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A selective, non-toxic CB$_2$ cannabinoid o-quinone with in vivo activity against triple negative breast cancer

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KEYWORDS: cannabinoid; breast cancer; quinone; CB$_2$; xenograft
ABSTRACT: Triple-negative breast cancer (TNBC) represents a subtype of breast cancer characterized by high aggressiveness. There is no current targeted therapy for these patients whose prognosis, as a group, is very poor. Here, we report the synthesis and evaluation of a potent antitumor agent in vivo for this type of breast cancer designed as a combination of quinone/cannabinoid pharmacophores. This new compound (10) has been selected from a series of chromenopyrazolediones with full selectivity for the non-psychotropic CB\textsubscript{2} cannabinoid receptor and with efficacy in inducing death of human TNBC cell lines. The dual concept quinone/cannabinoid was supported by the fact that compound 10 exerts antitumor effect by inducing cell apoptosis through activation of CB\textsubscript{2} receptors and through oxidative stress. Notably, it did not show either cytotoxicity on non-cancerous human mammary epithelial cells nor toxic effects in vivo suggesting that it may be a new therapeutic tool for the management of TNBC.

INTRODUCTION

Besides their well-known palliative actions in the treatment of chemotherapy-associated unwanted effects,\textsuperscript{1,2} cannabinoids have also demonstrated to have antitumor effects in numerous in vitro and in vivo models of cancer. In general, activity of cannabinoids can largely be accounted for by their effects on the endocannabinoid system that is currently becoming a very attractive target for anticancer therapies.\textsuperscript{3-8} As reported by various research groups, cannabinoids can reduce tumor growth and progression by modulating cancer cell proliferation, tumor angiogenesis and metastasis among other cancer hallmarks.\textsuperscript{4} The specific mechanisms by which cannabinoids inhibit tumor growth have not been fully elucidated, although it is known that these
mechanisms of action seem to depend on the specific type of tumor. In addition, there is evidence of upregulation of cannabinoid receptors and endocannabinoids in some tumor tissues compared to the corresponding normal tissues.\textsuperscript{4}

The antitumor properties of some phytocannabinoids [mainly $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC) and cannabidiol (CBD)],\textsuperscript{9–12} endocannabinoids [mainly anandamine (AEA)],\textsuperscript{13} and different synthetic cannabinoids (WIN 55,212-2, HU-210, CP 55,940, JWH-133 and JWH-105, among others) have been reported\textsuperscript{14–16} for a variety of tumor cells lines in culture and different animal models of cancer. Despite the recent advances in the field of cannabinoid ligands as antitumor agents, the clinical development of these compounds has been very limited likely due to their psychoactive properties which are produced by the activation of CB\textsubscript{1} receptors expressed in the central nervous system. Importantly, CB\textsubscript{2} receptors are mostly located in the immune system and poorly expressed in the brain and therefore their activation does not trigger psychoactivity. Therefore, considerable efforts have been recently focussed on the therapeutic potential of CB\textsubscript{2} ligands.\textsuperscript{17} If we refer to the anticancer application, most of the antitumor cannabinoids studied so far activate CB\textsubscript{1} and CB\textsubscript{2} receptors, and few of them (JWH-015,\textsuperscript{16} JWH-133,\textsuperscript{18} KM-233,\textsuperscript{19} HU-308\textsuperscript{20}) are only partially selective for CB\textsubscript{2} over CB\textsubscript{1} receptor. Therefore, the design, synthesis and functional characterization of fully selective CB\textsubscript{2} agonists may constitute an interesting strategy to explore the antitumor potential of cannabinoids avoiding the potential clinical problems associated to CB\textsubscript{1} activation.\textsuperscript{21}

Triple-negative breast cancer (TNBC) is a subtype of breast cancer characterized by the absence of immunohistochemical expression of estrogen, progesterone, and HER2 receptors.\textsuperscript{22} Although this population accounts for a low percentage of all breast tumors, it represents a disproportionate number of deaths.\textsuperscript{23} This is mainly due to the high aggressiveness of these
tumors and to the lack of targeted and effective therapies. Therefore, and despite the high responsiveness of some individuals, this group of patients has a very poor prognosis.\textsuperscript{23–26} For this reason, the discovery of new targets and drugs for the treatment of this disease is an urgent and essential clinical challenge.\textsuperscript{27} In this context, the therapeutic potential of cannabinoids, in particular those targeting selectively the different cannabinoid receptors, has been recently explored as a new hope for TNBC patients.\textsuperscript{11,28}

Our approach involves multitarget drugs for TNBC relying on molecules whose structure includes cannabinoid and quinone features in a single chemical entity. We previously designed CB\textsubscript{1} cannabinoid \textit{para}-quinones as antitumor agents for prostate cancer.\textsuperscript{29} The results obtained encouraged us to explore cannabinoid \textit{ortho}- and \textit{para}-quinones in TNBC in vitro and in vivo models.

