to stress (Blier and de Montigny, 1994; Blier and Ward, 2003; Joca *et al.*, 2003; Joca *et al.*, 2007).

Acute restraint is an uncontrollable stress situation that produces endocrine and autonomic responses characterized by increases in glucocorticoids levels, blood pressure and heart rate (HR) (Tavares and Correa, 2006; Hsu *et al.*, 2007). These responses are accompanied by activation of several brain structures (Pacak and Palkovits, 2001). In addition to physiological responses, animals submitted to restraint also develop behavioural changes reflected, for example, in reduced exploratory activity in an open field 24 h after stress (Kennett *et al.*, 1985a; 1987; Mechiel Korte and De Boer, 2003), increased immobility in a forced swimming test (Sevigi *et al.*, 2006) and reduced exploration of the open arms of an elevated plus-maze (EPM) (Guimaraes *et al.*, 1993; Padovan and Guimaraes, 2000). These stress-induced behavioural changes can be attenuated by systemic or intracerebral administration of anxiolytic and antidepressant drugs (Kennett *et al.*, 1985a; 1987; Guimaraes *et al.*, 1993; Padovan and Guimaraes, 2000; Mechiel Korte and De Boer, 2003). A possible effect of CBD on these changes, however, has not yet been investigated. Therefore, in the present work we tested the hypothesis that systemic administration of CBD would attenuate the acute physiological changes and the behavioural consequences of restraint stress. We also evaluated the involvement of 5-HT_{1A} receptors in the effects of CBD in this model.

Methods

Animals

The Institution's Animal Ethics Committee approved housing conditions and experimental procedures for animals. Male Wistar rats weighing 230–250 g were used. The animals were provided by our local Animal farm facility. After arriving at the Animal Care Unit of the Department of Pharmacology, School of Medicine of Ribeirão Preto, University of Sao Paulo, the animals were kept in groups of four per cage for 48 h. After this adaptation period they are housed individually in

plastic cages with free access to food and water under a 12 h light/dark cycle (lights on at 06:30 h) for a further 24 h period before being subjected to the surgical procedure (see below).

Surgical preparation

Twenty-four hours before being submitted to restraint stress, animals had a polyethylene catheter implanted into the femoral artery under anaesthesia (tribromoethanol, 250 mg kg⁻¹ i.p.), for the recording of arterial blood pressure and HR. The catheter was exposed on the dorsum of the animals and attached to the skin, allowing arterial pressure recordings from conscious animals.

Acute restraint

Experiment 1 In the morning period (between 7 a.m. and 12 a.m.), the animals were transferred to the experimental room in their home box. Mean arterial pressure (MAP) and HR were recorded using an HP-7754A amplifier (Hewlett Packard, Palo Alto, CA, USA) connected to a signal acquisition board (Biopac M-100, Goleta, CA, USA) and computer processed. After a few minutes of baseline recording, rats received a single i.p. injection of one of the following drugs: vehicle or CBD (1, 10 or 20 mg kg⁻¹). Thirty minutes later they were submitted to a 60 min restraint period in a small plastic cylindrical restraining tube (diameter = 6.5 cm and length = 15 cm). After the restraint period the animals returned to their cages. Each animal was submitted to only one session of restraint to prevent the development of stress tolerance (Guimaraes *et al.*, 1993).

Experiment 2 Based on the results obtained in the first experiment, we chose the dose of 10 mg kg⁻¹ of CBD to use in this second study. The protocol was similar to the first experiment except that before the restraint period the animals received, first, an i.p. injection of vehicle or WAY (0.1 mg kg⁻¹) followed, 30 min later, by a second injection of vehicle or CBD (10 mg kg⁻¹). As in experiment 1, 30 min after the last injection the animals were restrained for 60 min. One day later they were tested in the EPM. We also had a general control group of unrestrained animals treated with the saline+CBD vehicle that were tested 24 h later in the EPM.

The EPM test

The EPM test was conducted as described before (Padovan and Guimaraes, 2000). Briefly, the apparatus consisted of two opposite open arms (50 × 10 cm) crossed at a right angle by two arms of the same dimensions enclosed by 40 cm high walls with no roof. The maze was located 50 cm above the floor. Rodents naturally avoid the open arms of the EPM and anxiolytic compounds typically increase the exploration of these arms without changing the number of enclosed-arm entries (Pellow *et al.*, 1985; Carobrez and Bertoglio, 2005). The EPM was cleaned and dried before each session and the Ethovision software (Version 1.9, Noldus, Netherlands) was employed for behavioural analysis.

Data analysis

Mean arterial pressure and HR values were continuously recorded for 10 min before the 60 min restraint stress period.

Table 1 Basal values of the MAP and HR in vehicle (control), CBD and WAY100635 treated rats

Group		MAP (mmHg)	HR (bpm)
Control	<i>n</i> = 6	105 ± 2	338 ± 13
CBD 1 mg	<i>n</i> = 5	103 ± 3	363 ± 14
CBD 10 mg	<i>n</i> = 5	100 ± 3	349 ± 11
CBD 20 mg	<i>n</i> = 5	97 ± 3	369 ± 9
WAY100635	<i>n</i> = 5	99 ± 2	371 ± 14
		$F_{4,25} = 1.5, P > 0.05$	$F_{4,25} = 1.3, P > 0.05$

The values in the table represent the means ± SE. One-way ANOVA. CBD, cannabidiol; HR, heart rate; MAP, mean arterial pressure.

Data were expressed as means ± SEM of MAP or HR changes (respectively Δ MAP and Δ HR) sampled at 5 min intervals. Points sampled during the 10 min before restraint were used as control baseline value. MAP and HR changes were analysed using two-way ANOVA with treatment as independent factor and time as repeated measurement factor. When interaction between the factors was observed, groups were compared using one-way ANOVA followed by Bonferroni's *post hoc* test.

The per cent of entries ($100 \times \text{open}/\text{total entries}$) and time spent in the open arms ($100 \times \text{open}/\text{open} + \text{enclosed}$) of the EPM were calculated for each rat. These data, together with the number of enclosed arm entries, were analysed by one-way ANOVA followed by Bonferroni's *post hoc* test. Values of $P < 0.05$ were taken as showing statistically significant differences between means.

Drugs

The following drugs were used: CBD (THC Pharma, Frankfurt, Germany): 1, 10 or 20 mg kg⁻¹, suspended in polyoxyethyl- enesorbitan monooleate (Tween 80) 2%-saline (Resstel et al., 2006), WAY100635 (WAY, Sigma, St. Louis, MO, USA): 0.1 mg kg⁻¹ dissolved in saline (Kaster et al., 2005) and tribromoethanol (Aldrich, St. Louis, MO, USA). The solutions were prepared immediately before use and injected intraperitoneally in a volume of 1 mL kg⁻¹. The appropriate vehicles were used in each experiment.

Results

Experiment 1

Effects of CBD on cardiovascular responses to acute restraint Systemic injection of CBD (1, 10 and 20 mg kg⁻¹) did not affect baseline blood pressure ($F_{4,25} = 1.2, P > 0.05$) or HR ($F_{4,25} = 0.9, P > 0.05$) values when compared with vehicle control ($n = 6$, Table 1). As represented in Figure 1, acute restraint induced a marked and sustained increase of HR and MAP during the 60 min test. There were significant effects of treatment (MAP: $F_{3,240} = 93.5, P < 0.001$; HR: $F_{3,240} = 123, P < 0.001$), time (MAP: $F_{14,240} = 83.6, P < 0.001$; HR: $F_{14,240} = 27.9, P < 0.001$) and treatment versus time interaction (MAP: $F_{42,240} = 5.1, P < 0.01$; HR: $F_{42,240} = 7.9, P < 0.01$).

Cannabidiol decreased the stress-induced cardiovascular responses at doses of 10 and 20 mg kg⁻¹ (HR, $F_{3,16} = 19.9,$

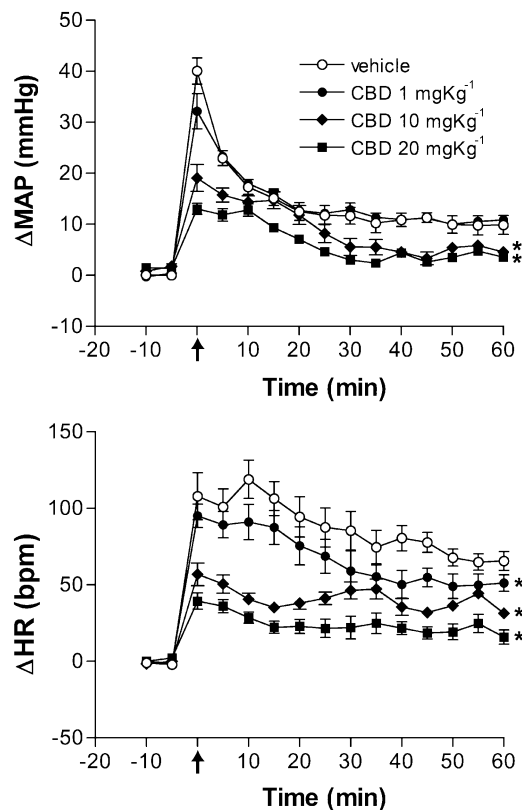


Figure 1 Effects of different doses of cannabidiol (CBD, 1, 10 or 20 mg kg⁻¹, *n* = 5 per group) or vehicle (Tween 80 2%-saline, 1 mL kg⁻¹, *n* = 6) on changes in mean arterial pressure (Δ MAP) and heart rate (Δ HR) of animals submitted to 60 min of restraint stress. The arrow indicates the beginning of the restraint period. Data shown represent the means ± SEM. * $P < 0.05$, compared with vehicle group; ANOVA followed by Bonferroni's *post hoc* test.

$P < 0.001$, MAP, $F_{3,16} = 14.6, P < 0.001$). The dose of 1 mg kg⁻¹ was also able to attenuate HR responses ($P < 0.05$). A dose-dependency was demonstrated by nonlinear regression analysis and showed a significant correlation between doses and attenuation of the increased MAP ($r^2 = 0.82, df = 13, P < 0.01$) and HR ($r^2 = 0.73, df = 13, P < 0.01$) (Fig. 2).

Experiment 2a

Effects of WAY100635 on CBD effects on acute cardiovascular responses to restraint WAY (*n* = 5) did not affect baseline values of blood pressure ($F_{4,25} = 1.2, P > 0.05$) or HR ($F_{4,25} = 0.9, P > 0.05$) compared with CBD (10 mg kg⁻¹, *n* = 6) and vehicle (*n* = 6, Table 1).

There were significant effects of restraint (MAP: $F_{3,270} = 35.95, P < 0.001$; HR: $F_{3,270} = 104.3, P < 0.001$), time (MAP: $F_{14,270} = 59.66, P < 0.001$; HR: $F_{14,270} = 66.4, P < 0.001$) and treatment versus time interaction (MAP: $F_{42,270} = 2.1, P < 0.01$; HR: $F_{42,270} = 1.9, P < 0.01$) (Fig. 3).

The decrease of the cardiovascular responses by systemic administration of CBD (10 mg kg⁻¹) was prevented by WAY (MAP: $F_{1,135} = 139.5, P < 0.001$; HR: $F_{1,135} = 290.3, P < 0.001$) (Fig. 3). The latter drug, by itself, had no effect on cardiovascular responses to restraint (MAP: $F_{1,135} = 1.3, P > 0.05$; HR: $F_{1,135} = 1.1, P > 0.05$) (Fig. 3).

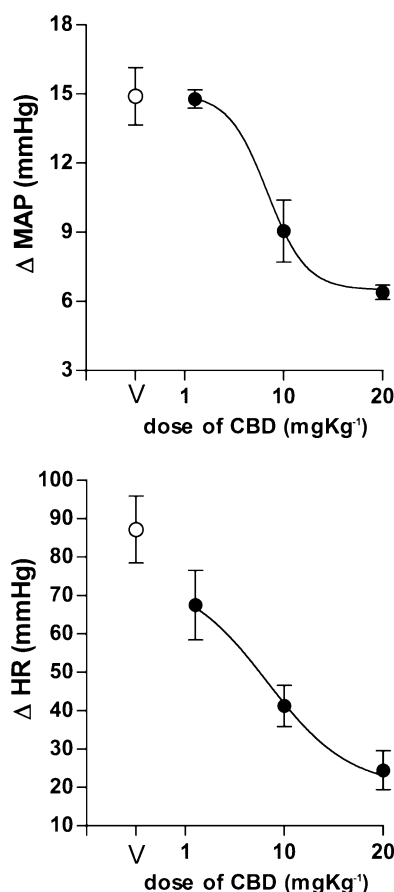


Figure 2 Mean arterial pressure (Δ MAP) and heart rate (Δ HR) changes in response to the injection of increasing doses of cannabidiol (CBD, 1, 10, 20 mg kg⁻¹, $n = 5$ /group) in rats. V: vehicle (Tween 80 2%-saline, 1 mL kg⁻¹, $n = 6$). Dose-effect curves were generated by nonlinear regression analysis. Data shown represent the means $\pm$ SEM of the variation of MAP and HR during the 60 min of acute restraint.

Typical experimental recordings showing the effects of CBD and WAY on cardiovascular responses observed during acute restraint can be seen in Figure 4.

