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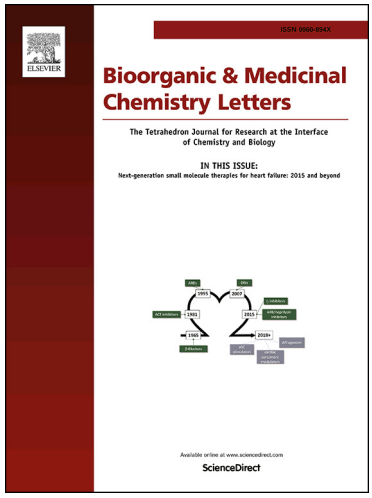

Divalent Cannabinoid-1 Receptor Ligands: A Linker Attachment Point Survey of SR141716A for Development of High-Affinity CB1R Molecular Probes

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Graphical Abstract

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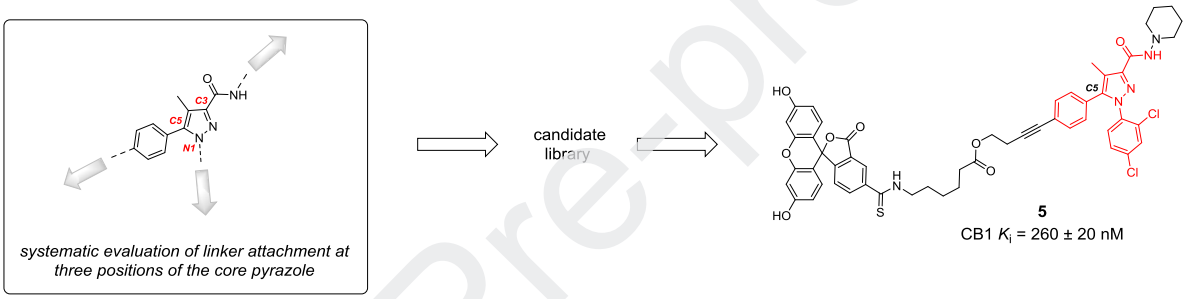
[d]*Current address: Department of Pharmacology and Toxicology, The University of Otago, Dunedin, New Zealand*



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ABSTRACT

The cannabinoid-1 receptor (CB1R) inverse agonist SR141716A has proven useful for study of the endocannabinoid system, including development of divalent CB1R ligands possessing a second functional motif attached via a linker unit. These have predominantly employed the C3 position of the central pyrazole ring for linker attachment. Despite this precedent, a novel series of C3-linked CB1R-D2R divalent ligands exhibited extremely high affinity at the D2R, but only poor affinity for the CB1R. A systematic linker attachment point survey of the SR141716A pharmacophore was therefore undertaken, establishing the C5 position as the optimal site for linker conjugation. This linker attachment survey enabled the identification of a novel divalent ligand as a lead compound to inform ongoing development of high-affinity CB1R molecular probes.

The CB1 cannabinoid receptor is an important therapeutic target, with involvement in the regulation of multiple neurotransmitters.[1] Unfortunately, undesired side-effects have limited the use of some clinically approved therapeutics and prevented approval of other candidates.[2,3] There remains a need for improved pharmacological tools to better characterize receptor binding and downstream signalling processes.[4,5] A growing body of evidence suggests that GPCRs not only function individually but are also able to form homodimeric, heterodimeric or higher-order oligomeric complexes that exhibit unique signalling and functional behaviour.[6,7] Interrogation of these multi-receptor complexes using pharmacological tools is important in order to understand their physiological significance. Additionally, direct targeting of GPCR dimers appears to offer potential for (i) selective modulation of specific signaling pathways associated with GPCR dimers, to avoid undesired therapeutic side effects, or (ii) for selective localisation of molecular probes to specific tissues co-expressing both constituent receptors in a GPCR heterodimer of interest.

The present study stemmed from a number of studies indicating that CB1 and D2 GPCRs are capable of forming hetereodimers that exhibit altered G-protein signaling with respect to the individual receptors.[8,9] Development of divalent ligands able to selectively bind CB1R-D2R heterodimers would provide a useful tool with which to investigate the complex cross-talk between the cannabinoid and dopamine signalling pathways.[10] Further, as these two GPCRs are predominantly co-expressed in neurons of the striatum,[11] a CB1R-D2R divalent ligand could potentially be used to target or study CB1 receptors in this particular region of the brain.[12,13] In concept, divalent molecular probes for GPCRs (Figure 1) are composed of a high affinity receptor ligand (Ligand A, *red*), conjugated via a linker (*green*) to a second GPCR ligand (Ligand B, *blue*) or functional tag (Tag, *yellow*). [6,7] The ligand binds to the receptor, while the linker extends out of the binding pocket to display either an additional ligand for simultaneous binding to a second GPCR (*left*), or a pharmacological tag for detection (*right*).[14,15] It should be noted that determination of the exact binding mode is non-trivial and other binding modes are possible. To be suitable for probe development, a ligand requires (a) high affinity and selectivity for the target receptor, (b) a suitable molecular motif that enables linker conjugation, and crucially (c) retention of the original pharmacological profile after linker attachment.