RESULTS

\textbf{Chemistry}. The initial 7-(1’,1’-dimethylheptyl)-dihydro-4,4-dimethylchromeno[4,3-c]pyrazol-9-ols 4-7 were synthesized from 5-(1’,1’-dimethyl-n-heptyl)-1,3-dihydroxybenzene (1) following the procedures previously reported by us.\textsuperscript{30} Scheme 1 outlines the synthesis of the \textit{ortho}- and \textit{para}-chromenopyrazolediones 8-13. The regio-controlled oxidation of the starting chromenopyrazoles to the corresponding 1,2- o 1,4-quinones was achieved by reaction under mild conditions with hypervalent iodine reagents.\textsuperscript{31} Only few methods have been reported for the preparation of \textit{ortho}-quinones by oxidation of phenols. In 2002, Pettus\textsuperscript{32} described the use of 2-iodoxybenzoic acid (IBX) as a regioselective oxidant of phenols. Since then, IBX-mediated oxidations have been extended to polyaromatic \textit{ortho}-quinones.\textsuperscript{33,34} This hypervalent iodine reagent was efficient in our hands for the preparation of the desired regioselective \textit{ortho}-
chromenopyrazoldiones 8-10. In what concerns the synthesis of the para-quinone derivatives 11-13, they were conveniently prepared in the presence of bis(trifluoro-acetoxy)iodobenzene (BTIB) as previously reported by us.  

![Chemical structure](image)

**Scheme 1.** Oxidation of chromenopyrazoles to the corresponding quinone derivatives 8-13.

Reaction conditions: (i) 3,3-Dimethylacrylic acid, CH$_3$SO$_3$H, P$_2$O$_5$, 70 °C, MW, 10 min; (ii) NaH, THF, MW, 46 °C, 20 min, then ethyl formate, THF, MW, 46 °C, 20 min; (iii) 2-Iodoxybenzoic acid, DMF, 30 min, room temperature; (iv) [Bis(trifluoroacetoxy)iodo]benzene, MeCN/ H$_2$O (6:1), 15 min, room temperature.
**Cannabinoid receptor affinity.** The affinities of the chromenopyrazolediones 8-13 for the cannabinoid receptors were determined through radioligand competition assays. Their ability to displace $[^3]$H]CP55940 from human cannabinoid CB$_1$ or CB$_2$ receptors (hCB$_1$, hCB$_2$) transfected into HEK293 EBNA cells was assessed. Standard cannabinoid ligand WIN55,212-2 was also tested for appraisal with compounds 8-13. Initially, the compounds were screened at a concentration of 40 µM. The affinity constant ($K_i$) of compounds able to displace the radioligand by more than 70% was determined from concentration–effect curves. These $K_i$ values are reported in Table 1.

**Table 1.** Binding affinity of chromenopyrazolediones 8-13 and the reference cannabinoid WIN55,212-2 for hCB$_1$ and hCB$_2$ cannabinoid receptors.

<table>
<thead>
<tr>
<th>Compd</th>
<th>$K_i$ (nM)$^a$</th>
<th>hCB$_1$</th>
<th>hCB$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>&gt; 40000</td>
<td>398 ± 49</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>&gt; 40000</td>
<td>597 ± 77</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>&gt; 40000</td>
<td>529 ± 26</td>
<td></td>
</tr>
<tr>
<td>11$^b$</td>
<td>324 ± 235</td>
<td>134 ± 21</td>
<td></td>
</tr>
<tr>
<td>12$^b$</td>
<td>14180 ± 5638</td>
<td>672 ± 191</td>
<td></td>
</tr>
<tr>
<td>13$^b$</td>
<td>8520 ± 3891</td>
<td>3665 ± 878</td>
<td></td>
</tr>
<tr>
<td>WIN55,212-2</td>
<td>45.6 ± 8.6</td>
<td>3.7 ± 0.2</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Values were obtained from competition curves using $[^3]$H]CP55940 as radioligand for hCB$_1$ and hCB$_2$ cannabinoid receptors and are expressed as the mean ± SEM of at least three experiments each performed in triplicate. $^b$ Ref. 29.
ortho-Chromenopyrazolediones 8-10 are fully selective towards CB₂ cannabinoid receptors. Their lack of affinity for CB₁ receptors (Kᵢ values higher than 40 µM) eliminates any side psychotropic side effect that would be derived from activation of central CB₁ receptors. To our knowledge, ortho-chromenopyrazolediones are the first fully selective CB₂ cannabinoid quinones reported so far. Quinone isomers afforded significant differences in their ability to bind the cannabinoid receptors. para-Chromenopyrazolediones 11-13 display affinity for both cannabinoid receptors, compound 8 providing the best binding values for both receptors.

**ADME parameters in silico.** Taking into account the fact that inappropriate absorption, distribution, metabolism, and excretion (ADME) are among the main determinants of drug development failures, these properties have been considered at this early stage of the drug discovery process. Nowadays, in silico approaches are widely accepted because of their fast and precise predictive potential.³⁵,³⁶ Pharmacokinetic properties of compounds 8-13 were predicted using QikProp (implemented in Maestro software) on each global minimum energy conformer. As presented in Table 2, chromenopyrazolediones follow Lipinski and Jorgensen pharmacokinetics rules.³⁷,³⁸ Due to their lipophilic nature, solubility of cannabinoids is a great challenge. Interestingly, the cannabinoid-quinones described herein display adequate solubility values. Almost all the predicted properties of the tested compounds are within the ranges predicted by QikProp for 95% of known oral drugs (Table 2). These data indicate that chromenopyrazolediones present a satisfactory druggability profile.
Table 2. Physicochemical descriptors calculated on the global minimum energy conformer of compounds 8-13 by QikProp 3.5 integrated in Maestro (Schrödinger, LLC, New York, USA).

