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Synthesis and biological evaluation of ferrocene-based cannabinoid receptor 2 ligands

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Abstract. Ferrocene analogues of known fatty acid amide hydrolase inhibitors and CB_2 ligands have been synthesized and characterized spectroscopically and crystallographically. The resulting bioorganometallic isoxazoles were assayed for their effects on CB_1 and CB_2 receptors as well as on FAAH. None had any FAAH activity but compound **3**, 5-(2-(pentyloxy)phenyl)-*N*-ferrocenylisoxazole-3-carboxamide, was found to be a potent CB_2 ligand (K_i = 32.5 nM).

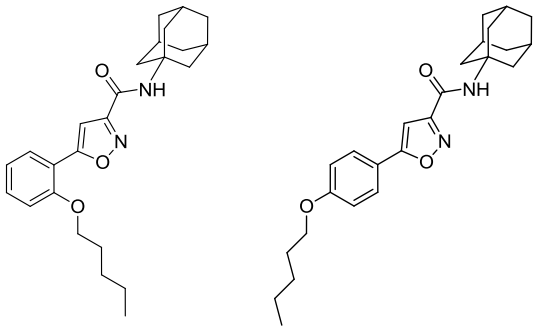
Keywords: isoxazole, ferrocene, cannabinoids, FAAH.

Introduction

N-Arachidonoylethanolamine (AEA), also known as anandamide, is an endogenous signaling mediator of the endocannabinoid system.[1] It is formed from a membrane precursor, termed *N*-arachidonoyl phosphatidylethanolamine, through multiple biosynthetic pathways.[2, 3] It has been identified that there is a basal amount of AEA in tissues. The equilibrium and circulation of AEA are regulated by several relevant synthases, hydrolases, and transporters.[4] Fatty acid amide hydrolase (FAAH) was demonstrated to be responsible for the main hydrolysis of AEA into arachidonic acid and ethanolamine.[5] There is evidence that increasing AEA levels can produce anti-inflammation, pain relief, injury repair, and neuroprotection by activating cannabinoid receptors CB_1/CB_2.[6-8] Therefore, stimulating CB_1/CB_2 receptors or inhibiting FAAH, thereby accumulating AEA, have been regarded as interesting therapeutic strategies for the treatment of pain, inflammation, nerve injury, or addiction.[4] Moreover, CB_2 inverse agonists have shown anti-inflammatory and anti-osteoporotic therapy in a manner dependent upon the migration of immune cells. [9-13]

Over recent decades, the development of CB_2 ligands and FAAH inhibitors has significantly progressed underpinned by numerous clinical trials of CB_2 agonists and FAAH inhibitors for the treatment of pain, inflammation, and central nervous system (CNS)-dependent disorders.[4, 14, 15] Recently, our work has identified 3-carboxamido-5-aryl-isozazole as a versatile scaffold for the design of CB_2 ligands

or FAAH inhibitors.[16-19] Of note, compound **1** (ALIAE218, Figure 1) showed potent affinity toward CB_2 receptors in the nanomolar range and significantly reduced dextran sulfate sodium (DSS)-induced colitis in mice.[16] Interestingly, the change of the pentoxy group of **1** from the ortho to the para position (**2**, ALIAE247, Figure 1) triggered a biological response switch from a CB_2 ligand to a FAAH inhibitor. Furthermore, the study of **2** in a model of DSS-induced colitis in mice identified its ability to alleviate inflammation.[18]



1 (ALIAE218)
K_i (CB_2) = 36 nM
IC_{50} (FAAH) > 10000 nM

2 (ALIAE247)
K_i (CB_2) > 1000 nM
IC_{50} (FAAH) = 8 nM

Figure 1. Structures of the known selective CB_2 ligand **1** and FAAH inhibitor **2**.

Discussion

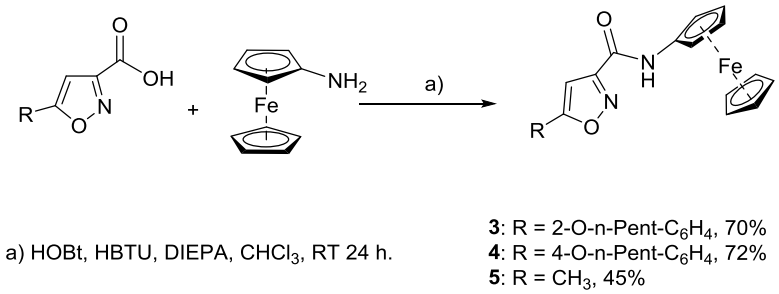
Ferrocene, known as an organometallic sandwich compound, has been used for drug design due to its suitable lipophilicity (logP = 2.66) facilitating membrane permeability, rotatable aromatic cyclopentadienyl ring conferring conformation diversity, and potential antioxidant capacity as well as its ability to generate reactive oxygen species (ROS) under certain conditions when suitable analogues undergo an activation process.[20-26] In addition, ferrocene and its derivatives have shown low toxicity in a wide range of tests in mammals (e.g. mice, rats, monkeys).[20] Recently,