Experiment 2b

Effects of CBD and WAY100635 on delayed behavioural consequences in the EPM induced by restraint Acute restraint induced a significant decrease in the percentage of open arm entries ($F_{3,25} = 7.72$, $P < 0.001$) compared with unrestrained controls ($n = 6$), when tested 24 h after stress (Fig. 5). CBD administration in restrained rats ($n = 6$) increased the percentage of open arm entries ($P < 0.001$, Fig. 5) compared with controls (vehicle-treated restrained animals, $n = 6$). This effect was prevented by pre-administration of WAY ($P < 0.001$, $n = 6$). No effect was found in animals treated only with WAY ($P > 0.05$, $n = 6$) (Fig. 5). There was also no effect on the percentage time spent in the open arms and in the number of enclosed arm entries ($P > 0.05$).

Discussion

The present results showed that (i) CBD reduced the pressor and tachycardic responses to restraint stress, in a dose-

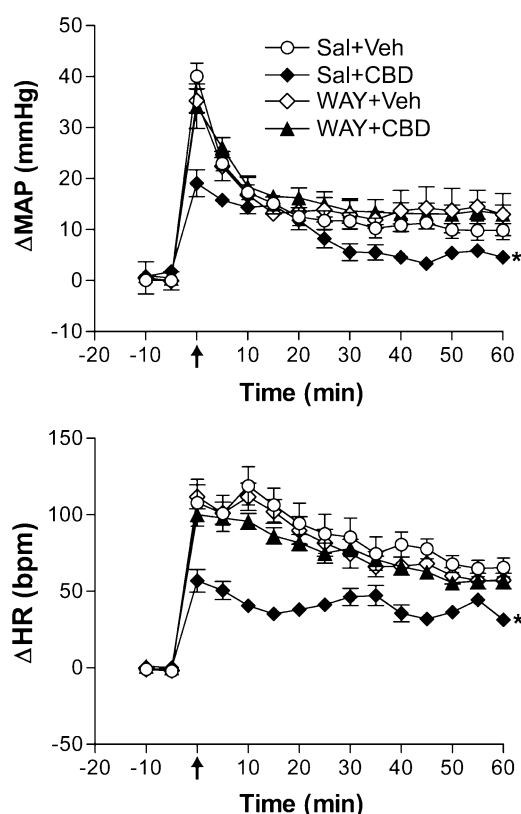


Figure 3 Effects of pre-treatment with of saline (Sal) or WAY100635 (WAY, 0.1 mg kg⁻¹) followed by second injection of vehicle (Veh, Tween 80 2%-saline) or cannabidiol (CBD, 10 mg kg⁻¹) immediately before a 60 min restraint period on increase in the mean arterial pressure (Δ MAP) and heart rate (Δ HR) induced by restraint stress. The arrow indicates the beginning of the restraint period. Data shown represent the mean $\pm$ SEM of five to six animals. * $P < 0.05$, compared with vehicle group; ANOVA followed by Bonferroni's *post hoc* test.

dependent manner; (ii) CBD attenuated the increased anxiety behaviour caused by a previous exposure to restraint; and (iii) effects of CBD on cardiovascular and behavioural responses to restraint can be blocked by WAY100635, a 5-HT_{1A} receptor antagonist.

Acute exposure to restraint stress has been shown to evoke several physiological and behavioural changes (Tavares and Correa, 2006; Hsu *et al.*, 2007) and, as expected, in the present study animals exhibited significant increases in MAP and HR during restraint. Moreover, they showed decreased exploration of the open arms of the EPM 24 h after restraint stress. These delayed behavioural consequences of acute restraint stress have been described in several models, including the open field and EPM (Kennett *et al.*, 1985a; Guimaraes *et al.*, 1993; Padovan and Guimaraes, 2000; Mechiel Korte and De Boer, 2003), and are sensitive to systemic and intra-cerebral injection of anxiolytic and antidepressant drugs (Kennett *et al.*, 1985a; Kennett *et al.*, 1987; Guimaraes *et al.*, 1993; Padovan and Guimaraes, 2000; Mechiel Korte and De Boer, 2003).

Cannabidiol did not induce any significant change in basal MAP and HR which agrees with the reported lack of significant cardiovascular effects of this drug (McQueen *et al.*, 2004;

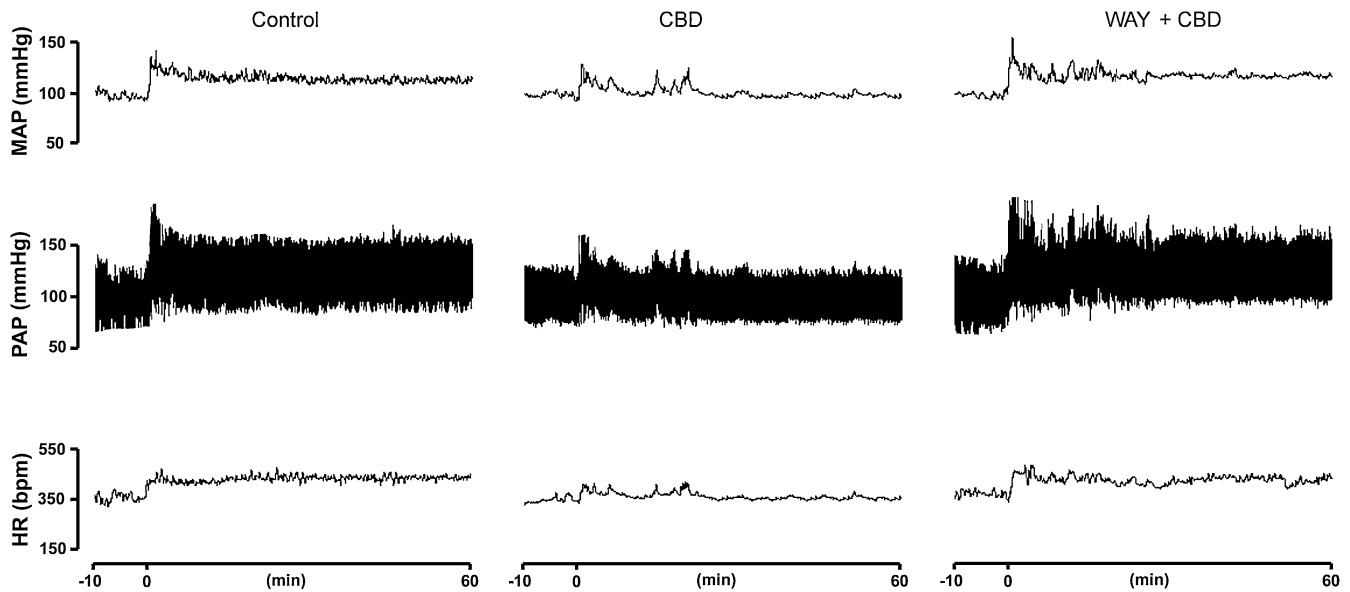


Figure 4 Mean arterial pressure (MAP), pulsatile arterial pressure (PAP) and heart rate (HR) individual recordings showing the cardiovascular changes evoked by acute restraint in animals treated with vehicle (control, Tween 80 2%-saline), cannabidiol (CBD) or cannabidiol after WAY100635 (WAY+CBD). The restraint period started at time 0.

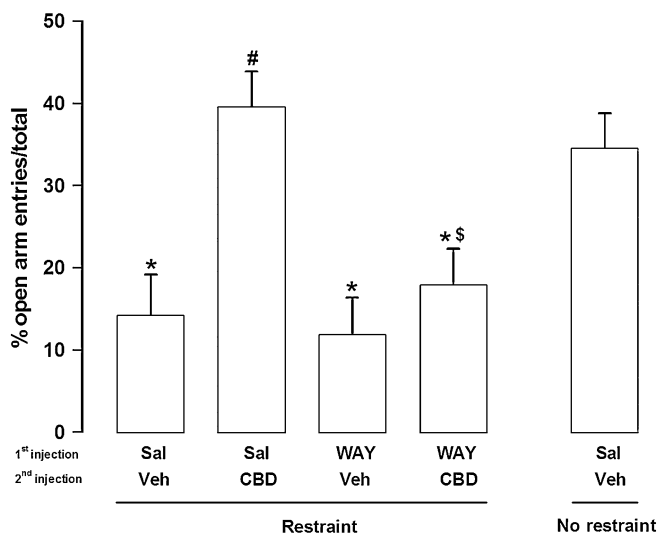


Figure 5 Effects in the elevated plus-maze (EPM) of a first systemic injection of saline (Sal) or WAY100635 (WAY, 0.1 mg kg⁻¹) followed by a second injection of vehicle (Veh, Tween 80 2%-saline) or cannabidiol (CBD, 10 mg kg⁻¹) immediately before a 60 min restraint period ($n=6$ per group). A non-stressed group (no-restraint) that received i.p. injections of saline followed by vehicle was used as general control. The EPM test was performed 24 h after the restraint period. Data represent the mean ($\pm$ SEM) per cent of open arm entries. * $P < 0.05$, compared with control group; # $P < 0.05$, compared with restraint-vehicle group; \$ $P < 0.05$, compared with restraint-CBD group; ANOVA followed by Bonferroni's *post hoc* test.

Resstel *et al.*, 2006). At low doses, it also does not interfere with memory and learning processes (Lichtman *et al.*, 1995; Fadda *et al.*, 2004; 2006). It is unlikely, therefore, that the attenuation of the cardiovascular and delayed behavioural responses to restraint depend on direct cardiovascular effects or memory impairment induced by the drug, but rather on an attenuation of the emotional response to stress. In agreement

with this proposal, acute administration of CBD has been shown to induce anxiolytic-like effects in several animal models, including the EPM, Vogel conflict test and contextual fear conditioning (Guimaraes *et al.*, 1990; Onaivi *et al.*, 1990; Moreira *et al.*, 2006; Resstel *et al.*, 2006). The effective doses of CBD in these previous studies were, in general, similar to ours. In the study by Guimaraes *et al.* (1990), however, CBD produced an inverted U-shaped dose-response curve, with the highest dose (20 mg kg⁻¹) being ineffective. The reasons for this difference are unknown, but may depend on the model used (Calabrese, 2008). As the EPM test is based on exploratory activity, it could be more prone to interference of non-specific drug effects that affects this parameter (Calabrese, 2008). In addition, at least for classical anxiolytics such as diazepam, the EPM is more sensitive than other models such as the Vogel conflict test (see Calabrese, 2008).

The mechanisms of the anxiolytic and anti-stress effects of CBD are not clear. It has a low affinity for cannabinoid receptors (Petitet *et al.*, 1998; Thomas *et al.*, 1998) although, under certain circumstances, it may act as an antagonist of CB₁- and CB₂-receptor agonists (Pertwee *et al.*, 2002). CBD could also block the reuptake and metabolism of anandamide, facilitating endocannabinoid-mediated neurotransmission (Watanabe *et al.*, 1998; Di Marzo *et al.*, 1999; Bisogno *et al.*, 2001; Mechoulam *et al.*, 2002). However, several effects of CBD have been shown to be independent of the endocannabinoid system (Hayakawa *et al.*, 2007). More recently, Russo *et al.* (2005) reported that CBD can displace the 5-HT agonist [³H]8-OH-DPAT from cloned human 5-HT_{1A} receptors expressed in Chinese hamster ovary cultured cells. Moreover, using signal transduction studies, this work also showed that CBD can act as an agonist at these receptors. The observation that CBD has agonistic properties at 5-HT_{1A} receptors has been supported by *in vivo* studies where the neuroprotective and anti-oxidative effects induced by CBD were blocked by pre-treatment with the 5-HT_{1A} antagonist WAY100135 (Mishima

et al., 2005; Hayakawa *et al.*, 2007). Our results corroborate these findings, by showing that the stress-attenuating effects induced by CBD were prevented by systemic pretreatment with WAY100635, a selective antagonist at these receptors. In agreement with our results, a recent study from our laboratory showed that CBD interacts with 5-HT_{1A} receptors in the dorsolateral periaqueductal gray to produce anxiolytic-like effects in the EPM (Campos and Guimaraes, 2008).

5-HT_{1A} receptors are widely distributed in the brain, especially in structures traditionally related to stress and anxiety, such as the raphe nuclei, hippocampus, prefrontal cortex, amygdala and hypothalamus (Chalmers and Watson, 1991). Although the role of 5-HT in anxiety is still a matter of intense debate (Millan, 2003), several pieces of evidence suggest that activation of 5-HT_{1A} receptors facilitates adaptation to stress (Kostowski *et al.*, 1992; Dekeyne *et al.*, 2000; Tsuji *et al.*, 2000; Blier and Ward, 2003; Joca *et al.*, 2003; Kagamiishi *et al.*, 2003; Rioja *et al.*, 2004; Joca *et al.*, 2007). For example, pre-administration of a 5-HT_{1A} agonist before an acute immobilization period blocked the stress-induced anxiogenic effect observed in an elevated T-maze test performed 24 h later (Rioja *et al.*, 2004). Similar results were reported by Tsuji *et al.* (2000), who observed that administration of 5-HT_{1A} receptor agonists before restraint stress attenuated, in a dose-dependent manner, the development of stress-induced anxiogenic effect observed 24 h later in the hole-board test of anxiety. Data from 5-HT_{1A} receptor knockout mice give further support to this hypothesis, as these animals display anxiogenic-like behaviour in different paradigms (Zhuang *et al.*, 1999; Tsetsenis *et al.*, 2007). It is proposed that the lack of this receptor would promote a bias in the processing of threatening cues which could render the animal more susceptible to the development of behavioural consequences of stress (Tsetsenis *et al.*, 2007). On the other hand, facilitation of this neurotransmission would mediate adaptation to stress (Graeff *et al.*, 1996). This suggestion is supported by results showing that behavioural adaptation to stress is accompanied by sensitization of 5-HT_{1A}-mediated neurotransmission (Kennett *et al.*, 1985b; 1987; Samad and Haleem, 2007) and that an increased expression of this receptor in the brain is associated with reduced anxiety-like behaviour (Kusserow *et al.*, 2004). Therefore, these studies support the possibility that activation of 5-HT_{1A} receptors protects animals against various emotional changes caused by stressful stimuli, perhaps by facilitating mechanisms involved in the ability to cope with the stressful situation.

