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Chemical modification of the naphthoyl 3-position of JWH-015: In search of a fluorescent probe to the cannabinoid CB_2 receptor

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Abstract—In silico modelling was used to guide the positioning of the fluorescent dye NBD-F on the cannabinoid CB_2 receptor agonist JWH-015. While the ultimate fluorescent conjugate lost extensive binding affinity to the cannabinoid CB_2 receptor, affinity and efficacy studies on the naphthoyl 3-position modified precursor molecules have provided new insight into structure–activity relationships associated with this position.

Investigation of receptor–ligand interactions is one of the principal tools employed in pharmacological research. Conventional methods, for example, the use of radioactively labelled ligands, still contribute a major aspect of this activity. However, these approaches are being increasingly replaced by fluorescence-based techniques,[1] allowing researchers to develop and exploit assays at the single cell (confocal microscopy)[2] and single molecule (fluorescence correlation spectroscopy, FCS) levels.[3] To fully exploit this technology, there remains a key requirement for the development of bioactive fluorescent probes for the receptor of interest. As such, we have recently demonstrated that fluorescently labelled small molecule ligands for the G-protein-coupled human adenosine A_1-receptor can be used to study their binding to the receptor in single living cells.[4] In continuation of our studies, we were eager to explore the potential to develop fluorescent molecules for non-biogenic amine-activated GPCRs and we selected the human cannabinoid CB_2 receptor as our target.

The human cannabinoid CB_2 receptor (hCB_2), first discovered in 1993,[5] has been implicated as a potential target in a number of diseases.[6] The hCB_2 receptor is found predominantly on the immune cells of the periphery. The other known class of cannabinoid receptors (CB_1) is mainly located within the CNS and is responsible for many mind-altering effects of cannabis use.[7] hCB_2 receptor systems have been shown, either by agonist-induced attenuation or by receptor up-regulation, to be important mediators in numerous diseases, including multiple sclerosis,[8] malignant disease[9] and neuropathic pain.[10] These diseases are not only severely debilitating but also offer limited pharmacotherapeutic choices available for treatment.

A high affinity fluorescent hCB_2 receptor ligand would allow a greater understanding of the hCB_2 receptor's pharmacology and its role in disease states through the use of modern fluorescence-based techniques that have been previously highlighted. Our efforts were initially concentrated towards conjugating the selective hCB_2 receptor agonist JWH-015 **1** with a fluorescent dye. JWH-015 **1** was chosen due to its nanomole affinity to hCB_2 receptors, high hCB_2/hCB_1 selectivity, extensive SAR and rapid synthetic accessibility.[11] The latter factor was key to us rejecting synthetically complex compounds, such as JWH-133, L-759633 and HU-308, as chemical starting points, despite their displaying a better CB_2 selectivity. In contrast to our approach taken during the development of fluorescent adenosine receptor ligands,[4] we were also keen to explore whether in silico modelling of JWH-015 **1** bound within the hCB_2 receptor, coupled with de novo drug design, would aid in the identification of a suitable position upon **1** that could tolerate the incorporation of a bulky fluorescent dye. Therefore, alongside the key advantages listed above, JWH-015 **1** possessed a number of benefits with regard

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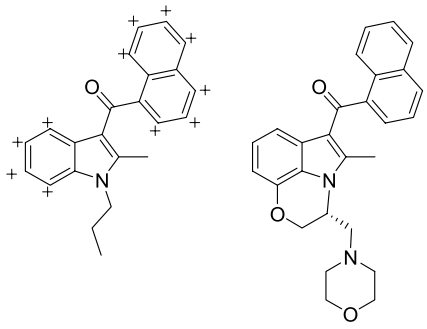
to the specific modelling strategy we planned to implement. More specifically, JWH-015 **1** displays a significant amount of structural homology to Win 55,212-2 **2**, a molecule which has been extensively studied by molecular modelling and mutational analysis techniques.[12] We therefore envisaged docking Win 55,212-2 **2** into an homology model of the hCB_2 receptor using previously published data concerning Win 55,212-2 **2** binding. Then, the structural similarities between JWH-015 **1** and Win 55,212-2 **2** could be utilised to superimpose common structural motifs (i.e., the naphthoyl rings) of the two molecules, to generate a rational model where JWH-015 is bound to the hCB_2 receptor.