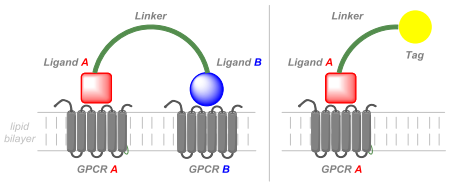


Figure 1. Design features for GPCR ligands binding divalently to two GPCRs (*left*) or enabling detection via a functional tag (*right*).

of appropriate ligands meeting the criteria above. In the case of the D2R, derivatives of the selective agonist 2-(N-phenylethyl-N-propyl)-amino-5-hydroxytetralin (PPHT) had been reported bearing both a fluorescent label[16] or an adenosine ($A_{2A}R$) receptor antagonist,[17] that both demonstrated high affinity at the D2R. For CB1, the selective inverse agonist SR141617A (Figure 2, **1**) that had received clinical approval for the treatment of obesity in 2006 as Rimonabant appeared to be an excellent candidate. Despite being later removed from the market due to psychological side-effects, the SR141716A diarylpyrazole scaffold has remained highly useful as a research platform. A wealth of structure-activity data exists for derivatives of **1** in both the scientific and patent literature, including different substituents at all positions of the pyrazole core (*highlighted in red*) and substitution of alternative (hetero)aromatic cores.[18,19]

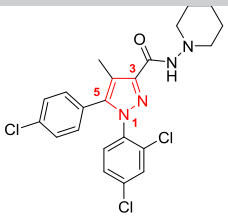
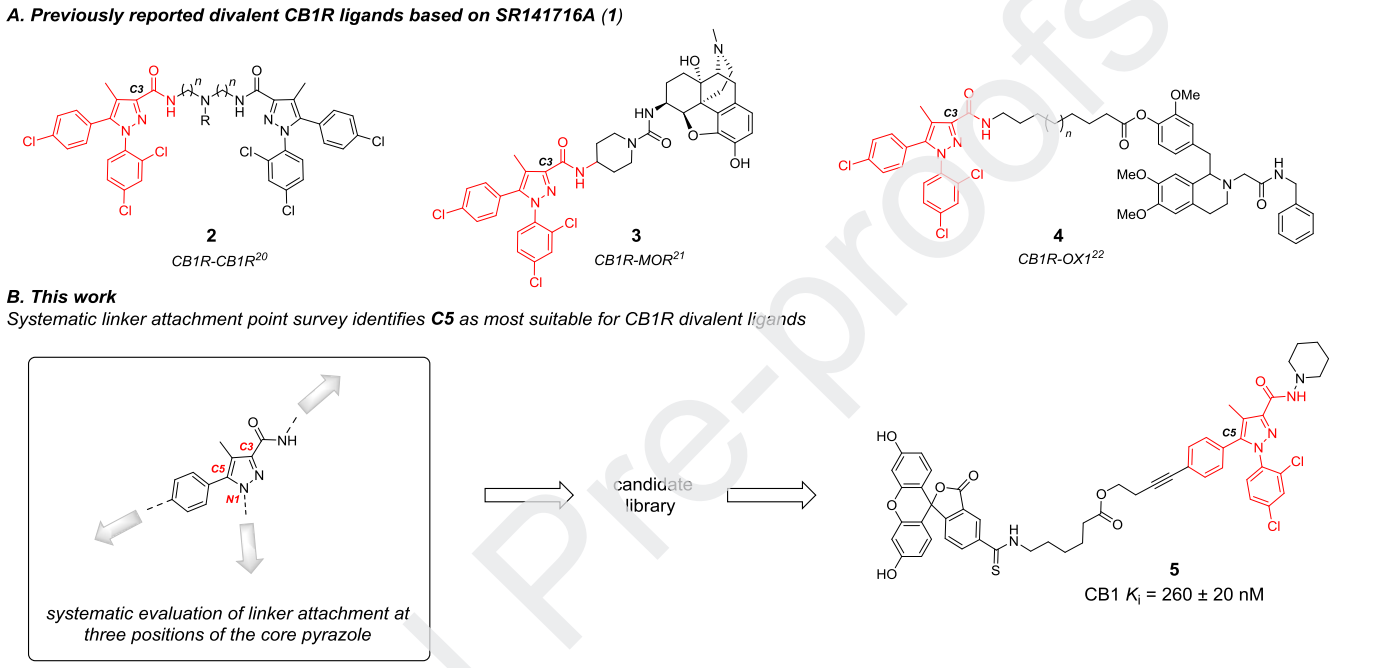


Figure 2. CB1R Ligand SR141716A (1). The pyrazole core (*red*) showing linker attachment positions N1, C3 and C5.