<table>
<thead>
<tr>
<th>Compd</th>
<th>QPlogS\textsuperscript{a}</th>
<th>QlogBB\textsuperscript{b}</th>
<th>QPlogHERG\textsuperscript{c}</th>
<th>QPPCaco\textsuperscript{d}</th>
<th>% Human oral absorption GI\textsuperscript{e}</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>-5.35</td>
<td>-1.09</td>
<td>-4.90</td>
<td>611</td>
<td>100</td>
</tr>
<tr>
<td>9</td>
<td>-5.62</td>
<td>-0.69</td>
<td>-5.11</td>
<td>1562</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
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<td>-0.65</td>
<td>-5.03</td>
<td>1976</td>
<td>100</td>
</tr>
<tr>
<td>11</td>
<td>-5.15</td>
<td>-1.01</td>
<td>-4.69</td>
<td>666</td>
<td>100</td>
</tr>
<tr>
<td>12</td>
<td>-5.36</td>
<td>-0.62</td>
<td>-4.90</td>
<td>1701</td>
<td>100</td>
</tr>
<tr>
<td>13</td>
<td>-5.78</td>
<td>-0.63</td>
<td>-5.23</td>
<td>1985</td>
<td>100</td>
</tr>
</tbody>
</table>

Range of 95% of drugs: \(a\)Predicted aqueous solubility [-6.5/0.5]; \(b\)Predicted log of the brain/blood partition coefficient [-3.0/1.2]; \(c\)HERG \(K^+\) Channel Blockage (log IC\textsubscript{50}) [concern below -5]; \(d\)Apparent Caco-2 cell permeability in nm/s [< 25 poor, > 500 excellent]; \(e\)Human Oral Absorption in GI [< 25% is poor].

**In vitro antiproliferative activity.** The antiproliferative activity of chromenopyrazolediones 8-13 was assessed against a human TNBC cell line (MDA-MB-231). Cell cultures were treated with different doses of the tested compound (8-13) for 48 h, and cell viability was then analysed by colorimetric measurements using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). All the cannabinoid-quinone derivatives (8-13) displayed potent growth inhibitory effects on triple-negative MDA-MB-231 cells (Figure 1) with low micromolar IC\textsubscript{50} values (Table 3).
**Figure 1.** Dose-response curves of antiproliferative effect of chromenopyrazolediones 8-13 against the human breast cancer cell line MDA-MB-231.

**Table 3.** Half-maximum inhibitory concentrations (IC$_{50}$) values for compounds 8-13.

<table>
<thead>
<tr>
<th>Compd</th>
<th>IC$_{50}$ (µM)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>8.5 ± 0.4</td>
</tr>
<tr>
<td>9</td>
<td>5.0 ± 0.8</td>
</tr>
<tr>
<td>10</td>
<td>2.8 ± 0.5</td>
</tr>
<tr>
<td>11</td>
<td>8.2 ± 1.1</td>
</tr>
<tr>
<td>12</td>
<td>5.8 ± 0.9</td>
</tr>
<tr>
<td>13</td>
<td>2.5 ± 0.1</td>
</tr>
</tbody>
</table>

$^a$Values are the mean ± SE of three independent experiments performed in triplicate
As shown in Figure 1 and Table 3, compounds 10 and 13 are the most potent inhibitors of cell proliferation. Due to its antiproliferative capacity and its CB$_2$ selective profile, compound 10 was selected to investigate the mode of action and to validate the concept quinone/cannabinoid.

Since triple negative breast cancer is a very heterogeneous disease itself,$^{39,40}$ we first analysed whether chromenopyrazoledione 10 was able to decrease the viability of other TNBC cell lines. The additional three TNBC cell lines tested (SUM149, SUM159 and MDA-MB-468) were sensitive to compound 10 as well (see Supporting information), with IC$_{50}$ values of 4.6, 4.1 and 17.3 µM, respectively.

**Cytotoxicity on human normal mammary epithelial cells.** Selective toxicity for cancer versus non-cancer cells is one of the main challenges of anticancer therapies. For this reason, we examined the growth inhibitory activity of compound 10 against human mammary epithelial cells (HMEC). At all tested concentrations, compound 10 did not display antiproliferative activity in this non-transformed mammary epithelial cell line (Figure 2). Compound 10 therefore demonstrated effective inhibition of TNBC cell growth in a concentration range that does not affect the proliferation of their non-transformed counterparts.
Figure 2. Effect of compound 10 on the viability of normal human mammary epithelial cells (HMEC). HMECs were treated with increasing concentrations of the drug. Cell viability was determined 48 h after treatment. Values are the mean ± SE of three independent experiments performed in duplicate. Data were assessed by one-way analysis of variance followed by the Student-Newman-Keuls.

Expression of CB₂ receptors in normal and in cancer cells. To determine whether a different expression of CB₂ is the molecular reason underlying the selectivity of compound 10 for cancer cells, we analysed CB₂ mRNA levels in all the cell lines tested in this study by real-time quantitative polymerase chain reaction (RTQ-PCR). CB₂ mRNA was detected in both transformed and non-transformed cells, but no correlation was observed between its relative expression and responsiveness to compound 10 (Figure 3). These results suggest that a
differential expression of the cannabinoid receptor is not the main reason explaining the lack of sensitivity of non-transformed cells to compound 10.