An in silico model of the hCB_2 receptor was therefore constructed by homology modelling to the 2.8 Å X-ray crystal structure of bovine rhodopsin.[13] The 3-naphthoylindole cannabinoid agonist Win 55,212-2 **2** (Fig. 1) was docked into the model utilising the previously published data to guide the positioning of the agonist.[12] The receptor–ligand complex was energy minimized and subjected to a 100 ps molecular dynamics simulation using the CHARMM force field.[14] Our model demonstrated similar receptor–ligand aromatic stacking interactions observed in previous hCB_2 models and indicated that the aromatic amino acids Phe 197, Phe 117 and Trp 194 interacted with Win 55,212-2 **2**, as previously reported by Song et al.[12] Structural alignment, by superimposing the naphthoyl moieties of both JWH-015 **1** and Win 55,212-2 **2**, deletion of the latter and further simulation produced a ligand–receptor model where JWH-015 **1** was located within the binding pocket (Fig. 2). Growing points for the addition of substituents onto JWH-015 **1** were determined (Fig. 1), based on previous SAR,[11b,c,d] and the molecule was subjected to de novo design using the program LigBuilder.[15]



JWH-015 (**1**) Win 55,212-2 (**2**)

Figure 1. Development of the fluorescent hCB_2 agonist. + Indicates a grow point used in LigBuilder.

The results of this exercise indicated a number of positions upon JWH-015 **1** that could potentially tolerate a bulky substituent, especially the 3-position of the naphthyl ring substituent. When we examined the structures generated by LigBuilder, one molecule that scored highly in its predicted hCB_2 receptor binding affinity possessed a substituent that closely resembled a known fluorescent dye (i.e., 7-nitrobenzoxadiazole, NBD). This molecule **3** was therefore chosen as our lead compound (Fig. 3).

Synthesis of the fluorescent congener was accomplished using modifications to a procedure by Bell et al.[16] The 2-methyl-1-*n*-propylindole derivative **5** was prepared by *N*-1 alkylation of commercially available 2-methylindole **4** using standard methodology (Scheme 1).

Meanwhile, commercially available 3-nitro-1,8-naphthalic anhydride **6** was selectively decarboxylated, via the

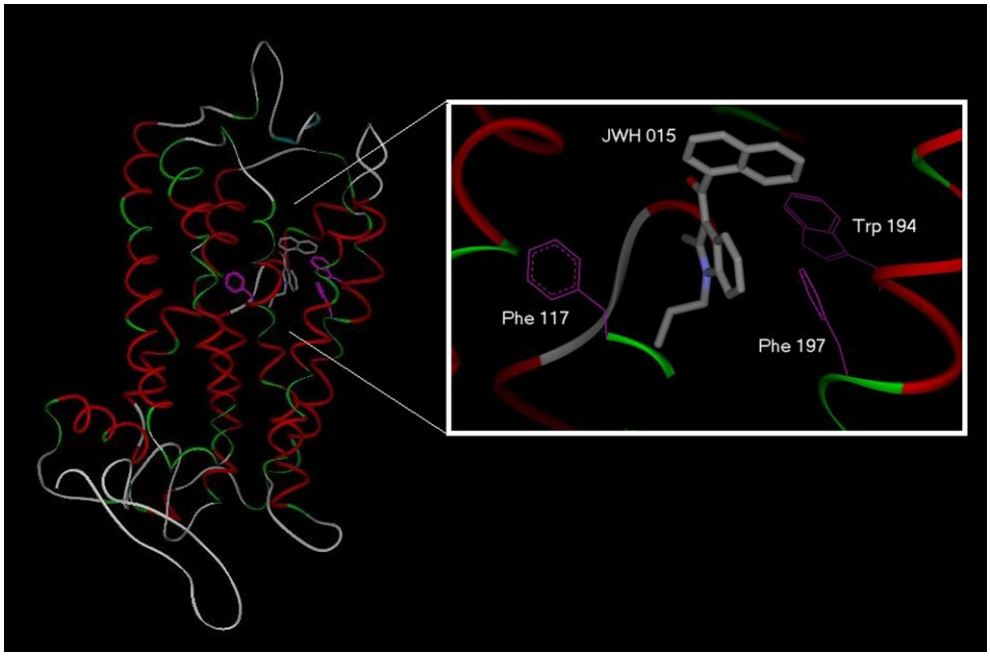
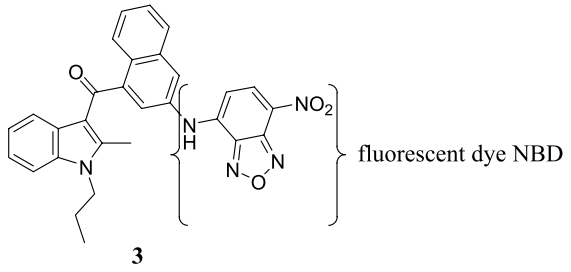