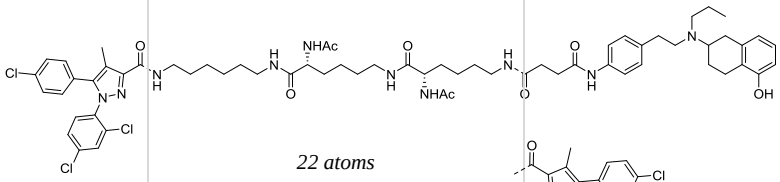
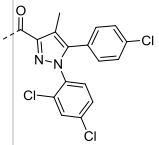
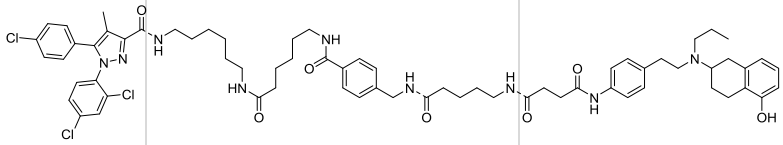
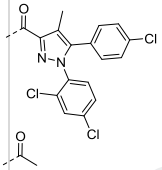
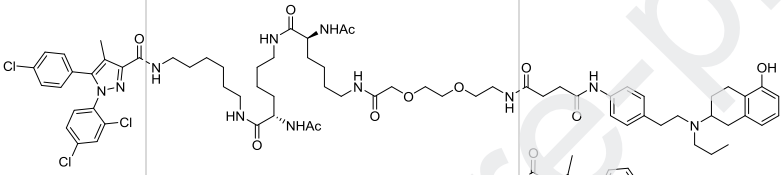
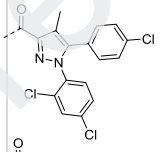
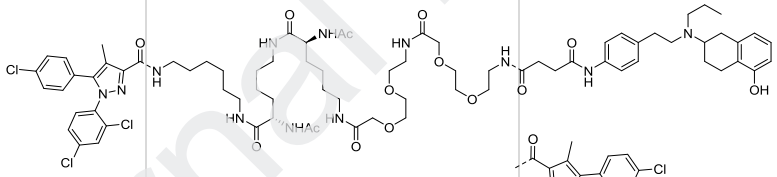
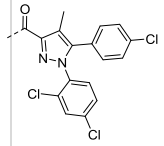
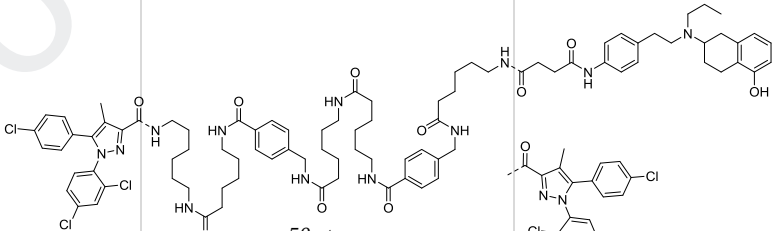
Scheme 1. CB1R probes based on SR141716A (**1**). Previously reported ligands[20–22] (A, *above*) and the current study (B, *below*).

Ligands based on this framework have been successfully applied towards the long-elusive goal of obtaining X-ray crystallographic data for the CB1R.[18–20] Importantly for our purposes, a number of studies towards divalent ligands based on **1** have been reported (Scheme 1A, **2-4**), dominated by linker conjugation to the SR141716A core via the C3 position.[21–26]

The initial goal of the current study was the preparation of a series of divalent CB1R-D2R ligands based on conjugation at the C3 position of **1**, to investigate CB1R-D2R heterodimerisation and signalling modulation. On the basis of previously reported work towards CB1R divalent ligands it appeared reasonable to expect that C3 linker attachment should deliver divalent ligands with good affinity for the CB1R. In the event however, all efforts to design C3-conjugated CB1R-D2 divalent ligands possessing useful CB1R affinity proved futile. In order to build a foundation of data to enable eventual design of high-affinity bivalent CB1R ligands, a systematic survey of possible alternative linker conjugation sites for the tetrazole core of SR141717A (**1**) at N1, C3, and C5 was undertaken (Scheme 1B), incorporating linkers varying in length and composition. This foundation eventually led to the identification of position C5 as the optimal conjugation point for **1**, and enabled the discovery of a novel CB1R ligand (**5**) incorporating a fluorophore as a lead compound to inform ongoing development of high-affinity *h*CB1R visualization tools.