![Figure 3](image.png)

**Figure 3.** Expression of CB$_2$ in transformed and non-transformed breast cancer cell lines. CB$_2$ mRNA expression in the indicated cell lines, as determined by real-time quantitative PCR. Results are expressed as arbitrary units vs CB$_2$ mRNA expression in HMEC that has been set as 1 unit.

**Molecular mechanism underlying the antiproliferative effect of compound 10.** With the aim of unveiling the molecular mechanisms involved in the growth inhibitory activity of compound 10, we studied its antiproliferative effect in the presence of different pharmacological tools [the CB$_1$-selective antagonist SR141716 (SR1), the CB$_2$-selective antagonist SR144528 (SR2), the GPR55-selective antagonist CDI16020046 (CDI), and the antioxidant α-tocopherol (α-Toc)]. We first verified that these compounds had no effect on their own on MDA-MB-231 cell viability (Figure 4). Then, cells were pre-incubated for 1 h with each of the mentioned compounds and then challenged with compound 10 for 48 h at IC$_{50}$ concentration. As shown in
Figure 4, SR2 and α-Toc were able to significantly prevent the inhibition of cell viability induced by 10, indicating that activation of the CB$_2$ receptor and generation of reactive oxygen species (ROS) were involved in the action of compound 10. This experiment confirms the CB$_2$ agonist nature and ROS production of compound 10. Conversely, neither SR1 nor CID altered the effect of compound 10 on cell viability, suggesting that neither CB$_1$ nor GPR55 are targets of this compound (Figure 4).

![Cell Viability Graph](Compd_10.png)

**Figure 4.** Molecular mechanisms involved in the growth inhibitory activity of compound 10. MDA-MB-231 cell viability was determined by the MTT test. CB$_1$-selective antagonist: SR141716 (SR1); CB$_2$-selective antagonist: SR144528 (SR2); GPR55-selective antagonist: CID16020046 (CDI); antioxidant: α-tocopherol (α-Toc). Results represent the mean ± S.E. of three different experiments performed in triplicate. Data were assessed by two-way analysis of variance followed by the Student-Newman-Keuls (*p < 0.05 and **p < 0.01 vs vehicle-treated cells; #p < 0.05 versus the compound alone).
To determine whether the decrease in cell viability elicited by compound 10 involves the induction of cancer cell death by apoptosis, we analysed the levels of caspase-3 by Western immunoblotting. The assay was performed following a 48 h treatment of cells with IC_{50} concentration of compound 10. Figure 5 shows an increase in active (i.e. cleavage) caspase-3 in response to compound 7, which confirms a proapoptotic effect of 10 on the TNBC cell line.

**Figure 5.** Analysis of caspase-3 protein expression. Western blot analysis of caspase 3 after 48 h exposure to compound 10 at IC_{50} concentration in MDA-MB-231 cells. The graph represents the densimetric analysis of protein levels. The optical density is relative to control cells set as 1. n = 3. Data were assessed by the Student’s t-test (*p<0.05 vs vehicle-treated cells).

**In vivo antitumor activity.** On the basis of its potent antiproliferative effect and its low toxicity in HMEC cells, compound 10 was selected for in vivo studies. To evaluate its capacity
to inhibit TNBC growth, tumor xenografts were generated in nude mice by subcutaneous inoculation of MDA-MB-231 human breast adenocarcinoma cells. Mice were treated intraperitoneally three times a week during four weeks with vehicle or 2 mg/kg of compound 10. As shown in Figure 6, compound 10 was able to effectively reduce the growth of triple-negative xenografts. Consequently, final tumor volume and tumor weight were significantly lower in mice treated with compound 10. Interestingly, the histopathological analysis of animal organs (liver, spleen, lungs and heart) showed no sign of toxicity in these animals (data not shown).

**Figure 6.** Compound 10 reduces tumor growth in vivo. (A) Effect of intraperitoneal administration of 10 on the growth of tumor xenografts generated in nude mice by injection of MDA-MB-231 human breast adenocarcinoma cells (mean ± SEM). Images show representative vehicle- and compound 10-treated tumors. (B) Effect of 10 administration on tumor weight. Data were assessed by two-way (with repeated measures) analysis of variance followed by the Student-Newman-Keuls (*p<0.05 vs. vehicle-treated tumors).
DISCUSSION

Cancer treatment is among the most promising therapeutic uses for cannabinoids, based on solid preclinical evidences clearly showing their anti-tumor actions in different cell and animal models of cancer. However, so far, only a pilot clinical trial has been reported for this indication in which Δ⁹-THC showed safety and a modest antitumor activity after intracerebral treatment of patients with recurrent glioblastoma multiforme, whereas a few more are presently in progress. Meanwhile, numerous pharmacological and biochemical studies are exploring in detail the use of cannabinoids in different type of tumors using several methodological strategies.

Since cancer is a complex multifactorial physiopathology triggered by deregulation of diverse cellular systems, successful cancer treatments should involve the modulation of multiple signalling pathways using combinations of different agents with different mechanisms of action. This is why we associated the antitumor action of quinones as producers of reactive oxygen species (ROS) with the antitumor cannabinoid activity in a single molecule. Quinone derivatives of phytocannabinoid have been already described; however, their antiproliferative effect was not attributed to a mechanism involving cannabinoid receptors since they did not bind to any of them.

