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Novel benzimidazole derivatives as selective CB2 agonists

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ABSTRACT

The preparation and evaluation of a novel class of CB2 agonists based on a benzimidazole moiety are reported. They showed binding affinities up to 1 nM towards the CB2 receptor with partial to full agonist potencies. They also demonstrated good to excellent selectivity (>1000-fold) over the CB1 receptor.

The endocannabinoid system has been extensively studied because of its implication in many biological processes such as metabolic regulation,[1,2] pain control,[3–5] cellular proliferation[6] and many other CNS functions.[7] These effects are mediated mainly via two subtypes of cannabinoids receptors, located either in the central nervous system (CB1) or in the peripheral tissues (CB2). The extensive pharmacology of cannabinoid receptors have been reviewed in the literature over the past few years.[8–12] Huge emphasis has been placed on the CB1 receptor due to its implication in many therapeutic applications. Selective CB1 antagonists are currently in clinical studies for the treatment of obesity and metabolic syndrome[1,2,13–15] or different types of addictions,[2,14,16] while CB1 agonists are being used for the treatment of multiple sclerosis, cancer, neuropathic and inflammatory pain.[17–19]

Despite the great homology between the CB1 and CB2 receptors,[20] the exact physiological roles of the CB2 receptor still remain to be fully defined. Recent studies have demonstrated that CB2 selective compounds were active in different neuropathic and inflammatory pain models.[21–25] Some neuroprotective roles have also been associated with CB2 agents,[26–29] that could lead to the prevention of some neurodegenerative diseases. Other studies have also highlighted potential roles for CB2 in cancer,[30,31] multiple sclerosis[32] and bone regeneration.[33,34] Furthermore, since the majority of CB2 receptors are distributed in peripheral tissues, centrally mediated side-effects would be greatly diminished with CB2 selective agents.

Several classes of natural or synthetic CB2 ligands have been reported in the literature.[35–42] In a high throughput CB1/CB2 screening campaign originated a few years ago at our company, several hit compounds were obtained from a variety of chemical series. This program enabled us to develop different selective CB2 ligands such as 1,2,3,4-tetrahydropyrroloindole agonists[43] or benzimidazole inverse agonists.[44] One of our first hits obtained was shown to be compound (**1**) (see Fig. 1). This benzimidazole derivative demonstrated very good binding affinity towards the CB2 receptor with decent selectivity over CB1 while showing partial agonist potency. This molecule represented a good starting template for SAR studies in order to increase the CB2 potency and CB1/CB2 selectivity of these new ligands. We report herein the synthesis and pharmacological evaluation of this novel class of benzimidazole ligands.

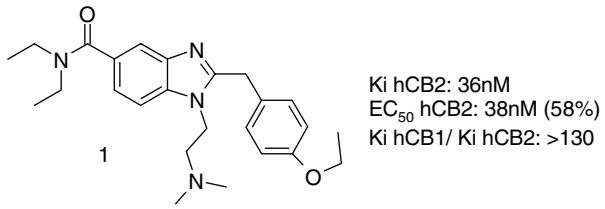

Figure 1. Initial benzimidazole hit ligand.

