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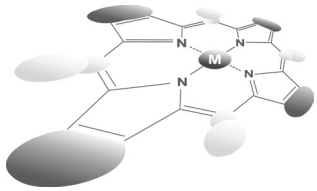

Synthesis of a novel CB_2 cannabinoid-porphyrin conjugate based on an antitumor chromenopyrazoledione

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ABSTRACT: With the objective of developing an antitumor agent, the synthesis of a chromenopyrazoledione conjugated to a tetraphenylporphyrin is described. A complete conformational analysis of the novel porphyrin conjugate was performed using *ab initio* Hartree–Fock calculations at the 6-31G* level. The novel conjugate (**14**) shows stronger absorption intensity for both Soret and Q-bands than the free *meso*-tetraphenylporphyrin. It binds weakly but selectively to the cannabinoid receptor type-2. During the synthetic approach, a new tetraphenylporphyrin, 5-[4-(3,5-dioxomorpholino)phenyl]-10,15,20-triphenylporphyrin (**10**), has been characterized.

KEYWORDS: tetraphenylporphyrin, chromenopyrazole, cannabinoid, bioconjugate, cancer, antitumor.

INTRODUCTION

Cancer is a multifactorial disease that involves numerous pathological processes. Therefore, the combination of different therapies represents a promising strategy in the treatment of malignant neoplasms. G-protein-coupled cannabinoid receptor type-1 (CB_1R) and type-2 (CB_2R) have emerged as promising therapeutic targets for cancer treatment [1]. We recently described chromenopyrazolediones with *in vivo* antitumor activity [2, 3]. The *para*-chromenopyrazoles were efficient for prostate cancer cell lines [3], while the *ortho*-chromenopyrazoles revealed to be potent for triple negative breast cancer [2]. Moreover, these later have shown selectivity for CB_2R which is an advantage due to the lack of unwanted psychoactive effects generated by activation of CB_1R in the brain. They exert antitumor effect by inducing cell apoptosis through activation of CB_2R and through oxidative stress. It is worthy to mention that these chromenopyrazolediones did not show cytotoxicity on organs such as liver, spleen, lungs, and heart *in vivo* [2].

Another effective and minimally invasive therapy for cancer treatment is the photodynamic therapy (PDT) [4, 5]. PDT has already been clinically approved for the treatment of various types of malignant disorders such as bladder, lung or esophageal cancer [6]. This technique involves the administration of a photosensitizer (PS) followed by its activation in the solid tumor by light irradiation at a specific wavelength. In the presence of tissue oxygen, the photoactive sensitizer triggers a series of photochemical processes that lead to direct cancer cell death and tumor microvascular damage [7, 8]. Different cell death pathways may be evoked by PDT: apoptosis, necrosis and autophagy [9]. The presence of high amount of collagen and lipid contributes to a preferential accumulation of the photosensitizer by malignant cell types. Therefore, this therapeutic procedure exerts a certain cytotoxic selectivity for cancer cells. In this

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context, the strategy proposed here consists in combining PDT with cannabinoid antitumor agents.

These last years, strategies have been explored in which porphyrins are conjugated to molecules showing preferential accumulation for tumor tissues or having affinity for receptors expressed in tumors [10]. Most intensive efforts have been generated for the use of carriers such as nanoparticles [11–13], liposomes [14], polymers [15], translocator protein [16], glycoprotein [17], antibodies [18], or cyclodextrins [19] to enhance the efficiency of the photosensitizers. Another strategy is the conjugation of a therapeutic drug to a porphyrin with two different approaches: combining a photosensitizer with a therapeutic agent or using porphyrins as carriers due to their ability to accumulate in cancer tissues as compared to normal tissues [20]. For instance, the photosensitizer temoporfin has been conjugated to non-steroidal anti-inflammatory compounds to improve the post-PDT treatment tumor regrowth [21]. The cytotoxic agent trilobolide, a sesquiterpene lactone inductor of nitroxic oxide, has been lately conjugated to porphyrin to increase its taking up by cancer cells [22]. The use of porphyrins as translocation vectors has also been examined with the anticancer agent doxorubicin that has been conjugated to porphyrazine through an acid-labile oxime linker [23].

The aim of the current study is the synthesis of a cannabinoid-porphyrin conjugate based on our antitumor chromenopyrazoledione (Fig. 1) [3]. During the course of this research, Bai *et al*. [24] reported the first CB_2R-targeted photosensitizer (IR700DX-mbc94). Phototherapy treatment using IR700DX-mbc94 greatly inhibited the growth of expressed CB_2R tumors but not tumors that were not expressing CB_2R [25].

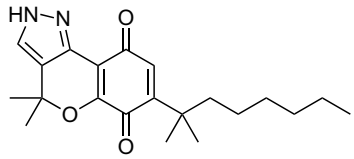


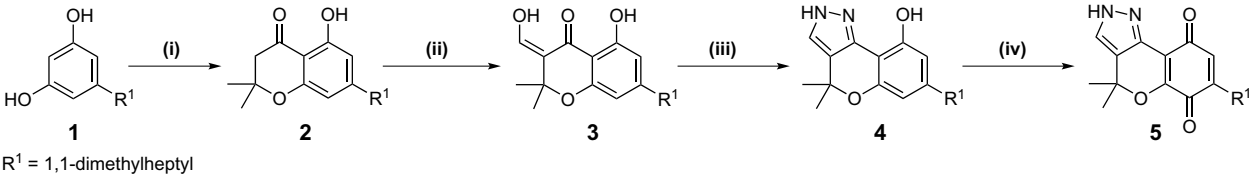
Fig. 1. *para*-Chromenopyrazoledione: an antitumoral agent