To prepare the initial series of proposed divalent CB1R-D2R ligands (Table 1), the C3 carboxylic acid chloride derivative of **1** was prepared by slightly modified literature methods[23] (*see* Supporting Information) and conjugated with a range of amine linkers, broadly following the earlier work of Zhang, Thomas, Fernandez-Fernandez and Portoghese.[20–22,24,25] The D2R ligand was incorporated through preparation of (±)-PPHT-NH_2 according to the synthesis described by Neumeyer[16] and subsequent coupling with a carboxylic acid terminated linker segment. The compounds prepared varied in linker length (22-50 atoms) and composition (lipophilic, hydrophilic, aromatic) in an effort to accommodate the differing chemical environments potentially encountered in accessing the receptor binding pocket.

To our disappointment, given the strong literature precedent for high affinity C3 derivatives, *no compounds* in the series were capable of reaching the minimum 75% radioligand displacement threshold at *h*CB1R to warrant further investigation ([^{3}H]-CP55,940 competition binding assay at 10 µM) indicating very poor affinity.

CB1R ligand	Linker	Secondary ligand		Displacement % (*h*CB1R)	*h*D2R K_i (nM)
	*22 atoms*		**5**	62	0.84
			6	11	
			**7**	41	
	*28 atoms*		**8**	33	2.2
			9	29	
			**10**	23	
	*31 atoms*		**11**	44	2.4
			12	23	
			**13**	37	
	*40 atoms*		**14**	33	1.1
			15	29	
			**16**	23	
	*50 atoms*		**17**	0	4.2
			18	0	
			**19**	0	

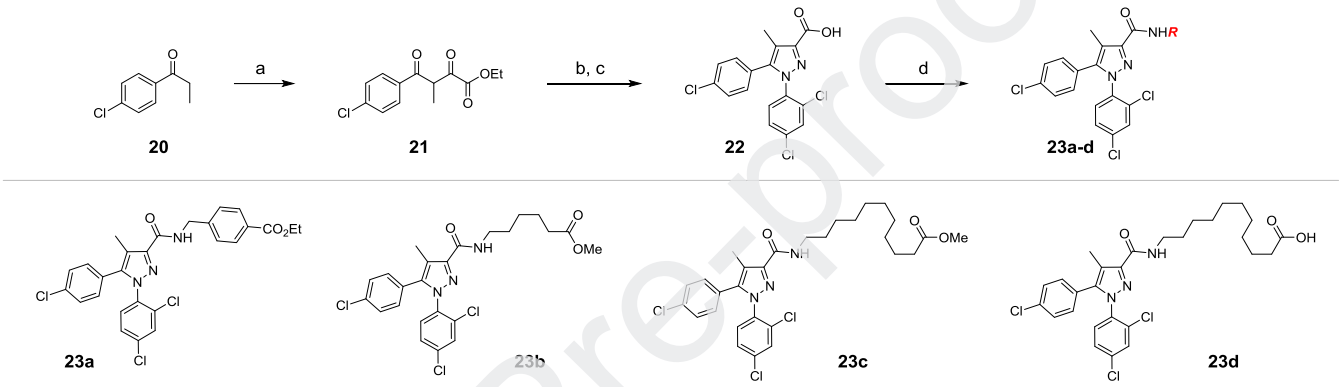
In [illegible] was generated for divalent CB1R-D2R ligands **5**, **6**, **11**, **17** and **18**. revealed that these ligands exhibited uniformly excellent affinity for the D2R, exceeding that of the parent agonist (±)-PPHT (*h*D2R K_i 13.3 nM).[16]

These data demonstrated that the linkers and D2R ligand used in this study were effective choices for preparation of high affinity divalent D2R ligands. Crucially however, the broad lack of useful CB1R affinity demonstrated by the more complex divalent ligands (**5**, **6**, **8**, **9**, **11**, **12**, **14**, **15**, **17** and **18**) highlighted the challenge of designing strongly-binding divalent ligands that incorporate a CB1R ligand. These latter findings may be due in part to direct entry of the D2R ligand into the receptor from the extracellular matrix, as opposed to entry via the hydrophobic lipid bilayer that is proposed to occur at CB1R.[26,27]

Although many SR141716A derivatives with differing substitution are known, to our knowledge there has been no systematic comparison of linker attachment sites, specifically directed towards the design of high-affinity divalent ligands. [illegible] of the SR141716A inverse agonist scaffold, in order to identify candidates suitable for further development of high-affinity divalent CB1R ligands.

