Bioorganic & Medicinal Chemistry 19 (2011) 5698–5707

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Fluorinated cannabinoid CB2 receptor ligands: Synthesis and in vitro binding characteristics of 2-oxoquinoline derivatives

Nashaat Turkman, Aleksander Shavrin, Roman A. Ivanov [†], Brian Rabinovich, Andrei Volgin, Juri G. Gelovani, Mian M. Alauddin [*]

Department of Experimental Diagnostic Imaging, The University of Texas, M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030, USA

ARTICLE INFO

Article history:
Received 10 May 2011
Revised 28 June 2011
Accepted 4 July 2011
Available online 5 August 2011

Keywords:
Central nervous system
Cannabinoid receptor 2
Multiple sclerosis
Neuroinflammation
Neurodegeneration

ABSTRACT

Cannabinoid receptor 2 (CB2) plays an important role in human physiology and the pathophysiology of different diseases, including neuroinflammation, neurodegeneration, and cancer. Several classes of CB2 receptor ligands, including 2-oxoquinoline derivatives, have been previously reported. We report the synthesis and results of in vitro receptor binding of a focused library of new fluorinated 2-oxoquinoline CB2 ligands. Twelve compounds, **13**–**16** **18**, **19**, **21**–**24**, **27**, and **28** were synthesized in good yields in multiple steps. Human U87 glioma cells expressing either *h*CB1 (control) or *h*CB2 were generated via lentiviral transduction. In vitro competitive binding assay was performed using [^{3}H]CP-55,940 in U87*h*CB1 and U87*h*CB2 cells. Inhibition constant (K_i) values of compounds **13**–**16**, **18**, **19**, **21**–**24**, **27**, and **28** for CB2 were >10,000, 2.8, 5.0, 2.4, 22, 0.8, 1.4, >10,000, 486, 58, 620, and 2400 nM, respectively, and those for CB1 were >10,000 nM. Preliminary in vitro results suggest that six of these compounds may be useful for therapy of neuropathic pain, neuroinflammatory diseases and immune disorders. In addition, compound **19**, with its subnanomolar K_i value, could be radiolabeled with ^{18}F and explored for PET imaging of CB2 expression.

1. Introduction

The hemp plant, *Cannabis sativa* L., known as marijuana, has been used for centuries as a therapeutic agent and recreational drug.[1,2] The active components of marijuana and their derivatives are classified as cannabinoids.[2] Two subtypes of the mammalian cannabinoid receptors have been identified: the CB1 and CB2 receptors. The CB1 receptor is primarily located in the central nervous system,[3,4] and the CB2 receptor is expressed in cells of the immune system, spleen, tonsils, and lymph nodes.[3,5,6] The CB1 and CB2 receptors are both G-protein coupled receptors and are involved in the inhibition of adenylate cyclase.

CB2 is believed to be devoid of psychoactivity, and has significant anti-inflammatory functions,[7] and inflammation is known to be a critical part of many types of neurodegenerative diseases.[8] CB2 receptors have been implicated in a range of leukocyte functions, and studies of CB2 receptors in leukocytes have shown results consistent with an anti-inflammatory and immunosuppressive role. This has also been supported by demonstrations that CB2 regulates inflammation in a diverse range of animal models, including gastrointestinal,[9] acute hindpaw,[10] and pulmonary inflammation.[11] CB2 expression in microglia in the brain was studied using human brain tissue taken from a patient with Alzheimer's disease. Microglia clustering at β-amyloid plaques expressed both CB2 receptor proteins and fatty acid amine hydrolase.[7] Ramirez et al.[12] replicated this finding and investigated the role of CB2 in the pathogenesis of a mouse model of Alzheimer's disease. CB2 receptor expression was also found to be up-regulated in sub-populations of microglia in a rat model of Huntington's disease.[13] CB2 receptors have been reported to be over-expressed in several cancer cells, including breast cancer,[14] skin cancer,[15] prostate,[16] and hepatocellular carcinoma.[17] Experimental evidence has shown that cannabinoids inhibit the growth of tumor xenografts in mice, tumor angiogenesis, and directly induce apoptosis in neoplastic cells.[14,15]

The CB2 receptor is an important target for therapeutic immune intervention, and, as such, research is currently focused on the development of CB2 selective ligands. WIN-55,212-2 was the first reported CB2 selective ligand, and it has been thoroughly investigated.[1] As a result, there are several different documented values for the binding affinity of WIN-55,212-2 at both CB1 and CB2 receptors.[18] While WIN-55,212-2 is extensively used for evaluating the receptor binding of potential cannabinoid ligands, it is not very useful pharmacologically as a selective ligand for either cannabinoid receptor because it has high affinity for both receptors.[1,9]

Subsequent to WIN-55,212-2, several other CB2 selective ligands have been synthesized, including cannabimimetic indoles, such as 1-(2,3-dichlorobenzoyl)-2-methyl-3-(2-[1-morpholino]ethyl)-5-methoxyindole (L768242)[19] and 1-propyl-2-methyl-3-

* Corresponding author. Tel.: +1 713 563 4872; fax: +1 713 563 4894.
E-mail address: alauddin@mdanderson.org (M.M. Alauddin).
† Present address: SiPix Imaging, Inc., Fremont, CA.

0968-0896/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2011.07.062

(1-naphthoyl)indole (JWH-015).[20] Indole L768242 has exceptionally high selectivity for the CB2 receptor (146-fold) with significantly high affinity at CB2 (inhibition constant [K_i] = 14 nM). The Huffman group has synthesized a variety of CB2 selective 1-deoxy and 1-methoxy-Δ^8-THC analogues, which include 1-deoxy-3-(1′,1′-dimethylbutyl)-Δ^8-THC (JWH-133), one of the most highly selective ligands for the CB2 receptor (K_i = 677 ± 132 nM at CB1 and K_i = 3.4 ± 1.0 nM at CB2).[21] 1-Methoxy-3-(1′,1′-dimethylhexyl)-Δ8-THC (JWH-229) is another highly selective ligand, with more than 170-fold selectivity for the CB2 receptor (K_i = 3134 ± 110 nM at CB1 and K_i = 18 ± 2 nM at CB2).[22] This group has also synthesized a large number of indole derivatives to identify CB2-specific ligands.[1,2,6,23] A variety of cannabimimetic indole derivatives have also been developed by the pharmaceutical companies, including Bristol-Myers Squibb and Abbott Laborat ories.[24,25]

In addition to traditional dibenzopyran-based cannabinoids[1,2,21,22] and cannabimimetic indoles,[6,24–26] a variety of potent cannabinoid ligands that are resorcinol derivatives[27,28] have been synthesized and reported. Two series of resorcinol dimethyl ethers were also reported by Wiley and others.[28] One of these compounds, O-1966A has very high (220-fold) selectivity for the CB2 receptor combined with very low affinity for the CB1 receptor (K_i = 5055 ± 984 nM at CB1 and K_i = 23 ± 2.1 nM at CB2). Mussinu et al.[29] described several highly CB2 selective tricyclic pyrazoles based upon CB1 antagonists SR141716A (Rimonabant®)[30] and the CB2 inverse agonist SR144528.[31,32] These pyrazoles are among the most highly selective ligands for the CB2 receptor to be reported to date, with selectivity ranging from 32- to 9810-fold depending on the substituent at the aromatic ring.

Iwamura et al. have synthesized several 2-oxoquinoline analogues with exceptionally high selectivity for the CB2 receptor.[33] Compound JTE-907 (**29**, Table 2, Supplementary data) in particular, is described as an inverse agonist in vitro for the CB2 receptor and possesses anti-inflammatory properties in vivo. JTE-907 (**29**) has modest affinity (K_i = 35.9 nM)[1] for the CB2 receptor, with an exceptionally high selectivity of 2760-fold at this receptor. Structural variations in these 2-oxoquinolines include an N-methyl substituent at the quinoline ring; variable alkoxy substituents in the 6, 7, and 8 positions of the quinoline moiety and substituents at the 3-position that vary from carboxylic acid groups to various amide and ester groups.[34] Compound **30** (Table 2, Supplementary data) in particular has very high CB2 receptor affinity (K_i = 0.014 nM) and extremely high selectivity (262,202-fold) for this receptor. Raitio et al. synthesized a new series of CB2 inverse agonists with responses comparable to SR144528,[34,35] based on the structure of 2-oxoquinoline JTE-907 (**29**, Table 2, Supplementary data) in which the piperonylamide end has been replaced with a variety of aromatic amide structures (**32**–**37**, Table 2, Supplementary data).[34]

Several other classes of compounds, including tricyclic pyrazoles,[30–32] sulfamoyl benzamides,[36] triarylbis-sulfones,[37] and arylsulfonamide,[38] have been synthesized and reported to be CB2 receptor ligands. However, among all the classes of CB2 ligands, 2-oxoquinoline derivatives appear to be the most efficient inverse agonists, with high binding affinity and selectivity for CB2 receptor.[34,35]

In order to develop novel CB2-specific ligands for therapy of neurological diseases and non-invasive imaging of CB2 receptor expression, we have rationally designed a focused library of potential CB2-specific compounds. We report synthesis of a new library of fluorinated analogues of 2-oxoquinoline as CB2 receptor ligands and results of their in vitro receptor binding in cell lines genetically engineered to express the CB1 or CB2 receptors. These studies demonstrate that some of the fluorinated 2-oxoquinoline analogues have high binding efficiency and selectivity for CB2 receptor, and these compounds may be suitable for treatment of neuropathic pain, inflammation, and immune disorders.

2. Results and discussion

2.1. Chemistry

The scheme for synthesis of compounds **13**, **14**, **15**, and **16** is shown in Figure 1 (Synthetic Scheme 1).

Compounds **2**–**12** were prepared following previously published methods[34,39,40] as shown in the synthetic Scheme 1 (Fig. 1) with minor modifications. Compound **2** was obtained in 80% yield and compounds **3** and **4** were obtained in 70% and 60% yields, respectively. Reduction of **3** and **4** using iron (Fe) powder and concd HCl produced compounds **5** and **6** in 80% and 83% yields, respectively. Compounds **7** and **8** were obtained from **5** and **6**, respectively, in 80% yields. The ethyl esters **7** and **8** were hydrolyzed by 2 M HCl acid to produce **9** and **10** in 73% and 75% yields, respectively. Intermediate compounds **2**–**10** were fully characterized by spectroscopic methods, and the ^{1}H NMR spectra were consistent with those reported previously.[39] Detailed experimental procedures for compounds **2**–**12** are presented in the Supplementary data.

Compounds **11** and **12** were prepared in situ and used without isolation and purification. Compounds **13**, **14**, **15**, and **16** were prepared from the intermediate acid chlorides **11** and **12** by reaction with commercially available 4-fluorobenzyl amine and 4-fluorophenyl ethylamine (Scheme 1). The yields for **13**, **14**, **15** and **16** were 76%, 70%, 86% and 96%, respectively.