we have performed the replacement of adamantanyl amine by aminoferrocene on a series of in-house dihydroquinoline-based CB_2 ligands. Our study implied that aminoferrocene-based compounds can replace adamantanyl amines in CB_2-targeting agents without significantly altering the binding affinity and efficacy of the molecules (Figure 2).[27] Given this initial success, we wished to expand this concept to other FAAH and CB_2 –based systems, namely isoxazoles. We anticipated that the formation of direct ferrocenyl anaolgues of **1** and **2**, namely **3** and **4** (*vide infra*) would suffice in this proof of principle study.

Figure 2. The principle of aminoferrocene acting as an adamantly amine bioisostere.

Results/Methodology.

Accordingly, we continued this research stream with a view to forming 3-carboxamido-5-ferrocenyl-isozazole-based modulators of the endocannabinoid system. These compounds were readily made by standard amide coupling protocols and were fully characterized by proton and carbon NMR spectroscopy, mass spectrometry and by elemental analysis. Analogue **5** was also made, but not tested, as it was an excellent candidate for obtaining solid state crystallization data, proving the presence and connectivity of the ferrocene moiety (Figure 3).



Scheme 1. Synthesis of isoxazole amides bearing a ferrocenyl unit.

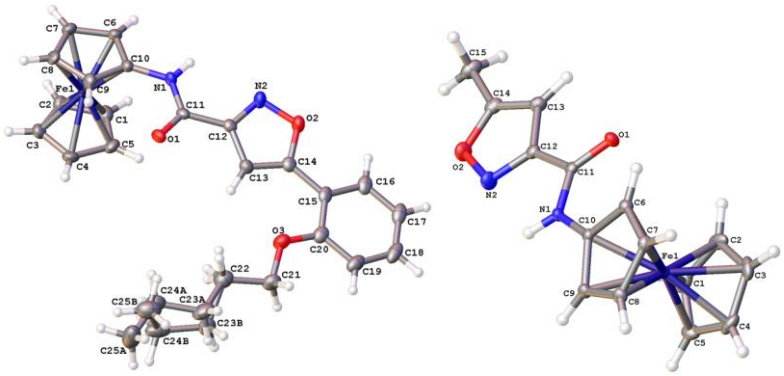


Figure 3. Solid state structures of analogues **3** and **5**.

The corresponding derivatives **3** and **4** (Scheme 1) were tested for their CB_2 vs CB_1 affinity in a binding assay, their efficacy toward CB_2, and their ability to inhibit FAAH.

The affinities of each synthesized compound for both CB_1 and CB_2 receptors were determined by a competitive radioligand displacement assay using the dual CB_1/CB_2 ligand [^{3}H]-CP55,940.[16, 28] Compounds displaying potent CB_2 affinity were further studied for their efficacy by cAMP assays using Chinese hamster ovary cells expressing CB_2 receptors (CHO-CB_2).[29, 30] Cells were treated with forskolin in order to activate adenylyl cyclase-dependent cAMP accumulation. Due to their influence on cAMP formation, CB_2 ligands can be classified as agonists that promote

cAMP accumulation and inverse agonists that inhibit cAMP production. As illustrated in Table 1, the replacement of the adamantanyl group of compound **1** (K_i = 36 nM) by a ferrocene unit (**3**, K_i = 32.5 nM) did not adversely affect its binding affinity toward CB_2 receptors. Interestingly, such a replacement improved the efficacy of cAMP formation (E_{max} from 242% to 400%, EC_{50} from 1046 nM to 221 nM). This observation is consistent with our previous conclusion that aminoferrocene-based compounds can replace adamantanyl amines in CB_2-targeting agents.[27] On the contrary, the introduction of a ferrocene unit to replace the adamantanyl group of compound **2** brought about a complete loss of FAAH inhibition probably due to the steric interaction which is induced by the slightly larger ferrocene unit rather than the adamantanyl group. Piomell and co-workers has studied the structure-activity relationship of carbamate-based FAAH inhibitors, which indicated the bulky groups (e.g., *exo*-2-norbornyl, adamantanyl) were unfavorable for FAAH inhibition.[31] Therefore, a ferrocene unit may be more sensitive to the steric interaction with FAAH active site in comparison to an adamantanyl group.

