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Brief Article

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4-Oxo-1,4-dihydropyridines as Selective CB2 Cannabinoid Receptor Ligands Part 2: Discovery of New Agonists Endowed with Protective Effect Against Experimental Colitis

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4-Oxo-1,4-dihydropyridines as Selective CB₂ Cannabinoid Receptor Ligands Part 2: Discovery of New Agonists Endowed with Protective Effect Against Experimental Colitis.

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ABSTRACT: Further to our earlier work on a the 4-0x0-1,4-dihydropyridine, we describe herein our strategy to get access to potent selective CB₂ receptor agonists. Thus, we designed and synthesized twenty nine compounds, evaluated on both hCB1 and hCB2 cannabinoid receptors and assessed eleven 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

CB₂ cannabinoid receptor is a G-protein coupled receptor that constitutes, with CB1 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 CB₂ 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 CB₂ 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 ACS Paragon Plus Environment

 CB_1/CB_2 agonists (Δ^9 -THC, 1 and HU-210, 2)⁸ as well as CB1 (Arachidonyl-2'-chloroethylamide, 3)9 and CB, (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) well FAAH (5-([1,1'-biphenyl]-4-yl)-N-(2as as (benzo[d][1,3]dioxol-5-yl)ethyl)isoxazole-3-carboxamide, 7 and URB597, $\mathbf{8}$)¹¹ and monoacylglycerol lipase (JZL184, $\mathbf{9}$) inhibitors.12 Unfortunately, some of these ligands are wellknown to exert undesirable psychotropic effects that are assumed to be central CB, receptor-mediated. Accordingly, CB, receptor selective agonists, with their nonpsychoactive profile, are likely to have therapeutic value in the treatment of IBD.

Therefore we aimed to develop new CB₂ selective agonists endowed with anti-inflammatory properties in the GI tract. Along this line, we recently described a series of 4-0x0-1,4-dihydropyridines as CB, 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, 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.

RESULTS AND DISCUSSION

Preliminary in vivo Studies. We first evaluated our lead CB₂ selective agonist **10** (*N*₃-(1-Adamantyl)-6-*tert*-butyl-1-pentyl-4-0x0-1,4-dihydropyridine-3-carboxamide, hCB₂: Ki = 29 nM and hCB₁: Ki > 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, i.p.), **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 CB₂ selective agonists,¹⁵ around the 4-0x0-1,4-dihydropyridine template, with improved in vivo activity.

Chart 1. Modest effect of compound 10 against experimental colitis.



In vitro Pharmacology. The affinities of target compounds **15-19**, **30-45** and **47-54** (table 1-2) against the human cannabinoid receptors (*h*CB₁ and *h*CB₂) were determined by a competitive radioligand displacement assay using [³H]-CP-55,940 as radioligand for both *h*CB₂ and *h*CB₁ receptors.¹⁶ All compounds were first screened at 1 µM concentration and Ki values were determined for those exhibiting a specific displacement superior to 60% either for *h*CB₁ or the *h*CB₂ (tables 1-2). In addition, we investigated their functional activity at CB₂ receptor using a [³⁵S]-GTPγS binding assay and *h*CB₂-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 *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 CB₂ receptor. For instance, replacing the bulky aliphatic adamantyl group by methylcyclopropyl (**18**) or phenyl (**19**) did not impact on CB₂ receptor affinity. On the other hand, these modifications led to compounds with good affinity at CB₁ (*h*CB₁: Ki = 176 nM for **18** and Ki = **88** nM for **19**) and therefore, to less CB₂ 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 CB₂ receptor while being devoid of CB₁ receptor affinity. Compound **32**, characterized by a 2adamantyl moiety, presented the best profile in our series with regard to affinity at CB₂ receptor and selectivity over CB₁ (*h*CB₂: Ki = 0.26 nM and S.I > 3846).

Table 1. C-3 and C-6 modulations: affinities and functional activities of 15-19 and 30-37 toward both cannabinoid receptors.



Cod	P	D	Binding affinity Ki (nM)		[³⁵ S]-GTPγS (<i>h</i> CB ₂)	
Сра	K2	к ₄	hCB2	hCB1	EC50 (nM)	Emax (%)
15	tert-butyl	$\sqrt{2}$	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	\sim	14.1 ± 1.8	176	27.8 ± 9.2	195 ± 11
19	tert-butyl	\square	9.2 ± 0.6	87.9	8.3±0.7	250 ± 9
30	methyl	$\sqrt{2}$	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	\bigcirc	24.4 ± 2.3	> 1000	49.9 ± 6.7	197 ± 3
34	methyl	\sim	> 1000	/	/	/
35	methyl		> 1000	/	/	/
36	methyl	\downarrow	> 1000	/	/	/
37	methyl	\bigcirc	> 1000	/	/	/
(R) - (+) WIN 55,212-2		3.1 ± 1.2	52.4 ± 1.2	89.6 ± 0.8	232 ± 2	

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 , the methyl at C-6 as well as the adamantyl group borne by the amide function should be retained.

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

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59 60 **38** characterized by an unsaturated alkyl chain, displayed no affinity at CB₂ receptor. In addition, the introduction of a trifluorobutyl chain (**45**) led to one of the most potent and selective ligands within this series (hCB_2 : Ki = 0.28 nM, S.I > 3571).

Table 2. *N*-1 and C-5 modulations: affinities and functional activities of 38-45 and 47-54 toward both cannabinoid receptors.