Compound **17** was synthesized in house according to a previously reported method[41] and reacted with the acid chlorides **11** and **12** as shown in Figure 2. The desired products **18** and **19** were obtained in 60% and 85% yields, respectively.

Synthesis of the compounds **20** and **21** is shown in Figure 3 (Synthetic Scheme 3).

Compound **20** was prepared by reaction of **12** with 4-aminophenyl ethylamine and obtained in 80% yield. The experimental details are identical as those described for preparation of compounds **13**–**16** (Scheme 1). Compound **20** was used for an alternative synthesis of **16** and synthesis of **21**.

Compound **16** was synthesized by two different methods: *Method 1*: The acid chloride **12** was reacted with 4-fluorophenyl ethylamine to produce **16** (as shown in Fig. 1). In this method, the yield of **16** was 97%; however, the method is not suitable for radiosynthesis using ^{18}F, because ^{18}F should be inserted into the benzene ring by a special method. Therefore, we explored an alternative method, as shown in Figure 3, which should be suitable for radiosynthesis of **16**. *Method 2:* In this method, diazotization of **20** was performed to produce an azo-salt compound in situ, which was converted to **16** by reaction with HF in pyridine. This reaction produced a mixture of two products, **16** and **21**, in 40% and 36% yields, respectively. The formation of **21** is quite interesting; in addition to fluorination of the aromatic ring, C_6-hydrogen was substituted by a nitro group. Thus, compound **21** was obtained as a by-product during diazotization of **20**, followed by reaction with HF in pyridine.

Synthesis of the compounds **22**–**24** is shown in Figure 4 (Synthetic Scheme 4).

The acid chlorides **11** and **12** were prepared in situ from **9** and **10** as described in Scheme 1, then coupled with the corresponding fluoroalkyl amines with two different carbon-chain lengths to produce compounds **22**–**24**. The yields of these compounds were 90–95%, almost quantitative.

Synthesis of compounds **27** and **28** is presented in Figure 5 (Synthetic Scheme 5).

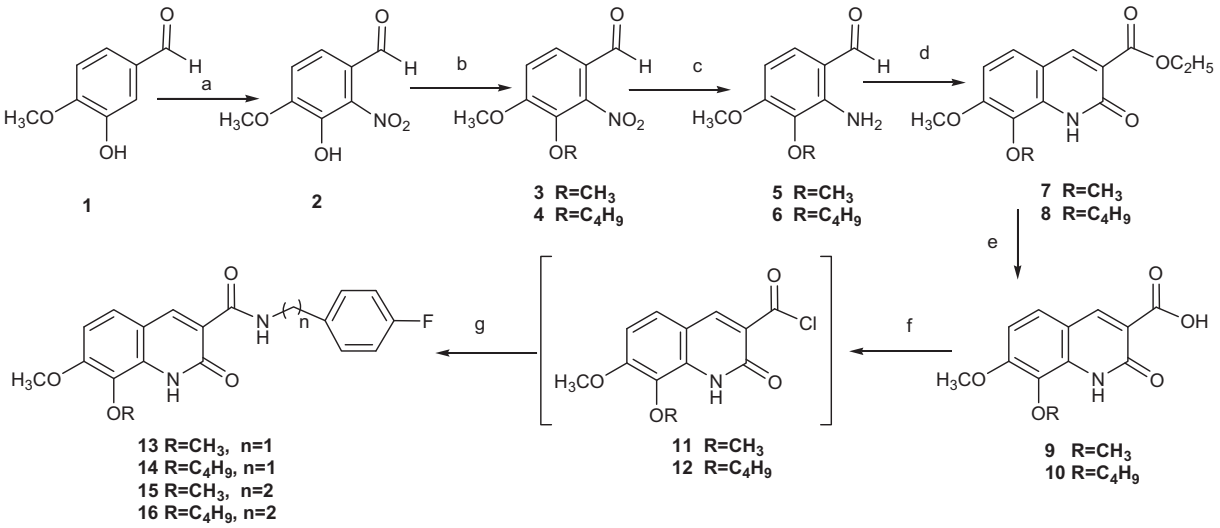


Figure 1. Scheme for synthesis of 7-methoxy-8-alkoxy-2-oxo-1,2-dihydroquinoline-3-carboxylic acid 4-fluoro-benzylamides **13**, **14** and 4-fluoro-phenethylamides **15** and **16**. Reagents and conditions: (a) DCM, −20 °C, NO_2BF_4, 24 h; (b) DMF, K_2CO_3, MeI or 1-bromobutane, 80 °C 24 h; (c) EtOH, Fe, HCl, H_2O, reflux 15 min; (d) EtOH, $CH_2(CO_2CH_3)_2$, piperidine, AcOH, reflux 16 h; (e) EtOH, HCl/H_2O, 60 °C, 16 h; (f) toluene, $SOCl_2$, reflux 3 h; (g) DCM, Et_3N, amine, rt, 1 h.

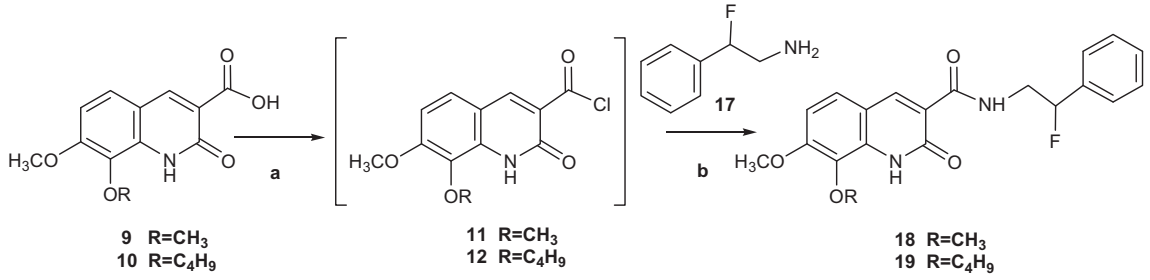


Figure 2. Scheme for synthesis of 7-methoxy-8-alkoxy-2-oxo-1,2-dihydroquinoline-3-carboxylic acid 2-fluoro-2-phenethylamides **18** and **19**. Reagents and conditions: (a) toluene, $SOCl_2$, reflux 3 h; (b) DCM, Et_3N, 17, rt, 1 h.

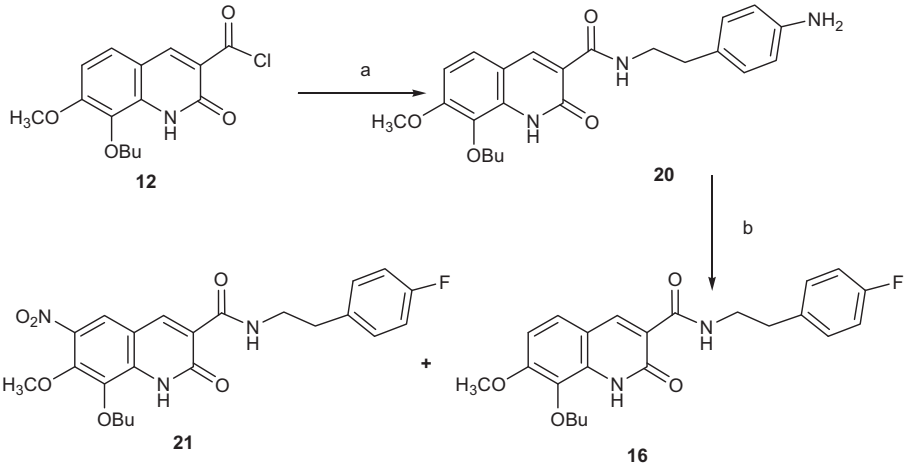


Figure 3. Scheme for synthesis of 7-methoxy-8-butyloxy-2-oxo-1,2-dihydroquinoline-3-carboxylic acid 4-amino-phenethylamide **20**, 7-methoxy-8-butoxyoxy-2-oxo-1,2-dihydroquinoline-3-carboxylic acid 4-fluoro-phenethylamide **16** and 6-nitro-7-methoxy-8-butyloxy-2-oxo-1,2-dihydroquinoline-3-carboxylic acid 4-fluoro-phenethylamide **21**. Reagents and conditions: (a) DCM, Et_3N, 4-aminophenylethylamine, rt, 30 min; (b) $NaNO_2$, pyridine·HF, rt, 1 h, 85 °C, 1 h.

The acid chloride **12** was prepared in situ as shown in Scheme 1, then coupled with the alkyne–amines following a previously published methodology[42] to produce **25** and **26**. Both compounds **25** and **26** were obtained in quantitative yields. Fluroethylazide was prepared following a previously reported method[43] and used without purification. Compounds **25** and **26** were then reacted with

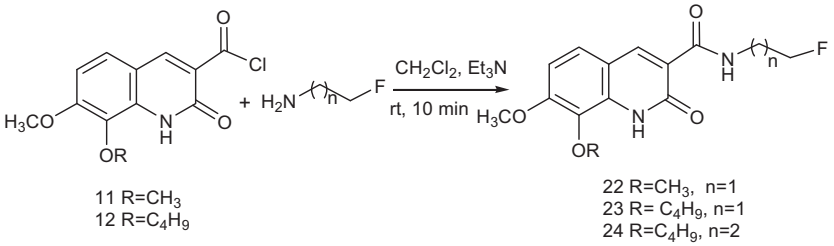


Figure 4. Scheme for synthesis of 7-methoxy-8-alkoxy-2-oxo-1,2-dihydroquinoline-3-carboxylic acid fluoro-alkylamides **22**–**24**.

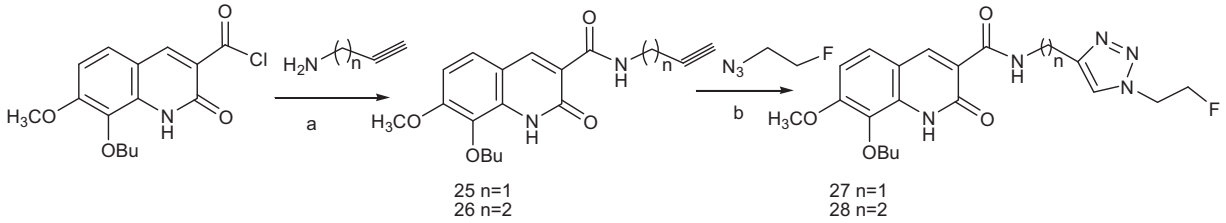


Figure 5. Scheme for synthesis of 7-methoxy-8-butoxy-2-oxo-1,2-dihydroquinoline-3-carboxylic acid-(fluoroethyl-triazolalkyl)amides **27** and **28**. Reagents and conditions: (a) CH_2Cl_2, Et_3N, rt; (b) $CuSO_4$, Na-ascorbate, DMF, rt.

fluoroethylazide in the presence of $CuSO_4$ and Na-ascorbate at room temperature using click chemistry to produce **27** and **28**. The yields in this step were 55% and 58%, respectively, for **27** and **28**.