The exact mechanism through which 5-HT_{1A} agonists induce their anxiolytic activity remains unclear. 5-HT_{1A} receptors are located presynaptically (somatodendritic autoreceptors) in 5-hydroxytryptaminergic cell bodies in the raphe nuclei of the brain stem and postsynaptically, predominantly in limbic structures such as the hippocampus and the hypothalamus (Verge *et al.*, 1985; 1986; Chalmers and Watson, 1991). It is still controversial whether the anxiolytic-like effects induced by acute systemic administration of 5-HT_{1A} agonists are due to the activation of the pre- or the post-synaptic receptors (File *et al.*, 1996; Lopez-Rubalcava, 1996). Moreover, depending on the structure where post-synaptic 5-HT_{1A} receptors are located, their activation may lead to anxiolytic or anxiogenic-like effects (Graeff *et al.*, 1996; Zan-

grossi *et al.*, 2001). Therefore, the exact mechanism of action of 5-HT_{1A} agonists as anxiolytic compounds is complex and still warrants further investigation.

In conclusion, the present findings indicate that CBD, by activating 5-HT_{1A} receptors, can attenuate physiological and behavioural responses to restraint stress. This finding raises the possibility that CBD could be useful for treating psychiatric disorders thought to involve impairment of stress-coping mechanisms, such as depression and post-traumatic stress disorder.

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Conflicts of interest

The authors state no conflict of interest.

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Neuroprotective effect of cannabidiol, a non-psychoactive component from *Cannabis sativa*, on β -amyloid-induced toxicity in PC12 cells

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Abstract

Alzheimer's disease is widely held to be associated with oxidative stress due, in part, to the membrane action of β -amyloid peptide aggregates. Here, we studied the effect of cannabidiol, a major non-psychoactive component of the marijuana plant (*Cannabis sativa*) on β -amyloid peptide-induced toxicity in cultured rat pheocromocytoma PC12 cells. Following exposure of cells to β -amyloid peptide (1 μ g/mL), a marked reduction in cell survival was observed. This effect was associated with increased reactive oxygen species (ROS) production and lipid peroxidation, as well as caspase 3 (a key enzyme in the apoptosis cell-signalling cascade) appearance, DNA fragmentation and increased intracellular calcium.

Treatment of the cells with cannabidiol (10^{-7} – 10^{-4} M) prior to β -amyloid peptide exposure significantly elevated cell survival while it decreased ROS production, lipid peroxidation, caspase 3 levels, DNA fragmentation and intracellular calcium. Our results indicate that cannabidiol exerts a combination of neuroprotective, anti-oxidative and anti-apoptotic effects against β -amyloid peptide toxicity, and that inhibition of caspase 3 appearance from its inactive precursor, pro-caspase 3, by cannabidiol is involved in the signalling pathway for this neuroprotection.

Keywords: Alzheimer's disease, apoptosis, β -amyloid, cannabidiol, cannabinoid, neuroprotection.

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Alzheimer's disease (AD) is the most common age-related neurodegenerative disorder (Koo *et al.* 1999). Regional neuronal degeneration, synaptic loss, presence of neurofibrillary tangles (NFTs) (Terry 1963) and senile plaques (Braak and Braak 1997) are specific hallmarks of this disease. While NFTs are the result of disposition of hyper-phosphorylated tau protein (Lee *et al.* 1991), senile plaques are complex extracellular lesions composed of a core of β -amyloid (A β) aggregates, surrounded by activated astrocytes, and dystrophic neuritis (Itagaki *et al.* 1989; Cotman *et al.* 1996).

Excessive accumulation of A β peptide has been proposed as a pivotal event in the pathogenesis of AD, although the precise mechanism by which A β induces neuronal death is still unknown (Hensley *et al.* 1994; Troy *et al.* 2001). Proposed mechanisms include production of oxygen free radicals (Behl *et al.* 1994), modification of cytosolic calcium homeostasis (Mattson 1992; Ueda *et al.* 1997), the Wnt pathway and activation of nuclear factor-kB (Green and Peers 2002; Caricasole *et al.* 2003). The cysteine proteases, known as caspases, are essential mediators of many of the pathways involved in executing the apoptotic programme

following A β accumulation (Gervais *et al.* 1999). Caspase-mediated apoptosis can be modulated by several agents, including antioxidants (Behl *et al.* 1994), calcium channel blockers (Weiss *et al.* 1994) and growth factors (Mattson *et al.* 1993).

Cannabinoids are a group of C₂₁ compounds occurring in the glandular hairs of *Cannabis sativa* (Indian hemp) and consequently in hashish and marijuana, the well known drug

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Abbreviations used: A β , β -amyloid; AD, Alzheimer's disease; [Ca²⁺]_i, changes in intracellular free calcium levels; CBD, cannabidiol; DCF, 2',7'-dichlorofluorescein; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulfoxide; H₂DCF-DA, dichlorofluorescein-diacetate; MDA, malonyl dialdehyde; MTT, 3(4,5-dimethylthiazol-2-yl)2,5-diphenyl-2H-tetrazolium bromide; NFTs, neurofibrillary tangles; O.D., optical density; PBS, phosphate-buffered saline; RFU, relative fluorescence unit; ROS, reactive oxygen species; Δ^9 -THC, Δ^9 -tetrahydrocannabinol.

of abuse (Samuelsson 1999; Nocerino *et al.* 2000). The most important representative cannabinoid is Δ^9 -tetrahydrocannabinol (Δ^9 -THC), which has psychoactive properties depending upon the interaction with the cannabinoid CB₁ receptors within the brain (Pertwee 1997). Good quality hashish contains 4–10% and marijuana 0.1–2.7% Δ^9 -THC (Samuelsson 1999). Cannabidiol, the main component of the glandular hairs (up to 15%), is a non-psychoactive cannabinoid; it exerts a plethora of pharmacological effects, including anti-convulsive, sedative, hypnotic, anti-psychotic, anti-nausea and anti-inflammatory actions (Mechoulam *et al.* 2002). Cannabidiol is a potent antioxidant compound and it has been recently proposed to have a neuroprotective role during ischemic damage (Hampson *et al.* 1998, 2000).

The aim of the present study is to evaluate the possible neuroprotective effect of cannabidiol on β -amyloid-induced neurotoxicity. For this purpose we evaluated the effect of cannabidiol on cell viability, reactive oxygen species (ROS) formation and membrane lipoperoxidation, as well as neuronal apoptosis in cultured rat pheochromocytoma PC12 cells exposed to A β .

Materials and methods

Materials

All the materials for cell culture were purchased from Biowittaker (Caravaggio, BG, Italy). Fetal calf serum and horse serum were from Hyclone (Logan, UT, USA). Human β -amyloid peptide (fragment 1–42) and CP55,940 $\{(-)-cis-3-[2-hydroxy-4-(, - dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexanol\}$ were from Tocris Cookson (Ballwin, UK). Antibody anti-caspase 3 was from Calbiochem (San Diego, CA, USA). Anti-mouse IgG was from Dako (Glostrup, Denmark). SR141716A [$(N$ -piperidin-1-yl)-5-(4-chlorophenyl)-1-2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride] was a gift from Dr Madaleine Mosse (SANOFI-Reserche). Cannabidiol, Vitamin E (DL- α -tocopherol) and all the other reagents used, unless otherwise stated, were from Sigma (Milan, Italy). A β fragment peptide was dissolved in pyrogen-free water, cannabidiol, vitamin E and SR141716A in dimethylsulfoxide (DMSO). The final DMSO concentration in the assay wells, irrespective of cannabinoid concentration, was always adjusted to 0.01%, a concentration that in preliminary experiments was found to have no effect on the response under study.

Cell culture

PC12 cells (American Tissue Culture Catalogue number CRL1721) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum, 15% horse serum, 2 mM glutamine, 100 U/mL penicillin and 100 μ g/mL streptomycin at 37°C in 5% CO₂/95% air. Confluent cells were detached, counted and seeded into Petri dishes (10 cm diameter) at a density of 1×10^6 cells/mL and allowed to adhere for 24 h at 37°C. Thereafter, the medium was replaced with fresh medium and cells were treated with A β (1 μ g/mL) in the presence or absence of cannabidiol (10^{-7} – 10^{-4} M, given immediately before A β). In some experiments, the effect of

cannabidiol (10^{-5} M) was evaluated in the presence of the CB₁ receptor antagonist SR141716A (10 min before cannabidiol). The concentration of A β was selected on the basis of our laboratory experience (i.e. a concentration which produced a submaximal effect on cell viability, lipid peroxidation and ROS formation) (data not shown).

MTT cell viability assay

Cell viability was determined using a 3(4,5-dimethylthiazol-2-yl)2,5-diphenyl-2H-tetrazolium bromide (MTT) conversion assay (Mosman 1983). Briefly, the cells were plated in 96-well culture plates at the density of 5×10^3 cells/well and allowed to adhere at 37°C for 2 h. Thereafter, the medium was replaced with fresh medium and the cells were incubated with A β peptide (1 μ g/mL) in the presence or absence of cannabidiol (10^{-4} – 10^{-7} M). After 24 h, 25 μ L MTT (5 mg/mL in DMEM) were added and the cells incubated for an additional 3 h at 37°C. After this time, the cells were lysed and the dark blue crystals solubilized with 125 μ L of a solution containing 50% (v/v) N,N, dimethylformamide, 20% (w/v) sodium dodecyl-sulfate, with an adjusted pH of 4.5. The optical density (O.D.) of each well was measured with a microplate spectrophotometer (Titertek Multiscan MCC/340; Labsystem) equipped with a 620 nm filter. The cell viability in response to the treatment with A β peptide in the presence or absence of cannabidiol was calculated as percentage of cell viability = (O.D. treated/O.D. control) $\times$ 100.

Measurement of reactive oxygen species (ROS)

The formation of ROS was evaluated by means of the probe 2',7'-dichlorofluorescein (DCF) according to the method described by Bass *et al.* (1983) and Le Bel *et al.* (1992). Briefly, PC12 cells were seeded at a density of 5×10^3 cells/well into 96-well plates and allowed to grow for 48 h. 2',7'-Dichlorofluorescein-diacetate (H₂DCF-DA, Sigma) was then added directly to the growth medium at a final concentration of 5 μ M and the cells incubated for 1 h at 37°C. H₂DCF-DA is a non-fluorescent permeant molecule which diffuses passively into cells; the acetates are then cleaved by intracellular esterases to form H₂DCF which is thereby trapped within the cell. In the presence of intracellular ROS, H₂DCF is rapidly oxidized to the highly fluorescent DCF. Therefore, cells were washed twice with phosphate-buffered saline (PBS), placed in fresh medium and treated with A β (1 μ g/mL) with or without cannabidiol (10^{-7} – 10^{-4} M) for 24 h. After treatment, cells were washed twice with PBS and the plates placed in a fluorescent microplate reader (LS 55 Luminescence Spectrometer; Perkin Elmer, Beaconsfield, Bucks, UK). Fluorescence was monitored using an excitation wavelength of 490 nm and an emission wavelength of 520 nm. Results were expressed as Relative Fluorescence Units (RFU). In some experiments, the effect of the cannabinoid receptor agonist CP55,940 (10^{-8} – 10^{-5} M) was also evaluated.

Lipid peroxidation assay

Malonyl dialdehyde (MDA), as the most abundant lipid peroxidation product from PC12 cells, was measured by the thiobarbituric acid colorimetric assay (Mihara and Uchiama 1978). Briefly, 24 h after treatment with A β (1 μ g/mL) (in the presence or absence of cannabidiol 10^{-4} – 10^{-7} M), the cells were washed three times with 1 $\times$ PBS, then scraped in 1 $\times$ PBS containing 0.5 mM EDTA and 1.13 mM butyl-hydroxytoluene. Cell lysis was performed by means of six cycles of freezing and thawing. To 450 μ L of cellular lysate was

added 1 mL 10% (w/v) trichloroacetic acid. After centrifugation at 1000 g for 10 min, 1.3 mL 0.5% (w/v) thiobarbituric acid were added and the mixture was heated at 100°C for 20 min. After cooling, MDA formation was recorded (Absorbance 530 nm and Absorbance 550 nm) in a Perkin Elmer (Massachusetts, MA, USA) spectrofluorimeter and the results are presented as ng MDA/ 1×10^6 cells.

Preparation of cytosolic fractions

Extracts of PC12 cells stimulated for 24 h with A β (1 μ g/mL) in the presence or absence of cannabidiol (10^{-7} – 10^{-4} M) were prepared as previously described (Esposito *et al.* 2002). Briefly, harvested cells (1×10^6) were washed twice with ice-cold PBS and centrifuged at 180 g for 10 min at 4°C. The cell pellet was resuspended in 100 μ L of ice-cold hypotonic lysis buffer [10 mM HEPES, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM phenylmethylsulfonyl fluoride, 1.5 μ g/mL soybean trypsin inhibitor, pepstatin A 7 μ g/mL, leupeptin 5 μ g/mL, 0.1 mM benzamidine, 0.5 mM dithiothreitol (DTT)] and incubated in ice for 15 min. The cells were lysed by rapid passage through a syringe needle five or six times and the cytoplasmic fraction was then obtained by centrifugation at 13 000 g for 1 min.