RESULTS AND DISCUSSION

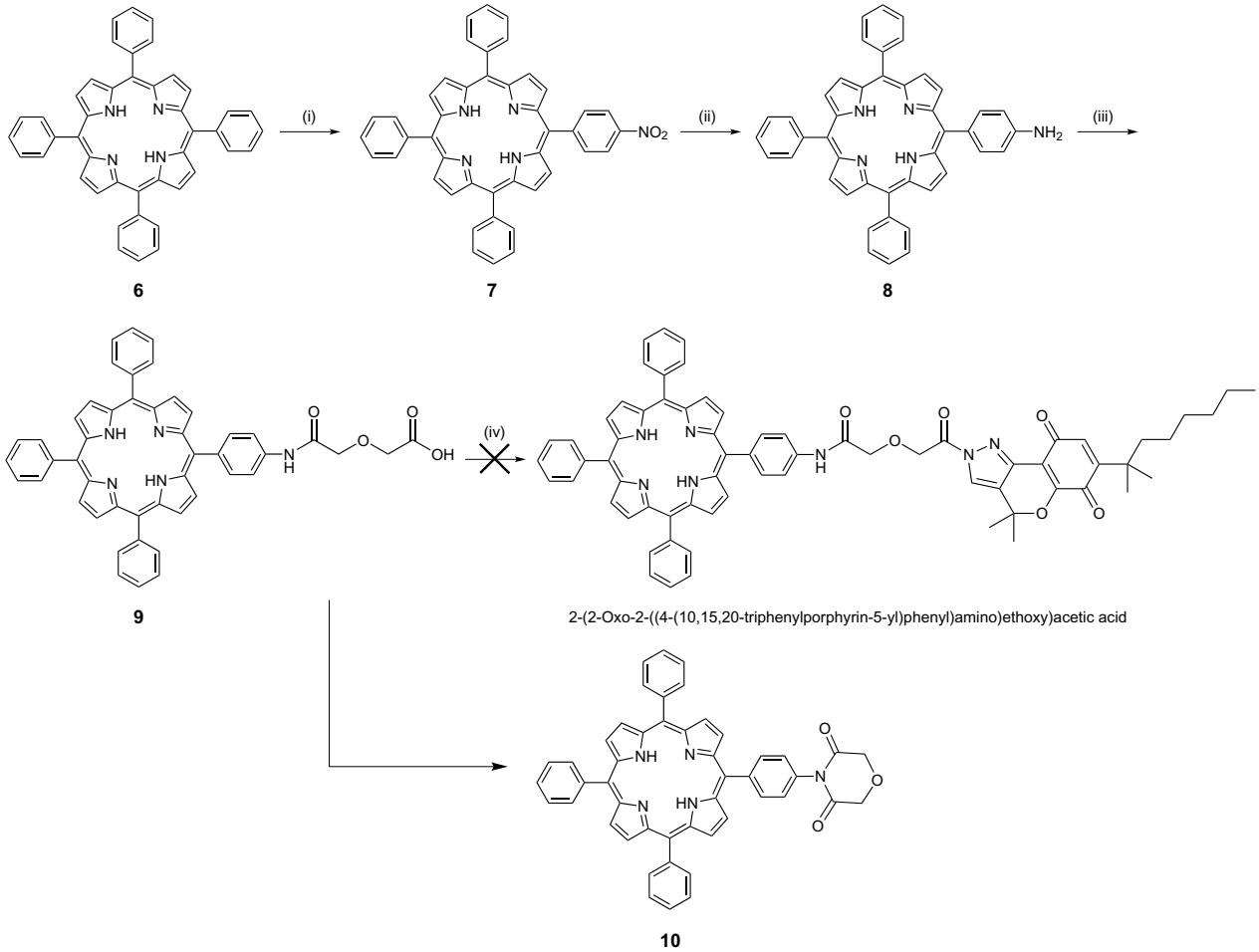
Synthesis

Firstly, 7-(1,1-dimethylheptyl)-1,4-dihydro-4,4-dimethylchromeno[4,3-*c*]pyrazol-6,9-dione (**5**) was synthesized according to the route previously described by us (Scheme 1) [3]. Preparation of the porphyrin moiety started from the commercially available *meso*-tetraphenylporphyrin (TPP, **6**) which was regioselectively *para*-nitrated to 5-(*p*-nitrophenyl)-10,15,20-triphenylporphyrin (**7**). The mononitro functionality was introduced using 1.8 equiv of sodium nitrite in the presence of TFA. This regiospecific mild procedure for electrophilic nitration at the *para* position of the phenyl groups in TPP was previously reported by Luguya *et al.* [26]. This approach provides selective control in the number of nitrated phenyl groups by varying the amount of sodium nitrite and the duration of the reaction. Nitroporphyrin **7** was then easily reduced with tin (II) chloride to obtain 5-(*p*-aminophenyl)-10,15,20-triphenylporphyrin **8** (Scheme 2).

The conversion of the amino group of porphyrin **8** to the carboxylic acid **9**, was achieved by reaction with diglycolic anhydride in DMF [27]. Unfortunately, the coupling of porphyrin **9** with chromenopyrazoledione **5** that was attempted through the following procedures was not achieved in our hands. We first proposed the conversion of the carboxylic acid **9** to the corresponding acid chloride by thionyl chloride followed by reaction with **5**. This procedure failed to give the desired amide. Then, different coupling reagents such as carbodiimides [carbonyldiimidazole (CDI)] or more potent coupling reagents such as phosphonium [(benzotriazol-1-yloxy)-tris[pyrrolidino] phosphonium hexafluorophosphate (PyBOP)] or uronium salts [hexafluorophosphate salt of the *O*-(7-azabenzotriazolyl)tetramethyl uranium (HATU)] in the presence of a base and dry DMF as solvent were also unsuccessful to give the desired cannabinoid-porphyrin conjugate. In most of these attempts, intramolecular cyclization of the 2-(2-amino-2-oxoethoxy)acetic acid group of compound **9** underwent the formation of a morpholine-3,5-dione affording porphyrin **10**. To our knowledge, 5-[4-(3,5-dioxomorpholino)phenyl]-10,15,20-triphenylporphyrin **10** has never been described in the literature. This intramolecular cyclization has scarcely



Scheme 1. Synthesis of 7-(1′,1′-dimethylheptyl)-dihydro-4,4-dimethylchromeno[4,3-*c*]pyrazol-6,9-dione. Reaction conditions. (i) 3,3-dimethylacrylic acid, CH_3SO_3H, P_2O_5, 70 °C, M.W., 10 min (81%). (ii) NaH, THF, M.W., 46 °C, 20 min then ethyl formate, THF, M.W., 46 °C, 20 min (76%). (iii) H_2N–NH_2, EtOH, 16 h, room temperature, (81%). (iv) [bis(trifluoro-acetoxy)iodo]benzene, ACN/H_2O (6:1), 15 min, room temperature (21%)



Scheme 2. Synthesis of porphyrin derivatives. Reaction conditions. (i) $NaNO_2$ (1.8 equiv), TFA, 25 °C, 3 min (49%). (ii) $SnCl_2$, conc. HCl, 65 °C, 1 h (96%). (iii) Diglycolic anhydride, DMF, rt, 24 h (85%). (iv) (a) $SOCl_2$, toluene, 120 °C, 30 min, MW; (b) chromenopyrazole **5**, NaH, CH_2Cl_2, rt, overnight

been studied in the literature in which the morpholine-3,5-dione was described as a by-product [28–30].