Figure 2. Molecular model of hCB_2. Inset shows detail of JWH-015 in the active site together with key interacting aromatic amino acids.



3

Figure 3. The lead molecule generated by the LigBuilder programme.

mercuric salt of the di-acid, to give the desired 3-nitronaphthoic acid.[17] The meta-substitution of this compound was confirmed by mp, ^{1}H and ^{1}H–^{13}C COSY NMR spectroscopy.[18] The 3-nitronaphthoic acid was then converted to its corresponding acid chloride **7** using $SOCl_2$. Lewis acid ($AlCl_3$) promoted electrophilic substitution at the C-3 position of **4**, affording the nitro derivative of JWH-015 **8**. Functional group transformation of the nitro group to the aryl amine **9** took place using catalytic hydrogenation. While the two low yielding reactions to give the acid chloride **7** (22%) and the 3-aroylindole **8** (19%) are unoptimised, both are comparable with previous literature preparations (see Refs. 17b and 11, respectively).

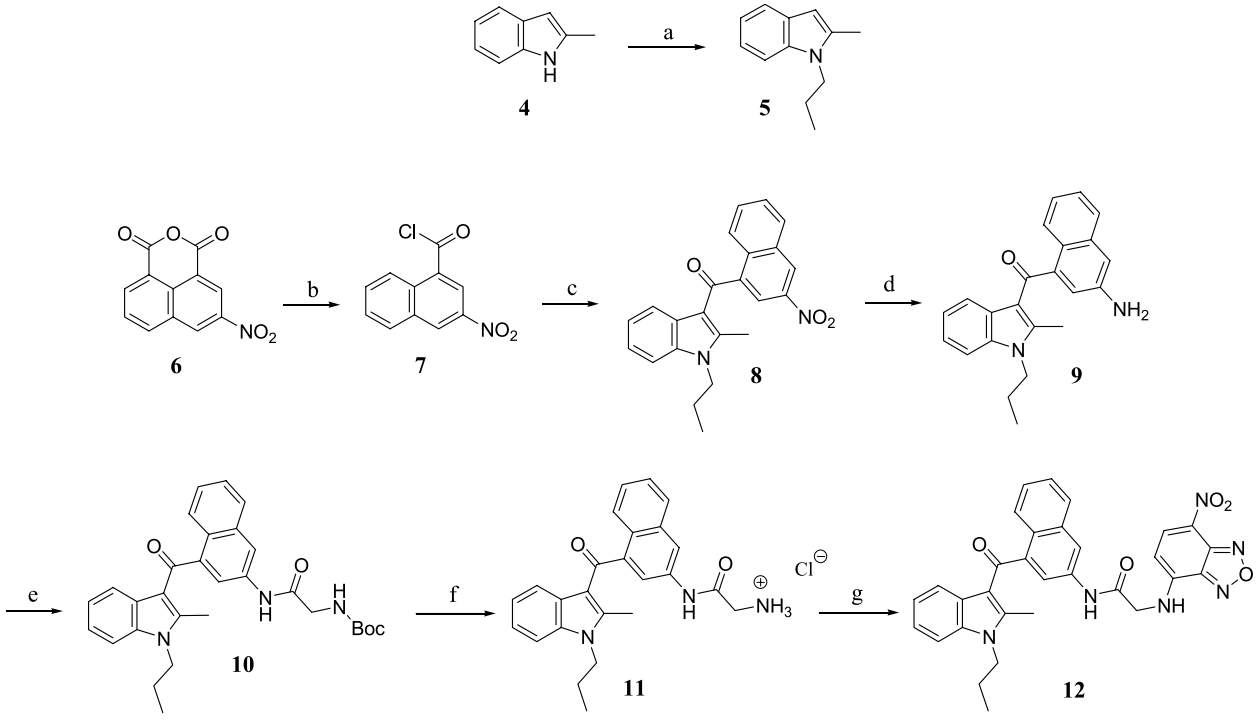
It is recognised that only non-fluorescent derivatives are formed when NBD fluoride is reacted with aromatic amines, due to a photoinduced electron transfer (PET) mechanism.[19] It is therefore necessary to insert a short functionalised linker between the aryl amine and the NBD fluorochrome. A short amide linker was chosen to keep the overall molecular size as close to that of the lead molecule **3** as possible and because this type of linker had been previously shown to completely negate the PET effect.[19] Therefore, *N*-Boc-protected glycine was coupled to **9** using standard carbodiimide chemistry. Acidolysis of the Boc group of **10** exposed the primary amine which, when coupled to the amine reactive NBD fluoride, gave the fluorescent congener **12**.[20]