Western blot analysis

Immunoblotting analysis of caspase 3 protein was performed on a cytosolic fraction of PC12 cells treated as described (Samali *et al.* 1999). Cytosolic fraction proteins were mixed with gel loading buffer (50 mM Tris, 10% SDS, 10% glycerol 2-mercaptoethanol, 2 mg bromophenol/ml) in a ratio of 1 : 1, boiled for 5 min and centrifuged at 10 000 g for 10 min. Protein concentration was determined and equivalent amounts (50 μ g) of each sample were separated under reducing conditions in 12% SDS–polyacrylamide minigel. The proteins were transferred onto nitrocellulose membrane according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked by incubation at 4°C overnight in high salt buffer (50 mM Trizma base, 500 mM NaCl, 0.05% Tween-20) containing 5% bovine serum albumin; they were then incubated for 1 h with anti-caspase 3 antibody (1 : 2000) (Chemicon, Temecula, CA, USA) for 1 h at room temperature, followed by incubation with horseradish peroxidase (HRP)-conjugate secondary antibody (Dako). The immune complexes were developed using enhanced chemiluminescence detection reagents (Amersham, Cologno Monzese, Italy) according to the manufacturer's instructions and exposed to Kodak X-Omat film. The protein bands of caspase 3 and pro-caspase 3 on X-ray film were scanned and densitometrically analysed with a GS-700 imaging densitometer (Bio-Rad Laboratories, Hercules, CA, USA). Results are expressed as the ratio pro-caspase/caspase 3 (a low ratio is indicative of apoptosis).

DNA fragmentation analysis

To prepare cellular DNA, PC12 cells (4×10^6 , incubated for 24 h with or without A β , alone, or in presence of cannabidiol 10^{-5} M and 10^{-4} M) were detached from 10 cm $\varnothing$ culture dishes and the cell suspension was centrifuged at 100 g for 10 min (Gschwind and Huber 1995). The cell pellet was then washed twice with ice-cold PBS and cellular DNA was isolated using a DNA isolation kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. A 20 μ L aliquot of each DNA sample was analysed on a 1.5% agarose gel containing ethidium bromide (1 μ g/mL) in TBE buffer (100 mM Tris, 90 mM boric acid, 1 mM EDTA) and run for 90 min

at 70 V. After electrophoresis, the DNA was visualized under UV light and photographed.

Measurement of intracellular calcium levels

Changes in intracellular free calcium levels ($[Ca^{2+}]_i$) in a suspension of PC12 cells were determined by the fura-2 method (Sandrasegare *et al.* 1994; Zhou *et al.* 1996). Upon binding calcium, fura-2 shifts its excitation wavelength from 380 to 340 nm, and the ratio of the fluorescence caused by excitation at 340 nm to that at 380 nm ($R_{340/380}$) reflects changes in $[Ca^{2+}]_i$ (Zhou *et al.* 1996). PC12 cells were incubated with 5 μ M fura-2/acetoxymethyl ester (fura-2/AM) in HBH (concentration in mM: NaCl 137, KCl 5.4, MgCl₂ 0.49, KH₂PO₄ 0.44, Na₂HPO₄ 0.64, NaHCO₃ 3, glucose 5.5, CaCl₂ 1.26 and HEPES 20, pH 7.4) in a 37°C incubator for 60 min, and washed twice before taking an aliquot for $[Ca^{2+}]_i$ measurement. Each aliquot contained 5×10^3 cells suspended in a total volume of 200 μ L HBH. Basal $[Ca^{2+}]_i$ values were obtained after 2 min of equilibration and $[Ca^{2+}]_i$ measurements were performed 2, 5, 10 and 15 min after A β administration. As A β produced a maximal increase 5 min after its administration, the effect of cannabidiol (10^{-6} – 10^{-4} M, administered 15 min before A β 1 μ g/mL) or vitamin E (10^{-5} M, used as a reference compound) was assessed at this time-point. Measurements were performed with a spectrofluorometer with an LS55 luminescence spectrometer (Perkin Elmer).

Statistical analysis

Results were expressed as the mean $\pm$ SEM of *n* experiments. Statistical analysis was determined with ANOVA and multiple comparisons were performed by Bonferroni's test, with $p < 0.05$ considered significant.

Results

Cell viability

MTT assay was used to detect cell viability. Incubation of PC12 cells with A β (1 μ g/mL) for 24 h caused $38.8 \pm 2.2\%$ of cell death (Fig. 1). Cannabidiol (10^{-7} – 10^{-4} M), added to the cells immediately before treatment with A β , significantly reduced cell death. The effect of cannabidiol was significant for all the concentrations tested. The cannabinoid receptor agonist CP55,940 also reduced A β -induced cell death (cell death: A β 37.0 ± 3.8 ; CP55,940 10^{-8} M + A β 13.6 ± 7.9 ; CP55,940 10^{-7} M + A β 21.7 ± 9.0 ; CP55,940 10^{-6} M + A β 8.5 ± 4.2 , $p < 0.05$, CP55,940 10^{-5} M + A β 6.6 ± 3.8 , $p < 0.05$, $n = 3$ –4).

Measurement of reactive oxygen species (ROS)

ROS accumulation, estimated using a converting reaction of the probe DCFH₂ to DCF, was significantly ($p < 0.001$) increased after incubation with A β (1 μ g/mL), as indicated by the increase in RFU (Fig. 2). Incubation of PC12 with cannabidiol (10^{-7} – 10^{-4} M) significantly inhibited A β -induced increase in ROS accumulation (Fig. 2). The effect of cannabidiol (10^{-5} M) was not significantly modified by the cannabinoid CB1 receptor antagonist SR141716A (10^{-5} M) (RFU: control 53 ± 5 ; A β 146 ± 11 ; cannabidiol +

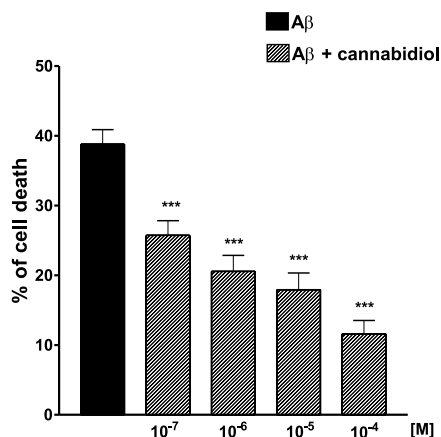


Fig. 1 Effect of cannabidiol (10^{-7} – 10^{-4} M) on β -amyloid ($A\beta$, 1 μ g/mL)-induced cell death. Cell death, evaluated by the reduction of the tetrazolium salt MTT, was assessed 24 h after incubation with $A\beta$. Cannabidiol was added immediately before $A\beta$. Results, expressed as the percentage of cell death, are the means $\pm$ SEM of five experiments in triplicate. Untreated cells were assumed to be vital (100% viability). *** p < 0.001 versus $A\beta$.

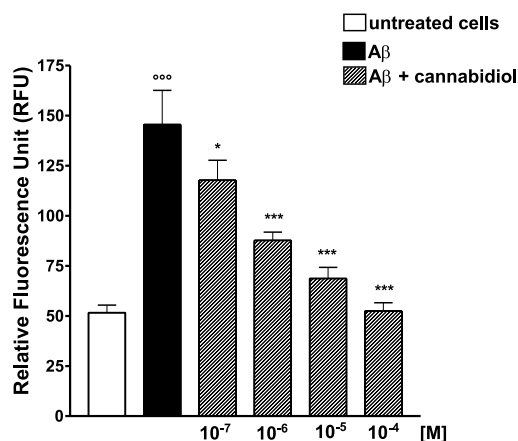


Fig. 2 Effect of cannabidiol (10^{-7} – 10^{-4} M) on β -amyloid ($A\beta$, 1 μ g/mL)-induced formation of reactive oxygen species (ROS). ROS formation, evaluated by the oxidation of 2',7'-dichlorofluorescein (H_2DCF) to the fluorescent 2',7'-dichlorofluorescein (DCF), was assessed 24 h after incubation with $A\beta$. Cannabidiol was added immediately before $A\beta$. Results are the means $\pm$ SEM of six experiments in triplicate. $^{***}p$ < 0.001 versus control (untreated cells); * p < 0.05 and *** p < 0.001 versus $A\beta$.

$A\beta$ 61 $\pm$ 11; $A\beta$ + cannabidiol + SR141716A 66 $\pm$ 6; n = 5). Given alone, SR141716A (10^{-5} M) did not modify ROS accumulation either in untreated cells or in cells incubated with $A\beta$ (data not shown). Cannabidiol did not affect viability in untreated cells (data not shown).

Lipid peroxidation assay

The thiobarbituric acid colorimetric assay, which measures malondialdehyde (MDA) levels, was used to quantify lipid

peroxidation. Exposure of PC12 cells to $A\beta$ (1 μ g/mL) caused a significant increase in MDA levels (indicating that lipid peroxidation occurs) (Fig. 3). Lipid peroxidation of $A\beta$ -treated cells was concentration-dependently reduced by cannabidiol (10^{-7} – 10^{-4} M). The effect of cannabidiol (10^{-5} M) was not significantly modified by the cannabinod CB_1 receptor antagonist SR141716A 10^{-5} M (MDA ng/ 10^6 cells: untreated cells 31 $\pm$ 3; $A\beta$ 85 $\pm$ 4; cannabidiol + $A\beta$ 45 $\pm$ 4; $A\beta$ + cannabidiol + SR141716A 42 $\pm$ 3; n = 5). Given alone, SR141716A (10^{-5} M) did not change MDA levels either in untreated cells (MDA ng/ 10^6 cells: untreated cells 31 $\pm$ 3; SR141716A 32 $\pm$ 3; n = 5) or cells incubated with $A\beta$ (MDA ng/ 10^6 cells: $A\beta$ 85 $\pm$ 4; $A\beta$ + SR141716A 81 $\pm$ 4; n = 5).

Apoptosis assay

We evaluated the appearance of the caspase 3 band in a cellular extract of PC12 cells by western blot analysis as a hallmark of apoptosis. In our time-course experiments, we showed that PC12 cells start to undergo to apoptosis 4 h after treatment with $A\beta$ (1 μ g/mL), but a marked apoptosis was evident at 6 h after treatment (Fig. 4a). Analysis of the pro-caspase/total caspase 3 ratio showed that $A\beta$ significantly induced apoptosis (as indicated by the decreased ratio) 6 h after treatment, and this increase was significantly counteracted by cannabidiol (10^{-4} M) (Fig. 4b). These results suggest that apoptosis in $A\beta$ -treated PC12 cells is mediated by caspase 3, and the protective action of cannabidiol may, at least in part, be attributed to inhibition of the caspase cascade.

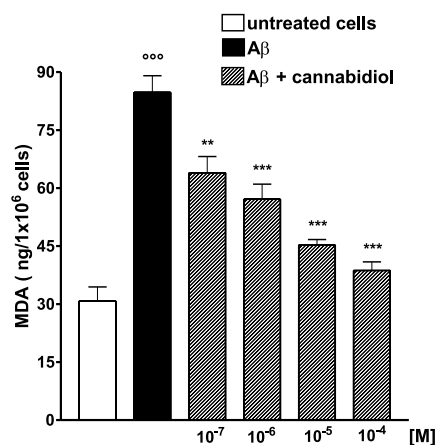


Fig. 3 Effect of cannabidiol (10^{-7} – 10^{-4} M) on β -amyloid ($A\beta$, 1 μ g/mL)-induced lipid peroxidation. Lipid peroxidation, evaluated by the thiobarbituric acid colorimetric assay [which measures malondialdehyde (MDA)], was assessed 24 h after incubation with $A\beta$. Cannabidiol was added immediately before $A\beta$. Results, expressed as concentration of MDA, are the means $\pm$ SEM of six experiments in triplicate. $^{***}p$ < 0.001 versus control (untreated cells); ** p < 0.01 and *** p < 0.001 versus $A\beta$.

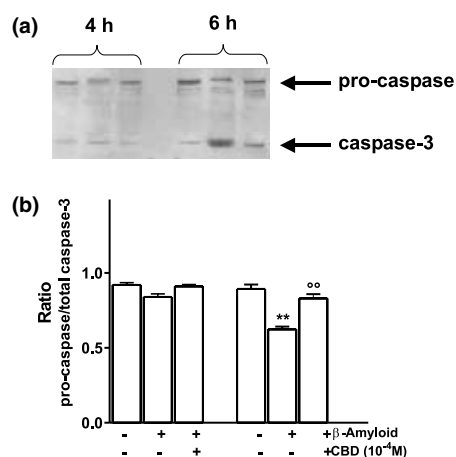


Fig. 4 Effect of cannabidiol (CBD, 10^{-4} M) on β -amyloid ($1 \mu\text{g/mL}$)-induced caspase 3 activation. (a) Representative western blot analysis of caspase 3 activation from its inactive precursor, pro-caspase 3, in PC12 cell homogenate treated with and without A β and in the presence of cannabidiol; arrows indicate cleaved (activated) caspase 3 at about 17 kDa and its precursor, pro-caspase, at about 43 kDa. (b) Ratio pro-caspase/total caspase 3 (a decreased ratio is indicative of apoptosis). ** $p < 0.01$ versus control cells (at 6 h), °° $p < 0.01$ versus β -amyloid (at 6 h), $n = 3$ separate experiments.

DNA fragmentation

Experiments on DNA fragmentation are shown in Fig. 5. Untreated PC12 cells showed no detectable DNA fragmentation. By contrast, DNA extracted from PC12 cells after 24 h of exposure to A β was clearly fragmented. Pretreatment with cannabidiol (10^{-5} M and 10^{-4} M) reduced A β -induced DNA ladders, the effect being more evident for the 10^{-4} M concentration.