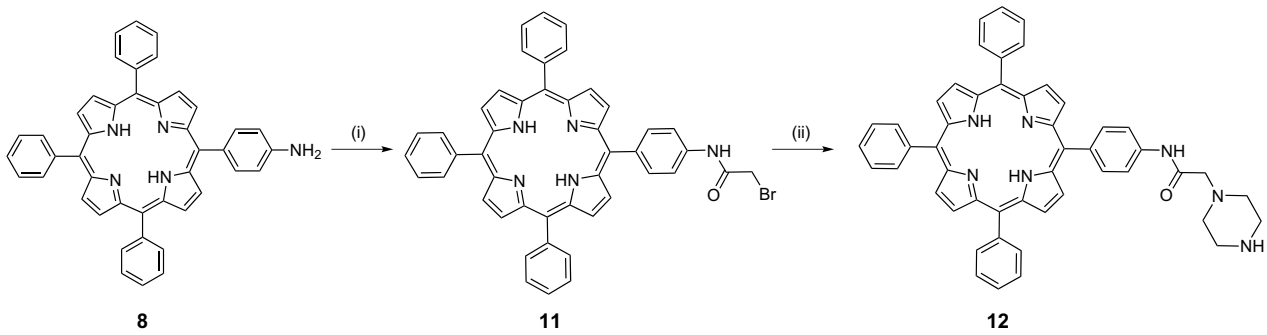
To avoid this intramolecular cyclization, another synthetic approach using butane-1,3-dione unstead of 1,1′-oxybis(ethane-2-one) as linker between the porphyrin and the chromenopyrazole was attempted without success.

After these consecutive synthetic failures in obtaining the desired cannabinoid-porphyrin conjugate, we decided to use the piperazine derivative **12** as starting material for the coupling with chromenopyrazole **5**. Interestingly, the piperazine moiety is an appropriate linker because of its low toxicity and biotransformation that involves several well-known metabolic reactions [31]. 5-(4α-piperazineacetylamidophenyl)-10,15,20-triphenylporphyrin (**12**) was previously described by Gaware *et al.* [32] as intermediate in the preparation of a conjugate of tetraphenylporphyrin with glucosamines. Thus, aminoporphyrin **8** was firstly acylated using bromoacetyl bromide to give 5-(4α-bromoacetylamidophenyl)-10,15,20-triphenylporphyrin (**11**). Then, a nucleophilic substitution with piperazine afforded the nucleophilic porphyrin intermediate 5-(4α-piperazineacetylamidophenyl)-10,15,-20-triphenylporphyrin (**12**) (Scheme 3). Finally, the synthesis of the porphyrin-cannabinoid conjugate **14** was achieved as depicted in Scheme 4. Acylation of chromenopyrazoledione **5** using bromoacetyl bromide afforded the substituted chromenopyrazole **13** that was then allowed to alkylate the piperazine intermediate **9** affording the desired conjugate **14**.

Attempts to directly link the aminoporphyrin **8** to compound **13** did not give the porphyrin-chromenopyrazoledione conjugate in our experiments. This fact may be due to the weak nucleophilicity of the aminoporphyrin [33].

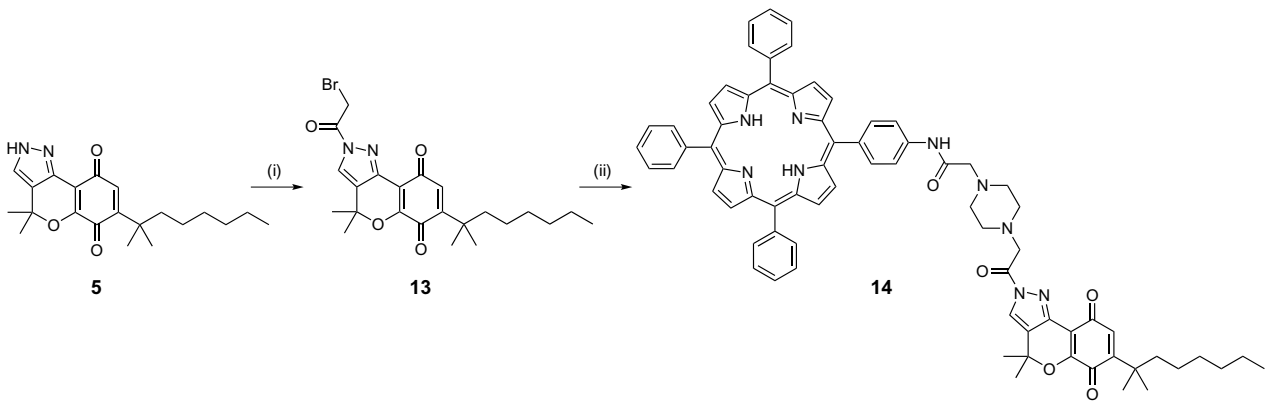
Conformational analysis of the porphyrin-chromenopyrazoledione conjugate

A complete conformational analysis of the novel porphyrin conjugate **14** was performed using *ab initio* Hartree–Fock calculations at the 6-31G* level as encoded in Spartan ´08 (Wave function, Inc., Irvine CA). As displayed in Fig. 2, the global minimum energy conformer



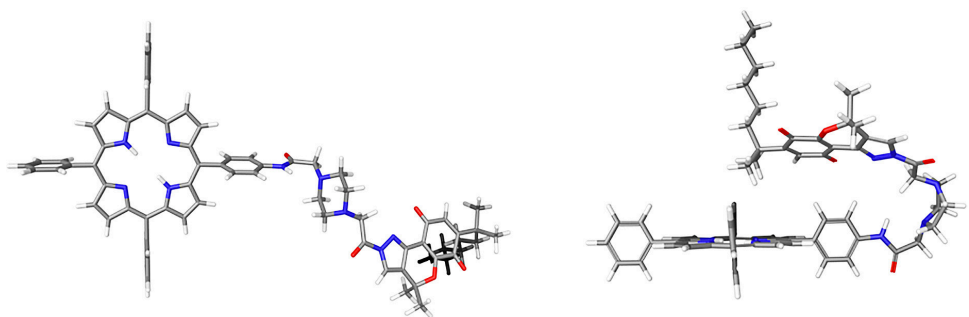
8 11 12

Scheme 3. Synthesis of the porphyrin-piperazine intermediate **12**. Reaction conditions. (i) Bromoacetyl bromide, Et_3N, CH_2Cl_2, 25 °C, 1 h (31%). (ii) Piperazine, CH_2Cl_2, 25 °C, 45 min (96%)