All new compounds were fully characterized by ^{1}H, ^{13}C, and ^{19}F NMR spectroscopy and high-resolution mass spectrometry. All compounds were analyzed by high-performance liquid chromatography (HPLC) for chemical purity and found to be greater than 98% pure. These compounds were used for an in vitro binding assay with the CB2 and CB1 receptors. HPLC chromatograms of the new compounds are presented in the Supplementary data.

2.2. In vitro studies

2.2.1. CB1 and CB2 expressing U87 glioblastoma cells lines

To evaluate the specific binding of the aforementioned fluorinated compounds to *h*CB2, U87 cells expressing either *h*CB1 (control) or *h*CB2 were generated via lentiviral transduction. The red fluorescent co-reporter, mKateS158A, was used for cells sorting. The resulting populations were 84% and 98% positive for CB1 and CB2, respectively, (Fig. 6).

2.2.2. In vitro receptor binding assay

This study was performed on the compounds **13**–**16**, **18**, **19**, **21**–**24**, **27**, and **28** using [^{3}H]CP-55,940, [side chain-2,3,4-^{3}H(N)], a radiolabeled CB2 receptor ligand, following a literature method.[6] The percent inhibition against [^{3}H]CP-55,940 and the K_i values were calculated using GraphPad Prism v4 software. The dissociation constant (K_d) and maximum binding (B_{max}) for [^{3}H]CP-55,940 were determined to be 4.9 nM and 791 fmol/mg protein, respectively (Fig. 7A), in CB2-expressing cells, and these values were in agreement with previously reported data.[6] The K_d and B_{max} for [^{3}H]CP-55,940 were determined to be 16.8 nM and 1773 fmol/mg protein in CB1-expressing cells (Fig. 7B).

Compounds **14**–**16**, **18, 19**, and **21** demonstrated high binding efficiency to CB2-expressing cell membrane preparations, as evidenced by inhibition of binding of [^{3}H]CP-55,940 (Fig. 7C). Other compounds, **13**, **22**–**24**, **27**, and **28**, did not show good binding for either CB2 or CB1; in particular, compounds **13** and **22** did not have any affinity for the CB2 receptor. Compounds **23**, **24**, **27**, and **28** demonstrated low levels of binding affinity for the CB2 receptor. Among these compounds, **23**, **24**, and **27**, had better affinity than **28**. Compounds **14**–**16**, **18**, **19**, and **21** did not have any significant competitive binding to CB1 receptor-expressing cell membranes (K_i > 10 μM), suggesting that these compounds are not CB1-specific receptor ligands. By contrast, the K_i values of some of these compounds for the CB2 receptor are in the low nanomolar and sub-nanomolar range, which suggests that they have high affinity to the CB2 receptor. In terms of selectivity, compounds **14**, **15**, **16**, **18**, **19**, and **21** have better selectivity for CB2, and **19** is the most selective one. Compound **21**, with a nitro substitution at the C_6-position, did not affect any binding efficiency to the CB2 receptor, but the substitution increased its specificity for CB2. The K_i values for CB2 and CB1 for compounds **13**–**16**, **18**, **19**, **21–24**, **27**, and **28** and their selectivity for CB2 are summarized in Table 1.

Multiple 2-oxo-quinoline derivatives containing various substituents at the R_1-, R_2-, and R_3-position have been previously reported[34,35] (Table 2, Supplementary data), which showed that most of the compounds had K_i values of sub-nanomolar concentrations,[35,44] much lower than those of our compounds. For example, compounds **29**–**31**, **41**–**43** and **46**–**49** have K_i values 0.01–0.08 nM, which is >10-fold lower than we found for our compounds. However, the K_i value of compound **29** was also reported to be 35.9 nM in another report[1] as opposed to 0.087 nM in separate reports.[35,44] Furthermore, the K_i value of the potent CB2 specific indole derivative L-768242 has been reported to be 14 nM[23]; and those of the traditional tetrahydrocannabibnoids (THC) JWH229 and JWH133 were reported to be 18.0 and 3.4 nM, respectively.[21] All these K_i values for the indole derivative and traditional THC derivatives are comparable to those of our fluorinated 2-oxoquiniline derivatives. For further confirmation, we performed an in vitro binding assay of compound **20**, which has an amino group at the *para*-position of the aromatic ring and a butyl group at the C_8-position, similar to the reported compound **39**, which has a pentyl group at C_8.[34] The K_i value of **20** was 22.3 nM, comparable to that of our fluorinated compound **18** (22 nM). In one paper, the binding efficiency of **39** was reported in terms of $-\log IC_{50}$; we have also calculated the $-\log IC_{50}$ of compound **20** for comparison. The reported $-\log IC_{50}$ of **39** is 8.5 ± 0.3, and that of our compound **20** is 8.8 ± 0.4, which are comparable to those reported previously.[34] Therefore, K_i values of our compounds are reliable and consistent with previously reported results.

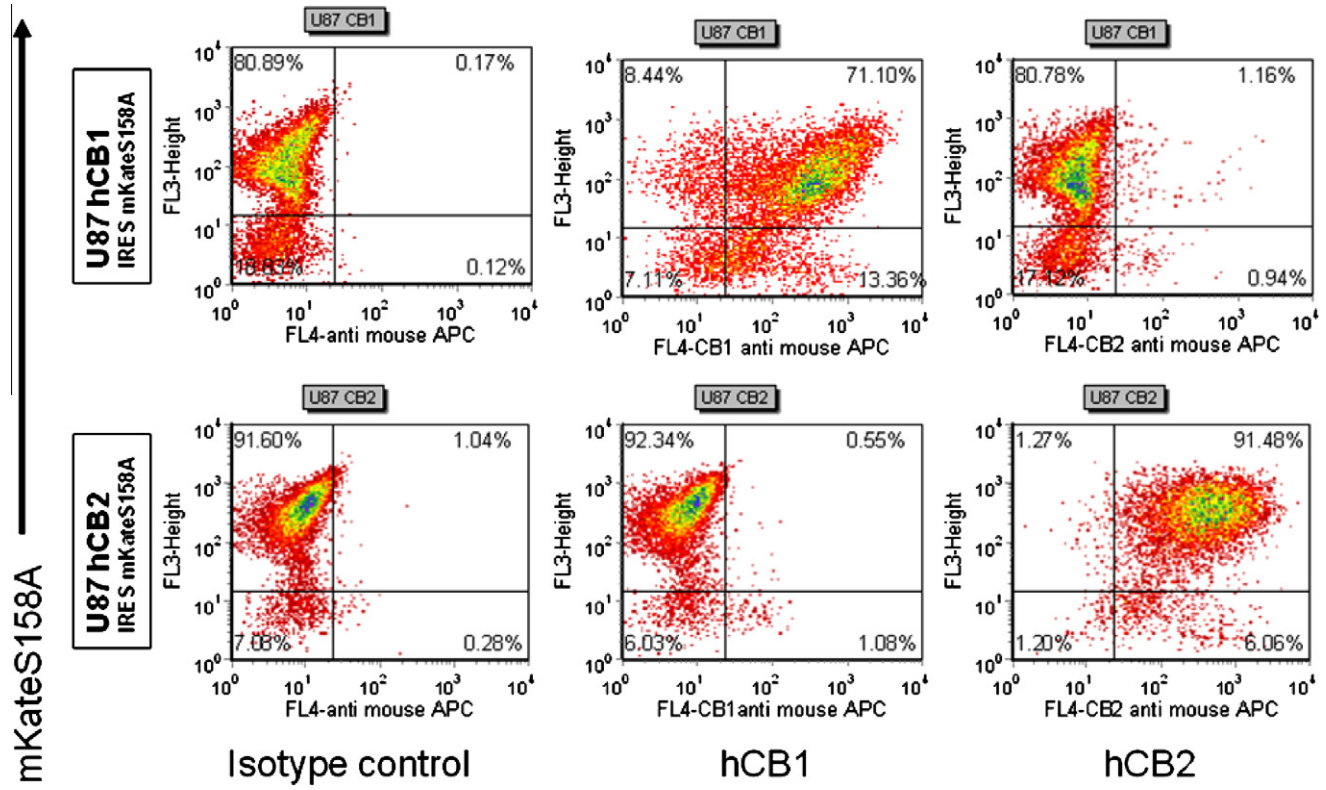


Figure 6. Expression of CB1 and CB2 in transduced U87 cells. U87 cells transduced with hCB1 (upper row) or hCB2 (lower row) and the red fluorescent co-reporter mKateS158A (*y*-axis), were assessed for expression of hCB1 and hCB2 (*x*-axis) via flow cytometry, respectively. Density plots are shown for each. Two-step staining was performed with an allophycocyanine (APC)-conjugated goat anti-mouse secondary antibody plus either an isotype control antibody (left column), anti-hCB1 (middle column) or an anti-hCB2 (right column).

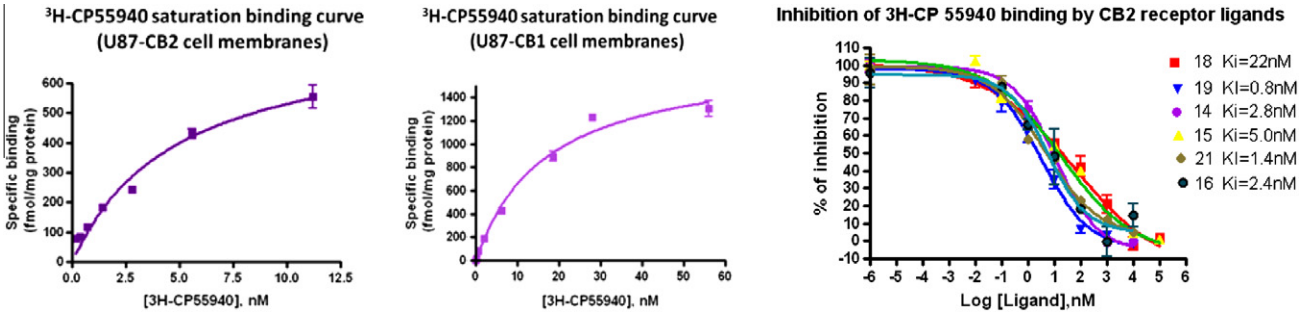


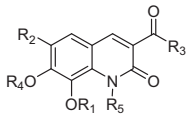
Figure 7. (A) Specific binding of [^{3}H]CP-55,940 to CB2-expressing cell membranes, (B) specific binding of [^{3}H]CP-55,940 to CB1-expressing cell membranes, (C) competitive inhibition of [^{3}H]CP-55,940 binding and K_i values for compounds **14**–**16**, **18**, **19**, and **21**.

