Brief Article

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4-Oxo-1,4-dihydropyridines as Selective CB_2 Cannabinoid Receptor Ligands Part 2: Discovery of New Agonists Endowed with Protective Effect Against Experimental Colitis

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S *Supporting Information*

ABSTRACT: Further on to our earlier work on the 4-oxo-1,4-dihydropyridine, we describe herein our strategy to get access to potent selective CB_2 receptor agonists. Thus, we designed and synthesized 29 compounds, evaluated on both hCB_1 and hCB_2 cannabinoid receptors, and assessed 11 of them in the TNBS-induced colitis model in mice. Compound **48** was found to be the most efficient of our series, exhibiting an exquisite protection against experimental colitis, superior to the one observed after treatment with Pentasa.

INTRODUCTION

CB_2 cannabinoid receptor is a G-protein coupled receptor that constitutes, with CB_1 cannabinoid receptor, the restricted family of cannabinoid receptors (CNRs), the two main targets of endocannabinoids (ECs).[1] The latter, including *N*-arachidonoylethanolamine (anandamide, AEA) and 2-arachidonoylglycerol, are lipid mediators that are biosynthesized on demand, and which, by their action on CNRs, exert a variety of physiological responses, including in the gut.[2] All the components of the endocannabinoid system have been identified in both normal and inflamed gastrointestinal (GI) tract. The CB_2 receptor is widely expressed in immune cells, such as macrophages, B and T cells,[3] which are involved in the inflammatory response during colitis.[4] In addition, it has been shown to be up-regulated in the inflamed colon,[5] while a reduction in FAAH expression accompanied by an elevation of the AEA levels has been reported.[6] These events have been hypothesized to counteract abnormal gut inflammation and hence to protect against colitis. Therefore, the endocannabinoid system, including CB_2 receptors, emerged as promising therapeutic target for the treatment of GI tract inflammatory conditions. Over the past few years, pharmacological modulation of both CNRs and ECs hydrolyzing enzymes were shown to protect against colitis.[7] Dual CB_1/CB_2 agonists (Δ^9-THC, **1**, and HU-210, **2**)[8] as well as CB_1 (arachidonyl-2′-chloroethylamide, **3**)[9] and CB_2 (JWH133, **4**, and AM1241, **5**)[5,9,10] selective agonists were shown to significantly reduce colon alterations in several experimental models of IBD in rodents. Similar results were obtained when using ECs membrane transport inhibitor **6** (VDM11) as well as FAAH (5-([1,1′-biphenyl]-4-yl)-*N*-(2-(benzo[*d*][1,3]dioxol-5-yl)-ethyl)isoxazole-3-carboxamide, **7**, and URB597, **8**)[11] and monoacylglycerol lipase (JZL184, **9**) inhibitors.[12] Unfortunately, some of these ligands are well-known to exert undesirable psychotropic effects that are assumed to be central CB_1 receptor mediated. Accordingly, CB_2 receptor selective agonists, with their nonpsychoactive profile, are likely to have therapeutic value in the treatment of IBD.

Therefore, we aimed to develop new CB_2 selective agonists endowed with anti-inflammatory properties in the GI tract. Along this line, we recently described a series of 4-oxo-1,4-dihydropyridines as CB_2 receptor selective ligands.[13] We showed that the C-6 substituent was able to govern the functional activity of these compounds, which act as agonists or inverse agonists depending on the presence of alkyl or aryl groups, respectively, at C-6 position. This work suggested that it was conceivable to design CB_2 ligands, based on a 4-oxo-1,4-dihydropyridine scaffold, with a predictable functional activity. These crucial findings gave us the opportunity to obtain CB_2 selective agonists of therapeutic interest in the treatment of colitis.

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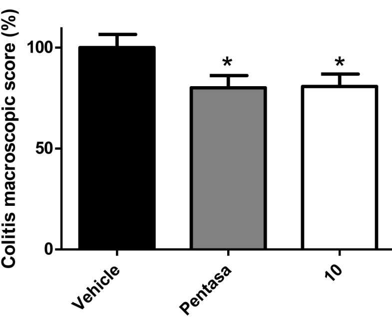
RESULTS AND DISCUSSION

Preliminary in Vivo Studies. We first evaluated our lead CB_2 selective agonist **10** (*N*3-(1-adamantyl)-6-*tert*-butyl-1-pentyl-4-oxo-1,4-dihydropyridine-3-carboxamide: hCB_2, K_i = 29 nM; hCB_1, K_i > 3000 nM)[13] in the TNBS-induced colitis model in mice (Chart 1).[14] Pentasa formulation of 5-aminosalicylic acid was used as positive control (150 mg/kg). After being administered once daily (10 mg/kg, ip), **10** was able to decrease colitis macroscopic scores by 19.3% (80.7% vs 100.0%, p = 0.04), thus having the same effect than our positive control Pentasa (19.1%, 80.1% vs 100.0%, p = 0.03). This promising result led us to prepare new CB_2 selective agonists,[15] around the 4-oxo-1,4-dihydropyridine template, with improved in vivo activity.