5 13 14

Scheme 4. Synthesis of the porphyrin-chromenopyrazoledione conjugate **14**. Reaction conditions. (i) Bromoacetyl bromide, Et_3N, CH_2Cl_2, 25 °C, 1 h (37%). (ii) Porphyrin **9**, Et_3N, CH_2Cl_2, 25 °C, overnight (12%)



(a) (b)

Fig. 2. (a) Global minimum energy conformer of compound **14** (ΔE: -0.11 Kcal/mol). (b) Higher energy conformer of compound **14** showed for comparison (ΔE: 4.55 Kcal/mol)

of conjugate **14** adopts an expanded spatial conformation whereas folded conformers (Fig. 2b) exert higher relative energy values. Nonetheless, these theoretical values are calculated under vacuum conditions. Physiological conditions may influence these conformations.

Photophysicochemical properties

The UV-vis spectra of the porphyrin-chromeno-pyrazoledione conjugate **14** and the free *meso*-tetraphenyl-porphyrin (TPP) were recorded at 0.1 mM. A Soret band with absorption maxima near 420 nm and medium Q-bands at 500–700 nm were observed for both porphyrins (Fig. 3). As clearly depicted in Fig. 3, compound **14** shows stronger absorption intensity for both Soret and Q-bands than the free TPP (Table 1). This suggests aggregation processes for the conjugate **14** that are less intense than for TPP. Broadening of Soret band is characteristic of π–π stacking and hydrophobic interactions in porphyrin systems. The aggregate formation is clearly affected by the ionic strength of the solvent (Fig. 3) [32, 34].

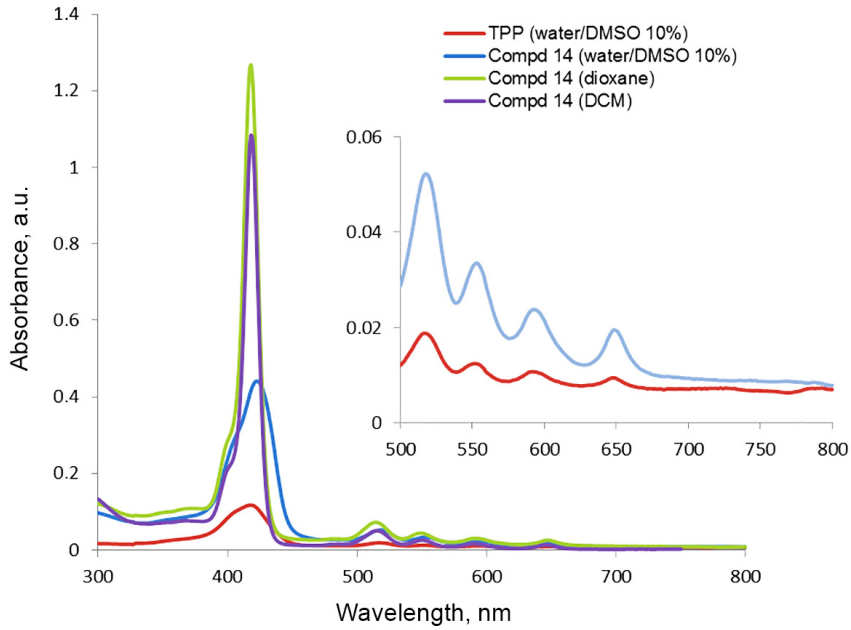


Fig. 3. UV-vis absorption spectra of **14** and tetraphenylporphyrin (TPP) at constant concentration (0.1 mM) in different solvents at room temperature

Table 1. Absorption maxima and molecular extinction coefficients of **14** and tetraphenylporphyrin (TPP) in different solvents at room temperature

	TPP (water/DMSO)		Compd **14** (water/DMSO)		Compd **14** (dioxane)		Compd **14** (DCM)	
	λ_{max}, nm	ε, M^{-1} cm^{-1}	λ_{max}, nm	ε, M^{-1} cm^{-1}	λ_{max}, nm	ε, M^{-1} cm^{-1}	λ_{max}, nm	ε, M^{-1} cm^{-1}
Soret band	418	17763	423	69244.8	418	201449	418	173102.4
	517	1984	518	6985.6	514	10276	515	7721
Q-band	552	968	553	3979.2	549	5752	550	3782
	591	688	592	2417.6	591	3564	590	2300
	648	479	648	1731.2	647	2809	646	1795

The fluorescence intensity of the TPP and compound **14** in aqueous solution at 0.1 mM did not show detectable emission. This absence of fluorescence may be caused by porphyrin-solvent interactions promoting non-radiative decay or self-aggregation of porphyrin molecules. Excitation of compound **14** dissolved in DCM (0.1 mM) at 418 nm resulted in the fluorescence spectrum displayed in Fig. 4.

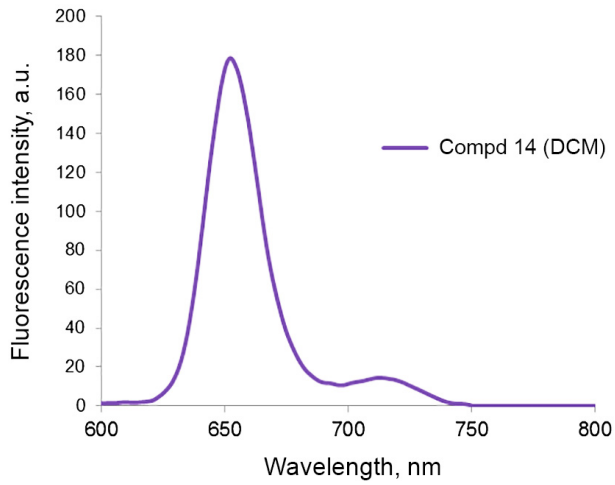


Fig. 4. Fluorescence spectrum of compound **14** (0.1 mM) under excitation with light of 418 nm in dichloromethane (slit width: 15–15 nm, and 1 cm path length)