		F	R4 N R2	Ð		
	R ₂	R ₄	Binding affinity		[³⁵ S]-GTPγS (<i>h</i> CB ₂)	
Cpd			Ki (nM)		EC50	Emax
			hCB2	hCB1	(nM)	(%)
38		Н	> 1000	/	/	/
39	\int_{0}^{0}	Н	> 1000	/	/	/
40	$\langle \rangle$	Н	> 1000	/	/	/
41		Н	> 1000	/	/	/
42	\bigcup	Н	24.1 ± 6.5	> 1000	47-4 ± 7-7	224 ± 6
43	\mathbf{c}	Н	195.8 ± 41.9	/	/	/
44	└ <u></u> ₀∽	Н	> 1000	/	/	/
45		Н	0.28 ± 0.18	> 1000	17.3 ± 7.7	22 4 ± 11
47	C_5H_{11}	Ι	20.8 ± 6.3	193.4	11.9 ± 0.8	299 ± 4
48	C_5H_{u}		36.5 ± 0.6	> 1000	114 ± 7	213 ± 36
49	$C_5H_{\rm n}$		54·3 ± 5·4	> 1000	39.9 ± 8.3	199 ± 12
50	$C_5H_{\rm u}$	Y C	> 1000	/	1	/
51	$C_5 H_{\rm u}$	A_	13.4 ± 0.6	105.1	88.5 ± 7.9	188 ± 7
52	$C_5 H_{\rm u}$	\bigcirc	189.4 ± 71.7	/	/	/
53	C_5H_{11}	\Box	> 1000	/	/	/
54	C_5H_{u}	NC	> 1000	/	/	/
(R)	(<i>R</i>) – (+) WIN 55,212-2		3.1 ± 1.2	52.4 ± 1.2	89.6 ± 0.8	232 ± 2

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 CB₂ but decreased selectivity, while a phenyl group (52) significantly decreased CB₂ 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 CB₂ receptor was completely abol-

ished. Surprisingly, when the *p*-methyl was replaced by an ethyl (**49**), the CB₂ activity was restored. Finally, a pyridine heterocycle (**48**) at C-5 was well tolerated with a Ki of 36 nM and no activity at CB₁ at 1μ M.

With regard to their functional activity, all compounds dose-dependently increased the [35 S]-GTP γ S binding between 188% to 258% (control value set at 100%), with EC₅₀ values in between 8.3 and 452 nM, which indicates that they behave as full agonists at CB₂ 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, since all C-5 substituted compounds show an agonist profile.

In vivo Pharmacology. A set of target CB₂ agonists, characterized by high CB₂ receptor affinity (*h*CB₂ Ki < 50 nM) and a selectivity index 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. The new CB2 receptor selective agonists attenuate colitis in TNBS-induced colitis (data are the mean \pm 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**, **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%, 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 TNF α and Il-1 β 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 TNF α and Il-1 β .

Chart 3. Histological damages (A), MPO activity (B), TNF α and IL-1 β mRNA levels (C, D) were measured in colon after TNBS-induced colitis and treatment with Pentasa (150 mg/kg) or compound 48 (10 mg/kg) (data are the mean ± SEM of 10 mice per group; *p < 0.05 vs TNBS and vehicle).



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 Å², 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 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 since both **10** and **48** were rapidly metabolized by mouse $(Cl_{int} > 200 \ \mu L.min^{-1}.mg^{-1})$ and human (1% of parent remaning after 60 min) hepatic microsomes.

Table 3. Determination and evaluation of selected physicochemical and in vitro DMPK parameters.

Compound	0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0	N V C ₅ H ₁₁ 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 $\mu M)^d$	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				

^aDetermined with ChemDraw Ultra 10.0. ^bAssessed by equilibrium dialysis at 37 °C. ^cCompounds were incubated (0 and 60 min) at 10 μ M in Caco-2 cell line. ^dPerformed on MDR1-MDCKII cell line, using cellular uptake of calcein AM. ^cCompounds were incubated (0 and 60 min) at 1 μ M in human liver microsomes (0.3 mg/mL). ^fCompounds were incubated at 1 μ M in mouse liver microsomes (0.3 mg/mL)

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-1,4dihydropyridine scaffold is suitable for an easy access to potent CB₂ receptor selective ligands. We have synthesized and evaluated, on both cannabinoid receptors, twenty nine 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, selective agonists. Among them eleven 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. Though this compound failed to act on TNF α and Il-1 β levels, it has drastically decreased macroscopic scores, histological damages and MPO activity. Future optimizations should be focused on improving DMPK profile of this series of compounds including metabolic stability.

EXPERIMENTAL SECTION

General Procedure for the Preparation of Compounds 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/H₂0 (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 H₂O-CHCl₃. The organic phase was washed both with water and brine, dried and evaporated. The crude material was chroma1

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59 60 tographied on silica gel (DCM/MeOH 95:5, v/v) to afford target compounds **48-54**.

N3-(1-Adamantyl)-6-methyl-4-oxo-1-pentyl-5-

(pyridin-4-yl)-1,4-dihydropyridine-3-carboxamide (48). White solid (70%); mp > 250 °C; 'H NMR (CDCl₃) 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 C₂₇ H₃₆ O₂ N₃ 434.2802, found 434.2788.

ASSOCIATED CONTENT

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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ABBREVIATIONS

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; hCB_{182} , human cannabinoid receptor 1 & 2

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4-Oxo-1,4-dihydropyridines as Selective CB₂ Cannabinoid Receptor Ligands Part 2: Discovery of New Agonists Endowed with Protective Effect Against Experimental Colitis.

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