Chart 1. Modest Effect of 10 against Experimental Colitis



In Vitro Pharmacology. The affinities of target compounds **15−19**, **30−45**, and **47−54** (Tables 1−2) against the human cannabinoid receptors (hCB_1 and hCB_2) were determined by a competitive radioligand displacement assay using [^{3}H]-CP-55,940 as radioligand for both hCB_2 and hCB_1 receptors.[16] All compounds were first screened at 1 μM concentration, and K_i values were determined for those exhibiting a specific displacement superior to 60% either for hCB_1 or hCB_2 (Tables 1−2). In addition, we investigated their functional activity at CB_2 receptor using a [^{35}S]-GTPγS binding assay and hCB_2-CHO cells membranes (Tables 1−2).[17]

Starting from compound **10**, first optimizations were focused on the moiety borne by the amide at C-3 position, keeping either the *tert*-butyl (**15−19**, Table 1) or methyl (**30−45**, Tables 1 and 2) group constant at the C-6 position. For compounds presenting a *tert*-butyl group at C-6, and regardless of the amide substituent, all evaluated compounds (**15−19**) possess very good affinity (ca. 10 nM) for the CB_2 receptor. For instance, replacing the bulky aliphatic adamantyl group by methylcyclopropyl (**18**) or phenyl (**19**) did not impact on CB_2 receptor affinity. On the other hand, these modifications led to compounds with good affinity at CB_1 (hCB_1: K_i = 176 nM for **18** and K_i = 88 nM for **19**) and therefore, to less CB_2 receptor-selective derivatives. Contrasting with this, compounds characterized by a methyl at C-6 and presenting a bulky aliphatic group at the amide function (**30−33**) displayed very high affinity for the CB_2 receptor while being devoid of CB_1 receptor affinity. Compound **32**, characterized by a 2-adamantyl moiety, presented the best profile in our series with regard to affinity at CB_2 receptor and selectivity over CB_1 (hCB_2: K_i = 0.26 nM and SI > 3846).

Surprisingly, for compounds **18** and **19**, when the *tert*-butyl group at C-6 was replaced by a methyl (**34** and **37**), the affinity for the CB_2 receptor was completely lost. These first results clearly suggest that, to achieve a good affinity and selectivity at CB_2 receptor, the methyl at C-6 as well as the adamantyl group borne by the amide function should be retained.

Table 1. C-3 and C-6 Modulations: Affinities and Functional Activities of 15−19 and 30−37 toward Both Cannabinoid Receptors

Cpd	R_3	R_1	Binding affinity Ki (nM)		[^{35}S]-GTPγS (hCB_2)	
			hCB_2	hCB_1	EC50 (nM)	Emax (%)
15	tert-butyl		10.2 ± 2.2	> 1000	49.2 ± 6.6	191 ± 11
16	tert-butyl		9.0 ± 2.0	548	47.1 ± 7.1	258 ± 6
17	tert-butyl		9.6 ± 0.7	562	452 ± 77	254 ± 16
18	tert-butyl		14.1 ± 1.8	176	27.8 ± 9.2	195 ± 11
19	tert-butyl		9.2 ± 0.6	87.9	8.3 ± 0.7	250 ± 9
30	methyl		15.2 ± 3.7	> 1000	53.1 ± 7.7	254 ± 23
31	methyl		6.4 ± 1.9	> 1000	84.2 ± 6.6	233 ± 15
32	methyl		0.26 ± 0.17	> 1000	38.4 ± 7.0	216 ± 12
33	methyl		24.4 ± 2.3	> 1000	49.9 ± 6.7	197 ± 3
34	methyl		> 1000	/	/	/
35	methyl		> 1000	/	/	/
36	methyl		> 1000	/	/	/
37	methyl		> 1000	/	/	/
(*R*) − (+) WIN 55,212-2			3.1 ± 1.2	52.4 ± 1.2	89.6 ± 0.8	232 ± 2

Next, we modified the *n*-pentyl chain at *N*-1 position by other alkyl chains (Table 2). Thus, we synthesized compound **42** featured by a tetrahydropyranylmethyl group that was shown to be important for the binding of indole series.[18] Even though this modification was tolerated, the replacement of the tetrahydropyranylmethyl group by other alkyl ether chains resulted in a decrease (**43**) or even loss (**39−41**, **44**) of the affinity. Similarly, compound **38**, characterized by an unsaturated alkyl chain, displayed no affinity at CB_2 receptor. On the other hand, the introduction of a trifluorobutyl chain (**45**) led to one of the most potent and selective ligands within this series (hCB_2: K_i = 0.28 nM, SI > 3571).