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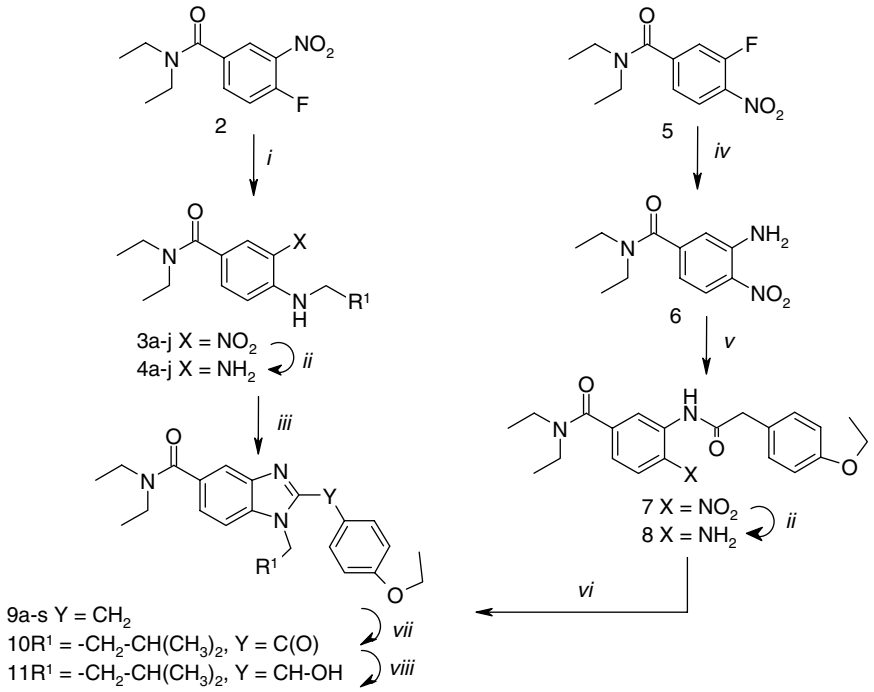
The synthetic strategy used was designed to maintain the benzimidazole core intact while modifying the lower side-chain, the diethyl amide (left-hand side) and the *p*-ethoxyphenyl part (right-hand side) of the molecule. The modifications were initially performed on the lower side-chain of the molecule by introducing various alkyl or aromatic substituents. Two different synthetic routes were developed in order to produce the desired molecules (Scheme 1). The first approach used a SnAr reaction involving the fluoro-nitro derivative (**2**) with different amines in refluxing ethanol. Reduction of compounds (**3a**–**j**) by catalytic hydrogenation afforded anilines (**4a**–**j**), which were then readily coupled with (4-ethoxyphenyl)acetic acid using a standard amide coupling strategy. The final cyclization under acidic conditions afforded the desired benzimidazole products (**9**) in relatively good yields (65–90%). Initial studies done at our site demonstrated that α-branched amines gave lower cyclization yields and the subsequent products gave poor binding results. In light of this, the focus was placed on amines free of any α-substituents. A more convergent approach could also be used by introducing initially the *p*-ethoxy moiety in the synthesis. *N*,*N*-Diethyl-3-fluoro-4-nitrobenzamide (**5**) was reacted with ammonium hydroxide to give the corresponding anilino product (**6**). The coupling of this deactivated aniline with (4-ethoxyphenyl)acetyl chloride could be performed in the presence of zinc dust,[45] which afforded the desired amide (**7**) in a good yield (82%). Reduction of the nitro group gave intermediate (**8**) that was alkylated with different aldehydes, followed by the acidic cyclization to give the analogous products (**9a**–**s**). The benzylic position could also be oxidized using MnO_2 to the corresponding ketone (**10**) in 71% yield. Subsequent reduction of the ketone afforded the corresponding alcohol (**11**).

It became evident throughout the initial screening campaign that the presence of the *p*-ethoxy group on the phenyl ring, on the right-hand side of the molecule, greatly enhanced the CB2 binding affinity for this class of compounds when compared to any other tested *para*-, *ortho*- or *meta*-substituents. Transformations of the *p*-ethoxy group into other ether functionalities were investigated (Scheme 2). The compounds were prepared following a similar procedure as described in Scheme 1. When the desired *para*-substituted phenylacetic acids were not commercially available, they could be prepared by the alkylation of methyl (4-hydroxyphenyl)acetate (**12**) with corresponding alkyl bromides, followed by hydrolysis of the methyl ester. Amide coupling of the acids (**13a**–**j**) with diamine intermediate (**4j**), followed by cyclization under acidic conditions afforded compounds **14a**–**j**.