Table 1. Affinities (K_i values), maximum efficacy (E_{max}), and/or half-maximal response (EC_{50}) toward *h*CB_2 and *h*CB_1 cannabinoid receptors and FAAH inhibition of compounds **1**-**4**. Selectivity ratios *h*CB_2 versus *h*CB_1, and cytotoxicity on CHO-WT, CHO-CB_2, and HT29 Cells.

Cpds	*h*CB_2 and *h*CB_1 binding assays[a]			CB_2 cAMP assay[a]		FAAH inhibition	Cytotoxicity assays % inhibition at 10 μM		
	CB_2 K_i (nM)	CB_1 K_i (nM)	Ratio CB_1/CB_2	E_{max} (%)[b]	EC_{50} (nM)	IC_{50} (nM)	CHO-WT	CHO-CB_2	HT29
1	36.0 ± 3.4[c]	> 1000[c]	> 83	242 ± 42	1046 ± 400	> 10000[c]	0%	0%	0 %
2	> 1000[c]	N. D.[d]	N. D.[d]	N. D.[d]	N. D.[d]	8[c]	0%	0%	0%
3	32.5 ± 11.4	2513.3 ± 731.6	77.3	400 ± 20	221 ± 52	> 10000	0%	0%	0%
4	> 1000	N. D.[d]	N. D.[d]	N. D.[d]	N. D.[d]	> 10000	1%	7%	10%

WIN55,212 (agonist)	6.9 ± 2.0	13.9 ± 4.0	2.0	45 ± 7	4.3 ± 1.1
AM630 (inverse agonist)	31.2 ± 12.4[e]	5152 ± 567[e]	165[e]	232 ± 79	785 ± 7

[a] Data represent the mean ± SEM of three or four experiments performed in duplicate or triplicate. [b] E_{max} values are expressed as a percentage of forskolin-induced cAMP production. [c] Data from ref 18. [d] N.D. means not determined. [e] Data from ref 9.

A model of CB_2 was built by homology with the crystallographically resolved complex of CB_1 with the CB_1 taranabant ligand (5u09) [32] in order to study the binding mode of compound **3**. The docking of compound **3** was realized using GOLD 5.1.[33] Compound **3** binds in a V shaped conformation, with a rather large number of hydrophobic contacts, as befits for a cannabinoid receptor (Figure 4). The ferrocene moiety is accommodated by a wide cavity at the extracellular end of the pocket and its pentyl tail points toward a narrower subpocket. Interestingly, the whole molecule is more remote from TM7 than from the other six helices. It does not form any hydrogen bond with the receptor, which is compensated for by the large number of favourable hydrophobic contacts and the orientation of the more hydrophilic parts toward the middle of the cavity. The ferrocene appears to be a key element in the positioning of the compound as it can only fit in the upper part of the cavity due to its width and therefore orients the whole binding mode of the molecule.

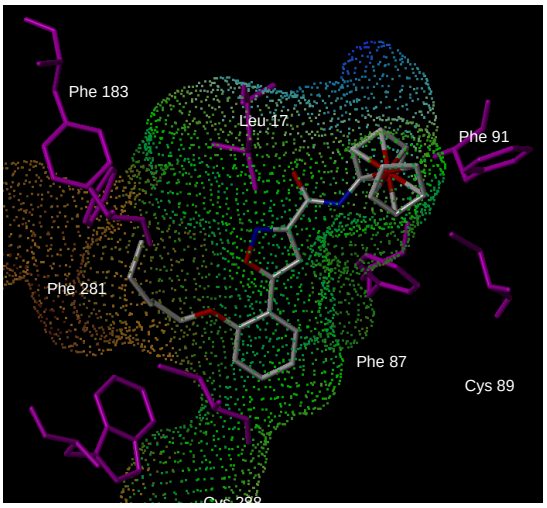


Figure 4: Putative binding mode of compound **3** into the CB_2 receptor

Experimental.

Chemistry.