Intracellular calcium levels

A β ($1 \mu\text{g/mL}$) produced a significant increase in $[\text{Ca}^{2+}]_i$ 2–15 min after its administration, with a maximal effect after 5 min (data not shown). Therefore, the effects of cannabidiol were assessed at this time point. A β -induced changes in $[\text{Ca}^{2+}]_i$ were reduced by both cannabidiol ($R_{340/380}$: basal 0.90 ± 0.02 ; A β 1.20 ± 0.06 ; A β + cannabidiol 10^{-6} M 1.10 ± 0.07 ; A β + cannabidiol 10^{-5} M 1.08 ± 0.07 ; A β + cannabidiol 10^{-4} M 0.91 ± 0.07 ; $n = 3$ –4, $p < 0.05$ for the 10^{-5} M and 10^{-4} M concentrations) and vitamin E 10^{-5} M ($R_{340/380}$: basal 0.90 ± 0.02 ; A β 1.20 ± 0.06 ; A β + vitamin E 0.93 ± 0.03 ; $n = 3$, $p < 0.05$).

Discussion

The accumulation of plaques containing A β is an invariant feature of AD pathology and there is abundant evidence suggesting that A β contributes to the aetiology of AD (Carlson 2003; Golde 2003). In the present paper we report that cannabidiol, a major non-psychotropic component of

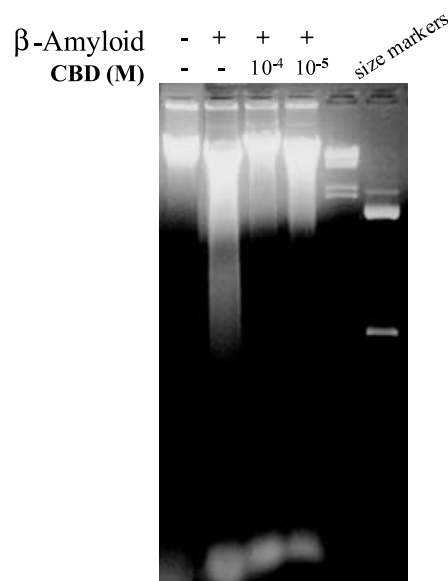


Fig. 5 DNA fragmentation in PC12 untreated cells and in PC12 cells treated with β -amyloid ($1 \mu\text{g/mL}$, alone or in the presence of cannabidiol 10^{-4} M and 10^{-5} M). Cellular DNA was extracted and visualized on agarose gel as described in the Materials and methods section. Each lane was loaded with the same amount of DNA. DNA size markers used were: (left) phage lambda DNA digested with Hind III restriction enzyme; (right) pBR322 DNA digested with Hinf I restriction enzyme.

marijuana, prevents A β -induced neurotoxicity, ROS production and lipid peroxidation; it also inhibits caspase 3 appearance (from the inactive precursor pro-caspase) in PC12 cells.

The MTT assay has been widely used as an index of cell survival and proliferation. Previous investigators have shown that cannabidiol produces a modest or no reduction in C6 glioma cell viability after 6 days of incubation (Jacobsson *et al.* 2000). Here, we have shown that exposure of PC12 cells to A β produces a reduction in cell viability, and that cannabidiol protects, in a concentration-dependent manner, A β -induced neurotoxicity. Consistent with these results, cannabidiol has been shown to reduce glutamate-, N-methyl-D-aspartate- and kainate-induced toxicity in rat cortical neurones (Hampson *et al.* 1998).

The cellular events involving free radical oxidative stress may be one basic pathway leading to cell degeneration. Increased oxidative stress and disturbed defensive mechanisms occur in the brain of AD patients, which might result in a self-propagating cascade of neurodegenerative events (Markesbery 1997). ROS have been shown to be involved in cell damage and death induced by amyloid peptides (Mattson *et al.* 1993; Brera *et al.* 2000) and antioxidant therapy has led to an improvement in AD patients. Excessive ROS can lead to a number of detrimental effects, including lipid peroxidation. In our experiments, PC12 cells exhibited

increased ROS levels and lipid peroxidation following exposure to A β peptide. The ability of A β to increase both ROS and lipid peroxidation has been documented previously in a number of neuronal cell types, including PC12 cells (Xiao *et al.* 2002; Onoue *et al.* 2002; Guan and Nordberg 2003). However, we have shown for the first time that cannabidiol reduced, in a concentration-dependent manner, A β -induced ROS accumulation and lipid peroxidation. Since antioxidants are known to attenuate A β -induced oxidative injury (Cash *et al.* 2002), it is likely that the previously reported antioxidant properties of cannabidiol could contribute to its beneficial effect. Other studies have shown that cannabidiol antagonizes the oxidative stress induced by retinoid anhydroretinol in lymphoblastoid cells (Chen and Buck 2000) and prevents hydrogen peroxide-induced oxidative damage in neuronal cultures (Hampson *et al.* 1998).

In contrast to Δ^9 -THC, cannabidiol is not a cannabinoid receptor agonist (Mechoulam *et al.* 2002); nevertheless, cannabidiol may enhance the endocannabinoid action at CB₁ receptors by its ability to block the uptake and the enzymatic degradation of the endocannabinoid anandamide (Bisogno *et al.* 2001). Furthermore, Petitet *et al.* (1998) have shown that cannabidiol, in the micromolar range, behaves as a CB₁ antagonist in the rat brain. The importance of CB₁ receptors in neuroprotection has been previously documented (Grundy *et al.* 2001; Van der Stelt *et al.* 2001; Marsicano *et al.* 2002). Milton (2002) showed that the endogenous cannabinoids anandamide and noladin ether have a protective effect on A β -induced toxicity through activation of CB₁ receptors in human teratocarcinoma cells. However, it is very unlikely that the protective (anti-oxidative) effect of cannabidiol observed here involves CB₁ receptors as the potent and selective CB₁ receptor antagonist, SR141716A, at a concentration previously shown to be fully effective (Esposito *et al.* 2001, 2002), did not show a protective effect (when administered alone), nor did it modify the protective action of cannabidiol. In agreement with our results, Molderings *et al.* (2002) showed (by using binding and PCR techniques) that rat pheochromocytoma PC12 cells are not endowed with CB₁ receptors. It is interesting to note that in these cells, we have shown that the cannabinoid agonist CP55,290 also has a protective effect on A β -induced toxicity, thus confirming the existence of a non-CB₁ mediated protective effect of cannabinoids.

Several experimental studies suggest an association between deposition of A β , oxidative stress and apoptosis associated with AD and ageing (Kokoszka *et al.* 2001; Marx 2001). The A β peptide has been shown to induce apoptosis in neurones, including PC12 cells, which may be responsible for neuronal death in AD (Martin *et al.* 2001; Troy *et al.* 2001). Apoptosis is associated with the activation of a family of aspartic acid-specific cysteine proteases, referred to as caspases (Nicholson and Thornberry 1997). There are at least 14 caspases identified in mammalian cells that are synthesized

as inactive precursor molecules, pro-caspase, and are converted by proteolytic cleavage to the active heterodimer (Thornberry and Lazebnik 1998). The activation of pro-caspase 3 to caspase 3 is a central event in the execution phase of apoptosis and appears to serve as the convergence point of different apoptotic signalling pathways (Nicholson and Thornberry 1997). In the present study, we have shown that cannabidiol increased pro-caspase 3 levels and, in parallel, reduced caspase 3 levels in A β -treated PC12 cells, suggesting that the cannabinoid could exert a protective role at the execution phase of apoptosis. The inhibitory effect of cannabidiol on apoptosis was strengthened by the observation that this cannabinoid inhibited the fragmentation of DNA (a hallmark of apoptosis) induced by A β . The fragmentation of DNA after A β has been previously documented (Gschwind and Huber 1995).

The pharmacological manipulation of neuronal calcium homeostasis may be a useful therapeutic approach in neurodegenerative pathologies, including AD, and a rise in [Ca²⁺]_i has been suggested to be responsible for toxic effects of A β (Mattson 2002). Previous investigators have shown that A β -induced neurotoxic actions in PC12 cells are associated with an increase in [Ca²⁺]_i and that this effect is blocked by antioxidants such as vitamin E (Zhou *et al.* 1996). In addition, it has been suggested that the increase in [Ca²⁺]_i induced by A β in PC12 cells is not the result of an influx of calcium through voltage-dependent calcium channels, but rather it is the result of a free radical-mediated process. In the present study we have shown that cannabidiol counteracted the A β -induced increase in [Ca²⁺]_i, which is in agreement with the antioxidant properties of this cannabinoid reported here and elsewhere (Hampson *et al.* 1998).

In summary, the results reported herein indicate, for the first time, that cannabidiol exerts a combination of neuroprotective, anti-oxidative and anti-apoptotic effects against A β insult. Inhibition of the activity of the apoptotic enzyme caspase 3 is likely to be involved in signalling pathways for the neurotrophic effect of cannabidiol. Given the low toxicity of cannabinol shown in humans (Cunha *et al.* 1980; Consroe *et al.* 1991), this non-psychoactive component of marijuana may play an important role in counteracting neuronal cell death occurring in Alzheimer's disease.

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REVIEW ARTICLE

Cannabidiol in Medicine: A Review of its Therapeutic Potential in CNS Disorders

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Cannabidiol (CBD) is the main non-psychotropic component of the glandular hairs of *Cannabis sativa*. It displays a plethora of actions including anticonvulsive, sedative, hypnotic, antipsychotic, antiinflammatory and neuroprotective properties. However, it is well established that CBD produces its biological effects without exerting significant intrinsic activity upon cannabinoid receptors. For this reason, CBD lacks the unwanted psychotropic effects characteristic of marijuana derivatives, so representing one of the bioactive constituents of *Cannabis sativa* with the highest potential for therapeutic use.

The present review reports the pharmacological profile of CBD and summarizes results from preclinical and clinical studies utilizing CBD, alone or in combination with other phytocannabinoids, for the treatment of a number of CNS disorders. Copyright © 2008 John Wiley & Sons, Ltd.

Keywords: cannabidiol; *Cannabis sativa*; CNS disorder; phytocannabinoid.

INTRODUCTION

Cannabidiol (CBD) is the main non-psychotropic component of the glandular hairs of *Cannabis sativa* and belongs to the so-called 'cannabinoids' (CBs). This term refers to a heterogeneous family of molecules usually exhibiting pharmacological properties by interacting with specific receptors. So far, two membrane receptors for CBs, both coupled to G protein and named CB1 and CB2, have been identified. While CB1 receptors are mainly expressed in the central and the peripheral nervous systems, CB2 receptors have been reported to be more abundantly detected in cells of the immune system (for review see Pertwee, 2006). Moreover, two orphan G protein-coupled receptors, GPR119 and GPR55, possibly activated by multiple different cannabinoid ligands (Brown, 2007), have been recently proposed as novel cannabinoid receptors. CBs can be separated into three different groups: endogenous (endocannabinoids), synthetic and phytocannabinoids. The latter group includes terpenophenolic substances extracted from *Cannabis sativa*, such as Δ^9 -tetrahydrocannabinol (Δ^9 -THC) and CBD. Cannabinoid pharmacology is a field rapidly expanding and the therapeutic properties of cannabinoid receptor agonists include analgesia, muscle relaxation, immunosuppressant, antiinflammatory and antiallergic effects, improvement of mood, stimulation of appetite, antiemesis, lowering of intraocular pressure, bronchodilatation, neuroprotection and antineoplastic effects (Pertwee, 2005). Despite the emerging evidence regard-

ing therapeutic activities of CBs, their effective introduction in clinical use is still controversial and strongly limited by the unavoidable psychotropic effects exhibited by many of them. In this context, much attention has been focused on CBD that constitutes up to 40% of the *Cannabis* extract, and represents one of the most promising candidates for clinical utilization due to its remarkable lack of any cognitive and psychoactive actions. Finally, CBD is regarded as an interesting putative lead compound to develop cannabinomimetic drugs, because of its excellent tolerability in humans (for review see Mechoulam and Hanus, 2002).

CBD PHARMACOLOGY

Cannabidiol was isolated from marijuana in the late 1930s, but only in the 1963 were its structure and stereochemistry first elucidated (Mechoulam and Shvo, 1963). Early studies focusing on CBD pharmacology started in the 1970s, with the first relevant finding concerning its hypnotic and anticonvulsant properties, published in 1981 (Carlini and Cunha, 1981). Since then, a large body of pharmacological effects has been demonstrated, both in preclinical and in clinical studies.

Up to the present, the molecular pharmacology of CBD has not yet been well defined and little is known about a possible CBD-receptor-mediated signalling pathway. Since it was previously demonstrated that CBD binds with a low affinity to both CB1 and CB2 cannabinoid receptors, much research was aimed at recognizing CB1 and CB2 independent modes of action for this phytocannabinoid. To date, different molecular targets have been proposed. The first evidence that CBD can bind to sites different from cannabinoid receptors was offered by the observation that natural CBD and the (+)-synthetic

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one both stimulate the type-1 vanilloid receptor (Bisogno *et al.*, 2001). Other studies indicated that CBD also binds to 5-HT_{1A} and such an interaction was suggested to account for the attenuation of cerebral infarction size occurring during ischemia (Mishima *et al.*, 2005) and also for its anxiolytic effect (Campos and Guimarães, 2008). It has been also reported that CBD may behave as an allosteric modulator at μ and δ opioid receptors, even if the effects occur at very high levels of phytocannabinoid, so that this modulation cannot be expected to contribute markedly to the CBD actions exerted *in vivo* (Kathmann *et al.*, 2006).