Cannabinoid receptor affinity

The cannabinoid binding affinity of the porphyrin-chromenopyrazoledione conjugate **14** was evaluated by radioligand competition experiments for both receptor types CB_1R and CB_2R. The porphyrin-piperazine intermediate **12** was also appraised in these assays. As depicted in Table 2, the new conjugate **14** displayed very low affinity for CB_2R and did not bind to CB_1R. Thus, compound **14** does not retain the affinity of its

Table 2. Binding affinity of the chromenopyrazole **5**, the porphyrin-chromenopyrazoledione conjugate **14**, the porphyrin intermediate **12** and the reference cannabinoid WIN55,212-2 for hCB$_1$R and hCB$_2$R

Compound	CB$_1$R K_i, μM[a]	CB$_2$R K_i, μM[a]
5	0.32 ± 0.23	0.13 ± 0.02
12	>40	>40
14	>40	13.79 ± 0.20
WIN55,212-2	0.04 ± 0.08	0.003 ± 0.002

[a]Values obtained from competition curves using [^{3}H] CP55940 as radioligand for hCB$_1$R and hCB$_2$R and are expressed as the mean ± SEM of at least three experiments.

chromenopyrazole precursor **5**. The TPP intermediate **12** did not show binding affinity for both receptor types.

EXPERIMENTAL

Chemistry

General methods and materials. Reagents and solvents were purchased from Sigma-Aldrich Co., Fluorochem, Acros Organics, Manchester Organics and Lab-Scan and were used without further purification or drying. Silica gel 60 F254 (0.2 mm) thin layer plates were purchased from Merck GmbH. Products were purified using flash column chromatography (Merck Silica gel 60, 230–400 mesh). The compounds were characterized by a combination of NMR experiments, HPLC-MS, and high-resolution mass spectrometry (HRMS). HPLC-MS analysis was performed on a Waters 2695 HPLC system equipped with a photodiode array 2996 coupled to Micromass ZQ 2000 mass spectrometer (ESI-MS), using a reverse-phase column SunFire™ (C-18, 4.6 × 50 mm, 3.5 μm) in 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. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker (300 and 75 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. Coupling constants are given in hertz (Hz). The purity of the novel compounds was determined by LC coupled to HRMS. The experiment was performed in a LC-MS hybrid quadrupole/time of flight (QTOF) analyzer equipped with an Agilent 1200 LC coupled to an Agilent 6500 Accurate Mass (1–2 ppm mass accuracy) using electrospray ionization in the positive mode (ESI$^+$). Elemental analyses of the compounds were performed using a LECO CHNS-932 apparatus. Deviations of the elemental analysis results from the calculated are within ±0.4%. UV-vis measurements were recorded on a Perkin-Elmer Lambda 25 UV-vis spectrometer. Fluorescence emission spectra for quantum yield were obtained using a SPEX FluoroMax spectrometer (Spectrocell Corporation, Oreland, PA, USA).

Synthesis

7-(1,1-Dimethylheptyl)-5-hydroxy-2,2-dimethyl-chroman-4-one (2) [3]. 5-(1,1-Dimethylheptyl)resorcinol (**1**) (2.50 g, 10.59 mmol) and 3,3-dimethylacrylic acid (1.59 g, 15.88 mmol) both dissolved in methanesulfonic acid (16 mL, 0.24 mmol) were added to P_2O_5 (1.20 g, 8.81 mmol) under nitrogen atmosphere. Then, the reaction mixture was stirred 8 h at 70 °C. Afterwards, water was added (50 mL) and the product was extracted with EtOAc (3 × 50 mL). The combined organic layers were dried over $MgSO_4$. The organic solvent was evaporated under reduced pressure and the crude was purified by column chromatography on silica gel (hexane/EtOAc, 5:1), obtaining the desired compound as a pale yellow solid. Yield 2.77 g (81%), mp 50–52 °C. ^{1}H NMR (300 MHz; $CDCl_3$; Me_4Si): δ_H, ppm 11.53 (s, 1H), 6.45 (d, J = 1.6 Hz, 1H), 6.37 (d, J = 1.6 Hz, 1H), 2.71 (s, 2H), 1.60–1.49 (m, 2H), 1.47 (s, 6H), 1.22 (s, 6H), 1.23–1.17 (m, 6H), 1.11–0.94 (m, 2H) 0.84 (t, J = 6.6 Hz, 3H). ^{13}C NMR (75 MHz; $CDCl_3$): δ_C, ppm 197.4, 162.6, 161.3, 159.5, 106.6, 105.7, 105.3, 78.9, 48.1, 44.0, 38.7, 31.7, 29.9, 22.6, 28.4, 26.7, 24.6, 14.0. HPLC-MS: [A, 80→95%], t_R: 4.94 min, (95%). MS (ES$^+$): m/z 319 [M + H]$^+$. Anal. calcd. for $C_{20}H_{30}O_3$: C, 75.43; H, 9.50. Found C, 75.52; H 9.64.

7-(1,1-Dimethylheptyl)-5-hydroxy-3-hydroxy-methylene-2,2-dimethylchroman-4-one (3) [3]. A solution of **2** (0.40 g, 1.25 mmol) in anhydrous THF (8 mL) was added to a vial containing dry sodium hydride (0.30 g, 12.57 mmol) under nitrogen atmosphere. The mixture was irradiated under microwave at 45 °C for 25 min. Subsequently, ethyl formate (2.88 mL, 37.70 mmol) was added to the sealed vial and it was irradiated under microwave at 45 °C for 25 min. Water was added and the product was extracted with EtOAc (3 × 5 mL). The combined organic layers were dried over $MgSO_4$ and the solvent was evaporated under reduced pressure. The crude was purified by column chromatography on silica gel (hexane/EtOAc, 4:1), to afford compound **3** as a yellow oil. Yield 0.33 g (76%). ^{1}H NMR (300 MHz; $CDCl_3$; Me_4Si): δ_H, ppm 13.49 (d, J = 11.7 Hz, 1H), 11.28 (s, 1H), 7.34 (d, J = 11.7 Hz, 1H), 6.47 (d, J = 1.6 Hz, 1H), 6.36 (d, J = 1.6 Hz), 1.58 (s, 6H), 1.56–1.46 (m, 2H), 1.22 (s, 6H), 1.14–1.28 (m, 6H), 1.10–1.04 (m, 2H), 0.84 (t, J = 6.5 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ_C, ppm 189.4, 162.7, 161.6, 161.5, 158.7, 114.4, 107.4, 106.2, 104.9, 78.3, 44.4, 38.8, 31.7, 29.9, 22.6, 28.4, 28.2, 24.6, 14.1. HPLC-MS: [A, 80→95%], t_R: 2.88 min, (97%). MS (ES$^+$): m/z 347 [M + H]$^+$. Anal. calcd. for $C_{21}H_{30}O_4$: C, 72.80; H, 8.73. Found C, 73.07; H 8.64.

