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Biological characterization of PM226, a chromenoisoxazole, as a selective CB_2 receptor agonist with neuroprotective profile

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ABSTRACT

Cannabinoids have emerged as promising neuroprotective agents due to their capability to activate specific targets, which are involved in the control of neuronal homeostasis and survival. Specifically, those ligands that selectively target and activate the CB_2 receptor may be useful for their anti-inflammatory and neuroprotective properties in various neurological disorders, with the advantage of being devoid of psychotropic effects associated with the activation of CB_1 receptors. The aim of this work has been to investigate the neuroprotective properties of 7-(1,1-dimethylheptyl)-4,4-dimethyl-9-methoxychromeno[3,4-d]isoxazole (PM226), a compound derived from a series of chromeno-isoxazoles and −pyrazoles, which seems to have a promising profile related to the CB_2 receptor. The compound binds selectively to this receptor with an affinity in the nanomolar range ($K_i = 12.8 \pm 2.4$ nM). It has negligible affinity for the CB_1 receptor ($K_i > 40000$ nM) and no activity at the GPR55. PM226 was also evaluated in GTPγS binding assays specific to the CB_2 receptor showing agonist activity ($EC_{50} = 38.67 \pm 6.70$ nM). *In silico* analysis of PM226 indicated that it has a good pharmacokinetic profile and a predicted ability to cross the blood-brain barrier. Next, PM226 was investigated in an *in vitro* model to explore its anti-inflammatory/neuroprotective properties. Conditioned media were collected from LPS-stimulated cultures of BV2 microglial cell line in the absence or presence of different doses of PM226, and then media were added to cultured M213-2O neuronal cells to record their influence on cell viability evaluated using MTT assays. As expected, cell viability was significantly reduced by the exposure to these conditioned media, while the addition of PM226 attenuated this reduction in a dose-dependent manner. This effect was reversed by co-incubating with the CB_2 antagonist SR144528, thus confirming the involvement of CB_2 receptors, whereas the addition of PM226 to neuronal cultures instead cultured BV2 cells was not effective. PM226 has also been studied in an *in vivo* model of mitochondrial damage generated by intrastriatal application of malonate in rats. MRI analysis showed that PM226 administration decreased the volume of the striatal lesion caused by malonate, effect that was confirmed after the histopathological evaluation (Nissl staining, Iba-1 immunostaining and TUNEL assay) of striatal sections derived from malonate-lesioned rats in the absence or presence of PM226. Again, the beneficial effects of PM226 were dependent on the activation of CB_2 receptors as they were reversed by blocking these receptors with AM630. Overall, PM226 has shown to have a promising neuroprotective profile derived from its ability to selectively activate CB_2 receptor, so that it could be a useful disease-modifying agent in those neurodegenerative pathologies in which the activation of these receptors may have therapeutic value.

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1. Introduction

Cannabinoid type-2 (CB_2) receptors, the so-called peripheral cannabinoid receptor type, were initially identified and characterized in immune tissues and cells [7], but further research confirmed their location also in the Central Nervous System (CNS) [3]. Within the CNS, the CB_2 receptor has been found in a few neuronal subpopulations [26,44,54], in oligodendrocytes [17,18], in neural progenitor cells [14], and predominantly in astrocytes and microglial cells, in particular when they become reactive [13]. Given this preferential location in activated glial elements, the function of CB_2 receptors has been related to the regulation by these cells of neuronal homeostasis and integrity, including: (i) the trophic role exerted by astrocytes consisting in the supply of metabolic substrates (*e.g.* lactate, ketone bodies) for neurons [5]; (ii) the generation, preferentially by astrocytes, of neurotrophins (*e.g.* GDNF), anti-inflammatory mediators (*e.g.* interleukin (IL)-10, IL-1 receptor antagonist) or prosurvival factors (*e.g.* transforming growth factor-β), which could potentially rescue damaged neurons [48,34]; (iii) the preservation of own astrocytes [19], which *per se* has been found to be beneficial for neurons, a fact also found with oligodendrocytes [33]; (iv) the control in the production of harmful mediators, such as the chemokine fractalkine by astrocytes [47], or the plethora of pro-inflammatory factors (*e.g.* tumor necrosis factor-α (TNF-α), IL-1β, nitric oxide) generated by reactive microglial cells [12,13,50]; and (v) the proliferation and migration of reactive microglial cells at lesion sites [9,55]. Therefore, these glia-related functions of CB_2 receptors situate these receptors in a promising position for being used pharmacologically as a target for attenuating neuroinflammation and neuronal deterioration that occur in neurodegenerative disorders [12,13].