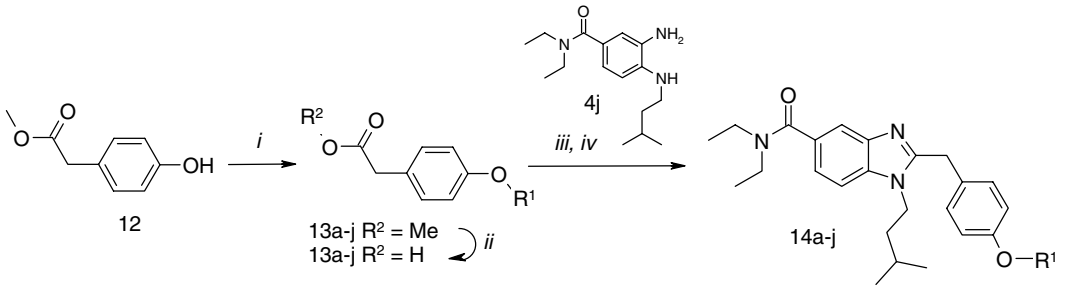
In order to maximize the diversity on the left-hand side of the molecule, two different templates were used. Modifications of the diethyl amide could be done starting from 3-fluoro-4-nitrobenzonitrile (**15**) (Scheme 3) following a similar synthetic route as the one described above. *p*-Ethoxy pyridine derivatives (right-hand side) were also prepared the same way in order to look at the potential effect on the CB2 binding. Hydrolysis of the nitriles (**18a**–**c**) under basic conditions into the corresponding acids (**19a**–**c**), followed by HATU (*N*,*N*,*N*′,*N*′-tetramethyl-*O*-(7-azabenzotriazole-1-yl) uranium hexafluorophosphate) coupling using different amines afforded final compounds (**20a**–**j**) in good yields (70–90%).

Diversity could also be created on the left-hand side starting from *N*-(4-fluoro-3-nitro-phenyl)-acetamide (**21**) in order to introduce different reversed-amide or urea functionalities (Scheme 4). Following a similar synthetic procedure, the benzimidazole core could be easily prepared. The *N*-acetyl amide benzimidazole (**24**) could be alkylated under phase transfer catalysis conditions. Removal of the acetate group followed by reaction with different acid chlorides of isocyanates afforded the final compounds (**26a**–**g**) in 75–90% yields. All final products were purified by reversed-phase chromatography with a water/acetonitrile gradient containing 0.05% TFA v/v and isolated as their corresponding TFA salts.[46]

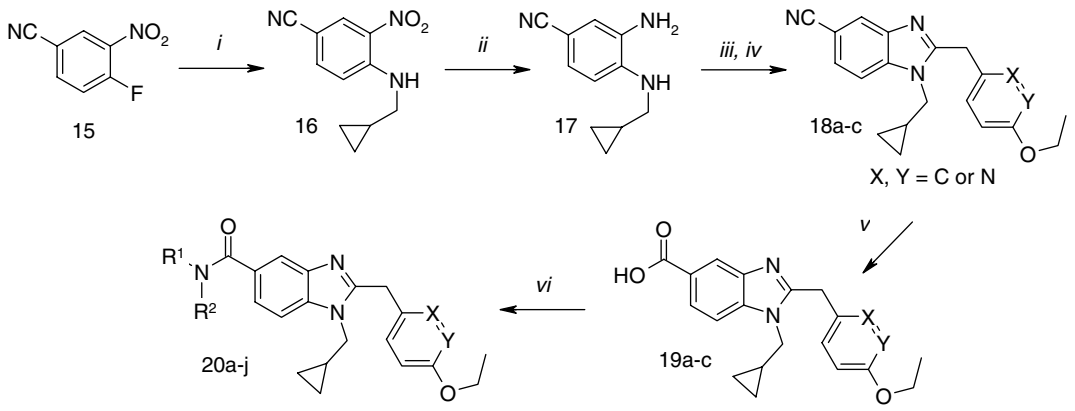
The CB1/CB2 binding results are summarized in Tables 1–4.[47] Only compounds that exhibited K_i hCB2 <100 nM were tested in the hCB2 GTPγ[^{35}S] assay.[48] The mixed CB1/CB2 agonists