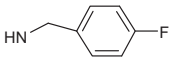
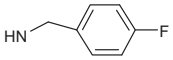
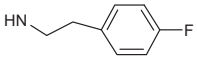
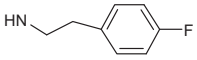
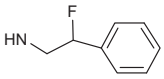
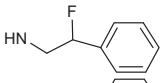
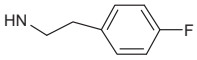
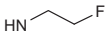
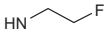
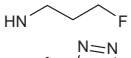
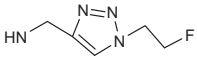
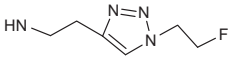
2.2.3. Structure–activity relationship

As described above (Table 1 and Fig. 7C), compounds **14**–**16**, **18**, **19**, and **21** demonstrated high binding affinity in the low-to-subnanomolar concentration range and high selectivity to the CB2 receptor. Compounds **13** and **22** did not show any binding affinity for CB2; however, compounds **23**, **24**, **25**, and **28** had some low levels of binding affinity for CB2. Compounds **13** and **14**, with a 4-fluoro-benzyl amide group (R_3) have in common a one-carbon chain between the amide bond and aromatic ring; only alkyl substituents (methyl vs butyl) at the C_8-position makes a significant difference in binding efficiency (K_i > 10,000 vs 2.8 nM) between these two compounds. This suggests that the larger group at the C_8-position enhances the binding affinity. By contrast, compounds **15** and **16**, with a two-carbon chain between the amide and the aromatic ring and a different alky substituent at the C_8-position, did not show a significant difference in binding efficiency. Therefore, the size of the substituent at C_8-position makes a significant difference in binding to CB2 for a compound with a methylene linker between the amide bond and phenyl ring. It has been reported that an ethylene linker between the amide bond and the phenyl ring (R_3) had 100-fold increase in potency compared with a methylene linker.[34] In the current series of newly synthesized fluorinated compounds, no such significant difference in binding efficiency was observed due to the different carbon-chain (methylene vs ethylene) between the amide and aromatic ring. For example, the K_i values of compounds **14** with a methylene and **16** with an ethylene linker but a butyl group at C_8 are comparable (2.8 vs 2.4 nM). A large difference in binding was observed between the methylene and ethylene linker only when the size of the substituent at the C_8-position was changed from the methyl to the butyl group.

The position of fluorine (in the aromatic ring vs side chain) also made a difference in binding efficiency. For example, compound **19**

Table 1
Inhibition constant (K_i) values of compounds **13**–**16**, **18**, **19**, **21**–**24**, **27**, and **28** using [^{3}H]CP-55,940 a radioligand for *h*CB1 and *h*CB2 cannabinoid receptors



Compd	R1	R2	R3	R4	R5	*h*CB2 K_i (nM)	*h*CB1 K_i (μM)	Selectivity CB2/CB1
13	Methyl	H		CH_3	H	>10,000	>10	>1
14	Butyl	H		CH_3	H	2.8 ± 0.4	>10	>3570
15	Methyl	H		CH_3	H	5.0 ± 1.1	>10	>2000
16	Butyl	H		CH_3	H	2.4 ± 0.7	>10	>4166
18	Methyl	H		CH_3	H	22 ± 4.1	>10	>454
19	Butyl	H		CH_3	H	0.8 ± 0.3	>10	>12500
21	Butyl	NO_2		CH_3	H	1.4 ± 0.4	>10	>7143
22	Methyl	H		CH_3	H	>10,000	>10	>1
23	Butyl	H		CH_3	H	486 ± 96	>10	>20.5
24	Butyl	H		CH_3	H	58 ± 14	>10	>172
27	Butyl	H		CH_3	H	620 ± 114	>10	>16
28	Butyl	H		CH_3	H	2400 ± 680	>10	>4

had a much higher binding efficiency (K_i = 0.8 nM) than compound **16** (K_i = 2.4 nM). The major difference between these two compounds is the position of fluorine: in the side chain rather than in the aromatic ring. Compounds with fluorine in the side chain and different substituents at the C_8-position had significantly different binding efficiency; for example, when the R_1 was changed from the butyl to methyl group, the K_i increased from 0.8 nM for **19** to 22 nM for **18**. This observation suggests that a larger group at the C_8-position (R_1) has better binding capability than a smaller group, irrespective of the other substituents, possibly due to the lipophylic nature of these compounds. Fluorine on the side chain rather than in the aromatic ring may also play a significant role in the π-stacking interactions between the ligand and aromatic amino acids of the receptor. Compounds with a 4-substituted-phenylethyl amide (R_3) with pentyl substituent at C_8-position (R_1) have been developed and reported earlier.[34,35] The presence of an electron withdrawing nitro group at the *para*-position of the aromatic ring has been shown to decrease potency through diminishing aromatic π-stacking interactions compared with other substituents at the *para*-position.[34] Thus, in our compounds, the presence of electronegative fluorine at the *para*-position of the aromatic ring may withdraw electron density of the π-electrons, reduce the stacking interaction, and diminish the potency of the compound compared with a fluorine in the benzylic position, which is consistent with the results in the literature.[34] As a result, compound **19** is 3-fold more potent than **16**. Our results suggest that substitution at the C_6-position improves selectivity for CB2 over CB1. Compound **21** has a nitro group at the C_6-postion, and this substitution makes the compound more selective for CB2, maintaining a low K_i value. This result is also consistent with the literature, because other C_6-substituted compounds **30**, **41**, and **42** (Table 2, Supplementary data) have been reported to be highly selective and sensitive CB2 ligands.[35,44]

Substitution of methyl for butyl at the C_8-position makes a significant difference in binding efficiency for the methylene-containing compounds (**13** and **14**), but no such difference was observed in the ethylene-containing compounds **15** and **16**. By contrast, there was a small difference in binding efficiency between the compounds containing a methylene and ethylene linker with a butyl group at the C_8-position, that is, an ethylene-containing compound had slightly better binding efficiency than those with a methylene-containing compound. There was a general trend that compounds containing a C_8-butyl substituent have better binding efficiency than those containing C_8-methyl. A nitro group at the C_6-position in compound **21** had a small influence that improved the binding efficiency compared with that of the C_6-unsubstituted compound **16**.

Substitution at the amide bond (R_3) makes a significant difference in binding efficiency of these 2-oxo-quinoline derivatives. Compounds with a straight chain substituent, such as fluoroethyl, showed no binding affinity; however, binding affinity increased with increasing carbon-chain length, which is consistent with the hydrocarbon-substituted compounds reported previously.[34] To further explore the structure–activity relationship with regard to π-stacking and charge or polarity on the R_3, we synthesized triazol-containing derivatives of 2-oxoquiniline. If these compounds had demonstrated improved binding characteristics to CB2, the rapid radiolabeling of these compounds could be achieved with click chemistry approaches using an alkyne derivative of 2-oxoquinoline and ^{18}F-labeled fluoroethylazide. However, the binding efficiency of these compounds was quite low. Thus, compounds **27** and **28** have K_i values of 620 and 2400 nM, respectively. These results suggest that substitution at the amide bond is critical for the binding efficiency of these compounds. In terms of specificity, compound **19** has the highest specificity for the CB2 receptor, and compounds **21**, **16**, and **14** are the next selective compounds for the CB2 receptor. Thus, given their binding efficiency and specificity to the CB2

receptor, the selected compounds **14**, **15**, **16**, **19**, and **21** require further in vivo studies in animal models.

Previously, a few ligand docking, mutation, and modeling studies have been reported for the CB2 receptor, that have used the structure of rhodopsin as a template to construct a model of the CB2 receptor for use in docking studies.[45] Based on docking studies with CB2 selective ligands, it was reported that hydrophobic and aromatic stacking interactions were more important than hydrogen bonding for CP-55,940 and WIN-55,212-2, and numerous aromatic stacking interactions were observed for the binding of WIN-55,212-2 at the CB2 receptor site.[46] It appears that hydrophobic and aromatic π-stacking interactions play similar roles in the newly synthesized library of compounds in binding with the CB2 receptor, and our results are in agreement with those previously reported for CP-55,940 and WIN-55,212-2.[46]

3. Conclusion

We have synthesized and tested a library of new fluorinated 2-oxoquinoline derivatives as CB2 receptor ligands. The results of our in vitro competitive radioligand binding studies suggest that some of these compounds are highly active and specific CB2 receptor ligands in the low nanomolar to sub-nanomolar affinity range and no significant binding to CB1 receptors. Therefore, these compounds may be useful for therapy of neuropathic pain, inflammation, and immune disorders and should be evaluated further in corresponding disease models in animals. In addition, compound **19** could be radiolabeled with ^{18}F and explored for PET imaging of CB2 expression. The studies are currently in progress.

4. Experimental

4.1. Chemistry

4.1.1. Reagents and instrumentation

All reagents and solvents were purchased from Aldrich Chemical Co. (Milwaukee, WI), and used without further purification unless otherwise specified. Tritiated CP-55,950 ([^{3}H]CP-55,940), with a specific activity of 174.6 mCi/μmol was purchased from PerkinElmer (USA). Thin-layer chromatography (TLC) was performed on pre-coated Kieselgel 60 F254 (Merck, Darmstadt, Germany) glass plates. Proton, ^{13}C, and ^{19}F NMR spectra were recorded on a Brucker 300 MHz spectrometer or a 600 MHz spectrometer using tetramethylsilane as an internal reference and hexafluorobenzene as an external reference, respectively, at The University of Texas M. D. Anderson Cancer Center. High-resolution mass spectra were obtained on a Brucker BioTOF II mass spectrometer at the University of Minnesota using the electrospray ionization (ESI) technique.

HPLC was performed with an 1100 series pump (Agilent, Germany), with a built-in UV detector operated at 214 or 254 nm, and a radioactivity detector with single-channel analyzer (Bioscan, Washington DC) with a C_{18} reverse-phase analytical column (4.6 × 250 mm; Alltech, Econosil). An acetonitrile/water (MeCN/H_2O) solvent system with various composition (depending on compound), was used for quality control analyses at a flow of 1 mL/min.