Years of research have allowed the development of numerous experimental tools to work *in vitro* and *in vivo* with the CB_2 receptor in relation with several physiological processes and pathological conditions [1,3,8,12,27,49]. This includes: (i) antibodies, despite of frequent problems of specificity [32]; (ii) genetically-manipulated laboratory animals, mainly mice having deficiency [6] or overexpression [16] of these receptors, and more recently GFP reporter mice to trace CB_2 receptor expression [46]; (iii) probes for genetic analysis [28]; and (iv) numerous chemicals including selective ligands with agonist [21] or antagonist/inverse agonist [25,31] properties, and more recently potential allosteric modulators [41]. Our laboratories have been involved in the development of new chemical entities with activity at the CB_2 receptor, for example, cannabinoid o-quinone derivatives with agonist activity at this receptor and potential against breast cancer [35] or some pyrrole derivatives with selective antagonist activity [43]. We have also been engaged in the development of a novel series of compounds based on a chromenoheterocycle scaffold. We conducted structure-activity studies generating a number of chromenopyrazoles and chromenoisoxazoles whose pharmacokinetic properties were predicted with the QikProp 3.5 tool, whereas their binding profile for the CB_2, and also for the cannabinoid type-1 (CB_1) receptor, was evaluated in competition studies, resulting to be highly selective for the CB_2 receptor. Docking studies with those derivatives having better affinity were also conducted. All these data have been included in a parallel publication [36]. We are particularly interested in studying the anti-inflammatory and cytoprotective potential of one of these compounds, 7-(1,1-dimethylheptyl)-4,4-dimethyl-9-methoxychromeno[3,4-d]isoxazole (PM226), given its promising pharmacokinetic and pharmacodynamic properties. Here, we present the chemical synthesis, binding characterization, pharmacokinetic analysis and *in vitro* and *in vivo* evaluation in models of neuronal damage for this chromenoisoxazole.

2. Materials and methods

2.1. Chemistry: general methods and materials

Reagents and solvents were purchased from Sigma-Aldrich Co. (Madrid, Spain), Fluorochem (Glossop, UK), Acros Organics (Madrid, Spain), Manchester Organics (Cheshire, UK) and Lab-Scan (Symta, Madrid, Spain) and were used without further purification or drying. Silica gel 60 F254 (0.2 mm) thin layer plates were purchased from Merck GmbH (VWR, Madrid, Spain). Microwave assisted organic synthesis was performed using the microwave reactor Biotage Initiator (NetInterlab, Madrid, Spain). Compounds **1**, **2** and PM226 were purified using flash column chromatography (Merck Silica gel 60, 230–400 mesh) or medium pressure chromatography using Biotage Isolera One (NetInterlab, Madrid, Spain) with prepacked silica gel columns (Biotage SNAP cartridges; Irida Ibérica, Madrid, Spain). The compounds were characterized by a combination of NMR experiments, HPLC–MS, high-resolution mass spectrometry (HRMS) and elemental analysis. HPLC–MS analysis was performed on a Waters 2695HPLC system equipped with a photodiode array 2996 coupled to Micromass ZQ 2000 mass spectrometer (ESI–MS), using a reverse-phase column SunFireTM (C-18, 4.6 × 50 mm, 3.5 μm) in a 10 min gradient A: CH_3CN/0.1% formic acid, B: H_2O/0.1% formic acid visualizing at λ = 254 nm. Flow rate was 1 mL/min. Elemental analyses of the compounds were performed using a LECO CHNS-932 apparatus (Sartorius, Alcobendas, Spain). Deviations of the elemental analysis results from the calculated are within ± 0.4%. ^1H, ^{13}C, HSQC and HMBC NMR spectra were recorded on a Bruker 300 (300 and 75 MHz), or a Varian 500 (500 and 126 MHz) at 25 °C. Samples were prepared as solutions in deuterated solvent and referenced to internal non-deuterated solvent peak. Chemical shifts were expressed in ppm (δ) downfield of tetramethylsilane. Coupling constants are given in hertzs (Hz). Melting points were measured on a MP 70 apparatus (Mettler-Toledo, Coslada, Spain). The synthesis of PM226 is depicted in Fig. 1A and consists in the following steps:

