Highly Selective, Amine-Derived Cannabinoid Receptor 2 Probes

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Abstract: The endocannabinoid (eCB) system is implied in various human diseases ranging from central nervous system to autoimmune disorders. Cannabinoid receptor 2 (CB_2R) is an integral component of the eCB system. Yet, the downstream effects elicited by this G protein-coupled receptor upon binding of endogenous or synthetic ligands are insufficiently understood—likely due to the limited arsenal of reliable biological and chemical tools. Herein, we report the design and synthesis of CB_2R-selective cannabinoids along with their *in vitro* pharmacological characterization (binding and functional studies). They combine structural features of HU-308 and AM841 to give chimeric ligands that emerge as potent CB_2R agonists with high selectivity over the closely related cannabinoid receptor 1 (CB_1R). The synthesis work includes convenient preparation of substituted resorcinols often found in cannabinoids. The utility of the synthetic cannabinoids in this study is showcased by preparation of the most selective high-affinity fluorescent probe for CB_2R to date.

Introduction

The recognition of (-)-Δ^9-*trans*-tetrahydrocannabinol as the main active ingredient in *Cannabis sativa* preparations[1] marks the beginning of a series of important discoveries that unraveled a lipid signaling system referred to as endocannabinoid (eCB) system.[2] The eCB system is found in all vertebrates and comprises (at least) two G protein-coupled receptors known as cannabinoid receptors 1 and 2 (CB_1R and CB_2R), endogenous lipidic ligands including *N*-arachidonoylethanolamine (also known as anandamide) and 2-arachidonoyl glycerol, as well as enzymes responsible for ligand metabolism.[3] The eCB system is implied in numerous processes such as memory, nociception and immune response,[4] and virtually all components of the eCB system have been considered as promising targets for the treatment of psychiatric disorders, autoimmune diseases, cancer and various other conditions.[5,6] Yet, the underlying mechanism of CB_1R- and CB_2R-mediated effects upon receptor modulation is insufficiently understood, especially in a tissue- and disease-dependent context. CB_2R in particular, commonly referred to as peripheral cannabinoid receptor, has been shown to be upregulated in brain microglia during neuroinflammation,[7] while its expression in cells of the healthy central nervous system is still under debate.[8] Investigations of CB_2R biology are made difficult due to low expression levels (if present at all), the inducible nature of CB_2R and the lack of reliable and selective protein detection tools, such as antibodies.[9] In general, functional derivatives of small molecules[10] are an alternative that may specifically engage targets and enable applications such as fluorescence-activated cell sorting and confocal microscopy (fluorescent probes), activity/affinity-based protein profiling (electrophilic or photoactivatable ligands)[11,12] and targeted protein degradation (PROTACs),[13] to name just a few.

The search for an irreversible CB_2R-selective ligand. In combination with protein engineering, small molecule ligands for CB_2R could help identifying a structural basis for CB_2R´s pronounced biased signaling.[14,15] Chemical probes that bind irreversibly would be particularly useful to investigate biological effects following continued receptor modulation, as stabilizing agents for crystallographic studies, and for antibody generation. However, despite decades of cannabinoid receptor research, no selective, irreversible binder for CB_2R has been reported.

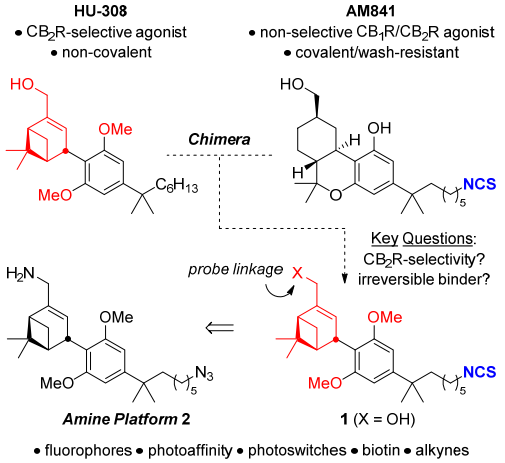

Figure 1. A hybrid of HU-308 and AM841 featuring structural elements responsible for CB_2R selectivity (pinene core, capped phenol/resorcinol) and irreversible binding (isothiocyanate).