Aminoferrrocene was purchased from TCI, UK and used as such. The two isoxazole acid precursors (i.e., 5-(2-pentyloxyphenyl)isoxazole-3-carboxylic acid and 5-(4-pentyloxyphenyl)isoxazole-3-carboxylic acid) were obtained using known synthetic procedures [16]. High resolution mass spectrometry (HRMS) was performed by the EPSRC National Mass Spectrometry Facility, University of Swansea. Elemental analyses were conducted by Stephen Boyer (London Metropolitan University). Reactions were carried out in a well vented fume hood. ^{1}H and ^{13}C-NMR spectra were recorded on Varian 500 MHz or 400 MHz spectrometers and chemical shifts are reported in ppm, usually referenced to TMS as an internal standard.

5-(2-(Pentyloxy)phenyl)-*N*-ferrocenylisoxazole-3-carboxamide, **3**.

5-(2-(Pentyloxy)phenyl)isoxazole-3-carboxylic acid (27.5 mg, 0.1 mmol) was combined with HOBt (6.8 mg, 0.05 mmol) , HBTU (57 mg, 0.15 mmol) and DIPEA (0.035 mL) in $CHCl_3$ (2 mL). The reaction mixture was stirred at room temperature for 45 min and then aminoferrocene (24.1 mg, 0.12 mmol) was added and the mixture was stirred for 24 hr. Thereafter the reaction mixture was washed successively with

NaOH (0.5 N, 20 mL), HCl (1 N, 20 mL) then H_2O (20 mL). The organic layer was dried with $MgSO_4$ and evaporated to a ca. 3 mL volume and purified by column chromatography. The orange band was eluted with a hexane: ethyl acetate (1:1) mixture, which was collected and evaporated to dryness. The yield was 34 mg, 70% of an orange solid. Crystallization by diffusion between CH_2Cl_2 and hexane provided the orange crystals.

^{1}H NMR ($CDCl_3$, 500 MHz): δ = 8.00-7.80 (2H, m, Ar), 7.44-7.39 (1H, m, Ar), 7.27 (1H,s, Ar), 7.07-6.93 (2H,m, Ar), 4.76 (2H, d, J = 1.9, 2CH), 4.23 (5H,s, Cp), 4.15 (2H, d, J = 1.9, 2CH), 4.09-3.92 (2H, m, CH_2), 1.96-1.91 (2H, m, CH_2), 1.48-1.42 (4H, m, $2CH_2$), 0.97 (3H, t, J = 7.1, CH_3). ^{13}C NMR ($CDCl_3$, 126 MHz): δ = 169.5, 165.2, 159.1, 156.0, 139.8, 131.8, 127.7, 120.6, 112.2, 110.0, 102.8, 69.4, 68.7, 64.9, 61.7, 28.8, 28.2, 22.4, 14.0. HRMS-ESI(m/z): found 481.1184 , calcd. for [$C_{25}H_{26}FeN_2O_3$ + Na]$^+$ 481.1185. Anal. Calcd (%) for $C_{25}H_{26}FeN_2O_3$: C, 65.51; H, 5.72; N, 6.11. Found (%): C, 65.41; H, 5.80; N, 6.12.

5-(4-(Pentyloxy)phenyl)-*N*-ferrocenylisoxazole-3-carboxamide, **4**.

The title compound was prepared by a coupling reaction. 5-(4-(Pentyloxy)phenyl)isoxazole-3-carboxylic acid (27.5 mg, 0.1 mmol) was reacted with HOBt (6.8 mg, 0.05 mmol), HBTU (57 mg, 0.15 mmol) and DIPEA (0.035 mL) in CH_2Cl_2 (2 mL). The reaction mixture was stirred at RT for 45 min. and then added aminoferrocene (24.1 mg, 0.12 mmol) stirred for 24 hr. Workup was as above. The orange band was eluted with a hexane: ethyl acetate (1:1) mixture, which was collected and evaporated to dryness. The yield was 35 mg, 72% (orange solid).

^{1}H NMR ($CDCl_3$, 500 MHz): δ = 7.94 (1H, s, Ar), 7.75-7.70 (2H, m, Ar), 7.27 (2H, s, Ar), 7.00-6.93 (2H, m, $2CH_2$), 6.89 (1H,s, Ar), 4.75-4.71 (2H, m, 2CH), 4.22 (5H, s, Cp), 4.06-4.01 (2H, m, 2CH) 1.83-1.76 (2H, m, CH_2), 1.45-1.42 (4H, m, $2CH_2$), 0.96 (3H, s, CH_3). ^{13}C NMR ($CDCl_3$, 126 MHz): δ = 172.0, 161.2, 159.1, 156.9, 127.6, 119.2, 115.1, 97.5, 93.2, 69.3, 68.3, 64.9, 61.7, 28.8, 28.1, 22.4, 14.0. HRMS-ESI(m/z): found 459.1333 , calc. for [$C_{25}H_{26}FeN_2O_3$ + H]$^+$ 459.1366. Anal. Calcd (%) for $C_{25}H_{26}FeN_2O_3$: C, 65.51; H, 5.72; N, 6.11. Found (%): C, 65.51; H, 5.82; N, 6.18.