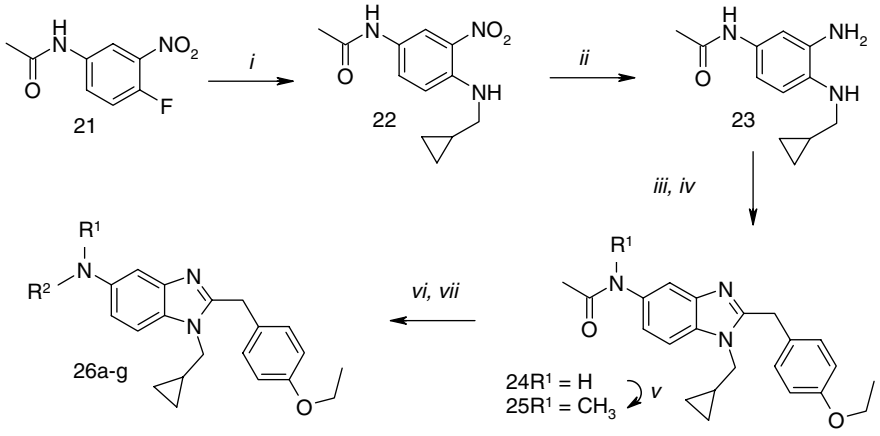
Scheme 1. Reagents and conditions: (i) $R^1CH_2NH_2$, Et_3N, EtOH, 75 °C, o/n; (ii) H_2, 10% Pd/C, EtOAc, rt, o/n; (iii) a—(4-ethoxyphenyl)acetic acid, HATU, DIPEA, DMF, rt, 3 h; b—DCE/HCl or glacial AcOH, 80 °C, 2 h; (iv) NH_4OH, EtOH, 65 °C, o/n; (v) (4-ethoxyphenyl)acetyl chloride, zinc dust, toluene, rt, o/n; (vi) a—aldehyde, BH_3-pyridine, DCE, AcOH, rt, 1 h; b—DCE/HCl or glacial AcOH, 80 °C, 2 h; (vii) MnO_2, dioxane, 65 °C, 24–48 h; (viii) $NaBH_4$, EtOH, rt, 1 h.



Scheme 2. Reagents and conditions: (i) R^1-Br, K_2CO_3, DMF, 100 °C, 8 h; (ii) LiOH, THF/H_2O, rt, 3 h; (iii) **4j**, HATU, DIPEA, DMF, rt, 3 h; (iv) DCE/HCl or glacial AcOH, 80 °C, 2 h.



Scheme 3. Reagents and conditions: (i) cyclopropylmethylamine, Et_3N, EtOH, 75 °C, o/n; (ii) H_2, 10% Pd/C, EtOAc, rt, o/n; (iii) acid, HATU, DIPEA, DMF, rt, 3 h; (iv) DCE/HCl or glacial AcOH, 80 °C, 2 h; (v) 20% KOH, EtOH, reflux, o/n; (vi) amine, HATU, DIPEA, DMF, rt, 2 h.

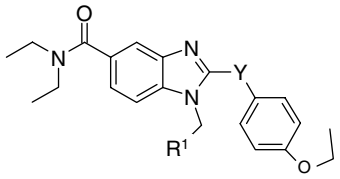


Scheme 4. Reagents and conditions: (i) cyclopropylmethylamine, Et_3N, EtOH, 75 °C, o/n; (ii) H_2, 10% Pd/C, EtOAc, rt, o/n; (iii) (4-ethoxyphenyl)acetic acid, HATU, DIPEA, DMF, rt, 3 h; (iv) glacial AcOH, 80 °C, 2 h; (v) Bu_4NBr, MeI, 50%KOH/ DCM, rt, o/n; (vi) 1 M HCl, EtOH, 120 °C, μwaves, 0.5 h; (vii) RC(O)Cl/ RNCO, Et_3N, DCM, rt, 1 h.

WIN55212-2[49] and Δ^9-THC were tested as standards for comparison purposes (Table 1). The results from Table 1 suggest that a wide variety of groups can be accommodated at the lower part of the molecule, with a preference for alkyl substituents (**9a**–**9f**). Increasing the bulk of the substituents also had the effect of increasing the CB1 binding affinity. Noticeable discrepancies between the CB1 and CB2 binding affinities were observed when polar atoms were introduced in the lower region of the molecule. Hydrophilic substituents such as pyridines (**9h**–**9i**), furan (**9k**), tetrahydropyran (**9l**), tetrahydrofurans (**9m**–**9n**) and 2-substituted piperidines (**9r**–**9s**) all showed good CB2 binding affinities while being essentially devoid of any CB1 binding affinity. However, charged groups at physiological pH, such as secondary amines (**9o**) or *N*-oxides (**9j**) at the position 4 of the substituent rings were not tolerated. Furthermore, the introduction of either a carbonyl (**10**) or alcohol (**11**) group at the benzylic position was also detrimental for the CB2 binding affinity. Even though the nature of the group seemed to exert an influence on the potency of the compounds, with E_{max} values ranging from 55% to 93%, no specific trend could be observed.