We hypothesized that hybrid structures derived from two prominent cannabinoid receptor ligands, namely HU-308 and AM841,[16,17] could serve as leads towards CB_2R-selective covalent ligands (**Figure 1**). HU-308 is a full agonist of CB_2R (cAMP assay: hCB_2R $EC_{50} \approx 6$ nM) and

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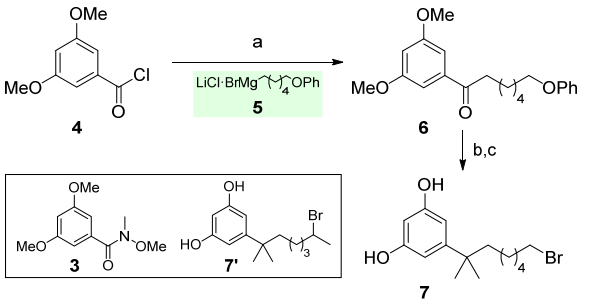
has been appreciated for its high affinity (hCB_2R K_i ≈ 20 nM) and pronounced selectivity over CB_1R.[15,16] The compound features an aliphatic, primary hydroxyl group and an arene bearing an aliphatic sidechain.[18] In contrast to classical cannabinoids as defined by the presence of a tricyclic benzochromene motif, HU-308 is a pinene derivative lacking the central pyran. HU-308 features two methyl ethers that were shown to be an important contributing factor to high CB_2R selectivity.[6] As a classical cannabinoid, AM841 includes a phenol along with a primary aliphatic alcohol. Its most notable feature is the electrophilic isothiocyanate at the terminus of the aliphatic sidechain, which can enable cross-linking of protein targets.[21] AM841 is a full agonist of both CB_1R and CB_2R (cAMP assay: hCB_1R EC_{50} ≈ 1 nM, hCB_2R EC_{50} < 1 nM) and has been described as a ligand that binds irreversibly to both receptors on the basis of radioligand saturation binding studies (CB_1R and CB_2R)[4,8] as well as mass spectrometry-based proteomics (CB_2R).[9] In these studies, the authors concluded that Cys6.47 (Ballesteros-Weinstein numbering),[24] a conserved amino acid present in both CB_1R and CB_2R, forms covalent adducts with the electrophilic isothiocyanate of AM841. Surprisingly, a recent structure resulting from co-crystallization of CB_1R with AM841 shows the aliphatic sidechain of the latter pointing away from Cys6.47.[25] While this observation renders a putative covalent adduct geometrically unlikely for CB_1R, it is important to note that the situation may be different in the case of CB_2R, for which a covalent interaction with AM841 had been shown by mass spectrometry.[22] Herein, we describe the design, synthesis and *in vitro* pharmacological evaluation of **1** (X = OH) and various derivatives, such as ethers and esters (X = OR) as well as amides (X = HNR). Our studies culminate in the identification of a privileged amine platform (**2**) for the modular preparation of highly selective CB_2R agonists.

Results and Discussion

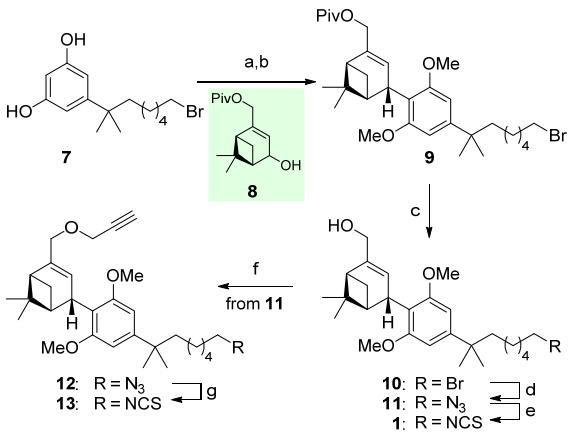
The synthesis of hybrid cannabinoid **1** featuring the pinene core and methyl ethers of HU-308 as well as the electrophilic side chain of AM841 commenced with preparation of known resorcinol derivative **7** (**Scheme 1**). The published route towards **7** involves organolithium addition to Weinreb amide **3** (62% yield), $TiMe_2Cl_2$-mediated conversion of ketone **6** into the corresponding geminal dimethyl derivative as described by Reetz[26] and subsequent global ether cleavage with BBr_3 to afford **7** (84% yield over two steps).[27] We found that **6** could be conveniently prepared by $Fe(acac)_3$ catalyzed cross coupling[28] of readily available acyl chloride **4** and Grignard reagent **5**[29] in 90% yield (~12.3 g). In our hands, subsequent conversion of ketone **6** into its geminal dimethyl derivative using $TiMe_2Cl_2$ (generated *in situ* from $TiCl_4$ and $ZnMe_2$) proceeded in 34% yield.[30] Global ether cleavage according to Tius´ BBr_3 protocol[27] proceeded cleanly as judged by thin layer chromatography. However, inspection of ^1H and ^{13}C NMR spectra revealed the presence of a side product identified as isomeric secondary alkyl bromide **7'** amounting to ca. 10% of the material. The outcome of the last two steps prompted us to devise an alternative synthetic strategy towards resorcinol derivatives that would avoid the observed isomerization (*vide infra*).