5-Methyl-*N*-ferrocenylisoxazole-3-carboxamide, **5**.

The title compound was prepared by a coupling reaction. 5-Methylisoxazole-3-carboxylic acid (127 mg, 1 mmol) was reacted with HOBt (68 mg, 0.5 mmol), HBTU (570 mg, 1.5 mmol) and DIPEA (0.35 mL) in $CHCl_3$ (20 mL). The reaction mixture was stirred at RT for 45 min. and then aminoferrocene (241 mg, 1.2 mmol) was added and the mixture was stirred for 24 hr. Work up was as above. An orange band was eluted with a hexane: ethyl acetate (6:4) mixture, which was collected and evaporated to dryness. The yield was 160 mg, 45% of an orange solid. Crystallization by diffusion between CH_2Cl_2 and hexane provided orange crystals.

^{1}H NMR ($CDCl_3$, 500 MHz): δ = 7.90 (1H, s, NH), 6.50 (1H, s, Ar), 4.72-4.69 (2H, m, 2CH), 4.20 (5H, s, Cp), 4.07-3.97 (2H, m, 2CH), 2.52 (3H, s, CH_3). ^{13}C NMR ($CDCl_3$, 126 MHz): δ = 171.5, 158.8, 156.9, 101.4, 93.3, 76.7, 69.3, 64.9, 61.6, 12.4. HRMS-ESI(m/z): found 311.0471, calcd. for [$C_{15}H_{14}FeN_2O_2$ + H]$^+$ 311.0477. Anal. Calcd (%) for $C_{15}H_{14}FeN_2O_2$: C, 58.09; H, 4.55; N, 9.03. Found (%): C, 58.20; H, 4.63; N, 9.13.

Biology.

Competition Binding Assay

Stock solutions of the compounds were prepared in DMSO at 10^{-2} M and further diluted with the binding buffer to the desired concentration, with a maximal DMSO concentration of 0.1%. Briefly, [^{3}H]-CP-55,940 (0.5 nM), nonselective human CB_1 and CB_2 cannabinoid receptor, were added to 6 μg of membranes from CB_1- or CB_2-overexpressing CHO cells in binding buffer (50 mM Tris-HCl, 5 mM MgCl2, 2.5 mM EDTA, 0.5 mg/mL BSA, pH 7.4). After 90 min at 30 °C, the incubation was stopped and the solutions were rapidly filtered over a UniFilter-96 GF/C glass fiber plate, presoaked in PEI (0.05%) on a Filtermate UniFilter 96-Harvester (PerkinElmer), and washed 10 times with icecold 50 mM Tris-HCl pH 7.4. The radioactivity on the

filters was measured using a TopCount NXT microplate scintillation counter (PerkinElmer) using 30 μL of MicroScint 40 (PerkinElmer). Assays were performed at least in triplicate in three independent experiments. The nonspecific binding was determined in the presence of 5 μM (R)- (+)-WIN 55,212-2 (Sigma).

Conclusion and future perspectives.

There is growing evidence that CB_2 inverse agonists can attenuate inflammation and osteoporosis through regulating the migration of immune cells.[9-13] Herein, we have shown, albeit limited to 2 examples, that the replacement of adamantylamine by an aminoferrocene is possible and may provide a new perspective for the design of potent CB_2 inverse agonists. This should be readily expandable to other ligand scaffolds other than isoxazoles and dihydroquinilones and to other metallocene-based derivatives. It is anticipated that other GPCR-based or enzyme inhibitor ligands might be designed incorporating a ferrocenyl moiety [34] with the potential for example to assist in X-ray protein studies [35] or to enable further Fe(II)/(III) redox chemistry [36-39].

Executive Summary

- It is possible to synthesize ferrocene analogues of known cannabinoid receptor ligands.
- These are characterized in both solution and in the solid state.
- Compound **3** retains binding and displays improved efficacy at the CB_2 receptor.

- Ongoing studies are looking at other uses of ferrocenes as bioisosteres in bioorganometallic chemistry.

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Financial & competing interests disclosure

The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed. No writing assistance was utilized in the production of this manuscript.

Disclosure. The work has been previously presented as part of a thesis, see http://sro.sussex.ac.uk/68599/.

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