However, although CBD displays very low affinity for both CB1 and CB2, it has been reported recently that it can operate as a CB2 receptor inverse agonist and this may, at least in part, contribute to its widely documented antiinflammatory properties (Thomas *et al.*, 2007).

Furthermore it is commonly recognized that many CBD effects are mainly due to its antioxidant activity as first demonstrated by Hampson *et al.* (1998) that exposed rat cortical neuron cultures to a toxic level of the excitatory neurotransmitter glutamate. On that occasion, the authors showed that CBD exerted a potent antioxidant activity, resulting in a more protective effect than either ascorbate or α -tocopherol, against glutamate-mediated neurotoxicity.

CBD PHARMACOKINETICS

The correct use of CBD in human therapy necessarily requires basic information related to pharmacokinetics. *Cannabis* derivatives are usually inhaled or orally administered. Other routes, including rectal, transdermic, eye drops, aerosols and intravenous have been used in a small number of studies so that the relevance of findings is limited. Recently, it was demonstrated in rabbits that sublingual administration of a solid CBD/beta-cyclodextrin complex may provide an alternative formulation for sublingual administration (Mannila *et al.*, 2007). The pharmacokinetics of CBD is quite complicated and in many aspects resembles that of Δ^9 -THC. Once taken orally, CBD bioavailability ranges between 13% and 19%, due to a marked first-pass effect, while the systemic bioavailability of inhaled CBD in a group of cannabis users was 31% (range 11–45%). The plasma pattern was similar to that of Δ^9 -THC. Daily oral doses of CBD 10 mg/kg/day chronically administered resulted in mean plasma concentrations of 5.9–11.2 ng/mL (Consroe *et al.*, 1991a). CBD is rapidly distributed when intravenously administered, and easily passes the blood–brain barrier. CBD shows a prolonged elimination; its terminal half-life is about 9 h, and it is excreted preferentially in the urine, both free and as its glucuronide compound (Samara *et al.*, 1990).

Cannabidiol impairs hepatic drug metabolism in several animal species, and inhibits mouse hepatic metabolism through the inactivation of specific cytochrome P450 belonging to the 2C and 3A subfamilies (Bornheim *et al.*, 1993). The metabolism of CBD showed biotransformation routes typically observed for cannabinoids (Harvey *et al.*, 1991a). It undergoes multiple hydroxylations, oxidations to carboxylic acids, beta-oxidation, conjugation and epoxidation (Harvey *et al.*, 1991a). Conjugation with fatty acids, first observed with Δ^9 - and Δ^8 -THC, provides a

potent means of increasing the lipophilicity and, hence, tissue accumulation (Leighty *et al.*, 1976). CBD-7-oic acid together with CBD-glucuronide represent the most abundant products of CBD metabolism detected in human urine (Harvey *et al.*, 1991b). Unlike Δ^9 -THC a remarkable percentage of unchanged CBD is excreted in the faeces (Wall *et al.*, 1976).

CBD TOXICITY

Cannabidiol exhibits very low toxicity in humans and in other species: the LD₅₀ after intravenous administration to rhesus monkeys was 212 mg/kg (Rosenkrantz *et al.*, 1981). The oral LD₅₀ has not been established, but in 1981 Rosenkrantz showed that an oral dose of CBD 20–50 times larger than the intravenous route is required to initiate severe intoxication (Rosenkrantz *et al.*, 1981). CBD does not cause relevant CNS alterations. Moreover, a large body of studies failed to reveal teratogenic or mutagenic effects induced by CBD (Dalterio *et al.*, 1984; Matsuyama and Fu, 1981).

PREPARATION CONTAINING CBD

Because of its very low toxicity in humans, a huge number of trials have been performed to assess the clinical efficacy of CBD in different pathologies. Starting from 2001, most of the trials have been carried out utilizing Sativex[®], the only commercially available preparation containing CBD/ Δ^9 -THC. At the moment, four different formulations of Sativex[®] are under investigation, including the high THC extract (Tetranabinex[®]), THC:CBD (narrow ratio), THC:CBD (broad ratio) and the high CBD extract (Nabidiox[®]) (No authors listed, 2003). Three Sativex[®] delivery systems exist: the oromucosal spray, sublingual tablets and inhaled (but not smoked) dosage forms.

In 2005 the oromucosal spray administration of Sativex[®] was approved for the treatment of multiple sclerosis symptoms (Perras, 2005).

It has been proved that co-administration of CBD and Δ^9 -THC may alter the pharmacological effect of the latter, potentiating some putative benefits, while attenuating some of its negative effects (Karniol *et al.*, 1974; Zuardi *et al.*, 1982; Russo and Guy, 2006).

CBD IN SOME CNS DISORDERS

The neuroprotective actions of CBD, mainly due to its antiinflammatory and antioxidant properties, have been well documented (Hampson *et al.*, 1998). Recently, a neuroprotective mechanism of CBD has also been confirmed in a mouse model of ischemia, where CBD explicates the cerebroprotective action via a cannabinoid receptor-independent myeloperoxidase-inhibiting mechanism (Hayakawa *et al.*, 2007), in addition to a 5HT_{1A} receptor action (Mishima *et al.*, 2005). It has also been demonstrated that CBD reverses binge ethanol-induced neurotoxicity, once again, via a cannabinoid receptor-independent antioxidant mechanism (Hamelink *et al.*,

2005). The potential of CBD to attenuate the excessive formation of peroxynitrites induced by glutamate also contributes to its neuroprotective effects, as demonstrated by the *in vitro* results pointing to the ability of CBD to induce the prevention of retinal apoptosis (El-Remessy *et al.*, 2003). These early data, together with the lack of psychotropic effects and the low toxicity, supports the notion that CBD may be considered a potentially useful therapeutic agent for the treatment of a number of neurological disorders.

Epilepsy

Work carried out in the early 1970s demonstrated that CBD possesses a rather potent protective effect against convulsions in a variety of animal models of epilepsy (Carlini *et al.*, 1973; Karler *et al.*, 1973; Karler and Turkani, 1980; Consroe and Wolkin, 1977a, 1977b). Indeed, some preclinical evidence showed that CBD was remarkably effective against focal seizures, including temporal lobe epilepsy and generalized convulsion induced by both electroshock and GABA receptor blockers. In these experimental paradigms of epilepsy the effects of CBD were considered almost equivalent to those exerted by antiepileptic compounds commonly used in human therapy. Interestingly, CBD was observed to potentiate the anticonvulsant activity of barbiturates and diphenylhydantoin. In this respect it is worth noting that phenytoin and CBD both display similar stereochemical requirements for anticonvulsant drug action (Tamir *et al.*, 1980). Based on these findings, CBD is regarded, among CBs, as the most promising candidate for antiepileptic therapy because of its powerful anticonvulsant properties in the absence of any relevant toxicity. However, besides a great deal of preclinical results, the available literature on CBD use as an anticonvulsant in human therapy is still limited, because it concerns a very few patients.

Many anecdotal reports have confirmed that CBD exerts considerable anticonvulsant activity and the results are efficacious in protecting against partial and tonic-clonic generalized seizures. Indeed, it has been reported that in epileptics who smoked cannabis to control seizures, interrupting cannabis use induced the reappearance of convulsions, whereas reverting to consumption limited symptoms.

Only one double-blind controlled trial was performed to explore the anticonvulsant activity of CBD in 15 patients suffering from frequent attacks of 'secondarily generalized epilepsy' with temporal focus, unresponsive to standard treatment (Cunha *et al.*, 1980). The results of this study indicated that 50% of patients taking CBD remained virtually symptoms-free for the duration of the trial, with no significant toxicity or side effects due to CBD administration. The rest of the patients under medication improved markedly, whereas subjects receiving the placebo remained unchanged. Despite these intriguing results the trial was never continued, in all probability because of the huge amounts of drug required.

Nowadays, CBD treatment has been also proposed as a promising therapeutic tool in monotherapy in children suffering from convulsions, resistant to conventional treatment, even though CBD was found to be ineffective against absence seizures produced in laboratory animals (Cortesi and Fusar-Poli, 2007). This suggestion has been advanced for the absence of tolerance

to anticonvulsant properties, also during prolonged treatment with CBD. This aspect is relevant in subjects who have been treated previously with a high-dose of anticonvulsants or with a combination of them. Moreover, the possibility of administering CBD by metered dose pump aerosol spray guarantees a very useful system of administration in children with a poor compliance.

Psychosis

Convergent evidence links the habitual use of *Cannabis* to a risk of developing schizophrenia or schizophrenia-like psychosis, especially in vulnerable subjects. This effect has been attributed to Δ^9 -THC, the main psychoactive constituent of the plant. Conversely, different preclinical studies have provided evidence for a potential antipsychotic activity of CBD, with a pharmacological profile resembling that of a neuroleptic drug. Indeed, the effects of CBD were similar to those produced by haloperidol in a rodent model predictive of antipsychotic activity, using different doses of drugs (Zuardi *et al.*, 1991). Both CBD and haloperidol reduced the occurrence of apomorphine-induced stereotypies, however, CBD did not provoke catalepsy, even at the highest doses. These results have supported the assumption that CBD possesses antipsychotic properties, without generating extrapyramidal side-effects, thus suggesting the hypothesis that CBD may work as an atypical antipsychotic (Zuardi *et al.*, 1991). According to this view, the pharmacological profile of CBD was reported to be comparable to that exhibited by clozapine. Interestingly both drugs, in contrast to conventional neuroleptics, induce c-fos expression in mesolimbic and mesocortical structures, but not in basal ganglia (Guimarães *et al.*, 2004).

The first available clinical data, related to this pathology, referred to CBD effects observed in a 19-year-old black woman with schizophrenia (Zuardi *et al.*, 1995). The administration of CBD resulted in a significant improvement of psychotic symptoms with an efficacy evaluated as equivalent to that of haloperidol. However, a subsequent clinical study aimed at verifying the efficacy of CBD monotherapy in patients with treatment-resistant schizophrenia (TRS) demonstrated only a mild improvement in one of three patients treated with CBD monotherapy, suggesting that such an approach has to be considered unsatisfactory for TRS (Zuardi *et al.*, 2006). On the other hand, findings have been published reinforcing the suggestions of previous work pointing to the antipsychotic properties of CBD. Indeed the results have demonstrated that smoking some strains of *Cannabis* containing more CBD, in addition to Δ^9 -THC, may be protective against psychotic-like symptoms induced by Δ^9 -THC alone (Morgan and Curran, 2008).

Anxiety

The results from several studies performed in the 1980s suggested that CBD displays sedative and antianxiety properties (Pickens, 1981; Musty, 1984; Musty *et al.*, 1984; Zuardi *et al.*, 1982). Such evidence prompted further investigations to be carried out, employing different paradigms, aimed at better defining its intrinsic anxiolytic efficacy. The findings confirmed that CBD

exerts significant anxiolytic activity and that antianxiety effects may exhibit an inverted U-shaped dose-response curve in rodents, with the higher doses being no longer effective (Guimaraes *et al.*, 1990, 1994). Moreover, using the elevated plus maze, anxiolytic effects were also reported for the three CBD derivatives: HU219, HU252, HU-291 (Guimaraes *et al.*, 1994). The mechanism by which CBD exerts its anxiolytic effects still remains not fully clarified. However, using a conditioned emotional paradigm, the Vogel conflict test, a model of anxiety based on the conflict between drinking and punishment, it was proved that CBD-mediated anxiolytic activity is independent of benzodiazepine receptors or of non-specific drug interference on the nociceptive threshold or water consumption (Moreira *et al.*, 2006). Recently, the binding at 5HT_{1A} receptor has been considered as a possible molecular target for its antianxiety activity. Indeed, evidence has been provided suggesting that activation of 5HT_{1A} receptors located in the dorsolateral periaqueductal gray could be one of the mechanisms of the anxiolytic effect observed with this compound after systemic administration (Campos and Guimarães, 2008).

In contrast to preclinical studies, few human trials have been conducted to evaluate the efficacy of CBD as an antianxiety agent. Oral administration of CBD in healthy volunteers was found to reduce the anxiogenic effects of Δ^9 -THC (Zuardi *et al.*, 1982). This property does not seem to implicate pharmacokinetic interactions with Δ^9 -THC, suggesting that CBD possesses intrinsic anxiolytic properties. Moreover, the anxiolytic effects of CBD were also observed in healthy volunteers submitted to a simulation of a public speaking test. On that occasion CBD (300 mg/per os) exhibited an efficacy comparable to that displayed by diazepam (10 mg/per os) and ipsapirone (5 mg/per os) (Zuardi *et al.*, 1993). Recently, in view of its anxiolytic effect, neuroimaging studies were performed to test whether CBD was able to affect neural functioning in brain regions usually implicated in the pathophysiology of anxiety. The findings demonstrated that CBD modulates neuronal activity in limbic and paralimbic areas, including orbitofrontal, cingulate and medial temporal cortex, and the insula (Crippa *et al.*, 2004).

In addition to anxiolytic actions, early investigations also demonstrated that CBD induces biphasic hypnotic effects in rats as well as enhances sleeping time, compared with placebo, in insomniacs (Monti, 1977; Carlini and Cunha, 1981). However, more recent results from animal studies rendered controversial previous data, since i.c.v. injections of CBD increased the extracellular level of dopamine in rat nucleus accumbens, suggesting that CBD can improve alertness, and might be regarded, on the contrary, as a potential therapeutic tool in sleep disorders such as hypersomnias (Murillo-Rodríguez *et al.*, 2006).