Due to the versatility of the chromenopyrazole scaffold for cannabinoid ligands as previously reported by us, we next explored its quinone derivatives. We recently reported the synthesis of para-quinone chromenopyrazoles that showed to be mixed CB₁/CB₂ receptors ligands with antiproliferative effects through CB₁ receptor activation for prostate cancer. In the present study,
ortho-quinone chromenopyrazoles (8-10) have been prepared using the appropriate regioselective oxidant, the 2-iodoxybenzoic acid (IBX). All of the para- and ortho-quinone chromenopyrazoles (11-13) displayed potent antiproliferative effects on triple-negative MDA-MB-231 breast cancer cells. They also showed to have satisfactory druggability profile. The most interesting feature was that the ortho-quinones (8-10) showed to be fully selective for the hCB2 receptor eliminating any psychotropic effect due to activation of CB1 receptor. Therefore, absence of centrally mediated CB1 effects of the ortho-compounds 8-10 represents high value for a potential clinical application. It is why one of these compounds (10) was selected to perform mechanistic and in vivo studies.

Compound 10 was evaluated in three additional TNCB cell lines due to the heterogeneity of cancer. It was also tested in normal mammary epithelial cells for toxicity evaluation. Interestingly, while compound 10 decreased the viability of breast cancer cells, it did not affect that of normal mammary epithelial cells. The selective toxicity of cannabinoids towards cancer cells versus normal cells was previously observed in phytocannabinoids such as Δ9-THC.9,12 Different expression levels could be a molecular reason underlying the selectivity of compound 10 for cancer cells. However, real-time quantitative PCR analysis showed no correlation between the relative expression of CB2 receptor and responsiveness to compound 10. Another explanation of this selectivity could be that redox status of cancer cells differs from normal cells. Therefore, tumor cells are more sensitive to oxidative stress than normal cells. Even though high ROS levels might be detrimental for normal cells as reviewed recently by Gorrini et al.46, in our system, compound 10 proved to be very efficient in reducing the viability of cancer cells in a concentration range that did not affect the viability of non-transformed cells.
Apart from Δ⁹-THC, other cannabinoids have been reported to possess antitumorigenic activity for TNBC.⁹,¹² The most studied are JWH-133,¹⁸ WIN55,212-2 and cannabidiol even though the molecular bases of the later are probably not related to activation of cannabinoid receptors but they are associated to ROS production.²⁸ In this context, it was of interest to study the mechanisms that contribute to the response of compound 10. By using selective antagonists of the CB₂ and CB₁ receptors in vitro, we confirmed that the antiproliferative effects of 10 were mediated by CB₂ receptor activation but not by CB₁ receptor activation. Thus, despite the significant but modest CB₂ affinity of compound 10 (sub-micromolar range), it showed antitumor activity through this receptor. These findings are in agreement with the antiproliferative studies performed with Δ⁹-THC (IC₅₀ = 5 µM) in the same cell line.⁹ Of interest, the results obtained using an antioxidant agent support the fact that compound 10 exerts part of its action through the induction of oxidative stress validating the proposed concept ROS/cannabinoid activity. We also showed that the putative cannabinoid receptor GRP55,⁴⁷–⁴⁹ highly expressed in the triple-negative MDA-MB-231 cell line, was not involved in compound 10 antitumor action.

Apoptosis is the main mechanism of cannabinoid- and quinone-induced cell death. The process of cell death produced by compound 10 has been confirmed to be apoptotic by measuring caspase-3 protein expression, a marker of apoptosis, by Western blotting.

The antiproliferative effect of compound 10 was also reproduced in vivo. Upon 4 weeks of treatment, significant reduction in tumor xenografts growth (MDA-MB-231) was observed. Moreover, the lack of toxicity of compound 10 in normal cells was further confirmed in vivo. Thus, the histopathological analysis of different organs of mice treated with compound 10 during one month revealed no signs of toxicity.
CONCLUSION

Although cannabinoids have gained broad interest in the field of cancer research, they have not yet been widely studied in clinical assays. One of the main reasons is the psychotropic effects they produce by activation of the CB₁ receptors expressed in the brain. Our study provides the first fully selective CB₂ cannabinoid quinone (10) producing antitumor responses in vitro and in vivo in TNBC cancer cells via CB₂ activation and ROS production without producing toxic effects.

EXPERIMENTAL

Chemistry

General Methods and Materials. All commercially available reagents and solvents were used without further purification. Column chromatography was performed using silica gel 60 (230-400 mesh). The purity of final compounds as determined by HPLC/MS and elemental analyses was ≥ 95%. Analytical 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 SunFireTM (C-18, 4.6 x 50 mm, 3.5 µm) and 5 min gradient A: MeCN/0.08% formic acid, B: H₂O/ 1% formic acid visualizing at λ = 254 nm. Elemental analyses of all synthesized compounds were performed using a LECO CHNS-932 apparatus. Deviations of the elemental analysis results from the calculated are within ± 0.4%. H and C NMR spectra were recorded on a Bruker 300 (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 (δ) downfield of tetramethylsilane. Melting points were determined on a MP70 Mettler Toledo apparatus. Chromenopyrazoles 4-7 were prepared according to procedures previously published by us. The preparation of compounds 11-13 has been reported by us.

7-(1’,1’-Dimethylheptyl)-1,4-dihydro-4,4-dimethylchromeno[4,3-c]pyrazol-8,9-dione (8).