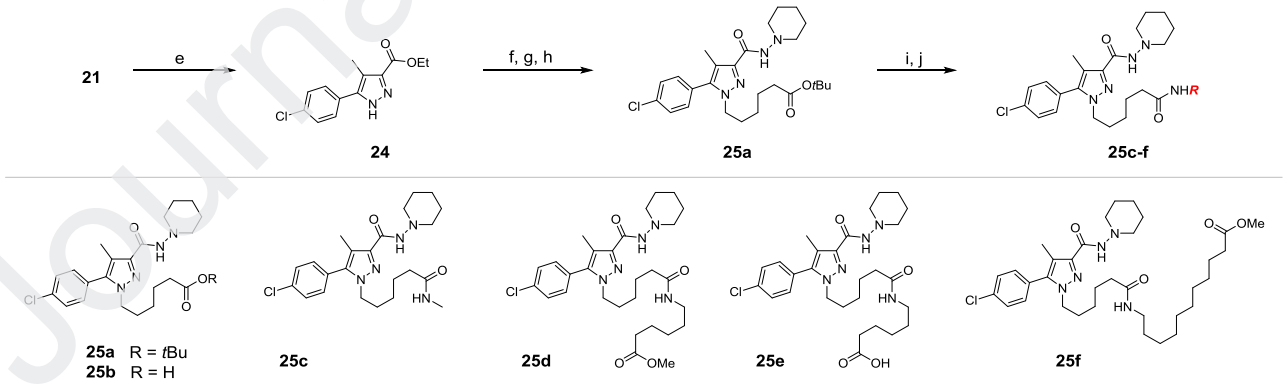
Two alternative sites for linker conjugation to **1** were identified as synthetically tractable for library preparation; the N1 and C5 positions of the pyrazole core (Scheme 1B). A series of simple ligands conjugated at C3 were also included for comparison. Synthetic intermediates adapted to bear a functional handle to enable linker conjugation were prepared using modifications of reported procedures (Schemes 2-4). Linker fragments of varying length and composition were then conjugated at the respective positions. The resultant SR141617A conjugates were again screened in an *h*CB1R radioligand competition binding assay at a single concentration of 10 µM. Concentration response curves were obtained only for those compounds capable of >75% radioligand displacement in the initial screen.

Scheme 2. **C3-Conjugated CB1R ligand series**. General preparative route (*top*) and derivative series prepared (*below*).



Reagents and conditions: a) LiHMDS (1.0 M in hexanes), Et_2O, 45 min, rt then diethyloxalate, rt, 16 h, 38%, b) 2,4-dichlorophenylhydrazine.HCl, EtOH, reflux, 16 h, 81%, c) KOH, MeOH/H2O, reflux, 16 h, quant., d) [for **23a** (87%), **23b** (79%) and **23d** (20%)]: oxalyl chloride, DMF (cat.), CH_2Cl_2, rt, 1 h then amine, DIPEA, CH_2Cl_2, rt, 16 h; [for **23c** (77%)]: EDCI.HCl, HOAt, DIPEA, 30 min, rt, then amine.

Scheme 3. **N1-Conjugated CB1R ligand series**. General preparative route (*top*) and derivative series prepared (*below*).



Reagents and conditions: e) LiHMDS (1.0 M in hexanes), Et_2O, 45 min, rt *then* diethyloxalate, rt, 16 h *then* N_2H_4.2HCl, EtOH, rt, 20 h, 78%, f) KOH, MeOH/H_2O, reflux, 5 h, quant., g) oxalyl chloride, DMF (cat.), CH_2Cl_2, rt, 0.5 h *then* 1-aminopiperidine, DIPEA, CH_2Cl_2, rt, 16 h, h) *tert*-butyl-6-bromohexanoate, K_2CO_3, DMF, rt, 24 h, 68%, i) TFA, CH_2Cl_2, rt, 0.5 h, **25b** (quant.), j) [for **25c** (89%) and **25f** (91%)]: HATU, amine, DMF, rt, 16 h; [for **25d** (65%)]: EDCI.HCl, HOAt, DIPEA, CH_2Cl_2, rt, 16 h; *then* [for **25e** (35%)]: KOH, MeOH/H_2O, rt, 16 h.