To complete the SAR, we investigated the impact of the C-5 substituent by introducing iodine, a cyclopropyl as well as various aromatic groups at this position (**47−54**, Table 2). Generally, this modification led to less potent and selective compounds. Introduction of iodine (**47**) and cyclopropyl (**51**) allowed to maintain a high affinity at CB_2 but decreased selectivity, while a phenyl group (**52**) significantly decreased CB_2 affinity. When this phenyl group was substituted in para position by a methyl group (**53**) or in meta position by a cyano (**54**) or a methylketone group (**50**), the affinity at CB_2 receptor

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Table 2. *N*-1 and C-5 Modulations: Affinities and Functional Activities of 38−45 and 47−54 toward Both Cannabinoid Receptors

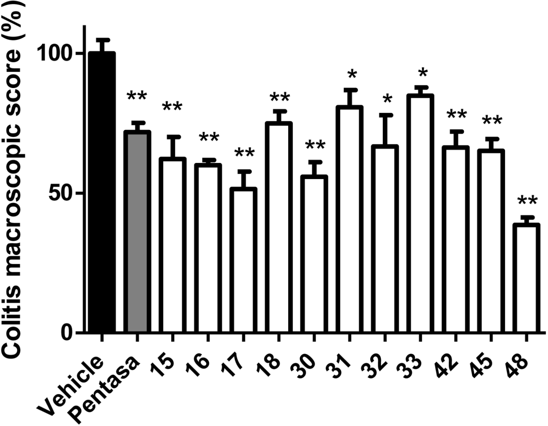
Cpd	R_2	R_4	Binding affinity Ki (nM)		$[^{35}S]$-GTPγS (hCB_2)	
			hCB_2	hCB_1	EC50 (nM)	Emax (%)
38		H	> 1000	/	/	/
39		H	> 1000	/	/	/
40		H	> 1000	/	/	/
41		H	> 1000	/	/	/
42		H	24.1 ± 6.5	> 1000	47.4 ± 7.7	224 ± 6
43		H	195.8 ± 41.9	/	/	/
44		H	> 1000	/	/	/
45		H	0.28 ± 0.18	> 1000	17.3 ± 7.7	224 ± 11
47	C_5H_{11}	I	20.8 ± 6.3	193.4	11.9 ± 0.8	299 ± 4
48	C_5H_{11}		36.5 ± 0.6	> 1000	114 ± 7	213 ± 36
49	C_5H_{11}		54.3 ± 5.4	> 1000	39.9 ± 8.3	199 ± 12
50	C_5H_{11}		> 1000	/	/	/
51	C_5H_{11}		13.4 ± 0.6	105.1	88.5 ± 7.9	188 ± 7
52	C_5H_{11}		189.4 ± 71.7	/	/	/
53	C_5H_{11}		> 1000	/	/	/
54	C_5H_{11}		> 1000	/	/	/
(*R*) − (+) WIN 55,212-2			3.1 ± 1.2	52.4 ± 1.2	89.6 ± 0.8	232 ± 2

was completely abolished. Surprisingly, when the *p*-methyl was replaced by an ethyl (**49**), the CB_2 activity was restored. Finally, a pyridine heterocycle (**48**) at C-5 was well tolerated with a K_i of 36 nM and no activity against CB_1 receptor at 1 μM.

With regard to their functional activity, all compounds dose-dependently increased the $[^{35}S]$-GTPγS binding between 188% and 258% (control value set at 100%), with EC_{50} values in between 8.3 and 452 nM, which indicates that they behave as full agonists at CB_2 receptor. Interestingly, while we previously showed that introducing an aromatic ring at C-6 position generally led to an inverse agonist profile,[13] the same kind of modifications at C-5 had no impact on the functional activity because all C-5 substituted compounds show an agonist profile.

In Vivo Pharmacology. A set of target CB_2 agonists, characterized by high CB_2 receptor affinity (hCB_2 K_i < 50 nM) and a selectivity index (over CB_1 receptor) of at least 10 (**15**−**18**, **30**−**33**, **42**, **45** and **48**), was selected to be evaluated in the mouse model of TNBS-induced colitis.[14] Thus, each compound was administered intraperitoneally once daily at the dosage of 10 mg/kg, starting three days before colitis induction. Pentasa granules mixed in food at the dosage of 150 mg/kg, and provided ad libitum during all the course of the experiment, was used as anti-inflammatory positive control (Chart 2).