Scheme 1. Synthesis of resorcinol **7**. Reagents and conditions: a) $Fe(acac)_3$ (3 mol%), THF, –78 °C, 90%. b) $TiCl_4$, $ZnMe_2$, CH_2Cl_2, –30 °C to rt, 34%. c) BBr_3, CH_2Cl_2, –78 °C to rt, quantitative as inseparable mixture of **7** and secondary bromide **7'** (ca. 9:1).

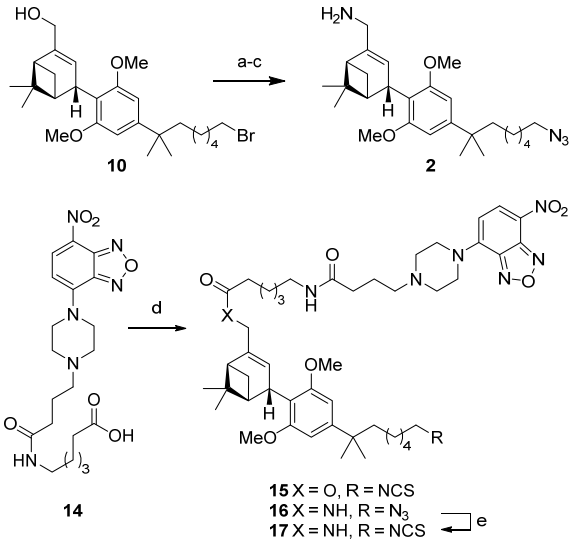
At this stage of the project, although the synthesis of **7** was suboptimal, we decided to venture ahead to collect initial pharmacological data. In analogy to the published synthesis of HU-308,[16] *p*TsOH-mediated Friedel-Crafts alkylation of **7** using allylic alcohol **8** derived from (+)-α-pinene followed by double methylation afforded **9** (**Scheme 2**). Ester reduction (DIBAL-H) and nucleophilic displacement of the derived alkyl bromide with sodium azide delivered **11** (93% yield over both steps), which was further elaborated into **1**. Alternatively, **11** was first propargylated prior to conversion of the azide to the corresponding isothiocyanate. The latter two-step sequence afforded bifunctional ligand **13** as putatively irreversible probe amenable to further derivatization for use in activity-based protein profiling experiments.

In a comparative study of HU-308 and its enantiomer HU-433, the authors noted that the latter exhibited higher CB_2R-mediated biological activity than HU-308. This contrasted the expectation based on radioligand binding studies, which attested HU-308's higher CB_2R affinity when compared to HU-433.[31] Therefore, **ent-1** was included in the present study in order to maximize the chance of identifying irreversible and selective CB_2R ligands. Compound **ent-1** was accessed by Friedel-Crafts alkylation with **ent-8** derived from (*1R*)-(−)-myrtenol and subsequent elaboration analogous to the sequence shown in **Scheme 2** (see SI).



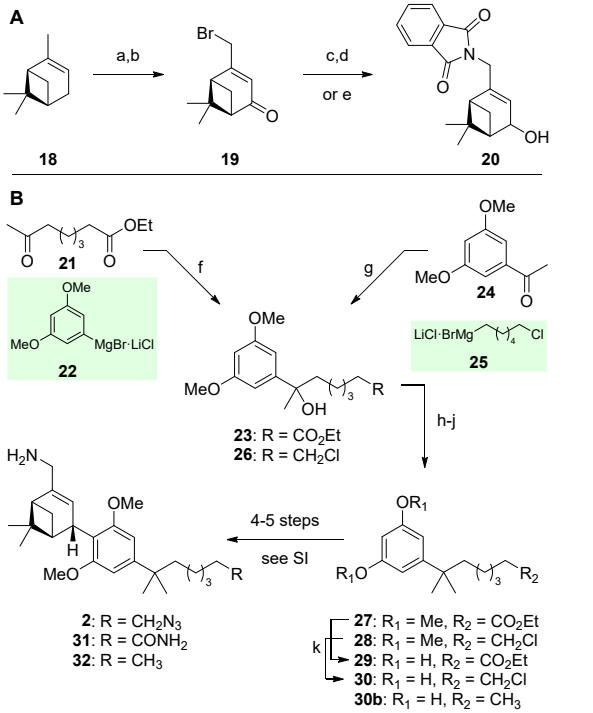
Scheme 2. Synthesis of electrophilic hybrid cannabinoids **1** and **13**. Reagents and conditions: a) *p*TsOH·H_2O (0.28 equiv), CH_2Cl_2, rt, 86%; b) Me_2SO_4, K_2CO_3, acetone, rt, 73%; c) DIBAL-H, CH_2Cl_2, 0 °C, 94%; d) NaN_3, DMF, rt, 99%; e) PPh_3, CS_2, THF, rt, 84%; f) propargyl bromide, Bu_4NHSO_4 (0.2 equiv), 50% aq. NaOH–PhMe, rt, 36%; g) PPh_3, CS_2, THF, rt, 81%.