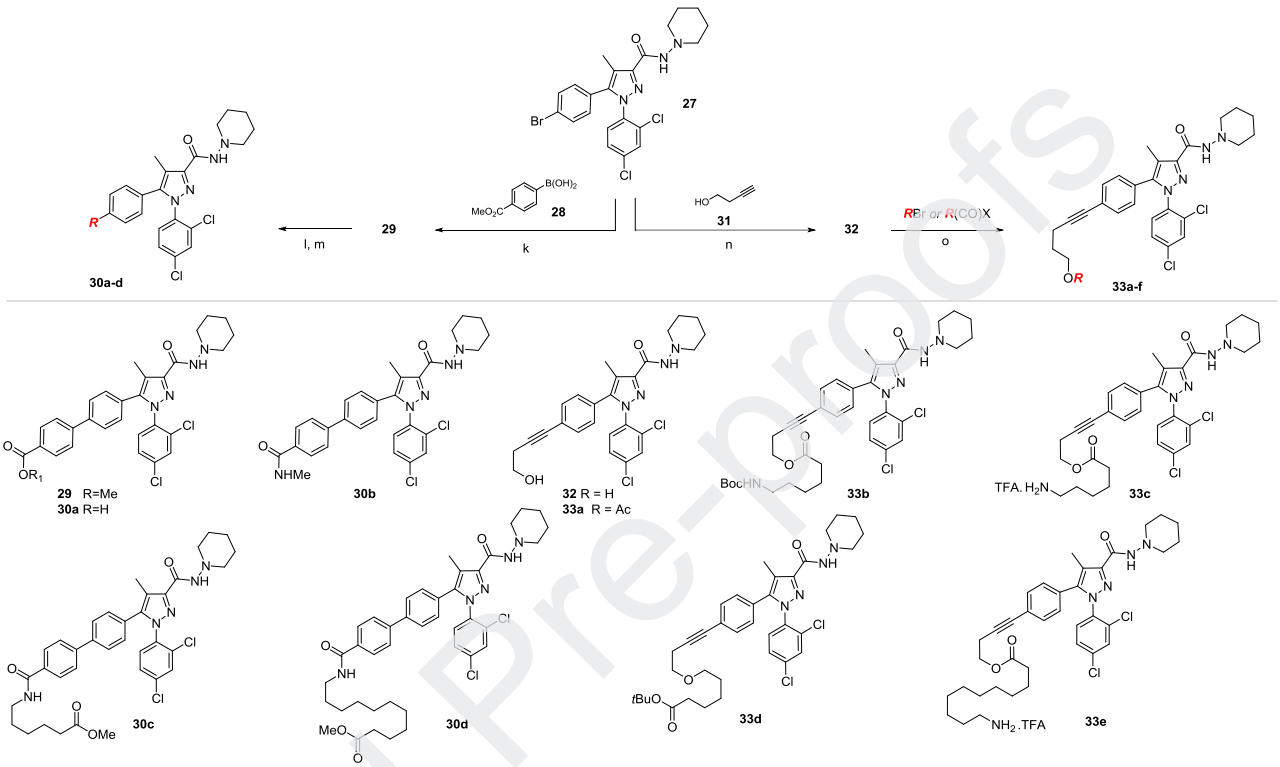
The C3-conjugated series of ligands prepared for the systematic survey (Scheme 2) were selected to probe in particular the effect of linker length on CB1R affinity. Although a large number of high- affinity C3 variants of this scaffold have been

use as covalent linker units. Target analogues were readily accessed via carboxylic acid **22**, prepared in three steps from 4-chloropropiophenone (**20**) by slightly modified literature methods.[23] Conversion of **22** to the acid chloride followed by coupling with aliphatic amines then afforded C3-conjugated derivatives **23a-d**.

Only a small number of reports describing N1-substituted analogues of **1** have been published, although a series of high-

tert-butyl ester **24** (Scheme 3) was prepared by condensation of β-keto ethyl glyoxalate **21** with hydrazine, followed by alkylation with 6-bromo *tert*-butylhexanoate. Removal of the *tert*-butyl group to give the corresponding acid **25b** then allowed the installation of linkers of varying length and composition, to give a series of N1-conjugated derivatives **25a-f**.

Scheme 4. **C5-Conjugated CB1R ligand series**. General preparative route (*above*) and derivatives prepared (*below*).