Chart 2. New CB_2 Receptor Selective Agonists Attenuate Colitis in TNBS-Induced Colitis[a]



[a]Data are the mean ± SEM of 10 mice per group; *$p < 0.05$; **$p < 0.01$ vs TNBS and vehicle.

Mice were euthanized three days after TNBS administration, and parameters reflecting the degree of colon inflammation were assessed. The colon of each mouse was examined, and damages were assessed by a semiquantitative scoring system. Mice that received the TNBS showed macroscopic colitis reflected by thickening of the bowel and ulceration areas. In this experiment, the positive control Pentasa was able to decrease colitis macroscopic scores by 28.2% (71.8% vs 100%).

Compounds **18** and **31**−**33** did not provide better results than the initial lead **10** (74.9−84.9% of colitis macroscopic scores attenuation). Compounds **15**, **42**, and **45** decreased macroscopic scores with the same magnitude than Pentasa (62.2%, 66.3%, 65.1%, respectively), while **16**, **17**, and **30** were shown to be more potent (60.0%, 51.5%, and 55.9%, respectively). Finally, compound **48** exerted a very strong protecting effect against colitis as indicated by the 61.4% decrease in colitis macroscopic score (38.6%). Because **48** was significantly more potent than the reference treatment, we sought to better characterize its properties. Thus, histological damages, MPO activity as well as TNFα and Il-1β mRNA were quantified. As can be seen from Chart 3, compound **48** significantly attenuated both histological scores (3.1 vs 4.9) and MPO activity (34.1% vs 73.7%) but no significant effect was observed on the mRNA expression of the two cytokines TNFα and Il-1β.

Preliminary in Vitro DMPK Profile. Initial lead **10** as well as **48** were profiled for key physicochemical properties, in vitro metabolic stability, plasma protein binding, and intestinal absorption, allowing a comparison between these two compounds (Table 3).[19] With a clogP of 5 and a tPSA below 50 Å^2, initial lead **10** displayed a high lipophilicity while having an acceptable molecular weight. Relative to **10**, compound **48** resides in less lipophilic space with a cLogP of 3.5 and a TPSA of 62, while keeping a reasonable molecular weight.

As for in vitro assessments (Table 3), plasma protein binding assay showed higher free levels for **48** than for **10** (7% vs 1%) and an improved permeability was measured for **48** as

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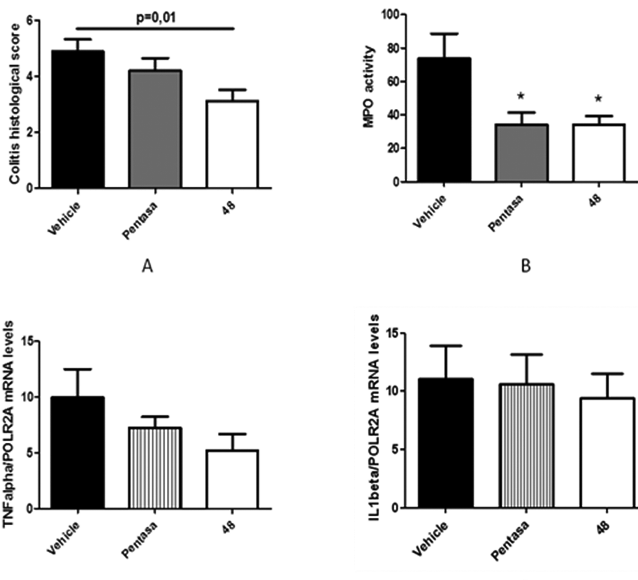
Chart 3. Histological Damages (A), MPO Activity (B), TNFα and IL-1β mRNA Levels (C, D) Were Measured in Colon after TNBS-Induced Colitis and Treatment with Pentasa (150 mg/kg) or 48 (10 mg/kg)[a]



[a]Data are the mean ± SEM of 10 mice per group; *$p < 0.05$ vs TNBS and vehicle.