7-(1,1-Dimethylheptyl)-2,4-dihydro-4,4-dimethylchromeno[4,3-*c*]pyrazol-9-ol (4) [3]. A solution of **3** (0.50 g, 1.44 mmol) and anhydrous hydrazine (0.11 mL, 3.61 mmol) in EtOH (9 mL) was stirred during 4 h at 40 °C. The solvent was evaporated under reduced pressure and the crude was purified by column chromatography on silica gel (hexane/EtOAc, 2:1) to furnish **4** as a yellow oil. Yield 0.40 g (81%). ^{1}H NMR (300 MHz; $CDCl_3$; Me_4Si): δ_H, ppm 7.32–7.29 (br s, 1H), 6.58 (d, J = 1.5 Hz, 1H), 6.51 (d, J = 1.5 Hz), 6.48 (s, 1H), 1.63 (s, 6H), 1.58–1.52 (m, 2H), 1.25 (s, 6H), 1.18 (s, 6H), 1.12–1.05 (m, 2H), 0.83 (t, J = 6.7 Hz, 3H). ^{13}C NMR (75 MHz; $CDCl_3$): δ_C, ppm 153.7, 153.5, 153.4, 144.1, 129.1, 123.4, 106.8, 106.5, 101.7, 77.0, 44.9, 38.4, 32.2, 30.4, 30.0, 29.3, 25.0, 23.1, 14.5. HPLC-MS: [A, 80→95%], t_R: 3.80 min, (98%). MS (ES$^+$): *m/z* 343 [M + H]$^+$. Anal. calcd. for $C_{21}H_{30}N_2O_2$: C, 73.65; H, 8.83. Found C, 74.01; H, 8.59.

7-(1,1-Dimethylheptyl)-1,4-dihydro-4,4-dimethylchromeno[4,3-*c*]pyrazol-6,9-dione (5) [3]**.** To a solution of 7-(1,1-dimethylheptyl)-1,4-dihydro-4,4-dimethylchromeno[4,3-*c*]pyrazol-9-ol (**4**) (130 mg, 0.38 mmol) in MeCN/H_2O (6:1, 2.5 mL) a solution of BTIB (490 mg, 1.14 mmol) in MeCN/H_2O (6:1, 2 mL) was added dropwise. The reaction mixture was stirred at room temperature for 15 min, neutralized with aqueous $NaHCO_3$ saturated solution, and extracted with diethyl ether. The organic layer was washed with H_2O, dried over $MgSO_4$ and concentrated. Column chromatography on silica gel (hexane/EtOAc, 1:2) afforded the title compound as a red solid. Yield 29 mg (21%). mp 85–86 °C. ^{1}H NMR (300 MHz; $CDCl_3$; Me_4Si): δ_H, ppm 8.41 (br s, 1H), 7.40 (s, 1H), 6.69 (s, 1H), 1.59–1.57 (br s, 6H), 1.55–1.48 (m, 2H), 1.30 (s, 6H), 1.27–1.23 (m, 6H), 1.19–1.12 (br s, 2H), 0.86–0.82 (m, 3H). ^{13}C NMR (75 MHz; $CDCl_3$): δ_C, ppm 184.1, 180.9, 160.2, 161.2, 137.8, 132.0, 130.4, 129.5, 113.8, 78.6, 43.3, 30.9, 29.6, 28.7, 27.4, 25.1, 23.2, 21.8, 14.0. HPLC-MS: [A, 70%→100%], t_R: 3.37 min (98%). MS (ES$^+$): *m/z* 357 [M + H]$^+$. Anal. calcd. for $C_{21}H_{28}N_2O_3$: C, 70.76; H, 7.92. Found C, 71.03; H, 8.24.

5-(4-Nitrophenyl)-10,15,20-triphenylporphyrin (7) [26]. To a solution of *meso*-tetraphenylporphyrin (TPP, **6**) (500 mg, 0.81 mmol) in TFA (25 mL) sodium nitrite (99 mg, 1.40 mmol) was added and the reaction mixture was stirred for 3 min at room temperature. After that, the crude was poured into water and extracted three times with CH_2Cl_2. The organic layers combined and washed with saturated aqueous $NaHCO_3$ and water. The mixture was dried over anhydrous Na_2SO_4 and the solvent was removed under vacuum. Flash column chromatography (CH_2Cl_2) provided the title compound as a purple solid. Yield 262 mg (49%). ^{1}H NMR (300 MHz; $CDCl_3$; Me_4Si): δ_H, ppm 9.03–8.99 (m, 2H), 8.86–8.79 (m, 6H), 8.59 (d, J = 8.1 Hz, 2H), 8.28 (d, J = 8.1 Hz, 2H), 8.19–8.12 (m, 6H), 7.81–7.64 (m, 9H), -2.77 (s, 2H). HPLC-MS: [iso 95%–5%], t_R: 10.0 min (94%). MS (ES$^+$): *m/z* 660 [M + H]$^+$. HRMS calcd. for $C_{44}H_{29}N_5O_2$: 659.2321. Found 659.2298.

5-(4-Aminophenyl)-10,15,20-triphenylporphyrin (8) [26]. 5-(4-Nitrophenyl)-10,15,20-triphenylporphyrin (**7**) (101 mg, 0.15 mmol) was dissolved in concentrated hydrochloric acid (10 mL) and, while stirring, tin(II) chloride (162 mg, 0.85 mmol) was carefully added. The mixture was heated to 65 °C for 1 h under nitrogen atmosphere. The crude was then poured into cold water and neutralized with ammonium hydroxide until pH 8. The aqueous solution was extracted with CH_2Cl_2 until colorless. The organic layers were combined, dried over Na_2SO_4, and the solvent was removed under reduced pressure. Flash column chromatography (CH_2Cl_2) afforded the title compound as a purple solid (92 mg, 96% yield). ^{1}H NMR (300 MHz; $CDCl_3$; Me_4Si): δ_H, ppm 8.93–8.91 (m, 2H), 8.79–8.67 (m, 6H), 8.22–8.19 (m, 6H), 8.10 (d, J = 7.8 Hz, 2H), 7.84–7.75 (m, 9H), 7.02 (d, J = 7.8 Hz, 2H), 4.02 (s, 2H), -2.69 (s, 2H). HPLC-MS: [iso 95%–5%], t_R: 6.13 min (99%). MS (ES$^+$): *m/z* 630 [M + H]$^+$. HRMS calcd. for $C_{44}H_{31}N_5$: 629.2579. Found 629.2583.