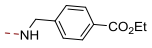
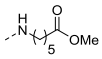
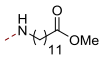
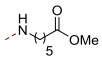
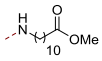
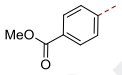
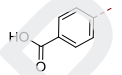
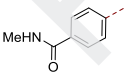
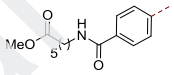
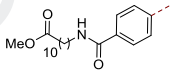
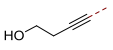
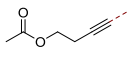
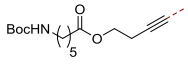
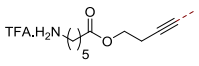
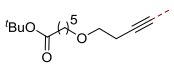
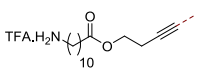


Reagents and conditions: k) 4-methoxyphenylboronic acid, $Pd(OAc)_2$ (10 mol %), PPh_3 (20 mol %), K_2CO_3, DME/H_2O (1:1), 80 °C, 16 h, 67%, l) LiOH, THF/H_2O, rt-reflux, 9 d, 87%, m) EDCI.HCl, DMAP (10 mol %), amine, CH_2Cl_2, rt, 16 h, n) 4-butyn-1-ol (**31**), $Pd(OAc)_2$ (10 mol %), PPh_3 (20 mol %), CuI (10 mol %), NEt_3, 16 h, 80 °C, 89%, o) [for **33a**]: Ac_2O, DMAP (10 mol %), CH_2Cl_2, rt, 1.5 h, 91%; [for **33b**]: EDCI.HCl, *N*-Boc 6-aminocaproic acid, CH_2Cl_2, rt, 16 h, 50%; *then* [for **33c**]: TFA, CH_2Cl_2, rt, 30 min; **33c** (quant.); [for **33d**]: *tert*-butyl-6-bromohexanoate, TBAI (10 mol %), NaH, DMF, 16 h, 80°C, 17%; [for **33e**]: EDCI.HCl, *N*-Boc 11-aminoundecanoic acid, CH_2Cl_2, rt, 16 h *then* TFA, CH_2Cl_2, rt, 30 min; **33e** (50% from **32**).

At the outset of this investigation, very few C5-conjugated derivatives were known.[35] The ligand Tocrifluor, derived from **1**, possesses a fluorescent C5-conjugated rhodamine tag,[37] although it has been reported to exhibit only moderate to low affinity for the CB1R.[5,36] The Makriyannis group have developed a C5-modified variant (AM6538) that enabled the determination of the first crystal structure of the CB1R,[37] and has been shown to exhibit long-lasting *in vivo* effects.[30]

Functionalization of the C5 substituent of SR141716A for linker conjugation was envisaged to be achieved through palladium-mediated cross-coupling of **27**, the brominated analogue of **1**. (Scheme 4). Aryl bromide **27** was accordingly prepared from 4'-bromopropiophenone, analogously to **1**, followed by coupling with either boronic acid **28**, or 3-butyn-1-ol (**31**), affording ester **29** or alcohol **32**, respectively. Linkers of varying length and composition were subsequently coupled to carboxylic acid **30a**, derived from hydrolysis of **29**, and alcohol **31**, affording a diverse range of C5-conjugated SR141716A derivatives.All SR141716A-linker conjugates prepared were initially screened for affinity at the *h*CB1R in a radioligand competition binding assay at a single concentration of 10 μM. Full concentration-response curves were then generated for those compounds capable of >75% radioligand displacement in the initial screen, for determination of the influence of the linker attachment position, length and composition on CB1R affinity (Table 2). Of the C3 SR141716A-linker conjugates **23a-d**, only **23a** and **23b** (K_i 8.2 and 13 nM, respectively) demonstrated high affinity at CB1R. Further extension of the C3 linker substituent to give **23c** and **23d** lowered CB1R affinity to the extent that these compounds failed to meet the 75% radioligand displacement threshold. It is possible that the high affinity of **23a** and **23b** is accounted for by the short linker acting as an effective isostere for the aminopiperidyl moiety of SR141716A (**1**). It should be noted that the low affinity of **23c-23d** is in contrast with the modest to high affinities previously reported for C3 long chain amide congeners of SR141716A (**2**, Scheme 1).[29]

T

Compound	*R*	Linker length (*atoms*)	Displacement[a] % (*h*CB1)	*h*CB1R K_i (nM)
C3 Series				
23a		9	*nd*[b]	8.2 ± 5.5
23b		8	*nd*	13 ± 3
23c		14	5	
23d		13	43	
N1 Series				
25a	O*t*Bu	-	37	
25b	OH	-	10	
25c	NHMe	-	3	
25d		8	9	
25e		7	33	
25f		13	13	
C5 Series				
29		7	*nd*	131 ± 22
30a		6	66	
30b		7	55	
30c		14	49	
30d		19	0	
32		5	98	180 ± 76
33a		7	100	11 ± 5
33b		16	79	106 ± 79
33c		12	59	1810 ± 210
33d		14	97	45 ± 33
33f		21	87	97 ± 80