Drawing from a report on biotin-conjugated HU-308, in which elongation along the allylic alcohol was tolerated by CB_2R,[32] fluorescent probe **15** was prepared by esterification of **1** with nitrobenzoxadiazole-derived acid **14** (**Scheme 3**). Since esters are prone to hydrolysis in biological environments, amide analogs of **15** were synthesized. To this end, amine **2** was prepared by a three-step sequence involving Mitsunobu reaction of allylic alcohol **10** with potassium phthalimide, bromide displacement with sodium azide and, finally, treatment with hydrazine. Subsequent amide formation and conversion of azide to isothiocyanate afforded fluorescent compounds **16** and **17** (see SI for fluorescence spectra).



Scheme 3. Synthesis of amine **2** and fluorescent derivatives **15**-**17**. Reagents and conditions: a) DIAD, PPh_3, phthalimide, THF, rt, 62%; b) NaN_3, DMF, rt, 88%; c) N_2H_4·H_2O, crotyl alcohol, EtOH, 75 °C, 73%; d) for **15**: **1**, EDC·HCl, DMAP, NEt_3, THF, rt, 90%; for **16**: **2**, EDC·HCl, NEt_3, THF, rt, 56%; e) PPh_3, CS_2, THF, rt, 74%.

In vitro pharmacological assessment of prepared compounds (*vide infra*) showed that amide derivatives **16** and **17** exhibited significantly increased binding affinity to hCB_2R compared to ester **15**, as well as sharply increased selectivity over hCB_1R. Interestingly, cannabinoids in which the primary alcohol is replaced by amines and their derivatives have not been studied in detail. The few examples include an acetamide derivative of Δ^8-tetrahydrocannabinol (Δ^8-THC) reported to be analgesically inactive[33] and more recent studies of fluorescent probes for the cannabinoid receptors.[34,35] A small number of amine analogs was evaluated in mice[36] and baboons,[37] but since these studies predate the discovery and cloning of cannabinoid receptors, no data on binding affinities are available. The apparent lack of structure-activity information and the observed high CB_2R affinity of ligands bearing the underexplored amide linkage prompted us to devise a concise synthetic strategy towards amide derivatives of HU-308. In addition, an expedient route to resorcinol derivatives that a) avoids contamination with constitutional isomers and b) allows for introduction of other functionalities in the side chain was developed (**Scheme 4**). The latter is of special interest regarding incorporation of polar functional groups to effect reduction of non-specific binding, an issue known to correlate with lipophilicity (logD).[38]



Scheme 4. A) Synthesis of *N*-protected allylic alcohol. Reagents and conditions: a) CrO_3, *N*-hydroxyphthalimide, acetone, rt, 44%; b) NBS, dibenzoyl peroxide, CCl_4, reflux, 62%; c) potassium phthalimide, DMF, rt, 85%; d) $CeCl_3$·$7H_2O$, $NaBH_4$, MeOH, –78 °C, 93%; e) DIBAL-H, Et_2O-hexanes; then acidic workup; then potassium phthalimide, DMSO, rt, 77% over 2 steps. **B**) Synthesis of resorcinol derivatives and further elaboration into allyl amines. Reagents and conditions: f) **22**, THF, –78 °C to rt, 92%; g) **25**, THF, –78 °C to rt, 60%; h) for **22**: $SOCl_2$, 0 °C; for **28**: HCl conc., rt; i) $AlMe_3$, CH_2Cl_2, –78 °C to rt; j) OsO_4 (cat.), oxone, DMF, rt, for **27**: 42% from **23**, for **28**: 67% from **26**; k) BBr_3, CH_2Cl_2, –78 °C to rt, 97% (**29**), 93% (**28**).

Following a patent procedure, (+)-α-pinene (**18**) was oxidized to verbenone by CrO_3 in presence of *N*-hydroxyphthalimide.[39] Subsequent allylic bromination under Wohl-Ziegler conditions afforded **19**. The two-step sequence involving bromide displacement and ketone reduction could be carried out in either order, although it proved convenient to first reduce ketone **19** and then subject the product bromide to displacement by azide. This order of events yielded allyl alcohol **20** in 77% yield over two steps and avoided capricious chemoselective ketone reduction in presence of phthalimide under Luche conditions.