The results of the *p*-ethoxy group substitutions are reported in Table 2. It seems that only the isopropoxy moiety (**14c**) gave similar CB2 binding affinity (4.5 nM) than compound **9a**. Smaller (**14a**–**b**) and/or bigger substituents (**14d**–**14i**) had a detrimental effect on the CB2 binding, with the exception of the phenoxy substituent (**14j**) which showed a comparable K_i (4.9 nM) to

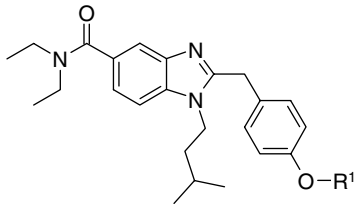
Table 1
Binding results of benzimidazole derivatives with lower part modifications



	R^1	Y	hCB2 K_i (nM)	hCB1 K_i (nM)	hCB2 EC_{50}* (nM)
Δ^9-THC	—	—	41 ± 2	2.9 ± 0.3	1.5 ± 0.1 (100%)
WIN5512-2	—	—	20 ± 3	140 ± 42	14 ± 3 (64%)
9a	$(CH_3)_2CH–CH_2–$	CH_2	4.5 ± 0.9	>5000	2.9 ± 0.2 (63%)
9b	Cyclopropyl	CH_2	4.1 ± 0.6	>5000	2.1 ± 0.7 (68%)
9c	Cyclobutyl	CH_2	1.6 ± 0.1	3115 ± 149	1.3 ± 0.1 (64%)
9d	Cyclopentyl	CH_2	1.0 ± 0.1	491 ± 27	0.7 ± 0.2 (79%)
9e	Cyclohexyl	CH_2	3.7 ± 1.3	110 ± 1.9	0.52 ± 0.04 (79%)
9f	1-Adamantane	CH_2	2.8 ± 0.2	406 ± 44	1.3 ± 0.2 (81%)
9g	Phenyl	CH_2	12 ± 3	4766 ± 348	1.1 ± 0.2 (88%)
9h	2-Pyridine	CH_2	39 ± 3	>5000	25 ± 5 (77%)
9i	4-Pyridine	CH_2	16 ± 2	>5000	5.0 ± 0.7 (78%)
9j	4-Pyridine-*N*-oxide	CH_2	588 ± 191	>5000	nd
9k	2-Furan	CH_2	11 ± 3	>5000	3.3 ± 0.9 (78%)
9l	4-Tetrahydropyran	CH_2	3.9 ± 0.9	1209 ± 53	1.0 ± 0.3 (89%)
9m	2-Tetrahydrofuran(*S*)	CH_2	15 ± 3	>5000	4.8 ± 1.1 (76%)
9n	2-Tetrahydrofuran(*R*)	CH_2	23 ± 4.5	>5000	8.3 ± 1.9 (62%)
9o	4-Piperidine	CH_2	2920 ± 78	>5000	nd
9p	2-Piperidine(*S*)	CH_2	75 ± 9	>5000	25 ± 6 (77%)
9q	2-Piperidine(*R*)	CH_2	35 ± 6	>5000	13 ± 2 (72%)
9r	2-*N*-Me-piperidine(*R*)	CH_2	8.9 ± 1.1	4315 ± 270	2.3 ± 0.3 (81%)
9s	2-*N*-Me-pyrrolidine(*R*)	CH_2	12 ± 2	>5000	3.4 ± 0.7 (93%)
10	$(CH_3)_2CH–CH_2–$	C=O	35 ± 3	>5000	25 ± 6 (55%)
11	$(CH_3)_2CH–CH_2–$	CH–OH	57 ± 4	>5000	69 ± 18 (70%)

nd, not determined.
* E_{max} are reported in brackets.

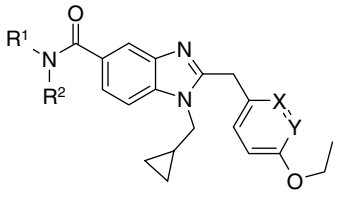
Table 2
Binding results of benzimidazole derivatives with *p*-ethoxy group replacements