Table 3. Determination and Evaluation of Selected Physicochemical and in Vitro DMPK Parameters for Key Compounds 10 and 48

Compound	10	48
Parameters		
MW (g/mol)	398.58	433.59
cLogP[a]	5.05	3.55
tPSA (Å^2)[a]	49.4	61.8
In vitro DMPK		
Human PPB (% free)[b]	1	7
A-B Caco-2 permeability (x 10^{-6} cm.s^{-1})[c]	9.8	49.9
P-gp inhibition (% at 10 μM)[d]	94.5	33.7
Metabolic stability (human liver microsomes, % parent remaining after 60 min)[e]	1	1
Mouse Cl_{int} (μl.min^{-1}. mg^{-1})[f]	>200	>200

[a]Determined with ChemDraw Ultra 10.0. [b]Assessed by equilibrium dialysis at 37 °C. [c]Compounds were incubated (0 and 60 min) at 10 μM in Caco-2 cell line. [d]Performed on MDR1-MDCKII cell line, using cellular uptake of calcein AM. [e]Compounds were incubated (0 and 60 min) at 1 μM in human liver microsomes (0.3 mg/mL). [f]Compounds were incubated at 1 μM in mouse liver microsomes (0.3 mg/mL).

compared to **10** in the in vitro CACO-2 assay. Moreover, in contrast to compound **10**, no interaction with P-gp was attributed to **48** as evidenced from the calcein-AM functional assay.

Unfortunately, compound **48** failed to increase metabolic stability in human and mouse liver microsomes because both **10** and **48** were rapidly metabolized by mouse (Cl_{int} >200 μL.min^{-1}·mg^{-1}) and human (1% of parent remaning after 60 min) hepatic microsomes.

Even though generally, **48** exhibited an improved in vitro DMPK profile relative to **10**, its low metabolic stability could represent a hindrance to oral administration.

■ CONCLUSION

The present study confirms that the 4-oxo-1,4-dihydropyridine scaffold is suitable for an easy access to potent CB_2 receptor selective ligands. We have synthesized and evaluated, on both cannabinoid receptors, 29 new compounds characterized by different moieties at *N*-1, C-3, C-5, and C-6 positions, allowing the description of sharp SAR and the discovery of very potent CB_2 selective agonists. Among them, 11 were evaluated in the TNBS-induced colitis model in mice and were found active in this experimental model. Compound **48** emerged as credible lead in this series with a strong protecting effect against colitis. Although this compound failed to act on TNFα and Il-1β levels, it has drastically decreased macroscopic scores, histological damages, and MPO activity. Even though **48** showed a better in vitro DMPK profile relative to **10**, future optimizations regarding this series should be focused on improving this profile, especially the metabolic stability.

■ EXPERIMENTAL SECTION

General Procedure for the Preparation of 48−54. Compound **47** (1 equiv), appropriate boronic acid (1.5 equiv), potassium phosphate (4 equiv), tricyclohexylphosphine (0.5 equiv), and palladium acetate (0.25 equiv) were suspended in a mixture of toluene/H_20 (3:2). The mixture was refluxed for 12 h, and the palladium acetate filtered. The resulting filtrate was then concentrated and the residue partitioned in $H_2O-CHCl_3$. The organic phase was washed both with water and brine, dried, and evaporated. The crude material was chromatographed on silica gel (DCM/MeOH 95:5, v/v) to afford target compounds **48−54**.

***N*3-(1-Adamantyl)-6-methyl-4-oxo-1-pentyl-5-(pyridin-4-yl)-1,4-dihydropyridine-3-carboxamide (48).** White solid (70%); mp >250 °C. ^{1}H NMR ($CDCl_3$) δ 10.00 (s, 1H), 8.70 (d, 2H, J = 5.5 Hz), 8.51 (s, 1H), 7.16 (d, 2H, J = 5.8 Hz), 3.96 (t, 2H, J = 7.7 Hz), 2.19 (s, 3H), 2.11 (m, 9H), 1.69 (m, 8H), 1.38 (m, 4H), 0.94 (t, 3H, J = 6.5 Hz). LC-MS (APCI+) m/z 434.3 (MH+). HRMS calcd for $C_{27}H_{36}O_2N_3$, 434.2802; found, 434.2788.

■ ASSOCIATED CONTENT

Supporting Information

Structures of endocannabinoid system-interacting ligands with protective effect against colitis, experimental procedures and spectroscopic data for all synthesized compounds, and detailed pharmacology. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

IBD, inflammatory bowel disease; CNRs, cannabinoid receptors; ECs, endocannabinoids; AEA, anandamide; 2-AG, 2-arachidonoylglycerol; GI, gastrointestinal; TNBS, 2,4,6-trinitrobenzenesulfonic acid; MPO, myeloperoxidase; Il-1β, interleukin-1β; SAR, structure−activity relationship; CHO, Chinese hamster ovary; $h\mathrm{CB}_{1\&2}$, human cannabinoid receptor 1 and 2

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