The revised resorcinol synthesis commenced with addition of Grignard reagent 22 to ketoester 21.[40] Alternatively, 25 was added to ketone 24. The resulting tertiary alcohols 23 and 26 were converted into their *gem*-dimethyl derivatives by modification of a reported procedure.[41] First, treatment with $SOCl_2$ (for 23) or conc. HCl (for 26) resulted in formation of the corresponding tertiary chlorides, which without purification were treated with $AlMe_3$ to install the *gem*-dimethyl group. The unpurified mixtures were subjected to oxone/cat. OsO_4 to effect oxidative degradation of the otherwise inseparable olefin byproducts (ca. 10%) formed by elimination of the tertiary alcohol over the course of installation of the *gem*-dimethyl group.[42] This procedure allowed for convenient isolation of the desired dimethyl resorcinols 27 and 28 (67% and 42% yield, respectively. Cleavage of the methyl ethers with BBr_3 cleanly afforded resorcinols **29** (93% yield) and **30** (97% yield) with no detectable formation of constitutional isomers. Each of these was then allowed to react with **20** and further elaborated into amines **2**, **31** and **32** following the logic outlined in **Scheme 2** (see SI). Additionally, known resorcinol **30b** was subjected to the same sequence to afford **32**. It is worth noting that THC derivatives bearing a terminal carboxylate in the side chain have been employed as haptens for antibody generation.[43]

With allylic amines **2**, **31**, and **32** in hand, straightforward derivatization reactions afforded a number of *aza*-HU-308 derivatives shown in **Figure 2**. Probes **33**-**35**, which may be activated upon irradiation,[44] were synthesized by amidation of the corresponding amines (**2**, **31**, and **32**) and recently applied in a study on CB_2R photoaffinity-labeling.[45] In this work, **33** successfully labelled the receptor upon irradiation (350 nm) as shown by in-gel fluorescence following fluorophore attachment.

Conjugation of 4-pentynoic acid and subsequent triazole formation with 1-azidoadamantane afforded compounds **36** and **37**. Amide **38** and sulfonamide **39** were prepared by condensation reactions with biotin and 1-butanesulfonyl chloride, respectively. Amide formation with tetra-*ortho*-chloro-azobenzene containing fatty acid FAAzo4[46] afforded photoswitch **40** and completed the set of amine-derived compounds tested in this study.

In vitro pharmacological characterization. All synthesized compounds were evaluated in radioligand binding studies with tritiated CP55,940 using membrane preparations of Chinese hamster ovary (CHO) cells overexpressing CB_2R (human and mouse) or human CB_1R, respectively. All experiments were run using aliquots of the same batches of isolated CHO-membranes expressing the respective receptor. In addition, widely used cyclic adenosine monophosphate (cAMP) assay was employed as described earlier[47] to assess functional activity of prepared HU-308 derivatives. Results are listed in **Table 1**.

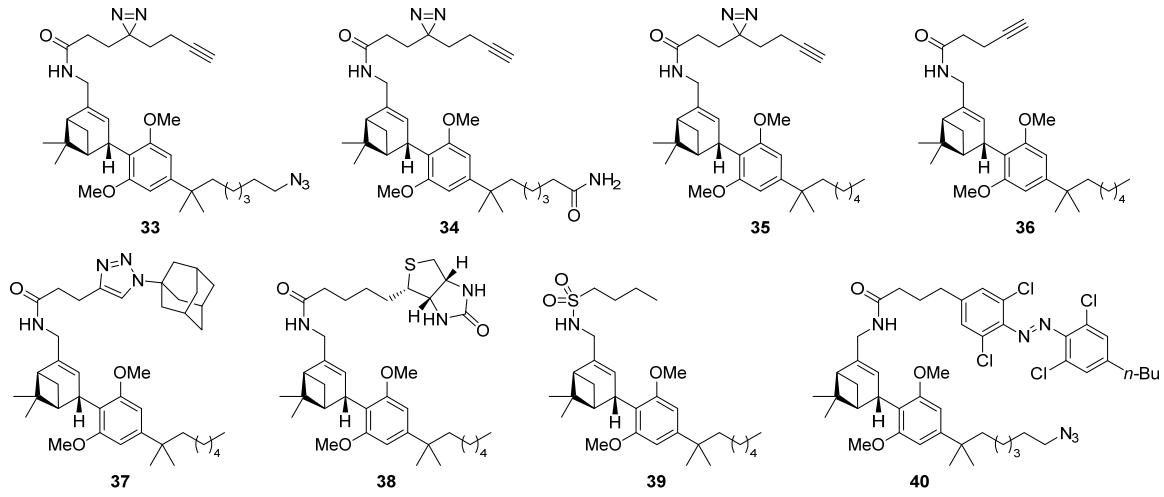
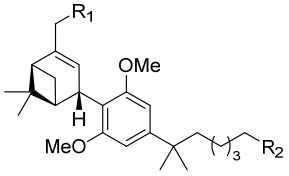