4.1.2. Chemical synthesis

4.1.2.1. Preparation of 7-methoxy-8-alkoxy-2-oxo-1,2-dihydroquinoline-3-carboxylic acid-(4-fluorobenzyl)amide: 13, 14. Both compounds **13** and **14** were synthesized following the same methodology; a representative method is described here. To a solution of the 4-fluorobenzylamine (0.187 mmol) in DCM (3.0 mL) was added triethylamine (37 μL), and the mixture was cooled to 0 °C. The acid chloride **11** or **12** (0.280 mmol) in DCM (0.5 mL) was added slowly and the mixture was stirred for 10 min; then the cooling bath was removed, and the mixture was stirred for an additional 20 min at rt. The solvent was removed under reduced pressure and the residue was purified by flash chromatography on a silica gel column using 5% MeOH/CH_2Cl_2. The solvent was evaporated on a rotary evaporator, and the product **13** was obtained as a white solid (powder) in 76% yield. ^1H NMR ($CDCl_3$) δ:10.04 (t, 1H, NH), 9.36 (s, 1H, NH), 8.92 (s, 1H, C_4–H), 7.50 (d, J = 8.4 Hz, 1H, C_5–H), 7.36 (m, 2H, aromatic), 7.03 (m, 2H, aromatic), 6.97 (d, J = 8.4 Hz, 1H, C_6-H), 4.66 (d, J = 6.6 Hz, 2H, N–CH_2), 4.02 (s, 3H, OMe), 3.99 (s, 3H, OMe). ^{13}C NMR ($CDCl_3$) δ: 163.66, 162.28, 162.05 (d, $J_{1\text{-}F}$ = 243.8 Hz), 154.51, 145.13, 134.47, 134.45, 133.28 (d, $J_{4\text{-}F}$ = 5.0 Hz), 129.33 (d, $J_{3\text{-}F}$ = 8.8 Hz), 125.63, 119.49, 115.38 (d, $J_{2\text{-}F}$ = 21.3 Hz), 114.29, 109.11, 61.08, 56.32, 42.81.^{19}F NMR ($CDCl_3$) δ: −114.66 (septet, AA'MM'X, J = 15.0 Hz, J = 9.0 Hz, J = 6.0 Hz, J = 3.0 Hz). High-resolution MS: calculated for $C_{19}H_{17}FN_2O_4$ (M+H) 356.1172, found 356.1169.

Compound **14** was purified on a silica gel column eluted with 70% EtOAc/hexane. The product **14** was obtained as a white solid in 50% yield. ^1H NMR ($CDCl_3$) δ: 8.55 (s, 1H, C_4–H), 7.60 (d, J = 8.4 Hz, 1H, C_5–H), 7.42–7.37 (m, 3H, aromatic), 7.10-7.04 (m, 3H, aromatic), 4.70 (d, J = 6.6 Hz, 2H, NCH_2), 4.27 (t, J = 6.6 Hz, 2H, OCH_2), 4.11 (s, 3H, OMe), 4.10 (s, 3H, OMe), 1.85–1.80 (m, 2H), 1.58–1.55 (m, 2H), 0.98 (t, J = 7.2 Hz, 3H, CH_3). ^{13}C NMR ($CDCl_3$) δ: 163.67, 162.21, 162.05 (d, $J_{1\text{-}F}$ = 243.8 Hz), 154.51, 145.13, 134.47, 134.45, 133.28 (d, $J_{4\text{-}F}$ = 5.0 Hz), 129.33 (d, $J_{3\text{-}F}$ = 8.8 Hz), 125.63, 119.49, 115.38 (d, $J_{2\text{-}F}$ = 21.3 Hz), 114.29, 109.13, 73.54, 56.27, 42.79, 32.21, 32.21, 19.13, 13.82. ^{19}F NMR ($CDCl_3$) δ: −114.4 6 (septet, AA'MM'X, J = 15.0 Hz, J = 9.0 Hz, J = 6.0 Hz, J = 3.0 Hz). High-resolution MS: calculated for $C_{22}H_{23}FN_2O_4$ (M+H) 398.1642, found 398.1649.

4.1.2.2. Preparation of 7-methoxy-8-alkoxy-2-oxo-1,2-dihydroquinoline-3-carboxylic acid-(4-fluorophenylethyl)amide: 15, 16. Both compounds **15** and **16** were synthesized following the same methodology; a representative method is described here. To a solution of the 4-fluorophenyl ethylamine (0.187 mmol) in DCM (3.0 mL) was added triethylamine (37 μL), and the mixture was cooled to 0 °C. The acid chloride **11** or **12** (0.280 mmol) in DCM (0.5 mL) was added slowly and the mixture was stirred for 10 min; then the cooling bath was removed, and the mixture was stirred for an additional 20 min at room temperature. The solvent was removed under reduced pressure, and the residue was purified by flash chromatography on a silica gel column using 5% MeOH/CH_2Cl_2. The solvent was evaporated on a rotary evaporator and the product **15** was obtained as a white solid in 86% yield. ^1H NMR ($CDCl_3$) δ: 9.70 (s, 1H, NH), 9.32 (s, 1H, NH), 8.88 (s, 1H, C_4–H), 7.50 (d, J = 8.4 Hz, 1H, C_5–H), 7.22–7.28 (m, 2H, aromatic), 6.95–7.05 (m, 3H, aromatic), 4.01 (s, OCH_3), 3.99 (s, 3H, OCH_3), 3.72 (q, 2H, J = 7.2 Hz, N–CH_2), 2.94 (t, J = 7.2 Hz, 2H, benzylic). ^{13}C NMR ($CDCl_3$) δ: 163.66, 162.28, 162.05 (d, $J_{1\text{-}F}$ = 243.8 Hz), 154.51, 145.13, 134.47, 134.45, 133.28 (d, $J_{4\text{-}F}$ = 5.0 Hz), 129.33 (d, $J_{3\text{-}F}$ = 8.8 Hz), 125.63, 119.49, 115.38 (d, $J_{2\text{-}F}$ = 21.3 Hz), 114.29, 109.11, 61.08, 56.32, 56.2, 42.81. High-resolution MS: calculated for $C_{20}H_{19}N_2FNaO_4$ (M+Na) 393.1227, found 393.1244.

Compound **16** was obtained in 96% yield. ^1H NMR ($CDCl_3$) δ: 9.65 (s, 1H, NH), 9.36 (s, 1H, NH), 8.85 (s, 1H, C_4–H), 7.43 (d, J = 8.4 Hz, 1H, C_5–H), 7.22–7.20 (m, 2H, aromatic), 6.96–7.00 (m, 2H, aromatic), 6.92 (d, J = 9.0 Hz, 1H, C_6–H), 4.12 (t, J = 6.6 Hz, 2H, OCH_2), 3.95 (s, 3H, OMe), 3.70–3.67 (m, 2H, N–CH_2), 2.90 (t, J = 7.2 Hz, 2H, benzylic), 1.80–1.75 (m, 2H), 1.52–1.45 (m, 2H), 0.97 (t, J = 7.2 Hz, 3H, CH_3). ^{13}C NMR ($CDCl_3$) δ: 163.5, 162.4, 162.2, 160.7, 154.4, 144.9, 134.8 (2C), 133.4, 132.4, 130.2 130.1, 125.2, 119.3, 115.3, 115.2, 114.3, 109.1, 73.5, 56.2, 41.0, 34.9, 32.1, 19.1, 13.8. ^{19}F NMR ($CDCl_3$) δ: -116.9. High-resolution MS: calculated for $C_{23}H_{25}N_2FNaO_4$ (M+Na) 435.1718, found 435.1788.

4.1.2.3. Preparation of 2-fluoro-2-phenylethylamine: 17. Compound **17** was prepared following a literature method[41] with modifications. Briefly, to a stirred solution of mandelonitrile (1.33 g, 1.0 mmol, 1.0 equiv) in dry DCM (5 mL) at 0 °C was added diethylaminosulfure trifluoride (DAST) (1.77 g, 1.1 mmol, 1.1 equiv) in DCM (2 mL). The resulting mixture was stirred at 0 °C and stirred for 30 min, diluted with DCM (15 mL), and treated with saturated $NaHCO_3$. The organic layer was collected and dried over $MgSO_4$. After removal of the solvent under vacuum, the crude product was chromatographed on a silica gel column and eluted with 10% EtOAc/hexane to afford α-fluorophenylacetonitrile as yellow oil (0.88 g) in 65% yield. The ^{1}H NMR spectrum was consistent with the literature,[41] and ^{19}F NMR showed a doublet at −167.4 ppm with J = 48 Hz. Finally, the cyano-group was reduced to the corresponding amine by treatment with 1 M borane–THF solution in THF at 0 °C for 40 min and quenched the reaction mixture with ethanolic hydrochloric acid, then concentrated under reduced pressure. The desired 2-fluorophenylethyl amine was triturated with CH_3CN and then recrystallized from ethanol/acetonitrile to afford **17** as colorless crystals in 49% yield. ^{1}H NMR ($CDCl_3$) δ: 7.50 (m, 5H, aromatic), 5.8 (dddd, J = 48.0, 8.4, 7.2, 4.5, 3.0 Hz, 1H, benzylic), 3.50 (m, 2H, methylene). ^{19}F NMR ($CDCl_3$) δ: −183 (dm). MS: 154 (M+H).

4.1.2.4. Preparation of 7-methoxy-8-alkoxy-2-oxo-1,2-dihydroquinoline-3-carboxylic acid-(2-fluoro-2-phenyl ethyl)amide: 18, 19. Both compounds **18** and **19** were prepared using the same methodology, a representative method is described here. To a solution of the 2-fluoro-2-phenylethylamine (0.187 mmol) in DCM (3.0 mL) was added triethylamine (37 μL), and the mixture was cooled to 0 °C. The acid chloride **11** or **12** (0.280 mmol) in DCM (0.5 mL) was added slowly and the mixture was stirred for 10 min; then the cooling bath was removed and the mixture was stirred for an additional 20 min at rt. The solvent was removed under reduced pressure, and the residue was purified by flash chromatography on a silica gel column using 5% MeOH/CH_2Cl_2. The solvent was evaporated on a rotary evaporator, and the product **18** was obtained as a white solid (powder) in 75% yield. ^{1}H NMR ($CDCl_3$) δ: 10.00 (t, J = 6 Hz, 1H, NH), 9.32 (s, 1H, NH), 8.89 (s, 1H, C_4–H), 7.35–7.55 (m, 6H, aromatic H & C_5–H), 6.97 (d, J = 9.0 Hz, 1H, C_6–H), (dddd, J = 48.0 Hz, 8.4 Hz, 7.2 Hz,4.5 Hz, 3.0 Hz, 1 H, benzylic), 4.05–4.20 (m,1H, N–CH_2), 4.03 (s, 3H, OMe), 4.00 (s, 3H, OMe), 3.58–3.84 (m, 1H, N–CH_2). ^{13}C NMR ($CDCl_3$) δ: 163.4, 162.1, 154.1, 144.7, 144.6, 133.4, 132.3, 129.5 (2C), 129.3, 125.2, 120.0, 115.7 (2C), 114.2, 109.0, 73.7, 56.3, 41.4, 35.0, 29.8, 28.1, 22.4, 13.8. ^{19}F NMR ($CDCl_3$) δ: −182.71 (dt, J = 47.6 Hz, J = 30.8 Hz, J = 14.0 Hz). High-resolution MS: calculated for $C_{20}H_{19}N_2FNaO_4$ (M+Na) 393.1227, found 393.1258.