7-(1,1-Dimethylheptyl)-5-hydroxy-3-hydroxymethylene-2,2-dimethylchroman-4-one (compound 1): It was synthesized as described by Cumella et al. [10].

7-(1,1-Dimethylheptyl)-4,4-dimethyl-4*H*-chromeno[3,4-*d*]isoxazol-9-ol (compound 2): a solution of **1** (0.12 g, 0.35 mmol) and hydroxylamine hydrochloride (49 mg, 0.71 mmol) in ethanol (4 mL) was refluxed for 45 min. After cooling the mixture, the crude was filtered and washed with cold ethanol. After removal of the solvent, the crude was purified by chromatography on silica gel (hexane/ethyl acetate, 2:1) to obtain **2** as a pale yellow solid (0.11 g; 91%); mp: 109–111 °C; ^1H NMR (300 MHz, $CDCl_3$) δ: 8.12 (s, 1H, 3-H), 6.57 (d, *J* = 1.6 Hz, 1H, 8-H), 6.55 (d, *J* = 1.4 Hz, 6-H), 1.64 (s, 6H, $OC(CH_3)_2$), 1.59–1.47 (m, 2H, 2′-H), 1.24 (s, 6H, $C(CH_3)_2$), 1.17–1.09 (br s, 8H, 3′-H, 4′-H, 5′-H, 6′-H), 0.82 ppm (t, *J* = 7.0 Hz, 3H, 7′-H); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 160.2 (9-C), 156.6 (9b-C), 153.6 (5a-C), 151.7 (7-C), 145.4 (3-C), 119.4 (9a-C), 109.5 (8-C), 108.1 (6-C), 105.9 (3a-C), 78.0 ($OC(CH_3)_2$), 44.5 (2′-C), 38.5 ($C(CH_3)_2$), 31.9, 30.1, 24.8 (3′-C, 4′-C, 5′-C), 29.2 ($C(CH_3)_2$), 28.9 ($OC(CH_3)_2$), 22.9 (6′-C), 14.3 ppm (7′-C); HPLC–MS: [A, 80% → 95%], t_R: 2.77 min, (99%); MS (ES^+, *m*/*z*) 344 $[M+H]^+$; Anal. calcd. for $C_{21}H_{29}NO_3$: C 73.44, H 8.51, found: C 73.81, H, 8.59.

7-(1,1-Dimethylheptyl)-9-methoxy-4,4-dimethyl-4*H*-chromeno[3,4-*d*]isoxazole (PM226): A solution of **2** (30 mg, 0.09 mmol), in anhydrous THF (2 mL) was added dropwise to a precooled suspension of sodium hydride (3 mg, 0.13 mmol) in anhydrous THF (1 mL) under nitrogen atmosphere. The resulting yellow solution was stirred for 10 min at room temperature. Then, iodomethane (16 μL, 0.26 mmol) was rapidly added. The reaction mixture was refluxed for 1 h. The crude was diluted with diethyl ether, filtered and concentrated under vacuum. Column