To a solution of 7-(1’,1’-dimethylheptyl)-1,4-dihydro-4,4-dimethylchromeno[4,3-c]pyrazol-9-ol (4) (30 mg, 0.08 mmol) dissolved in DMF (1.5 mL) was added solid IBX (36 mg, 0.13 mmol), and the resulting mixture was stirred at room temperature for 30 min. The mixture was then extracted with EtOAc, washed with water and brine. The solvent was removed under vacuum.

The resulting residue was purified by chromatography on a silica gel column (hexane/EtOAc 2:1) to furnish the title compound (8) as a red solid (9 mg, 29% yield); mp: 90.2 °C; 1H-NMR (CDCl3) δ: 8.03 (bs, 1H, NH), 7.66 (s, 1H, 3-H), 7.12 (s, 1H, 6-H), 1.65 (s, 6H, OC(CH3)2), 1.51-1.49 (m, 2H, 2’-H), 1.37 (s, 6H, C(CH3)2), 1.29-1.27 (m, 6H, 3´H, 4´-H, 5´-H), 1.21-1.18 (m, 2H, 6´H), 0.84 ppm (t, J = 7.1 Hz, 3H, 7´FH); 13C-NMR (CDCl3) δ: 180.1 (9FC), 179.6 (8FC), 164.4 (5a-C), 163.8 (7-C), 142.0 (6-C), 138.5 (9b-C), 131.5 (3-C), 130.3 (3a-C), 110.9 (9a-C), 79.7 (OC(CH3)2), 45.9 (2´-C), 31.2 (1´-C), 30.0, 29.3 y 28.1 (3´-C, 4´-C and 5´-C); 25.8 (C(CH3)2), 24.9 (OC(CH3)2), 22.5 (6´-C), 13.6 ppm (7´-C); HPLC/MS: [A, 80% → 100%], tR: 2.03 min (99%), MS (ES+, m/z) 357 [M+H]+. Anal. Calcd. for C21H28N2O3: C, 70.76 ; H, 7.92. Found: C, 70.41 ; H, 7.57.

7-(1’,1’-Dimethylheptyl)-2,4-dihydro-2,4,4-trimethylchromeno[4,3-c]pyrazol-8,9-dione (9).

Prepared from 7-(1’,1’-dimethylheptyl)-2,4-dihydro-2,4,4-trimethylchromeno[4,3-c]pyrazol-9-ol (5) (45 mg, 0.12 mmol) and IBX (53 mg, 0.18 mmol) by following the procedure described for 8. Column chromatography on silica gel (hexanes/EtOAc 1:1) afforded 9 as a red solid (11 mg, 24
% yield); mp: 86.1 °C; \(^1\)H-NMR (CDCl\(_3\) \(\delta\): 7.28 (s, 1H, 3-H), 6.73 (s, 1H, 6-H), 3.89 (s, 3H, NCH\(_3\)), 1.64 (s, 6H, OC(CH\(_3\))\(_2\)), 1.57-1.55 (bs, 2H, 2'-H), 1.29 (s, 6H, C(CH\(_3\))\(_2\)), 1.22-1.18 (m, 6H, 3'-H, 4'-H, 5'-H), 1.10-1.03 (m, 2H, 6'H), 0.81 ppm (t, \(J = 7.0\) Hz, 3H, 7'-H); \(^{13}\)C-NMR (CDCl\(_3\) \(\delta\): 182.3 (9-C), 180.6 (8-C), 165.1 (5-a-C), 160.3 (7-C), 139.8 (9b-C), 137.0 (6-C), 132.2 (3-C), 129.9 (3a-C), 113.8 (9a-C), 83.0 (OC(CH\(_3\))\(_2\)), 42.3 (NCH\(_3\)), 41.0 (2'-C), 33.1 (1'-C), 32.0, 30.9 and 29.6 (3'-C, 4'-C and 5'-C), 28.1 (C(CH\(_3\))\(_2\)), 22.9 (OC(CH\(_3\))\(_2\)), 21.8 (6'-C), 14.1 ppm (7'-C); HPLC/MS: [A, 80% \(\rightarrow\) 100%], tR: 2.07 min (96%), MS (ES+, m/z) 371 [M+H]+. Anal. Calcd. for C\(_{22}\)H\(_{30}\)N\(_2\)O\(_3\): C, 71.32; H, 8.16. Found: C, 71.19; H 7.82.