	R^1	hCB2 K_i (nM)	hCB1 K_i (nM)	hCB2 EC_{50}* (nM)
9a	Ethyl	4.5 ± 0.9	>5000	2.9 ± 0.2 (63%)
14a	H	799 ± 169	>5000	nd
14b	Methyl	17 ± 4	>5000	7.3 ± 1.8 (43%)
14c	Isopropyl	4.5 ± 0.3	4679 ± 190	2.4 ± 0.6 (79%)
14d	Cyclobutyl	44 ± 15	>5000	24 ± 5 (73%)
14e	Cyclopentyl	43 ± 8	>5000	18 ± 1 (83%)
14f	Cyclohexyl	526 ± 125	>5000	nd
14g	CF_3	25 ± 7	>5000	11 ± 2 (29%)
14h	$–CH_2–CF_3$	57 ± 15	>5000	65 ± 18 (68%)
14i	$–CH_2$-cyclopropyl	53 ± 8	4190 ± 149	43 ± 8 (75%)
14j	Phenyl	4.9 ± 0.2	2396 ± 124	3.2 ± 0.1 (66%)

nd, not determined.
* E_{max} are reported in brackets.

Table 3
Binding results of benzimidazole derivatives with diethyl amide group replacements



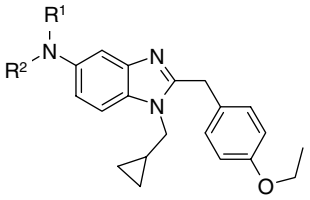
	R^1	R^2	X	Y	hCB2 K_i (nM)	hCB1 K_i (nM)	hCB2 EC_{50}* (nM)
9b	Ethyl	Ethyl	CH	CH	4.1 ± 0.6	>5000	2.1 ± 0.7 (68%)
20a	Ethyl	H	CH	CH	63 ± 6	>5000	52 ± 18 (53%)
20b	H	H	CH	CH	>5000	>5000	nd
20c	Propyl	Methyl	CH	CH	7.6 ± 2.1	>5000	3.2 ± 0.4 (54%)
20d	Propyl	Propyl	CH	CH	2.8 ± 0.6	2608 ± 245	2.0 ± 0.1 (67%)
20e	$–(CH_2)_4–$	—	CH	CH	14 ± 2	>5000	6.2 ± 1.2 (51%)
20f	$–(CH_2)_5–$	—	CH	CH	6.9 ± 1.7	>5000	3.0 ± 0.4 (45%)
20g	Cyclohexyl	Methyl	CH	CH	11 ± 5	>5000	4.2 ± 0.4 (60%)
20h	$CF_3CH_2–$	$CF_3CH_2–$	CH	CH	5.6 ± 1.0	4534 ± 134	2.0 ± 0.4 (51%)
20i	Ethyl	Ethyl	N	CH	27 ± 4	>5000	22 ± 5 (63%)
20j	Ethyl	Ethyl	CH	N	133 ± 25	>5000	nd

nd, not determined.
* E_{max} are reported in brackets.

compound **9a**, probably coming through an additional π–π interaction with the receptor binding pocket.

Most of the diethyl amide modifications reported in Table 3 showed CB2 K_i values in the same range as compound **9b**. Linear (**20c**–**d**), cyclic (**20e**–**g**) or fluorinated (**20h**) bis-alkyl substituents were all well tolerated demonstrating probably the presence of a large binding pocket at the receptor site. Reducing the size of the group to a mono ethyl (**20a**) showed about a 10-fold decrease in CB2 binding affinity, whereas having only a primary amide on the left-hand side (**20b**) resulted in a total loss of activity. The introduction of a nitrogen atom in the benzyl ring on the right-

Table 4
Binding results of benzimidazole derivatives with left-hand side modifications



	R^1	R^2	hCB2 K_i (nM)	hCB1 K_i (nM)	hCB2 EC_{50}* (nM)
24	Acetyl	H	>5000	>5000	nd
25	Acetyl	Me	1349 ± 359	>5000	nd
26a	H	Me	4342 ± 117	>5000	nd
26b	$-C(O)CH_2CH(CH_3)_2$	Me	4.0 ± 0.7	>5000	2.7 ± 0.4 (54%)
26c	–C(O)-2-thiophene	Me	5.0 ± 1.3	2483 ± 271	2.6 ± 0.3 (51%)
26d	$-C(O)N(CH_3)_2$	Me	41 ± 3	>5000	36 ± 5 (55%)
26e	$-C(O)N(CH_2CH_3)_2$	Me	5.9 ± 0.7	>5000	2.0 ± 0.1 (48%)
26f	$-C(O)NHCH(CH_3)_2$	Me	5.7 ± 1.2	>5000	6.4 ± 1.5 (51%)
26g		Me	4.0 ± 0.6	3475 ± 206	3.3 ± 0.3 (60%)

nd, not determined. Abbreviations: Me, methyl.
* E_{max} are reported in brackets.

hand side of the molecule did not have any positive influence on the CB2 binding, as compounds **20i** and **20j** showed a 6.5- to 33-fold decrease in the binding affinity, respectively.