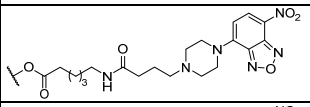
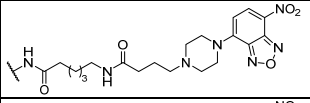
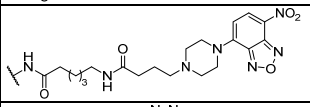
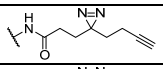
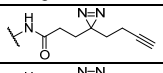
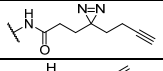
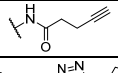
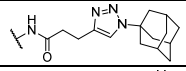
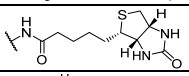
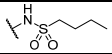
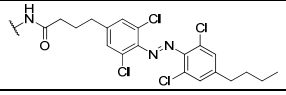
Figure 2. Additional *aza*-HU-308 derivatives tested in this study. Compounds were prepared by condensation reactions of the corresponding allylic amines. **37** was synthesized from **36** by copper-catalyzed cycloaddition. See SI for experimental details.

Compounds **1**, **10** and **11** share the free allylic alcohol and exhibit distinct groups terminating the dimethylheptyl chain. These compounds emerged as high affinity hCB_2R binders (K_i hCB_2R < 13 nM) with at least 86-fold selectivity over hCB_1R. In line with Mechoulam's report,[31] compounds **ent-1**, **ent-10-Cl** and **ent-11** showed lower hCB_2R affinity than their enantiomers by a factor of ca. 10 (hCB_2R K_i < 121 nM) but retained high selectivity over hCB_1R (hCB_1R K_i > 3.1 μM). Nitrobenzofurazan-derived compounds **15**-**17** were identified as highly promising hCB_2R-selective fluorescent probes given their complete selectivity over CB_1R. Importantly, exchanging the potentially vulnerable ester functionality in **15** for an amide linkage resulted in analogs **16** and **17** with significantly increased CB_2R affinity and remarkable selectivity over CB_1R (ratio hCB_1R K_i/hCB_2R K_i > 2000). The primary amide in **35** proved detrimental to receptor selectivity. Comparing compounds **33-35** differing only in the side chain, the terminal azide emerged as most favorable for achieving high hCB_2R-selectivity. Introduction of said terminal azide (**33**) results in ca. 10-fold increase in selectivity compared to its saturated analog **35**.[35] Adamantyl-substituted triazole and biotin derivatives **37** and **38** exhibited decreased hCB_2R affinity along with low selectivity over hCB_1R. Sulfonamide **39** showed affinity comparable to **35**, while azobenzene derivative **40** (as undefined mixture of *trans*- and *cis*-isomers) was found to be a hCB_2R-selective high-affinity ligand. Interestingly, all compounds tested showed a preference for human CB_2R over the mouse isoform. Human and mouse CB_2R share 86% sequence identity within the ligand binding domain differing only in amino acids 72 and 261. While hCB_2R contains amino acids Ser72 and Val261, mCB_2R displays Asn72 and Ala261 residues. These alterations seem to create a binding pocket less effective in accommodating HU-308 derivatives.

In the functional cAMP assay, all compounds were identified as full agonists of both human and mouse CB_2R with relative EC_{50} values resembling the trends observed in the radioligand binding assay, albeit not as pronounced. With the exception of **10**, **11** and to some extent **40**, all compounds bearing a terminal NCS or N_3 group were functionally inactive at hCB_1R (**12**: partial hCB_1R agonist with 58% efficacy and hCB_1R EC_{50} ≈ 1.4 μM). Comparison of compounds **15** and **17** shows that the higher binding affinity of amide **17** also translates into higher potency in cAMP assay for both human and mouse CB_2R.

Table 1. *In vitro* pharmacological assessment of HU-308 derivatives.[a]