7-(1',1'-Dimethylheptyl)-1-ethyl-1,4-dihydro-4,4-dimethylchromeno[4,3-c]pyrazol-8,9-dione (10). Prepared from 7-(1',1'-dimethylheptyl)-1-ethyl-1,4-dihydro-4,4-dimethylchromeno[4,3-c]pyrazol-9-ol (6) (20 mg, 0.05 mmol) and IBX (22 mg, 0.08 mmol) by following the procedure described for 5. Column chromatography on silica gel (hexanes/EtOAc 4:1) afforded 10 as a red solid (6 mg, 30 % yield); mp: 94.8 °C; \(^1\)H-NMR (CDCl\(_3\) \(\delta\): 7.49 (s, 1H, 3-H), 7.11 (s, 1H, 6-H), 4.04 (q, \(J = 7.1\) Hz, 2H, NCH\(_2\)CH\(_3\)), 1.49 (s, 6H, OC(CH\(_3\))\(_2\)), 1.35 (t, \(J = 7.1\) Hz, 3H, NCH\(_2\)CH\(_3\)), 1.30-1.26 (m, 2H, 2'-H), 1.22 (s, 6H, C(CH\(_3\))\(_2\)), 1.19-1.16 (m, 6H, 3'-H, 4'-H, 5'-H), 1.03-1.01 (bs, 2H, 6'-H), 0.87 ppm (t, \(J = 6.9\) Hz, 3H, 7'-H); \(^{13}\)C-NMR (CDCl\(_3\) \(\delta\): 182.9 (9-C), 181.0 (8-C), 161.5 (5a-C), 159.3 (7-C), 138.2 (9b-C), 135.9 (6-C), 131.0 (3-C), 129.7 (3a-C), 112.3 (9a-C), 80.1 (OC(CH\(_3\))\(_2\)), 47.1 (NCH\(_2\)CH\(_3\)), 40.6 (2'-C), 35.3 (1'-C), 31.1, 29.8 and 28.5 (3'-C, 4'-C and 5'-C), 27.0 (OC(CH\(_3\))\(_2\)), 25.3 (C(CH\(_3\))\(_2\)), 22.1 (6'-C), 14.9 (NCH\(_2\)CH\(_3\)), 13.7 ppm (7'-C); HPLC/MS: [A, 80% \(\rightarrow\) 100%], tR: 3.97 min (98%), MS (ES+, m/z) 385 [M+H]+. Anal. Calcd. for C\(_{23}\)H\(_{32}\)N\(_2\)O\(_3\): C, 71.84; H, 8.39. Found: C, 71.93; H 8.06.

7-(1',1'-Dimethylheptyl)-1,4-dihydro-4,4-dimethylchromeno[4,3-c]pyrazol-6,9-dione (11). Anal. Calcd. for C\(_{21}\)H\(_{28}\)N\(_2\)O\(_3\): C, 70.76; H, 7.92. Found: C, 71.03; H 8.24.
In silico ADME calculations. A set of 34 physico-chemical descriptors was computed using QikProp version 3.5 integrated in Maestro (Schrödinger, LLC, New York, USA). The QikProp descriptors are shown in Table 2. The 3D conformations used in the calculation of QikProp descriptors were generated using the program Spartan ’08 (Wave function, Inc., Irvine CA) as follows: the structure of each molecule was built from the fragment library available in the program. Then, ab initio energy minimizations of each structure at the Hartree-Fock 6-31G* level were performed. A conformational search was next implemented using Molecular Mechanics (Monte Carlo method) followed by a minimization of the energy of each conformer calculated at the Hartree-Fock 6-31G* level. The global minimum energy conformer of each compound was used as input for ADME studies with QikProp.

Pharmacological assays

Binding assays. Membranes from transfected cells with human CB₁ or CB₂ expressed cannabinoid receptors (RBHCB1M400UA and RBXCB2M400UA) were supplied by Perkin-Elmer Life and Analytical Sciences (Boston, MA). The membrane protein added was 8 µg/well for CB₁ assays and 4 µg/well for CB₂ assays. The assay was developed in binding buffer (50 mM TrisCl, 5 mM MgCl₂·H₂O, 2.5 mM EDTA, 0.5 mg/mL BSA and pH = 7.4 for CB₁ binding; 50 mM TrisCl, 5 mM MgCl₂·H₂O, 2.5 mM EGTA, 1 mg/mL BSA and pH = 7.5 for CB₂ binding) in...
a final volume of 200 µL for CB₁ binding and 600 µL for CB₂ binding. The radioligand was [³H]-CP55940 (PerkinElmer) used at a concentration of Kᵦ x 0.8 nM following the recommendations indicated in previous publication.⁵⁰ 96-Well plates and the tubes necessary for the experiment were previously siliconized with Sigmacote (Sigma). Membranes were suspended in the corresponding buffer and were incubated with the radioligand and each compound (10⁻⁴-10⁻¹¹ M) for 90 min at 30 ºC. 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 polyethyleneimine 0.05%. After filtering, the filter was washed nine times with ice-cold binding buffer, dried and a melt-on scintillation sheet (MeltilexTM 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 Kᵦ values were expressed as mean ± SD of at least three experiments performed in triplicate for each point.

Cell culture and viability. MDA-MB-231 cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA) and maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 5 units/mL penicillin, and 5 mg/mL streptomycin. SUM149, SUM159, and MDA-MD-468 cells were kindly donated by Dr. Moreno-Bueno (Instituto de Investigaciones Biomédicas Alberto Sols, Madrid, Spain). SUM149 and SUM159 cells were maintained in Ham's F-12 medium with 5% FBS, 5 µg/ml insulin, 0.5 µg/ml hydrocortisone. MDA-MD-468 was maintained in DMEM with 10%FBS. Human mammary epithelial cells (HMEC) were obtained from Cambrex and grown in mammary epithelial growth medium (Cambrex, East Rutherford, NJ) according to the manufacturer’s instructions. Cannabinoid ligands were prepared
in DMSO. Control incubations had the corresponding DMSO content. Cells were cultured at a density of 5000 cells/cm² in a 24-well plate. They were transferred to a serum-free medium or a 0.5% FBS medium (HMEC) at least 6 h before treatment with the compounds at concentrations ranging from 0 to 25 µM for 48 h. When indicated, cells were pre-incubated with SR141716 (SR1), SR144528 (SR2), CDI16020046 (CDI), and the antioxidant α-tocopherol (α-Toc) for 1 h before treatment with the compound 7. Finally, the MTT reduction assay was carried out to evaluate the effects of the different compounds on cell viability and to determine the IC₅₀.