Compound **19** was purified on silica gel column using 70% EtOAc/Hexane. The product **19** was obtained as a white solid in 50% yield. ^{1}H NMR ($CDCl_3$) δ: 10.00 (t, J = 6 Hz, 1H, NH), 9.17 (s, 1H, NH), 8.88 (s, 1H, C_4-H), 7.28–7.49 (m, 6H, aromatic & C_5–H), 6.95 (d, J = 9.0 Hz, 1H, C_6–H), 5.70 (dm, J_{HF} = 48 Hz 1H, benzylic), 4.14–4.19 (m, 3H, O-CH_2 & N–CH_2), 3.99 (s, 3H, O–CH_3), 3.65–3.85, (m, 1H, N–CH_2), 1.76-1.84 (m, 2H), 1.50–1.57 (m, 2H), 1.02 (t, J = 7.2 Hz, 3H, CH_3). ^{13}C NMR ($CDCl_3$) δ: 163.96, 162.02, 154.45, 145.06, 137.78, 137.63, 133.56, 132.42, 128.67, 128.37, 125.64, 125.58, 125.36, 119.35, 114.2, 109.09, 92.71 (d, $J_{1\text{-}F}$ = 172.5 Hz), 73.56, 56.30, 45.40 (d, $J_{2\text{-}F}$ = 24.0 Hz), 32.26, 19.15, 13.8. ^{19}F NMR ($CDCl_3$) δ: −183.11 (dt, J=47.6 Hz, J = 30.8 Hz, J = 14.0 Hz). High-resolution MS: calculated for $C_{23}H_{25}N_2FNaO_4$ (M+Na) 435.1696, found 435.1702.

4.1.2.5. Preparation of 7-methoxy-8-butyloxy-2-oxo-1,2-dihydroquinoline-3-carboxylic acid 4-amino-phenethylamide: 20. Compound **20** was prepared by similar method as described for compounds **13–16** (Scheme 1). Briefly, the acid chloride **12** (1.0 equiv) in anhydrous DCM was added drop-wise to a solution of 4-aminophenylethylamine (1.5 equiv) and triethylamine (2.0 equiv) in dry DCM at 0 °C. The ice bath was removed in 15 min and the reaction mixture stirred at room temperature (rt) for 16 h. The reaction was quenched in a brine solution and the product was extracted with DCM, dried over $MgSO_4$, and then concentrated under vacuum at ambient temperature. The crude residue was chromatographed on a silica gel column eluting with 10% MeOH/CH_2Cl_2 to give the desired product **20** in 96% yield. ^{1}H NMR ($CDCl_3$) δ: 9.67 (s, 1 H), 9.13 (s, 1H), 8.88 (s, 1H), 7.48 (d, J = 8.4 Hz, 1H), 7.10 (d, J = 7.8 Hz, 2H), 6.95 (d, J = 8.4 Hz, 1H), 6.69 (d, J = 7.2, 2H), 4.16 (t, J = 6.6 Hz, 2H), 4.00 (s, 3H), 3.70–3.69 (m, 2H), 3.61 (br s, 2H), 2.86 (t, J = 7.2 Hz, 2 H), 1.83–1.80 (m, 2H), 1.55–1.52 (m, 2H), 1.02 (t, J = 7.2 Hz, 3H). ^{13}C NMR ($CDCl_3$) δ: 163.4, 162.1, 154.1, 144.7, 144.6, 133.4, 132.3, 129.5 (2C), 129.3, 125.2, 120.0, 115.7 (2C), 114.2, 109.0, 73.7, 56.3, 41.4, 35.0, 29.8, 28.1, 22.4, 13.8. MS: calculated for $C_{22}H_{25}N_3O_4$ (M+Na) 395.1643, found 395.1648.

4.1.2.6. Preparation of 7-methoxy-8-butyloxy-2-oxo-1,2-dihydroquinoline-3-carboxylic acid-(4-fluorphenethyl)amide and 6-nitro-7-methoxy-8-butyloxy-2-oxo-1,2-dihydroquinoline-3-carboxylic acid-(4-fluorphenethyl)amide: 16, 21. Compound **16** was prepared by two different methods.

Method 1: This method is a direct coupling of the 4-fluorophenyl ethylamine with the acid chloride **12**, similar to the preparation of other compounds, **13–16**. *Method 2:* To a solution of 7-methoxy-8-butyloxy-2-oxo-1,2-dihydroquinoline-3-carboxylic acid *p*-aminophenethylamide **20** (20 mg, 1 equiv) in 125 μL of pyridine·HF was added 5.5 mg of sodium nitrite (0.08 mmol, 1.5 equiv). The reaction mixture was stirred at rt for 1 h and then at 85 °C for an additional hour. The reaction mixture was quenched with ice-water (10 mL), and the product was extracted with ethyl acetate, dried over $MgSO_4$, and then the solution was concentrated under vacuum. The crude residue was chromatographed on silica gel column eluting with 2:1 EtOAc/hexane to give 8.0 mg of the desired product **16** (40% yield) and 8 mg of its 6-nitro derivative **21** (36% yield). The NMR and mass spectra of **16** produced by this method were identical with those of **16** obtained from method 1.

Compound **21** was isolated as a by-product in 36% yield during the preparation of **16** using method 2. ^{1}H NMR ($CDCl_3$) δ: 9.63 (s, 1H, NH), 9.48 (s, 1H, NH), 9.29 (s, 1H, C_4–H), 7.76 (s, 1H, C_5–H), 7.22–7.19 (m, 2H, aromatic), 6.98 (t, J = 8.4 Hz, 2H, aromatic), 4.32 (t, J = 7.2 Hz, 2H, O–CH_2), 4.03 (s, 3H, OMe), 3.71–3.68 (m, 2H, N–CH_2), 2.91 (t, J = 7.2 Hz, 2H, benzylic), 1.81–1.76 (m, 2H), 1.51–1.45 (m, 2H), 0.98 (t, J = 7.2 Hz, 3H, CH_3). ^{13}C NMR ($CDCl_3$) δ: 162.4 (2C), 160.8 (2C), 151.3, 141.2, 140.1, 136.4, 134.7 (2C), 133.4 130.2, 130.1, 123.2, 115.3, 115.2, 108.0, 107.3, 74.2, 56.8, 41.2, 34.8, 29.7, 19.0, 13.7. ^{19}F NMR ($CDCl_3$) δ: −116.8. High-resolution MS: calculated for $C_{23}H_{24}N_3FO_6Na$ (M+Na) 480.1718, found 480.1593.

4.1.2.7. Preparation of 7-methoxy-8-alkoxy-2-oxo-1,2-dihydroquinoline-3-carboxylic acid-(fluoroalkyl)amide: 22–24. Compounds **22–24** were synthesized following the same methodology as described in Scheme 4; a representative method is described here. To a solution of the 2-fluoroethylamine (0.187 mmol) in DCM (3.0 mL) was added triethylamine (37 μL), and the mixture was cooled to 0 °C. The acid chloride **11** or **12** (0.280 mmol) in DCM (0.5 mL) was added slowly, the mixture was stirred for 10 min; then the cooling bath was removed, and the mixture was stirred for an additional 30 min at rt. The solvent was removed under reduced pressure, and the residue was purified by flash chromatography on a silica gel column using 5% MeOH/CH_2Cl_2. The solvent was evaporated on a rotary evaporator and

the product **22** was obtained as a white solid (powder) in 90% yield. ^{1}H NMR ($CDCl_3$) δ: 9.94 (br t, 1H, NH), 9.55 (s, 1H, NH), 8.88 (s, 1H, C_4–H), 7.48 (d, J = 9.0 Hz, 1H, C_5–H), 6.97 (d, J = 9.0 Hz, 1H, C_6–H), 4.65 (dt, $J_{1\text{-}F}$ = 47.4 Hz, J_2 = 4.8 Hz, 2H, F–CH_2), 3.99 (s, 6H, OMe), 3.85 (q, J_1 = 10.5 Hz, J_2 = 5.4 Hz, 1H, N–CH_2), 3.76 (q, J_1 = 10.5 Hz, J_2 = 5.4). ^{13}C NMR ($CDCl_3$) δ: 164.01, 162.26, 154.55, 145.03, 133.40, 133.30, 125.63, 119.34, 114.23, 109.03, 82.45 (d, $J_{1\text{-}F}$ = 161.3 Hz), 61.10, 56.32, 40.00 (d, $J_{3\text{-}F}$ = 21.1 Hz). ^{19}F NMR ($CDCl_3$) δ: −223.06 (m). High-resolution MS: calculated for $C_{14}H_{15}FN_2O_4Na$ (M+Na) 317.0914, found 317.0909.

Compound **23** was also purified on a silica gel column eluted with 70% EtOAc/hexane and obtained as a white solid in 92% yield. ^{1}H NMR ($CDCl_3$) δ: 9.91 (br t, 1H, NH), 9.17 (s, 1H, NH), 8.88 (s, 1H, C_4–H), 7.47 (d, J = 9.0 Hz, 1H, C_5–H), 6.96 (d, J = 9.0 Hz, 1H, C_6–H), 4.60 (dt, $J_{1\text{-}F}$ = 47.6 Hz, J_2 = 5.7 Hz, 2H, F-CH_2), 4.17 (t, J = 6.6 Hz, 2H, OCH_2), 3.99 (s, 3H, OMe), 3.62 (q, J_1 = 12.9 Hz, J_2 = 6.9 Hz, 2H, N-CH_2), 1.80 (m, 2H), 1.58 (m, 2H), 0.98 (t, J = 7.2 Hz, 3H, CH_3). ^{13}C NMR ($CDCl_3$) δ: 164.00, 162.08, 154.45, 145.03, 133.55, 132.38, 125.36, 119.28, 114.20, 108.07, 82.43 (d, $J_{1\text{-}F}$ = 168.3 Hz), 73.54, 56.28, 39.97(d, $J_{2\text{-}F}$ = 21.1 Hz), 32.21, 19.13, 13.83. ^{19}F NMR ($CDCl_3$) δ: −223.18 (m). High-resolution MS: calculated for $C_{17}H_{21}FN_2O_4$ Na (M+Na) 336.1485, found 336.1485.