CBD and control of movement disorders

Anecdotal evidence has suggested a potential beneficial role of CBD, alone or in combination with Δ^9 -THC, in different neurodegenerative diseases, including Parkinson's disease (PD) and Huntington's disease (HD), two chronic illnesses due to degenerative processes involving specific nuclei of the basal ganglia, resulting in the abnormal regulation of movements. Both disorders have been

scantly investigated from the clinical point of view, whereas, at the preclinical level, the accumulated findings appear more exhaustive and convincing for the possible medical utilization of CBD to improve symptoms and/or delay disease progression. According to recent preclinical findings, plant-derived cannabinoids were able to prevent neuronal damage induced by 6-hydroxydopamine unilateral injection into the nigra, pars compacta (Lastres-Becker *et al.*, 2005). This effect appeared not to implicate CB receptor mediation while, more likely, it might be due to antioxidant activity, possibly combined with the capability to modulate glial responses, relevant to neural survival. In rats with hemiparkinsonism, generated by the nigral administration of 6-hydroxydopamine, the neuroprotective effects exerted by CBD reverted dopaminergic transmission impairment, by reducing dopaminergic cell death, rather than by enhancing the functional turnover of the surviving neurons (Lastres-Becker *et al.*, 2005). Early human reports demonstrated a dose-related improvement (ranging from 20% to 50%) in parkinsonian patients treated with oral doses of CBD (100–600 mg/day over a 6 week period) (Consroe *et al.*, 1986). Conversely, in a more recent controlled trial of a mixture of Δ^9 -THC/CBD (2.5 mg/1.25 mg per capsule), it failed to exert any beneficial effect either on parkinsonism or levodopa-induced dyskinesias (Carroll *et al.*, 2004). Unfortunately no subsequent trials were performed in an attempt to clarify such conflicting results. Certainly, in comparison with the relevance of preclinical data, the limited clinical evidence suggests that human studies should be carried out to verify for good the potential for a future clinical application of CBD in PD.

Similarly, based on anecdotal accounts and the results of preliminary clinical observations CBD was considered to be a compound with therapeutic potential also against hyperkinetic disorders. Indeed, CBD was demonstrated to mitigate apomorphine-induced turning behaviour in 6-hydroxydopamine injected rats, an animal model of hyperkinetic movement disorders, while, on the other hand, it was able to potentiate hypokinesia generated by tetrabenazine (Consroe *et al.*, 1988). More recently CBD was found to protect striatal neurons against the *in vivo* toxicity of 3-nitropropionic acid, a mitochondrial toxin that replicates some biochemical alterations occurring in HD (Sagredo *et al.*, 2007).

Cannabidiol was investigated for its efficacy in HD, alone or as an add-on drug to the approved therapy with neuroleptics (Consroe *et al.*, 1991b). CBD, at an average daily dose of 10 mg/kg/day for 6 weeks, was neither symptomatically effective nor toxic compared with placebo, in neuroleptic-free patients with HD. Considering the negative results on both the therapeutic and the safety measures, there is a question about the dose as well as the duration of the trial. Since these results cannot be considered conclusive, further trials, utilizing CBD alone or in combination with Δ^9 -THC, should be performed to evaluate clinically the real antihyperkinetic value of these molecules.

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by selective loss involving motoneuronal cells in the cortex, brainstem and spinal cord. Recent reports confirm that in the pathophysiology of ALS neuroinflammation and oxidative stress play a crucial role (Turner and Talbot, 2008). Based on this evidence, it is possible to speculate that CBD, because of its antiinflammatory and

antioxidative properties, could be a promising option to improve disturbances and prolong survival in ALS patients. This is strongly reinforced by the observations that Δ^9 -THC was able to delay impairment and prolong survival in a mouse transgenic model of ALS, and that similar results were achieved when cannabinal was used (Weydt *et al.*, 2005). Furthermore, these findings have to be considered together with anecdotal reports that recreational smoking of marijuana does improve symptomatology in ALS subjects.

CBD and experimental model of Alzheimer diseases

To date very promising results have been achieved in the control of β -amyloid ($A\beta$) toxicity by utilizing CBD. Although at present it is still not fully clear the precise biochemical mechanisms by which $A\beta$ exerts its detrimental effects, nevertheless its role in inducing neuronal damage and in mediating neuroinflammation. Following this idea, it has been demonstrated that CBD protected differentiated PC12 neuronal cells from $A\beta$ exposure, through a combination of antioxidant, antiinflammatory and antiapoptotic effects (Iuvone *et al.*, 2004). The CBD antioxidant effect accounts mainly for the survival of cultured neurons, with a potency higher than α -tocopherol. Moreover it helps to attenuate $A\beta$ neurotoxicity, even with a mechanism not possessed by classical antioxidants. In fact, CBD administration results in a blunting of $A\beta$ -induced GSK-3 β activation, the key enzyme of the WNT/ β -catenin pathway, so preventing the hyperphosphorylation of tau proteins and, consequently, neurofibrillary tangle formation.

Cannabidiol decreases the phosphorylation of the stress-activated protein kinase, P38 MAPK, so preventing translocation to the nucleus of NF- κ B and the subsequent transcription of important pro-inflammatory genes,

including that encoding for iNOS protein (Esposito *et al.*, 2006). The CBD antiinflammatory properties were also confirmed in an *in vivo* study on mice inoculated with $A\beta$, where CBD dose-dependently inhibited reactive gliosis, by impairing glial cell activation and pro-inflammatory mediator release (Esposito *et al.*, 2007).

Despite these encouraging preclinical results, the therapeutic potential of CBD in AD is already being investigated. Clinical trials to explore the beneficial effects of Δ^9 -THC/CBD mixture in AD patients are presently in progress.

CONCLUSION

Cannabidiol exhibits an impressive plethora of actions, including anticonvulsive, sedative, hypnotic, antipsychotic, antiinflammatory and neuroprotective properties. Many of which may be of therapeutic relevance as well as serving as leads for pharmaceutical development. CBD is a compound well tolerated in humans, with a profile of very low toxicity, and devoid of psychoactive and cognitive effects. Preliminary studies highlight these remarkably important properties and encourage further experiments to be performed in more complex systems in order to clarify the mechanism(s) responsible for its molecular and cellular actions. At the same time, since most of the investigations suggesting CBD as a novel medicine with substantial neuroprotective potential have been carried out in the most part in animal or then cellular models, then more clinical trials able to validate its beneficial properties are warranted. The results generated by these trials might allow that the current promising expectatives can progress from the present preclinical evidence to a practical therapeutic application.

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Synthesis and Pharmacological Evaluation in Mice of Halogenated Cannabidiol Derivatives

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Six halogenated derivatives of cannabidiol (CBD, **1**) substituted on the aromatic ring at the 3' and/or 5' position, 3'-chloro- (**2**), 3',5'-dichloro- (**3**), 3'-bromo- (**4**), 3',5'-dibromo- (**5**), 3'-iodo- (**6**) and 3',5'-diiodo-CBD (**7**) were synthesized and their pharmacological effects of barbiturate-induced sleep prolongation, anticonvulsant effects and locomotor activity were evaluated by intravenous (i.v.) injection in mice. **2** (10 mg/kg, i.v., 69 ± 10 min) significantly prolonged pentobarbital-induced sleeping time by 3.1-fold, compared to control (22 ± 2 min), although other **1** derivatives used did not significantly affect the sleeping time. **2**, **4** and **6** (10 mg/kg, i.v.) significantly prolonged hexobarbital-induced sleeping time by 2.0-, 2.0- and 2.3-fold, respectively, compared with control (52 ± 5 min). On the other hand, **1** and all halogenated derivatives did not significantly prolong barbiturate-induced sleeping time. The monohalogenated derivatives, **2**, **4** and **6** were able to prolong pentobarbital and hexobarbital-induced sleeping time, although the dihalogenated derivatives, **3**, **5** and **7** did not exhibit a prolongation of the sleeping time. All halogenated derivatives of **1** except for brominated derivatives (**2**, **3**, **6**, **7**) tended to prolong tonic seizure latency induced by pentylenetetrazol. **1** and its halogenated derivatives did not exhibit any prolongation of seizure latency induced by picrotoxin or strychnine. Maximal electroshock test demonstrated that **1** and **4** exhibited almost the same potency in their anticonvulsant effects, although other **1** derivatives **2**, **3**, **5**, **6** and **7** did not show significant effect up to a dose of 63 mg/kg, i.v. The ED₅₀ values (mg/kg, i.v.) of **1** and **4** were 38 and 44, respectively. **1** and **4** also showed anticonvulsant effect in minimal and maximal electroshock-threshold tests. **2**, **4** and **6** tended to decrease the total distance (horizontal activity) and number of rearings (vertical activity) of mice, whereas **3**, **5** and **7** tended to increase the number of rearings. However, the effects of all derivatives were not statistically significant from the control. **2** and **4** were the most potent derivatives on pharmacological activities among the synthetic cannabinoids examined in the present study. These results indicate that monohalogenation of **1** leads to some modification of the pharmacological profile of CBD.

Key words cannabidiol; halogenated cannabidiol derivative; anticonvulsant; barbiturate-induced sleep prolongation; locomotor activity

Cannabinoids are C₂₁ compounds composed of C, H and O, and Δ^9 -tetrahydrocannabinol (Δ^9 -THC), cannabinol (CBN) and cannabidiol (CBD, **1**) are known as three major cannabinoids in marihuana.¹⁾ In previous studies, we reported synthesis of halogenated Δ^9 -THC and CBN derivatives and its pharmacological evaluation in mice.²⁾ We indicated that these halogenated cannabinoid derivatives had some psychotropic activity in mice, and the pharmacological effects of monohalogenated derivatives of Δ^9 -THC and CBN were comparable to those of parent compounds, whereas dihalogenated derivatives were much less active.

1 is known to possess barbiturate-induced sleep prolonging and anticonvulsant effects,³⁾ although this cannabinoid is devoid of the psychomimetic activity observed in THC. The preclinical studies suggest that **1** is a useful drug in treatment of epilepsy.⁴⁾ It is important to know the structure–activity relationship of halogenated cannabinoids for better understanding of cannabinoid effects.

The present study was designed to determine pharmacological effects in mice of halogenated derivatives of **1** on the aromatic ring.

Experimental

1 was isolated and purified from cannabis leaves according to the method of Aramaki *et al.*⁵⁾ Its purity used was determined to be at least 98% by gas chromatography. Sodium pentobarbital and sodium hexobarbital were from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan) and pentylenetetrazol (PTZ) and strychnine nitrate were purchased from Aldrich Chem. Co. Inc. (Mil-

waukee, WI). 18-Crown-6, *m*-chloroperbenzoic acid (MCPBA), picrotoxin and sodium barbitol were obtained from Wako Pure Chemical Ind. (Osaka, Japan). All other chemicals and solvents used were of analytical reagent grade.

Proton nuclear magnetic resonance (¹H-NMR) spectra were recorded on a JEOL JNM-GSX400 Fourier transform (FT)-NMR spectrometer using tetramethylsilane as an internal standard. ¹H-NMR chemical shift assignments were based on those of **1** reported previously.⁶⁾ The following abbreviations are used: s=singlet, d=doublet, t=triplet, m=multiplet and br=broad. The mass spectra (MS) were recorded on a JEOL JMS-SX-102A mass spectrometer.

General Halogenation Procedure We carried out the synthesis of halogenated derivatives (**2**, **3**, **4**, **5**) according to the method of Umemoto *et al.*⁷⁾ The synthesis of **6** and **7** followed a method described previously for halogenated derivatives of THC and CBN.²⁾

3'-Chloro-CBD (**2**) and 3',5'-Dichloro-CBD (**3**): **2**; 158 mg from **1** (254 mg 56% yield) as a yellow oil. MS *m/z*: 348 (M⁺). ¹H-NMR (CDCl₃) δ : 0.89 (3H, t, C₅-H), 1.78 (3H, s, C₁₀-H), 2.58 (2H, t, C₁-H), 3.97 (1H, m, C₃-H), 4.41, 4.55 (1H $\times$ 2, s, C₉-H), 5.54 (1H, brs, C₂-H), 6.31 (1H, s, C₅-H). **3**; 170 mg from **1** (1.589 g, 8% yield) as a yellow oil. MS *m/z*: 382 (M⁺). ¹H-NMR (CDCl₃) δ : 0.91 (3H, t, C₅-H), 1.77 (3H, s, C₁₀-H), 2.83 (2H, t, C₁-H), 4.04 (1H, m, C₃-H), 4.42, 4.53 (1H $\times$ 2, s, C₉-H), 5.46 (1H, brs, C₂-H).

3'-Bromo-CBD (**4**) and 3',5'-Dibromo-CBD (**5**): **4**; 1.42 g from **1** (1.67 g, 68% yield) as a yellow oil. MS *m/z*: 392 (M⁺). ¹H-NMR (CDCl₃) δ : 0.90 (3H, t, C₅-H), 1.78 (3H, s, C₁₀-H), 2.59 (2H, t, C₁-H), 4.00 (1H, m, C₃-H), 4.41, 4.55 (1H $\times$ 2, s, C₉-H), 5.54 (1H, brs, C₂-H), 6.33 (1H, s, C₅-H). **5**; 247 mg from **1** (380 mg, 54% yield) as a yellow oil. MS *m/z*: 470 (M⁺). ¹H-NMR (CDCl₃) δ : 0.92 (3H, t, C₅-H), 1.77 (3H, s, C₁₀-H), 2.92 (2H, t, C₁-H), 4.07 (1H, m, C₃-H), 4.41, 4.54 (1H $\times$ 2, s, C₉-H), 5.46 (1H, brs, C₂-H).