#	R_1		R_2	hCB_2R K_i [nM]	mCB_2R K_i [nM]	hCB_1R K_i [nM]	ratio K_i hCB_1R/ hCB_2R	hCB_2R EC_{50} [nM] (%efficacy)	mCB_2R EC_{50} [nM] (%efficacy)	hCB_1R EC_{50} [nM] (%efficacy)
1		**1**		13.1	158	2670	204	0.6 (95)	1.7 (95)	> 10000
2		*ent*-**1**		121	1320	>10000	>83	11.6 (95)	65 (97)	> 10000
3		**10-Br**		7.8	59	670	86	0.9 (99)	0.7 (98)	178 (80)
4		*ent*-**10-Cl**		56	760	3150	56	7.5 (98)	9.0 (98)	> 10000
5		**11**		3.7	40	1130	305	0.6 (99)	0.5 (96)	100 (79)
6		*ent*-**11**		33	428	3860	117	3.0 (95)	5.5 (97)	> 10000
7		**12**		116	1080	>10000	>86	0.5 (101)	1.9 (97)	28.4 (41)
8		**13**		45	530	6160	137	0.2 (95)	1.8 (94)	1380 (58)
9		**15**		900	1580	>10000	>11	41 (95)	>10000	> 10000
10		**16**		4.2	n/a	>10000	>2381	n/a	n/a	n/a
11		**17**		4.7	78	>10000	>2128	0.5 (97)	2.6 (97)	> 10000
13		**33**		9.3	88	3890	418	0.9 (98)	0.7 (100)	> 10000
12		**34**		96	417	357	4	17.0 (96)	168 (97)	120 (106)
14		**35**		151	3220	6430	43	1.9 (97)	13.9 (93)	> 10000
15		**36**		7.8	128	780	100	0.5 (101)	0.3 (100)	> 10000
16		**37**		1380	>10000	1340	1	197 (91)	128 (85)	> 10000
17		**38**		88	314	2760	31	1.6 (100)	1.2 (100)	> 10000
18		**39**		147	5400	>10000	>68	1.8 (99)	16.2 (102)	> 10000
19		**40**		41	389	3630	89	9.3 (99)	12.9 (96)	650 (82)

[a] Dissociation constants (Ki) were determined by radioligand binding assay using membrane preparations of CB_1R/CB_2R overexpressing CHO cells with [^{3}H]-CP55,940 as described previously and are given as average values from one up to six independent experiments each performed in triplicate. cAMP assays were performed with CHO cells expressing human CB_1R and CB_2R receptor variants as described.[47] Efficacies are expressed as percentages relative to the effect evoked by CP55,940 (1 μM). EC_{50} values are averages from one to two independent experiments each performed in triplicate.

Docking studies. Docking agonist **17** into the recently published X-ray crystal structure of antagonist bound CB_2R (PDB code: 5ZTY)[48] suggests a favorable hydrogen bond between the amide group of **17** (N-H as H-bond donor) and the carbonyl of Ser72 (H-bond acceptor) (**Figure 3**). This finding may explain the observed increased binding affinity of **17** (and other amide analogs) compared to ester **15**. The hypothesis is further corroborated by pairwise comparison of compounds **11**/**12** and **1**/**13**. In both cases, analogs bearing a free hydroxy group as potential H-bond donor (**1**, **11**) exhibit higher binding affinity than the corresponding propargyl ether analogs (**12**, **13**).

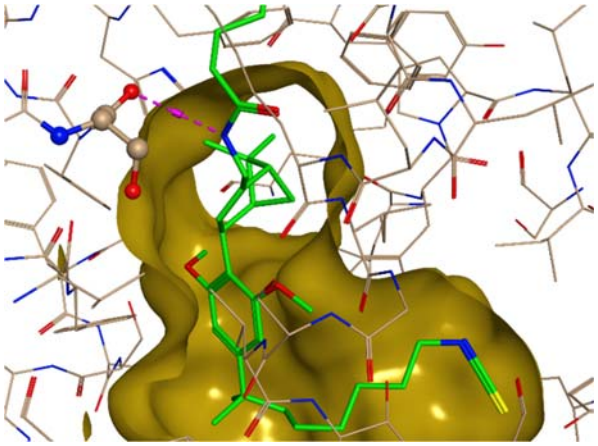


Figure 3 Docking pose of **17** within the crystal structure of CB_2R determined in complex with antagonist AM10257 (PDB code: 5ZTY). The model suggests formation of a hydrogen bond (magenta) between **17** and Ser72 (bold ribbon).

Probing for irreversible binding. Earlier reports describe saturation binding experiments to show AM841's ability to covalently bind both CB_1R and CB_2R via Cys6.47.[17,23] In these experiments, membranes expressing wildtype CB_1R and CB_2R were preincubated with AM841 or DMSO control. Following excessive washing steps, saturation binding experiments with tritiated CP55,940 revealed decreased receptor density (B_{max}) for pretreated membranes compared to control. In addition, performing the same experiment with receptor variants in which Cys6.47 was mutated to serine, alanine or leucine did not show reduction of B_{max} when comparing pretreated membranes to control. As orthosteric covalent binders are expected to reduce the number of available binding sites, these observations led to the conclusion that AM841 is an irreversible binder of both CB_1R and CB_2R.