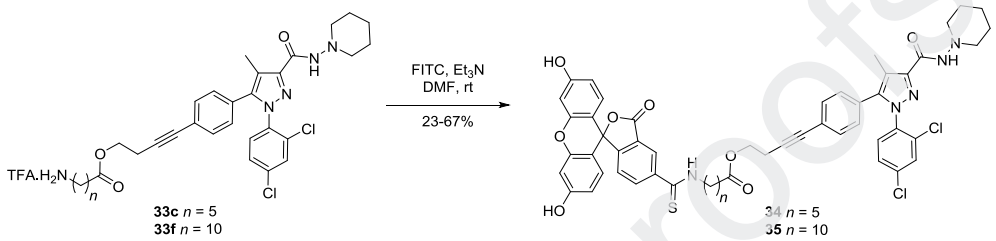
[a] Radioligand [^{3}H]-CP 55,940 displacement at 10 µM, *n*=3; [b] nd = not determined

the displacement threshold in the initial screen. This prevented a meaningful assessment of N1 as a position for linker attachment and suggests that alkyl N1-conjugated analogues of SR141716A are poor candidates for molecular probe development.

With the exception of control **29**, C5-biaryl SR141716A-linker conjugates **30a-d** possessed poor CB1 affinity, in line with data reported for the biaryl Tocrifluor T117.[5,37] In contrast, we were pleased to discover that all derivatives of alcohol **32** retained appreciable affinity upon conjugation of a linker. CB1R affinity of the parent alcohol **32** (K_i 180 nM) was found to be improved by either acetylation (**33a**, K_i 11 nM) or etherification (**33d**, K_i 45 nM), suggesting that use of these functional groups for subsequent linker attachment might allow retention of good

c acid linker fragment, coupled via an ester linkage, corroborated this hypothesis (K_i 106 nM). Removal of the Boc group to give **33c** saw a dramatic 20-fold loss of affinity (K_i 1810 nM) indicating that the amine group, positively charged at physiological pH, could not be accommodated at this position. In contrast, extension of the linker to 16 atoms to give **33f** proved to be a decisive modification, as amine **33f** (K_i 97 nM) displayed similar high CB1 affinity to **33b**. Although the ester linkage could potentially be labile to hydrolysis in aqueous media, it was anticipated, and borne out subsequent affinity assays, that this functionality would be robust enough for SAR development, especially in the context of likely localisation to the lipid bilayer prior to engagement with the membrane-bound CB1R.

Table 3. Synthesis and biological characterization of fluorescent CB1R probes **34** and **35**.



Compound	Linker length (*atoms*)	*h*CB1R K_i (nM) ± SEM
34	12	260 ± 20
35	21	2110 ± 1000

These data suggested that conjugation of a linker to the C5 position of the SR141716A pharmacophore via an ester bond was a suitable basis for the further development of CB1 molecular probes. Informed by the completed linker attachment survey, two CB1 fluorescent probes based on C5 conjugation of SR141716A (**1**) were prepared, by coupling of fluorescein isothiocyanate (FITC) to the 5 and 10-carbon C5 linker conjugates **33c** and **33f** (Table 3). The affinity of the fluorescent probes **34** and **35** were then determined through radioligand competition binding assays at *h*CB1. The affinity of the 17 atom linker congener **35** (K_i 2110 nM) was modest. However compound **34** bearing an 12-atom linker was found to display a useful level of affinity for CB1 (K_i 260 nM) incurring only a small loss of affinity from precursor **33b** (K_i 97 nM) despite the addition of the large fluorophore. We anticipate that **34** or closely related derivatives will have potential to become useful fluorescent probes for ongoing study of the CB1R.

In conclusion, investigation into the synthesis of divalent CB1R ligands based on the pharmacophore of SR141716A (**1**) was carried out. Despite strong literature precedent, linker conjugation at the C3 position did not lead to divalent compounds with useful levels of CB1R affinity. A systematic survey of alternative linker attachment positions on the SR141716A pharmacophore was therefore undertaken. Derivatives conjugated at the C5 position via a hydrophobic ester linkage were identified as the optimal foundation for ongoing development of divalent or multifunctional molecular probes for the CB1R. These findings enabled the discovery of a novel high affinity CB1R molecular probe **34** incorporating a fluorescein subunit, based on linker conjugation at C5. We anticipate that the findings of this study will have broad utility in informing future design of divalent or multifunctional high-affinity molecular probes for the cannabinoid CB1R.

ASSOCIATED CONTENT

Supporting Information

The supporting information is available free of charge at ACS websites under the DOI blank.

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Notes

The authors declare no competing financial interest

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