Similar observations can be drawn from the results of Table 4 where compounds bearing smaller groups (**24**–**26a**) showed much lower CB2 binding affinities than those bearing either bulkier amide (**20b**–**c**) or urea (**20d**–**g**) moieties. The binding interactions at this specific site seem to be mainly hydrophobic and non-specific since it can accommodate different groups and/or functionalities. The left-hand part of the molecule also seem to have less influence on the nature of the ligand since all the reported modifications in Tables 3 and 4 only resulted in compounds showing partial agonism (E_{max} 45–67%).

In conclusion, these molecules, based on a benzimidazole core represent new scaffolds in the development of cannabinoid agonists. These ligands demonstrated good binding affinities with decent potencies towards the CB2 receptor, along with excellent selectivity over the CB1 receptor. Further investigations of this new class of ligands are currently underway in our laboratories in order to look at their potential biological application.

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46. All products gave satisfactory analytical characterization showing purity >95% as determined by HPLC using a Zorbax C-18 column (λ = 215, 254 and 280 nm). ^{1}H NMR spectra were obtained from a 400 MHz Varian Unity Plus spectrometer. Mass spectra were obtained on a Micromass Quattro micro API or an Agilent 1100 Series LC/MSD instrument using loop injection. Selected analytical characterizations: Compound **9a**: ^{1}H NMR (DMSO-d_6) δ 7.80 (d, 1H), 7.67 (s, 1H), 7.42 (d, 1H), 7.25 (d, 2H), 6.91(m, 2H), 4.46 (s, 2H), 4.31 (t, 2H), 3.97 (q, 2H), 3.16 (b, 4H), 1.61 (m, 1H), 1.35 (m, 2H), 1.28 (t, 3H), 1.07 (b, 6H), 0.85 (m, 6H); 422.29 (MH+ monoisot.); Compound **9b**: ^{1}H NMR (DMSO-d_6) δ 7.59 (d, 1H), 7.30 (s, 1H), 7.08 (d, 1H), 6.91 (d, 2H), 6.53 (d, 2H), 4.14 (s, 2H), 3.96 (d, 2H), 3.58 (q, 2H), 3.04 (b, 2H), 2.80 (b, 2H), 0.90 (t, 3H), 0.78 (b, 7H), 0.10–0.03 (m, 4H); 406.17 (MH+ monoisot.); Compound **14j**: ^{1}H NMR (CD_3OD) δ 7.84 (d, 1H), 7.72 (s, 1H), 7.54 (dd, 1H), 7.33 (m, 4H), 7.10 (t, 1H), 6.98 (m, 4H), 4.57 (s, 2H), 4.41 (dd, 2H), 3.55 (br s, 2H), 3.27 (br s, 2H), 1.69 (m, 1H), 1.51 (m, 2H), 1.24 (br s, 3H), 1.11 (br s, 3H), 0.96 (s, 6H); 470.32 (MH+ monoisot.); Compound **20h**: ^{1}H NMR (CD_3OD) δ 8.00 (d, 1H), 7.79 (s, 1H), 7.59 (d, 1H), 7.27 (s, 2H), 6.95 (d, 2H), 4.56 (s, 2H), 4.36 (m, 6H), 4.02 (q, 2H), 1.37 (t, 3H), 1.24 (m, 1H),0.61 (m, 2H), 0.48 (m, 2H); 514.22 (MH+ monoisot.); Compound **20i**: ^{1}H NMR (CD_3OD) δ 8.13 (d, 1H), 7.94 (d, 1H), 7.69 (s, 1H), 7.53 (dd, 1H), 7.45 (d, 1H), 7.40 (dd, 1H), 4.80 (s, 2H), 4.37 (d, 2H), 4.04 (q, 2H), 3.58–3.46 (m, 2H), 3.26–3.19(m, 2H), 1.32 (t, 3H), 1.25–1.15 (m, 4H), 1.13–1.00 (m, 3H), 0.59–0.52 (m, 2H), 0.45–0.38 (m, 2H); 407.27 (MH+ monoisot.); Compound **26g**: ^{1}H NMR (CD_3OD) δ 0.51 (m, 2H), 0.66 (m, 2H), 1.28 (m, 1H), 1.41 (m, 7H), 1.55 (m, 2H), 3.28 (s, 3H), 3.34 (m, 4H), 4.07 (q, 2H), 4.39 (d, 2H), 4.57 (s, 2H), 7.01 (m, 2H), 7.30 (m, 2H), 7.39 (dd, 1H), 7.44 (d, 1H), 7.90 (d, 1H); 447.36 (MH+ monoisot.).
47. General procedure for the CB1/CB2 binding assay: cannabinoids membranes are thawed at 37 °C, passed 3 times through a 25-gauge blunt-end needle, diluted in the cannabinoid binding buffer (50 mM Tris, 2.5 mM EDTA, 5 mM $MgCl_2$, and 0.5 mg/mL BSA fatty acid free, pH 7.4) and 80 μL aliquots containing the appropriate amount of protein are distributed in 96-well plates. The IC_{50} of compounds (150 μL) are evaluated from 10-point dose–response curves done with 70 μL of ^{3}H-CP55, 940 at 20,000 to 25,000 dpm per well (0.17–0.21 nM) in a final volume of 300 μL. The total and non-specific binding are determined in the absence and presence of 0.2 μM of HU210 (150 μL). The plates are vortexed