Briefly, cells were incubated for 2 h at 37°C with 0.3 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma, St Louis, MO, USA) and then were solubilized with 100 µl isopropanol. Absorbance at 570 nm was measured with a plate spectrophotometer (ELX 800 Bio-Tek Instruments, INC).

Real-time quantitative PCR (RTQ-PCR). RNA was isolated with Trizol Reagent (Invitrogen), and cDNA was obtained with Transcriptor Reverse Transcriptase (Roche Applied Science, Penzberg, Germany). The primers used for RTQ-PCR amplification were: human CB₂ receptor, sense 5’-CTGTTTCACTGGCAGCTTG-3’, and antisense 5’-AGCTGCATGCAAAGACCAC-3’. Each value was adjusted by using TBP RNA levels as a reference.

Western Blot Analysis. MDA-MB-231 human breast cancer cells were treated with DMSO (vehicle), or compound 10 for two days. Cells were then harvested and lysed. Protein concentrations were quantified by the Bradford method with bovine serum albumin as the standard. Equal amounts of total cellular protein extract (25 µg) were separated by electrophoresis in SDS-polyacrylamide gels (12%) and transferred to PVDF membranes. After blocking with 5% nonfat milk, membranes were incubated at 4°C with the desired primary antibodies overnight at the following dilutions: anti-caspase-3 (1:500 Cell Signaling), and β-actin
(1:5000; Sigma). Subsequently, membranes were incubated with appropriate secondary antibodies. Luminograms were obtained with an enhanced chemiluminescence detection kit (Amersham Life Sciences, Arlington Heights, IL) and densitometric analysis was performed with Quantity One software (Bio-Rad, Hercules, CA, USA).

In vivo antitumor assays. All procedures involving animals were performed with the approval of the Complutense University Animal Experimentation Committee according to European official regulations. Tumors were induced in nude mice by subcutaneous injection of 5 × 10^6 MDA-MB-231 human breast adenocarcinoma cells in PBS. When tumors reached an average size of 200 mm^3, animals were randomly assigned to different groups. At this point, animals started to receive vehicle or compound 10 (2 mg/kg/d) in 100 µl of sesame oil thrice a week by intraperitoneal injection. Tumors were routinely measured with an external caliper, and volume was calculated as \((4\pi/3) \times (\text{width}/2)^2 \times (\text{length}/2)\). After 4 weeks of treatment, animals were sacrificed. Tumors were excised, weighted and snap frozen for further analysis. Organs (liver, spleen, heart and lungs) were also collected and fixed in 4% PFA for histopathological analysis by H&E staining.

Statistics. Data were assessed by the Student’s t-test, as well as by the analysis of variance (one-way or two-way) followed by the corresponding post-hoc test (Student-Newman-Keuls test), as required.

ASSOCIATED CONTENT

Viability assays of compound 10 in different TNBC cell lines (SUM149, SUM159 MDA-MB-231 and MDA-MB-468). This material is available free of charge via the Internet at http://pubs.acs.org
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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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ABBREVIATIONS

CB, cannabinoid; TNBC, triple-negative breast cancer; HMEC, normal human mammary epithelial cells, THC, tetrahydrocannabinol; CBD, cannabidiol; AEA, anandamide; IBX, 2-iodoxybenzoic acid; BTIB, bis(trifluoroacetoxy)iodobenzene; DMF, dimethylformamide; HEK, human embryonic kidney; EBNA, epstein-barr nuclear antigen-1; Compd, compound; Ref, literature reference; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ADME, administration, distribution, metabolism, excretion; SR1, SR141716; SR2, SR144528; CDI, CDI16020046; α-Toc, α-tocopherol; ROS, reactive oxygen species; S.E., standard error;
Veh, vehicle; SEM, standard error of the mean; HPLC, high performance liquid chromatography; MS, mass spectrometry; mp, melting point; ES, electrospray; ESI, electrospray ionization; BSA, bovine serum albumine; EDTA, ethylenediaminetetraacetic acid; PFA, paraformaldehyde.

REFERENCES


(22) Chiorean, R.; Braicu, C.; Berindan-Neagoe, I. Another review on triple negative breast cancer. Are we on the right way towards the exit from the labyrinth? The Breast 2013, 22, 1026–1033.


(42) Guzmán, M.; Duarte, M. J.; Blázquez, C.; Ravina, J.; Rosa, M. C.; Galve-Roperh, I.; Sánchez, C.; Velasco, G.; González-Feria, L. A pilot clinical study of Delta9-


Table of Contents graphic
Oxidation of chromenopyrazoles to the corresponding quinone derivatives 8-13. Reaction conditions: (i) 3,3-Dimethylacrylic acid, CH$_3$SO$_3$H, P$_2$O$_5$, 70 ºC, MW, 10 min; (ii) NaH, THF, MW, 46 ºC, 20 min, then ethyl formate, THF, MW, 46 ºC, 20 min; (iii) 2-Iodoxybenzoic acid, DMF, 30 min, room temperature; (iv) [Bis(trifluoroacetoxy)iodo]benzene, MeCN/ H$_2$O (6:1), 15 min, room temperature.

169x107mm (300 x 300 DPI)
CB₂ mRNA expression for different cell lines:

- FMEC
- SUM-149
- SUM-159
- MDA-MB-231
- MDA-MB-468