Compound **24** was obtained as a white solid in quantitative yield after purification. ^{1}H NMR ($CDCl_3$) δ: 9.75 (br t, 1H, NH), 9.16 (s, 1H, NH), 8.87 (s, 1H, C_4–H), 7.47 (d, J = 9.0 Hz, 1H, C_5–H), 6.95 (d, J = 9.0 Hz, 1H, C_6–H), 4.60 (dt, $J_{1\text{-}F}$ = 47.6 Hz, J_2 = 5.7 Hz, 2H, F–CH_2), 4.17 (t, J = 6.6 Hz, 2H, OCH_2), 3.99 (s, 3H, OMe), 3.62 (q, J_1 = 12.9 Hz, J_2 = 6.9 Hz, 2H, N-CH_2), 2.13–2.00 (m, 2H), 1.81 (m, 2H), 1.52 (m, 2H), 1.00 (t, J = 7.2 Hz, 3H, CH_3). ^{13}C NMR ($CDCl_3$) δ: 169.79, 162.15, 154.36, 144.36, 133.45, 132.38, 125.32, 119.43, 114.26, 109.07, 82.01 (d, $J_{1\text{-}F}$ = 164.3 Hz), 73.54, 56.27, 35.92 (d, $J_{3\text{-}F}$ = 5.2 Hz), 32.22, 30.55 (d, $J_{2\text{-}F}$ = 19.6 Hz), 19.13, 13.83. ^{19}F NMR ($CDCl_3$) δ: −226.82 (m). High-resolution MS: calculated for $C_{18}H_{23}FN_2O_4Na$ (M+Na) 373.1540, found 373.1573.

4.1.2.8. Preparation of 7-methoxy-8-butoxy-2-oxo-1,2-dihydroquinoline-3-carboxylic acid-(alkyl-alkyne)amide: 25, 26. Compounds **25** and **26** were prepared by the same method as described in Scheme 5 following a previously published method.[42] The acid chloride **12** (0.280 mmol) in DCM (0.5 mL) was added slowly to a solution of either 2-propyne amine or 3-butyne amine (0.187 mmol) in DCM (3.0 mL), which was cooled to 0 °C, and the mixture was stirred for 10 min. The cooling bath was removed, and the reaction mixture was stirred for an additional 3 h at rt. The solvent was removed under reduced pressure, and the residue was purified by flash chromatography on a silica gel column using 70% EtOAc/hexane. Compound **25** was obtained in quantitative yield; its ^{1}H NMR spectrum was consistent with that reported in the literature.[42]

Compound **26** was obtained as a white solid in quantitative yield. ^{1}H NMR ($CDCl_3$) δ: 9.90 (br t, 1H, NH), 9.18 (s, 1H, NH), 8.87 (s, 1H, C_4–H), 7.46 (d, J = 9.0 Hz, 1H, C_5–H), 6.95 (d, J = 9.0 Hz, 1H, C_6–H), 4.15 (t, J = 6.9 Hz, 2H, OCH_2), 3.99 (s, 3H, OMe), 3.65 (q, J_1 = 12.9 Hz, J_2 = 6.6 Hz, 2H, N–CH_2), 2.54 (ddd, J = 9.3 Hz, J = 6.9 Hz, J = 2.7 Hz, 2H), 2.06 (t, J = 2.7 Hz, 2H), 1.81 (m, 2H), 1.52 (m, 2H), 1.00 (t, J = 7.2 Hz, 3H, CH_3). ^{13}C NMR ($CDCl_3$) δ: 163.67, 162.11, 154.37, 144.93, 133.47, 132.36, 125.33, 119.44, 114.25, 109.06, 81.70, 73.54, 69.76, 56.28, 38.36, 32.24, 19.51, 19.13, 13.83. High-resolution MS: calculated for $C_{19}H_{22}N_2O_4Na$ (M+Na) 365.1477, found 365.1475.

4.1.2.9. Preparation of 2-fluorethylazide. This compound was prepared according to a previously reported method.[43] Briefly, 2-fluoroethyl bromide (0.028 mmol) was reacted with sodium azide (0.029 mmol) in DMF (10 mL) for 48 h at rt. The solution was filtered and used without further purification.

4.1.2.10. Preparation of 7-methoxy-8-butoxy-2-oxo-1,2-dihydroquinoline-3-carboxylic acid-((1-(2-fluoroethyl)-1*H*-1,2,3-triazol)alkyl)amide: 27, 28. Compounds **27** and **28** were prepared from **25** and **26**, respectively, following a method similar to that described in Scheme 5. A representative procedure is described here. To a solution of fluoroethylazide (1.5 mL, 2.8 mM, in DMF) was added propargyl amide **25**. In a separate flask was dissolved copper(II) sulfate pentahydrate (25.0 mg, 0.10 mmol) in water (1.0 mL) followed by addition of sodium ascorbate (56 mg, mmol). This solution was transferred to the above reaction mixture and stirred at rt for 12 h. The solvent was removed under a stream of air; the residue was redissolved in DCM and subjected to column chromatography using 10% MeOH/CH_2Cl_2. The solvent was evaporated on a rotary evaporator and the product **27** was obtained as a white solid in 55% yield. ^{1}H NMR ($CDCl_3$) δ: 10.00 (br t, 1H, NH), 9.14 (s, 1H, NH), 8.89 (s, 1H, C_4-H), 7.58 (s, 1H, triazole-H), 7.49 (d, J = 9.0 Hz, 1H, C_5–H), 6.60 (d, J = 9.0 Hz, 1H, C_6–H), 4.88 (t, J = 4.7 Hz 1H, N–CH), 4.81 (m, 2H), 4.70 (m, 2H), 4.60 (m, 1H, F–CH), 4.16 (t, J = 6.6 Hz, 2H, OCH_2), 3.99 (s, 3H, OMe), 1.81 (m, 2H), 1.52 (m, 2H), 1.00 (t, J = 7.2 Hz, 3H, CH_3). ^{13}C NMR ($CDCl_3$) δ: 163.84, 161.93, 154.45, 145.84, 145.03, 133.54, 132.39, 125.35, 123.20, 119.34, 114.19, 109.09, 81.51 (d, $J_{1\text{-}F}$ = 171.0 Hz), 73.55, 56.29, 50.50 (d, $J_{2\text{-}F}$ = 20.0 Hz), 35.26, 32.22, 19.12, 13.33. ^{19}F NMR ($CDCl_3$) δ: −221.6 (m). High-resolution MS: calculated for $C_{20}H_{24}FN_5O_4Na$ (M+Na) 440.1710, found 440.1694.

Compound **28** was obtained as a white solid in 58% yield. ^{1}H NMR ($CDCl_3$) δ: 9.79 (br t, 1H, NH), 9.17 (s, 1H, NH), 8.86 (s, 1H, C_4–H), 7.58 (s, 1H, triazole-H), 7.46 (d, J = 9.0 Hz, 1H, C_5–H), 6.95 (d, J = 9.0 Hz, 1H, C_6–H), 4.87 (t, J = 4.7 Hz 1H, N–CH), 4.70 (m, *overlapped,* 2H, N–CH–CH–F), 4.60 (m, 1H, F-CH), 4.15 (t, J = 6.6 Hz, 2H, OCH_2), 3.98 (s, 3H, OMe), 3.82 (q, J_1 = 12.9 Hz, J_2 = 6.9 Hz, 2H, N-CH_2), 3.10 (t, J = 6.9 Hz, 2H), 1.81 (m, 2H), 1.52 (m, 2H), 1.00 (t, J = 7.2 Hz, 3H, CH_3). ^{13}C NMR ($CDCl_3$) δ: 163.71, 162.07, 154.36, 145.74, 144.86, 133.45, 132.37, 125.31, 122.33, 119.49, 114.24, 109.06, 81.61 (d, $J_{1\text{-}F}$ = 172.0 Hz), 73.53, 56.28 50.41 (d, $J_{2\text{-}F}$ = 20.0 Hz), 33.04, 32.22, 26.02, 19.12, 13.33. ^{19}F NMR ($CDCl_3$) δ: −221.4 (m). High-resolution MS: calculated for $C_{21}H_{26}FN_5O_4Na$ (M+Na) 454.1867, found 454.1869.

4.2. In vitro studies

4.2.1. Engineering and flow cytometric evaluation of human CB1 and CB2-expressing U87 glioblastoma cells

pCR4-TOPO-*h*CB1 and pPCR-Script Amp SK(+)-*h*CB2, encoding the human CB1 (accession number NM016083) and *h*CB2 (accession number BC069722) were purchased from Open Biosystems (Huntsville, AL), respectively. CB1 and CB2 were subcloned into pDONR222 via BP reaction following amplification with attB1 and attB2 flanked primers and then recombined into a Gateway-adapted lentivirus encoding an internal mscv LTR (Ref = http://www.ncbi.nlm.nih.gov/pubmed/20730500) and the red fluorescent protein mKateS158A downstream of an emcv IRES (pLV4312-CB2). Virus was packaged in 293-FT cells using pMD2.G (VSV.G env) and pCMV-deltaR8.91 and concentrated 50× using Amicon Ultra-15 100,000 NMWL centrifugal concentration units (Millipore, Billerica, MA). Concentrated viral supernatants were used to transduce the human glioblastoma cell line U87 (ATTC, Manassas, VA) via spinfection for 2 h at 2200 RPM/30 °C. CB1+/mKateS158A+ and CB2+/mKateS158A+ U87 cells were sorted on a FACSAria cell sorter (BD Biosciences, San Jose, CA) based on expression of the co-reporter mKateS158A. Cell surface expression of hCB1 and hCB2 was assessed via flow cytometry on a FACSCalibur (BD Biosciences, San Jose, CA). Primary antibodies specific for CB1 (Clone 368302) and CB2 (Clone 352114) were obtained from R&D Systems (Minneapolis, MN). Secondary staining was

performed using a goat anti-mouse allophycocyanin conjugated polyclonal antibody (Jackson ImmunoResearch, West Grove, PA).

4.2.2. Receptor binding assay

This study was performed on the compounds **13**–**16**, **18**, **19**, **21–24**, **27**, and **28** using [^{3}H]CP-55,940, [side chain-2,3,4-^{3}H(N)], a radiolabeled CB2 receptor ligand, as follows. U87 cells (transduced with CB2 receptor) were cultured on Petri-dishes until 80–90% confluence was achieved. Then cells were washed with PBS, scraped, and centrifuged. The cell pellet was homogenized in the binding buffer (50 mM Tris–HCl, 5 mM $MgCl_2$, and 2.5 mM EDTA (pH = 7.4). The homogenate was divided into aliquots (30–50 μg protein) and then incubated with a fixed concentration of the radiolabeled ligand [^{3}H]CP-55,940 (2.8 nM) and different concentrations of the compounds for 1 h at 4 °C. After incubation, aliquots were filtered through GF/C filters, washed with TRIS buffer (4 mL × 5), and radioactivity on the filters was counted in a scintillation counter. The K_d and Bmax for [^{3}H]CP55940 were calculated to be 4.9 nM and 791 fmol/mg protein, respectively. The percent inhibition against [^{3}H]CP-55,940 and the K_i were calculated using GraphPad Prism v4 software. The binding assay for CB1 receptor was performed following the same methodology as that described for the CB2 receptor binding assay.