and incubated for 60 min at room temperature, filtered through Unifilters GF/B (presoaked in 0.1% polyethyleneimine) with the Tomtec or Packard harvester using 3 mL of wash buffer (50 mM Tris, 5 mM $MgCl_2$, 0.05% BSA, pH 7.0). The filters are dried for 1 h at 55 °C. The specific binding (SB) is calculated as TB-NS, and the SB in the presence of various ligands is expressed as percentage of control SB. Values of IC_{50} and Hill coefficient (n_H) for ligands in displacing specifically bound radioligand are calculated in Activity base with ExcelFit. The concentration of compounds to use and dilutions are also calculated with Activity base. The radioactivity (cpm) is counted in a TopCount (Packard) after adding 65 μL/well of MS-20 scintillation liquid.

48. GTPγ[^{35}S] binding on cloned human CB2 receptors in Sf9 cell membranes: human CB2 assay was performed in a buffer consisting of 50 mM Hepes, 20 mM NaOH, 100 mM NaCl, 1 mM EDTA, 5 mM $MgCl_2$, 0.1% BSA, and 15 μM GDP. The pH was set at 7.4 at room temperature. The washing buffer consisted of 50 mM Tris, 5 mM $MgCl_2$, 50 mM NaCl, pH 7.4, at 4 °C. Compounds were tested in 10-point dose–response curves. The assay, performed in 96-well plates, consisted of 300 μL, containing 150 μL of buffer alone or compound at varying concentrations, 80 μL of membranes (5 μg of protein/well) mixed with 56 μM of GDP (15 μM final). Finally, 70 μL of the tracer GTPγ[^{35}S] (100,000 to 130,000 dpm/well) is added to start the reaction. Eight wells were used to define basal (negative control) binding and 8 for positive control (maximal binding) using 1 μM WIN55,212-2. Plates were then mixed by hand on an orbital mixer and incubated for 1 h at room temperature. Filter plates were presoaked in deionized water. Filtration was performed with a Packard cell harvester using 3× 1 mL of wash buffer. Filter plates were then dried at 55 °C for 1.5 h before adding 50 μL of Microscint 20 (Packard Biosciences) scintillation fluid. Filter plates were counted in a Packard Top Count. The cpm values of GTPγ[^{35}S] binding in the 8 wells containing GTPγ[^{35}S] and membranes were averaged to define basal binding (BB) and the values of the 8 wells containing 1 μM WIN55,212-2 were averaged to define GTPγ[^{35}S] maximal binding (MB). The stimulation of GTPγ[^{35}S] binding observed for each concentration of compound (cpm ECC) was expressed as a percentage of maximal effect elicited by 1 μM WIN55,212-2. GTPγ[^{35}S] specific binding is calculated by subtracting the BB.

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