2-{2-Oxo-2-[(4-(10,15,20-triphenylporphyrin-5-yl)phenyl)amino]ethoxy}acetic acid (9) [27]. To a solution of aminoporphyrin **8** (340 mg, 0.53 mmol) in DMF (3 mL) diglycolic anhydride (93 mg, 0.80 mmol) was added. The reaction was stirred at room temperature overnight. The crude was diluted with $CHCl_3$ and hexane until precipitation occurred. The precipitate was filtered and washed with water to remove residual anhydride and then dried under vacuum to obtain the title compound as a purple solid. Yield 340.9 mg (85%). ^{1}H NMR (300 MHz; $CDCl_3$; Me_4Si): δ_H, ppm 9.01–8.94 (m, 2H), 8.82–8.79 (m, 6H), 8.31–8.16 (m, 10H), 7.78–7.64 (m, 9H), 4.61 (s, 2H), 4.45 (s, 2H), -2.75 (br s, 2H). HRMS calcd. for $C_{48}H_{35}N_5O_4$: 745.2689. Found 745.2701.

5-[4-(3,5-Dioxomorpholino)phenyl]-10,15,20-triphenylporphyrin (10). A solution of compound **9** (40 mg, 0.05 mmol) in toluene (2 mL) and $SOCl_2$ (6 µL, 0.08 mmol) was heated at 120 °C under microwave irradiation conditions for 30 min. The solvent was removed under vacuum and the corresponding acyl chloride was used for the next step without further purification. A solution of **5** (14 mg, 0.04 mmol) in anhydrous CH_2Cl_2 (1 mL) was added to a precooled suspension of NaH (3 mg, 0.12 mmol) in CH_2Cl_2, the mixture was stirred for 10 min under nitrogen atmosphere. After that, the acyl chloride (30 mg, 0.04 mmol), dissolved in anhydrous CH_2Cl_2 (1 mL), was rapidly added and the reaction was stirred for 30 min. The reaction mixture was then diluted with CH_2Cl_2 and washed with water and brine. The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. Column chromatography on silica gel (MeOH/CH_2Cl_2, 1:12) afforded the title undesired compound as a purple solid. Yield 15 mg (52%). ^{1}H NMR (300 MHz; CDCl3; Me_4Si): δ_H, ppm 8.99–8.87 (m, 2H), 8.80–8.72 (m, 6H), 8.23–8.15 (m, 8H), 7.97 (d,

J = 8.0 Hz, 2H), 7.82–7.74 (m, 9H), 4.39 (s, 4H), -2.88 (s, 2H). HPLC-MS: [iso 95%–5%], t_R: 4.31 min (99%). MS (ES^+): *m/z* 758 $[M + H]^+$. HRMS calcd. for $C_{48}H_{33}N_5O_3$: 727.2583. Found 727.2602.

5-(4α-Bromoacetylamidophenyl)-10,15,20-triphenylporphyrin (11) [32]. A solution of aminoporphyrin **8** (600 mg, 0.95 mmol) in CH_2Cl_2 (10 mL) and Et_3N (0.29 mL, 2.01 mmol) was stirred under N_2 atmosphere. Bromoacetylbromide (0.11 mL, 1.33 mmol) was added dropwise at room temperature and the reaction mixture was stirred for 1 h. The crude was diluted in CH_2Cl_2, washed with water and brine and extracted three times. The combined organic layers were dried over Na_2SO_4 and concentrated under vacuum. Flash column chromatography on silica gel (CH_2Cl_2) afforded the title compound as a purple solid. Yield 220 mg (31%). ^1H NMR (300 MHz; CDCl3; Me_4Si): δ_H, ppm 8.84–8.79 (m, 2H), 8.74–8.68 (m, 6H), 8.37–8.34 (br s, 1H), 8.26–8.11 (m, 10H), 7.79–7.63 (m, 9H), 4.22 (s, 2H), -2.81 (br s, 2H). HPLC-MS: [iso 95%–5%], t_R: 6.27 min (99%). MS (ES^+): *m/z* 751 $[M + H]^+$. HRMS calcd. for $C_{46}H_{32}BrN_5O$: 749.1790. Found 749.1814.

5-(4α-Piperazineacetylamidophenyl)-10,15,20-triphenylporphyrin (12) [32]. A mixture of bromoacetylated porphyrin **11** (50 mg, 0.06 mmol) and piperazine (34 mg, 0.39 mmol) in CH_2Cl_2 (3 mL) were stirred at room temperature for 1 h under N_2 atmosphere. The reaction mixture was then diluted with CH_2Cl_2 and washed with water and brine. The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. Column chromatography on silica gel (MeOH/CH_2Cl_2, 1:12) afforded the title compound as a purple solid. Yield 29 mg (96%). ^1H NMR (300 MHz; $CDCl_3$; Me_4Si): δ_H, ppm 9.07–8.99 (br s, 1H), 8.88–8.73 (m, 8H), 8.21–8.09 (m, 10H), 7.87–7.70 (m, 9H), 4.07 (s, 2H), 2.95–2.89 (br t, 4H), 2.73–2.68 (br t, 4H), 2.43 (br s, 1H), -2.82 (s, 2H). HPLC-MS: [A, 60%→95%], t_R: 2.42 min (90%). MS (ES^+): *m/z*) 756 $[M + H]^+$. HRMS calcd. for $C_{50}H_{41}N_7O$: 755.3373. Found 755.3351.