In analogy, we proceeded to evaluate compounds **1** and **ent-1** for their ability to reduce B_{max}. To this end, membrane preparations of hCB_2R-overexpressing CHO-cells were incubated with DMSO control or putatively covalent compounds **1** (90 nM) and **ent-1** (790 nM) at concentrations corresponding to ca. 6-fold K_i for 60 minutes. Following excessive washing steps to remove non-covalently bound material, determination of receptor density (B_{max}) using tritiated CP55,940 would allow for indirect proof of irreversible bond formation for orthosteric ligands. As shown in **Figure 4**, saturation binding of [^{3}H]-CP55,940 using membranes preincubated with **1** or DMSO (**Figure 4A**), and **ent-1** or DMSO (**Figure 4B**) revealed similar maximum specific binding (B_{max}) within either set of experiments. Thus, **1** and **ent-1** are not considered irreversible, orthosteric ligands of hCB_2R. This outcome is in line with a separate experiment aiming to detect covalent bond formation of fluorescent electrophilic ligand **17**. In this experiment, membranes of hCB_2R overexpressing CHO-cells were incubated with **17** (up to 10 µM). When proteins of the sample were resolved by polyacrylamide gel electrophoresis, no fluorescent band corresponding to CB_2R protein was detected.

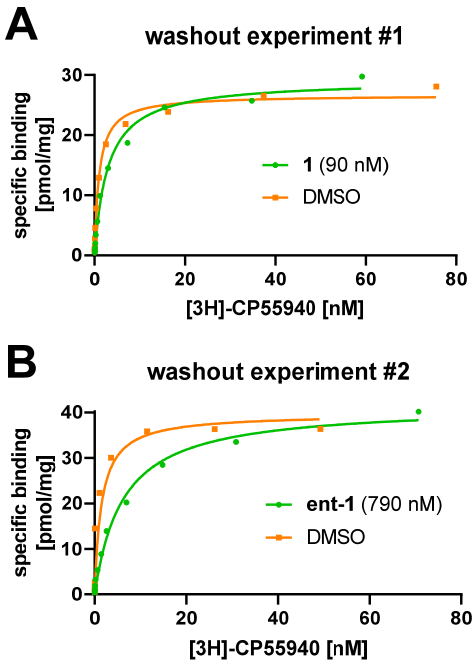


Figure 4 Washout experiments probing for irreversible binding. Saturation binding of [^{3}H]-CP55,940. Specific binding in dependence on radioligand concentration determined using membranes pretreated with 6x K_i of ligands **1**, **ent-1** or DMSO.

Conclusions

Enabled by a practical synthesis of functionalized resorcinols, the amine derivatives identified in this study allow modular and convenient access to highly CB_2R-selective ligands. Conjugates prepared from amine **13** in particular, bearing a terminal azide in the dimethylheptyl side chain, emerged as ligands with the highest affinity and selectivity for hCB_2R across the series. The promise of this approach is showcased by the synthesis of various functional CB_2R ligands, most notably fluorescent probe **16**. This exceptionally selective fluoroprobe binds hCB_2R with a K_i of 4 nM as determined by radioligand binding assay and shows no detectable interaction with hCB_1R. Thus, our initial quest for selective CB_2R ligands that bind irreversibly by merging structural motifs found in HU-308 and AM841 afforded useful hybrid cannabinoids of type **1** and its enantiomer **ent-1**. However, studies of these ligands and their derivatives do not support the formation of covalent adducts to CB_2R.

Docking studies using the recently published crystal structure of inactive state hCB_2R suggest the marked affinity of the amide analogs is due to a hydrogen bond between the amide N-H and Ser72, which is not available to the corresponding ester derivatives. Interestingly, the chemotype presented displays intrinsic preference for

human CB_2R over the mouse isoform. This finding is important in the light of transferability of preclinical *in vivo* data generated using commonly employed mouse models.

We believe the *in vitro* pharmacological data presented for amide derivatives of HU-308 is a valuable addition to the structure-activity-relationship knowledge of cannabinoids. Our work serves as blueprint for the preparation of selective CB_2R ligands, incorporating functional elements tailored to a given biological experiment. Ongoing work in our laboratories focuses on additional applications of the ligands described and will be reported as results become available.

Acknowledgements

EMC is grateful to ETH-Zürich and F. Hoffmann-La Roche for support of the research program. We thank Prof. James Frank for providing a sample of FAAzo4 and Dr. Marjolein Soethoudt for evaluation of electrophilic fluoroprobe **17**. Dr. Sylwia Huber, Eric Kusznir and Dr. Arne Rufer are gratefully acknowledged for the generation of absorption, excitation and emission spectra.

Keywords: cannabinoid receptor 2 • CB_2R • electrophilic ligands • fluorescent probe • chemical probe • endocannabinoid system

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