Acknowledgments

This work was supported by the Developmental Projects Program of the Center for Advanced Biomedical Imaging Research (CABIR) at MD Anderson Cancer Center, and grants: 1 U24 CA126577 01 (NIH) and CA 106672 (NIH).

Supplementary data

Supplementary data (experimental details on compounds **2**–**12**, HPLC chromatograms of the new compounds, and a list of compounds with K_i values previously published in the literature (with references)) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.07.062.

References and notes

1. Marriott, K.-S.; Huffman, J. W. *Curr. Top. Med. Chem.* **2008**, *8*, 187.
2. Huffman, J. W. *Mini Rev. Med. Chem.* **2005**, *5*, 641.
3. Howlett, A. C.; Barth, F.; Bonner, T. I.; Cabral, G.; Casellas, P.; Devane, W. A.; Felder, C. C.; Herkenham, M.; Mackie, K.; Martin, B. R.; Mechoulam, R.; Pertwee, R. G. *Pharmacol. Rev.* **2002**, *54*, 161.
4. Munro, S.; Thomas, K. L.; Abu-Shar, M. *Nature* **1993**, *365*, 61.
5. Gareau, Y.; Dufresne, C.; Gallant, M.; Rochette, C.; Sawyer, N. N.; Slipetz, D. M.; Tremblay, N.; Weech, P. K.; Metters, K. M.; Labelle, M. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 189.
6. Huffman, J. W.; Dai, D.; Martin, B. R.; Compton, D. R. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 563.
7. Ashton, J. C.; Glass, M. *Curr. Neuropharrm.* **2007**, *5*, 73.
8. Clarkstone, A. N.; Rahman, R.; Appeton, I. *Curr. Opin. Investig Drugs* **2004**, *5*, 706.
9. Masa, F.; Monory, K. *J. Endocrinol. Invest.* **2006**, *29*, 47.
10. Conti, S.; Costa, B.; Colleoni, M.; Parolaro, D.; Giagnoni, G. *Br. J. Pharmacol.* **2002**, *135*, 181.
11. Berdyshev, E.; Boichot, E.; Corbel, M.; Germain, N.; Lagente, V. *Life Sci.* **1998**, *63*, 125.
12. Ramirez, B. G.; Blazquez, C.; Gomez del, P. T.; Guzman, M.; de Cebalos, M. L. *J. Nerosci.* **2005**, *25*, 1904.
13. Fernandez-Ruiz, J.; Romero, J.; Velasco, G.; Tolon, R. M.; Ramos, J. A.; Guzman, M. *Trends Pharmacol. Sci.* **2006**, *30*, 30.
14. Qamri, Z.; Preet, A.; Nasser, M. W.; Bass, C. E.; Leone, G.; Barsky, S. H.; Ganju, R. K. *Mol. Cancer Ther.* **2009**, *8*, 3117.
15. Blasquez, C.; Carracedo, A.; Barrado, L.; Real, P. J.; Fernandez-Luna, J. L.; Velasco, G.; Malumbres, M.; Guzman, M. *FASEB J.* **2006**, *20*, 2633.
16. Sarfaraz, S.; Afaq, F.; Adhami, V. M.; Malik, A.; Mukhtar, H. *J. Biol. Chem.* **2006**, *28*, 39480.
17. Xu, X.; Liu, Y.; Huang, S.; Liu, G.; Xie, C.; Zhou, J.; Fan, W.; Li, Q.; Wang, Q.; Zhong, D.; Miao, X. *Cancer Genet. Cytogenet.* **2006**, *171*, 31.
18. Felder, C. C.; Joyce, K. E.; Briley, E. M.; Mansouri, J.; Mackie, K.; Blond, O.; Lai, Y.; Ma, A. L.; Mitchell, R. L. *Mol. Pharmacol.* **1995**, *48*, 443.
19. Showalter, V. M.; Compton, D. R.; Martin, B. R.; Abood, M. E. *J. Pharmacol. Exp. Ther.* **1996**, *278*, 989.
20. Gallant, M.; Dufresne, C.; Gareau, Y.; Guay, D.; Leblanc, Y.; Prasit, P.; Rochette, C.; Sawyer, N.; Slipetz, D. M.; Tremblay, N.; Metters, K. M.; Labelle, M. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 2263.
21. Huffman, J. W.; Liddle, J.; Yu, S.; Aung, M. M.; Abood, M. E.; Wiley, J. L.; Martin, B. R. *Bioorg. Med. Chem.* **1999**, *7*, 2905–2914.
22. Huffman, J. W.; Bushell, S. M.; Miller, J. R. A.; Wiley, J. L.; Martin, B. R. *Bioorg. Med. Chem.* **2002**, *10*, 4119.
23. Huffman, J. W.; Padgett, L. W. *Curr. Med. Chem.* **2005**, *12*, 1395.
24. Hynes, J.; Leftheris, K.; Wu, H.; Pandit, C.; Chen, P.; Norris, D. J.; Chen, B. C.; Zhao, R.; Kiener, P. A.; Chen, X.; Turk, L. A.; Patil-Koota, V.; Gillooly, K. M.; Shuster, D. J.; McIntyre, K. W. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 2399.
25. Dart, M.; Frost, J.; Tietje, K.; Daza, A.; Grayson, G.; Fan, Y.; Mukherjee, S.; Garrison, T. R.; Yao, B.; Meyer, M. *Symposium on the Cannabinoids*, International Cannabinoid Research Society; Burlington, VT, **2006**, p.125.
26. Wiley, J. L.; Compton, D. R.; Dai, D.; Lainton, J. A. H.; Phillips, M.; Huffman, J. W.; Martin, B. R. *J. Pharmacol. Exp. Ther.* **1998**, *285*, 995.
27. Brizzi, A.; Brizzi, V.; Cascio, M. G.; Bisogno, T.; Sirianni, R. D.; Marzo, V. *J. Med. Chem.* **2005**, *48*, 7343.
28. Wiley, J. L.; Beletskaya, I. D.; Ng, E. W.; Dai, Z.; Crocker, P. J.; Mahadevan, A.; Razdan, R. K.; Martin, B. R. *J. Pharmacol. Exp. Ther.* **2002**, *301*, 679.
29. Mussinu, J. M.; Ruiu, S.; Mule, A. C.; Pau, A.; Carai, M. A.; Loriga, G.; Murineddu, G.; Pinna, G. A. *Bioorg. Med. Chem.* **2003**, *11*, 251.
30. Rinaldi-Carmona, M.; Barth, F.; Heaulme, M.; Shire, D.; Calandra, B.; Congy, C.; Martinez, S.; Maruani, J.; Neliat, G.; Caput, D.; Ferrara, P.; Soubrie, P.; Breliere, J. C.; Le Fur, G. *FEBS Lett.* **1994**, *350*, 240.
31. Rinaldi-Carmona, M.; Barth, F.; Millan, J.; Derocq, J. M.; Casellas, P.; Congy, C.; Oustric, D.; Sarran, M.; Bouaboula, M.; Calandra, B.; Portier, M.; Shire, D.; Breliere, J. C.; Le Fur, G. L. *J. Pharmacol. Exp. Ther.* **1998**, *284*, 644.
32. Shire, D.; Calandra, B.; Bouaboula, M.; Barth, F.; Rinaldi-Carmona, M.; Casellas, P.; Ferrara, P. *Life Sci.* **1999**, *65*, 627.
33. Iwamura, H.; Suzuki, H.; Ueda, Y.; Kaya, T.; Inaba, T. *J. Pharmacol. Exp. Ther.* **2001**, *296*, 420.
34. Raitio, K. H.; Savinainen, J. R.; Vepsalainen, J.; Laitinen, J. T.; Poso, A.; Jarvinen, T.; Nevalainen, T. *J. Med. Chem.* **2006**, *49*, 2022.
35. Raitio, K. H.; Salo, O. M. H.; Nevalainen, T.; Poso, A.; Järvinen, T. *Curr. Med. Chem.* **2005**, *12*, 1217.
36. Worm, K.; Zhou, Q. J.; Saeui, C. T.; Green, R. C.; Cassel, J. A.; Stabley, G. J.; DeHaven, R. N.; James, N. C.; LaBuda, C. J.; Koblish, M.; Little, P. J.; Dolle, R. E. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 2830.
37. Lunn, C. A.; Fine, J. S.; Rojas-Triana, A.; Jackson, J. V.; Fan, X.; Kung, T. T.; Gonsiorek, W.; Schwarz, M. A.; Lavey, B.; Kozlowski, J. A.; Narula, S. K.; Lundell, D. J.; Hipkin, R. W.; Bober, L. A. *J. Pharmacol. Exp. Ther.* **2006**, *316*, 780.
38. Ermann, M.; Reither, D.; Walker, E. R.; Mudhi, I.; Jenkis, J. E.; Noya-Marino, B.; Brewer, M. L.; Taylor, M. G. *Biorg. Med. Chem. Lett.* **2008**, *18*, 1725.
39. Evens, N.; Muccioli, G. G.; Houbrechts, N.; Lambert, D. M.; Verbruggen, A. M.; Laere, K. V.; Bormans, G. M. *Nucl. Med. Biol.* **2009**, *36*, 455.
40. Gao, M.; Wang, M.; Miller, K. D.; Hutchins, G. D.; Zheng, Q.-H. *Bioorg. Med. Chem.* **2010**, *18*, 2099.
41. LeTourneau, M. E.; McCarthy, J. R. *Tetrahedron Lett.* **1984**, *25*, 5227.
42. Raitio, K. H.; Savinainen, J. R.; Jarvinen, T.; Nevalainen, T.; Jarvinen, T.; Vepsalainen, J. *Chem. Biol. Drug Des.* **2006**, *68*, 334.
43. Glaser, M.; Årstad, E. *Bioconjug. Chem.* **2007**, *18*, 989.
44. Muccioli, G. G.; Lambert, D. M. *Curr. Med. Chem.* **2005**, *12*, 1361.
45. Salo, O. M. H.; Raitio, K. H.; Savinainen, J. R.; Nevalainen, T.; Lahtela-Kakkonen, M.; Laitinen, J. T.; Jarvinen, T.; Poso, A. *J. Med. Chem.* **2005**, *48*, 7166.
46. Rhee, M.-H.; Nevo, I.; Bayewitch, M. L.; Zagoory, O.; Vogel, Z. *J. Neurochem.* **2000**, *75*, 2485.