2-(2-Bromoacetyl)-7-(1,1-dimethylheptyl)-1,4-dihydro-4,4-dimethylchromeno[4,3-*c*]pyrazol-6,9-dione (13). Compound **5** (35 mg, 0.10 mmol) was dissolved in anhydrous CH_2Cl_2 (3 mL) and stirred under N_2 atmosphere. Et_3N (30 μL, 0.21 mmol) was added, followed by dropwise addition of bromoacetylbromide (13 μL, 0.15 mmol) at room temperature. Stirring was continued at room temperature for 1 h. The reaction mixture was diluted in CH_2Cl_2, then washed with water and brine and the product was extracted three times. The combined organic layers were then dried over Na_2SO_4 and the solvent was removed under vacuum. Column chromatography on silica gel (hexane/EtOAc, 1:2) afforded the title compound as an orange oil. Yield 17 mg (37%). ^1H NMR (300 MHz; $CDCl_3$; Me_4Si): δ_H, ppm7.74 (s, 1H), 6.85 (s, 1H), 4.52 (s, 2H), 1.64–1.59 (br s, 6H), 1.54–1.47 (m, 2H), 1.38 (s, 6H), 1.33–1.27 (m, 6H), 1.22–1.17 (br s, 2H), 0.99–0.87 (m, 3H). HPLC-MS: [A, 80% → 95%], t_R: 4.16 min (93%). MS (ES^+): *m/z* 477 $[M + H]^+$. HRMS calcd. for $C_{23}H_{29}BrN_2O_4$: 476.1311. Found 476.1328.

Porphyrin-chromenopyrazoledione conjugate (14). Compound **12** (14 mg, 0.02 mmol) dissolved in anhydrous CH_2Cl_2 (2 mL) was stirred in Et_3N (3 μL, 0.02 mmol) under N_2 atmosphere for 5 min. Compound **13** (17 mg, 0.04 mmol), dissolved in anhydrous CH_2Cl_2 (2 mL), was rapidly added and the reaction was stirred overnight at room temperature. The reaction mixture was diluted in CH_2Cl_2, then washed with water and brine and the product was extracted three times. The combined organic layers were then dried over Na_2SO_4 and the solvent was removed under vacuum. Column chromatography on silica gel (MeOH/CH_2Cl_2, 1:12) afforded the title compound as a purple solid. Yield 2.50 mg (12%). ^1H NMR (300 MHz; $CDCl_3$; Me_4Si): δ_H, ppm 9.19–9.14 (br s, 1H), 8.91–8.79 (m, 8H), 8.24–8.11 (m, 10H), 7.81 (s, 1H), 7.67–7.54 (m, 9H), 6.79 (s, 1H), 4.13 (s, 2H), 3.88 (s, 2H), 2.88–2.76 (br t, 4H), 2.63–2.60 (br t, 4H), 1.72–1.63 (m, 6H), 1.58–1.46 (m, 2H), 1.41 (s, 6H), 1.41–1.34 (m, 6H), 1.30–1.21 (br s, 2H), 0.85 (t, *J* = 7.0 Hz, 3H), -2.78 (s, 2H). ^{13}C NMR (75 MHz; $CDCl_3$): δ_C, ppm 182.7, 181.3, 170.1, 162.8, 161.5, 143.6, 139.4, 137.6, 136.1, 134.9, 134.0, 131.7, 130.9, 130.2, 128.8, 127.3, 126.5, 121.0, 119.7, 118.1, 114.3, 77.5, 62.6, 54.6, 54.2, 46.7, 44.0, 31.6, 29.9, 28.1, 26.9, 25.3, 22.8, 22.0, 14.1. HPLC-MS: [A, 60%→95%], t_R: 11.28 min (91%). MS (ES^+): *m/z* 1152 $[M + H]^+$. HRMS calcd. for $C_{73}H_{69}N_9O_5$: 1151.5422. Found 1151.5456.

Cannabinoid binding experiments

Membranes from transfected cells with human cannabinoid receptors (RBHCB1M400UA and RBXCB2M400UA) were supplied by Perkin-Elmer Life and Analytical Sciences (Boston, MA). The protein concentration for the CB_1R membranes was 8.0 $mg.mL^{-1}$, whereas for the CB_2R membranes the protein concentration was 4.0 $mg.mL^{-1}$ or 3.6 $mg.mL^{-1}$ depending on the batch. The commercial membranes were diluted (approximatively 1:20) with the binding buffer (50 mM TrisCl, 5 mM $MgCl_2$. H_2O, 2.5 mM EDTA, 0.5 $mg.mL^{-1}$ BSA and pH = 7.4 for CB_1R binding; 50 mM TrisCl, 5 mM $MgCl_2.H_2O$, 2.5 mM EGTA, 1 $mg.mL^{-1}$ BSA and pH = 7.5 for CB_2R binding). The final membrane protein concentration was 0.4 $mg.mL^{-1}$ of incubation volume and 0.2 mg/mL of incubation volume for the CB_1R and the CB_2R assays, respectively. The radioligand used was $[^3H]$-CP55940 (PerkinElmer) at a concentration of membrane $K_D \times 0.8$ nm, and the final volume was 200 μL for CB_1R binding and was 600 μL for CB_2R binding. 96-Well plates and the tubes necessary for the experiment were previously siliconized with Sigmacote (Sigma).

Membranes were resuspended in the corresponding buffer and were incubated with the radioligand and each compound (10^{-4}–10^{-11} M) for 90 min at 30 °C. Non-specific

binding was determined with 10 μM WIN55212-2 and 100% binding of the radioligand to the membrane was determined by its incubation with membrane without any compound. Filtration was performed by a Harvester® filtermate (Perkin-Elmer) with Filtermat A GF/C filters pretreated with polyethylenimine 0.05%. After filtering, the filter was washed nine times with binding buffer, dried and a melt-on scintillation sheet (Meltilex™ A, Perkin Elmer) was melted onto it. Then, radioactivity was quantified by a liquid scintillation spectrophotometer (Wallac MicroBeta Trilux, Perkin-Elmer). Competition binding data were analyzed by using GraphPad Prism program and K_i values are expressed as mean ± SEM of at least three experiments performed in triplicate for each point.

CONCLUSIONS AND FUTURE PERSPECTIVES

With the purpose of developing an antitumor agent, chromenopyrazoledione **5** was conjugated to a tetraphenylporphyrin derivative. This macrocycle may confer to our cannabinoid a more specific tumor tissue delivery and may enable the development of target-selective phototherapy approaches. The novel conjugate **14** binds weakly but selectively to CB_2R. Further studies involving **14** will consist of *in vivo* assays to study its metabolism processes. Additionally, the synthetic design in this study provided a methodology to prepare a new tetraphenylporphyrin, 5-[4-(3,5-dioxomorpholino) phenyl]-10,15,20-triphenylporphyrin (**10**).

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