3'-Iodo-CBD (**6**) and 3',5'-Diiodo-CBD (**7**): **6**; 440 mg from **1** (617 mg, 51% yield) as a yellow oil. MS *m/z*: 440 (M⁺). ¹H-NMR (CDCl₃) δ : 0.90 (3H, t, C₅-H), 1.81 (3H, s, C₁₀-H), 2.59 (2H, t, C₁-H), 3.93 (1H, m, C₃-H),

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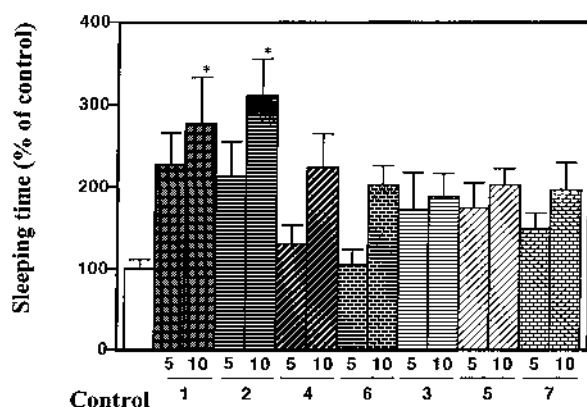
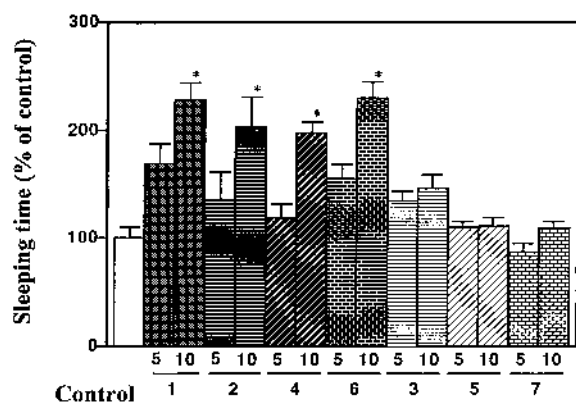
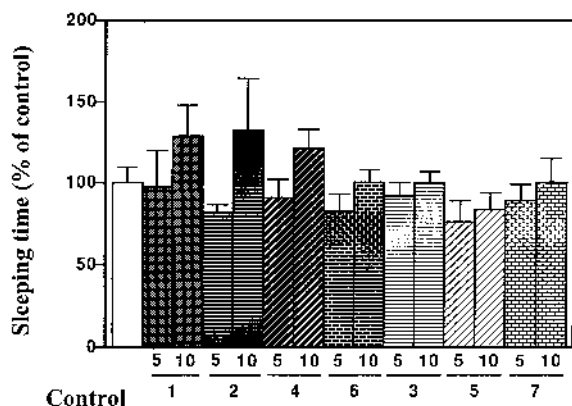
A : Pentobarbital-induced sleeping time**B : Hexobarbital-induced sleeping time****C : Barbital-induced sleeping time**

Fig. 1. Effects of CBD and Its Halogenated Derivatives on Pentobarbital-, Hexobarbital- and Barbital-Induced Sleeping Time in Mice by I.v. Injection

Pentobarbital, hexobarbital and barbital were injected to mice i.p. at 40, 100 and 300 mg/kg, respectively, 20 min after the injection of the cannabinoids (5 or 10 mg/kg, i.v.). The mean sleeping times in control mice were 22 ± 2 , 52 ± 5 and 133 ± 13 min (i.v.). Each group consisted of 5–15 mice. 1, CBD; 2, 3'-Cl-CBD; 3, 3',5'-di-Cl-CBD; 4, 3'-Br-CBD; 5, 3',5'-di-Br-CBD; 6, 3'-I-CBD; 7, 3',5'-di-I-CBD. * Significantly different from control ($p < 0.01$).

separately into vertical and horizontal components. The horizontal activity measured the total distance of an animal's movement. In this index, as well as for the vertical activity, the time period for recording the mouse behaviors was divided into total terms of 60 min for each component. Both activities tended to increase upon injection of 5 and 7, although significant activity was not observed. The horizontal and vertical activities for the groups treated with 1, 2, 3, 4, 5,

Table 2. Effects of CBD and Its Halogenated Derivatives on PTZ-, Picrotoxin- and Strychnine-Induced Seizures in Mice by I.v. Injection

Treatment	n	Latency for PTZ-induced seizures (s)	
		Clonic seizures	Tonic seizures
Control	21	139 ± 17	393 ± 53
1	11	286 ± 50*	695 ± 107
2	10	233 ± 30	667 ± 110
4	10	115 ± 13	361 ± 52
6	10	189 ± 29	641 ± 92
3	10	144 ± 53	522 ± 130
5	10	122 ± 13	376 ± 57
7	10	221 ± 41	679 ± 127

Treatment	n	Latency for Picrotoxin-induced seizures (s)	
		Clonic seizures	Tonic seizures
Control	8	327 ± 17	357 ± 23
1	8	302 ± 20	360 ± 25
2	8	303 ± 20	351 ± 20
4	8	293 ± 19	389 ± 38
6	8	387 ± 18	469 ± 40
3	8	312 ± 16	368 ± 27
5	8	312 ± 21	392 ± 44
7	8	327 ± 15	400 ± 32

Treatment	n	Latency for Strychnine-induced seizures (s)	
		Tonic seizures	
Control	16	162 ± 15	
1	8	136 ± 7	
2	8	161 ± 10	
4	12	238 ± 59	
6	8	156 ± 25	
3	8	128 ± 7	
5	11	180 ± 28	
7	8	162 ± 23	

PTZ (120 mg/kg, s.c.), picrotoxin (10 mg/kg, s.c.) and strychnine (2 mg/kg, i.p.) were injected into mice 20 min after the i.v. injection of the cannabinoids (10 mg/kg) or vehicle (physiological saline containing 1% Tween 80). * Significantly different from control ($p < 0.05$).

Table 3. Anticonvulsant Effects of CBD and Its Halogenated Derivatives Against MES in Mice

Treatment	Peak effect time (min)	MES seizures ^{a)} ED ₅₀ mg/kg (95% confidence limits)
1	15	38 (26–56)
2	15	>100
4	15	44 (37–53)
6	15	>100
3	15	>100
5	15	>100
7	15	>100

a) 50 mA, 0.2 s duration. Anticonvulsant effects of 1 and its halogenated derivatives against MES in mice were measured using a tonic extensor as end point by E.C. stimulator MK-800 15 min after the i.v. injection of the cannabinoids. E.C. stimulator MK-800 conditions: output, 50 mA; output time, 0.2 s; pulse interval, 10 msec; pulse width, 5.0 msec. Each group consisted of 8–26 mice.

6 and **7** were 2680 ± 404 , 2574 ± 315 , 3054 ± 553 , 2616 ± 430 , 2579 ± 905 , 3994 ± 652 and 4364 ± 795 cm (total distances), and 53 ± 16 , 41 ± 9 , 35 ± 14 , 49 ± 12 , 69 ± 22 , 88 ± 15 and 87 ± 18 (number of rearings), respectively. The horizontal and vertical activities for the control were 3812 ± 471 cm (total distances) and 52 ± 8 (number of rearings), respectively.

Discussion

The pharmacology and toxicology of **1** is important for at least two reasons: first, **1** is a major constituent of marihuana, though it causes no significant psychic effects and has low toxicity;¹²⁾ secondly, the preclinical evaluation of its anticonvulsant properties suggests that the drug may be a useful anti-epileptic agent.¹³⁾ The clinical potential of **1** is enhanced by its apparent lack of toxicity; for example, in man, massive intravenous doses of **1** do not produce the psychotoxic and cardiovascular effects of marihuana or Δ^9 -THC.¹⁴⁾ However, animal studies have demonstrated that **1** can prolong barbiturate sleeping time, which was subsequently shown to be the result of the ability of **1** to inhibit barbiturate metabolism by the liver.¹⁵⁾

Since the pioneering synthetic work of Adams,¹⁶⁾ it has been evident that structural modifications of the cannabinoid molecule profoundly influence the potency of this class of compounds. A major advancement in this field was achieved when the search brought about a therapeutic cannabinoid, devoid of THC-like psychotropic effects.¹⁷⁾ To attain this aim necessitates further knowledge on the structure–activity relationship of **1** derivatives. Charalambous *et al.*¹⁸⁾ and Martin *et al.*¹⁹⁾ reported the pharmacological effects of halogenated derivatives of Δ^8 - and Δ^9 -THC, although systematic evaluation of these effects of halogenated THC had not been car-

ried out at that time. Martin *et al.*¹⁹⁾ reported the enhanced and decreased potency of 2-iodo- Δ^8 -THC and 4-bromo- Δ^8 -THC, respectively, in their pharmacological effects such as rectal temperature, analgesia, spontaneous activity and immobility index. Previous studies in our laboratory demonstrated that halogenated derivatives of CBN and Δ^9 -THC exhibited cannabimimetic activity to some extent.²⁾

In the present study, we synthesized six halogenated derivatives of **1** on the aromatic ring and their pharmacological activities in mice were compared with those of **1** using the anticonvulsant effect, barbiturate-induced sleep prolongation and locomotor activity as indices for systematic evaluation. All halogenated derivatives were successfully synthesized by halogenation of **1** with potassium halides, 18-crown-6 and MCPBA in the methylene chloride as previously reported.²⁰⁾ Comparison of pharmacological effects of CBD and its halogenated derivatives in mice investigated in the present study are summarized in Table 5. The results indicate that introduction of halogens modified the pharmacological effects of **1**. Compound **2** significantly prolonged pentobarbital-induced sleeping time. Monohalogenated derivatives of **1** (10 mg/kg, i.v.) also significantly prolonged hexobarbital-induced sleeping time. In particular, **1** and **2** prolonged both hexobarbital- and pentobarbital-induced sleeping time, but not barbital-induced sleeping time. Dihalogenated derivatives also showed the prolonging effect of pentobarbital-induced sleep, but to lesser extents. These results suggest that **1** and its monohalogenated derivatives prolonged barbiturate-induced sleep by inhibition of the hepatic metabolism of barbiturates. The lack of prolongation effect of **1** and its halogenated derivatives on barbital-induced sleeping time may be related to the fact that metabolic degradation does not seem to be important for termination of action of barbital. **1** showed a significant anticonvulsant effect against PTZ-induced clonic seizures, although halogenated derivatives of **1** were not active in protecting against picrotoxin- and strychnine-induced seizures. Some of the halogenated derivatives of **1** tended to prolong seizure latency induced by PTZ (**2**, **6**, **7**), that by picrotoxin (**6**) and that by strychnine (**4**), whereas the anticonvulsant effect was statistically not significant. Compound **1** is known to possess anticonvulsant effects against MES-induced seizures.^{3b,13)} In the present study, **4** showed almost the same potency in the MES-test, although other halogenated derivatives were less active than **1**. The previous studies from our laboratories also

Table 4. Minimal and MES-Threshold Tests for CBD and 3'-Bromo-CBD

Treatment	Minimal electroshock		MES	
	Control (mA)	Treated (mA)	Control (mA)	Treated (mA)
1	7.4	8.4	10.9	13.7
4	7.4	8.4	10.9	13.4

Values are median-effective electroshock. Threshold against minimal and MES seizures in mice were measured by a tonic extensor as end point by E.C. stimulator MK-800 15 min after the i.v. injection of **1** and **4**. Each group consisted of 8 mice.

Table 5. Comparison of Pharmacological Effects of CBD and Its Halogenated Derivatives in Mice

Compound	Barbiturate synergism			Anticonvulsant effect				Locomotor activity			
	Pentobarbital	Hexobarbital	Barbital	PTZ		Picrotoxin		Strychnine	MES	Rearing	Total distance
				Clonic	Tonic	Clonic	Tonic				
1	++**	++**	±	++*	+	±	±	—	+	—	—
2	++**	++**	±	+	+	±	±	±	—	—	—
4	+	++**	±	±	±	±	±	±	+	—	—
6	+	++**	±	+	+	+	+	±	—	+	—
3	+	+	±	±	+	±	±	—	—	±	±
5	+	±	±	±	±	±	±	±	—	±	±
7	+	±	±	+	+	±	±	±	—	+	±

++; Implies significant prolongation of barbiturate-induced sleep or seizure latency against both clonic and tonic seizures induced by convulsants. +; Implies tendency to prolong barbiturate-induced sleep or seizure latency against both the clonic and tonic seizures, and tendency to increase locomotor activity of mice. —; Implies tendency to shorten seizure latency against both clonic and tonic seizures induced by convulsants, and decrease of locomotor activity of mice. ±; No change. Significantly different from control: *, $p < 0.05$; **, $p < 0.01$.

demonstrated that brominated derivatives of CBN and Δ^9 -THC showed relatively higher pharmacological effects in some cases.²⁾ The lack of significant effects of **1** and its halogenated derivatives on locomotor activity of mice indicated that change in this activity may be related to the central nervous system effect of cannabinoids.

The results indicate that pharmacological effects of monohalogenated derivatives such as **2** were comparable to those of **1**; introduction of bromine may be especially useful for modification of the anticonvulsant effect of **1**; whereas dihalogenated derivatives were much less active. In conclusion, we synthesized six **1** halogenated on the aromatic ring, and evaluated that some pharmacological effects of **1** could also be modified by its halogenation as previously studied in the CBN and Δ^9 -THC.

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