The hCB_2 receptor affinity for compounds **8**, **9**, **11** and **12** was measured by a competitive displacement assay against [^{3}H]CP55, 940 (a high affinity cannabinoid agonist) on membranes prepared from CHO cells expressing the hCB_2 receptor.[21] The results of this assay are given in Table 1.

Table 1. Results of in vitro assays using the hCB_2 receptor

Compound number	K_i CB_2[a] (nM)	% [^{35}S]GTP-γ-S binding from basal at 1 μM[c]
JWH-015		
1	36 (31–42) (lit.[b] 14 ± 5)	142 (± 19)*
8	143 (56–364)	134 (± 8)*
9	191 (59–622)	151 (± 22)*
11	420 (359–492)	Not tested
12	25% displacement at 10 μM	Not tested

[a] Results in parentheses are 95% confidence intervals for three individual experiments run in triplicate.
[b] Result from Ref. 11c.
[c] Results in parentheses are the standard error of the mean for three individual experiments run in triplicate.
* $P < 0.05$ compared to basal binding (100%).



4 5 6 7 8 9 10 11 12

Scheme 1. Regents: (a) 1-bromopropane, NaH, DMF (97%); (b) (i) NaOH, water, HgO. (ii) $SOCl_2$ (22%); (c) **5**, DCM, $AlCl_3$ (19%); (d) Pd/C, EtOH, H_2 (66%); (e) Boc-glycine, DCC, DMAP, DCM (67%); (f) MeOH, AcCl (50%); (g) NBD-F, MeCN, $NaHCO_3$ buffer 8.3 (46%).

The results show that the naphthyl 3-position of JHW-015 **1** is tolerant to limited chemical modification. As the steric bulk at this position increases in this series of molecules, affinity towards the hCB_2 receptor decreases accordingly. Loss of affinity is greatest (>250-fold) for the bulky NBD fluorescent conjugate **12**, which showed a limited displacement of [^{3}H]CP55, 940 from the receptor at 10 μM. The two compounds, which retained the greatest affinity for the hCB_2 receptor (compounds **8** and **9**), were subsequently tested in a [^{35}S]GTP-γ-S-binding assay to assess if they retained the agonist profile of the original lead molecule JWH-015 **1**. The results in Table 1 show that compounds **1**, **8** and **9** were able to significantly increase [^{35}S]GTP-γ-S binding above basal levels when tested at a concentration of 1000 nM, indicating agonist properties of all three compounds. None of the three compounds were able to significantly raise [^{35}S]GTP-γ-S binding above the basal level when used at 10 nM concentration. ANOVA was unable to detect any significant statistical difference between **1**, **8** and **9** at a concentration of 1000 nM.

Notwithstanding its low affinity, the fluorescent conjugate **12** was assessed in confocal microscopy experiments to observe if the compound exhibited any discernable membrane binding to CHO cells expressing the hCB_2 receptor.[21] When conjugate **12** (100 nM) was applied to live CHO cells expressing the hCB_2 receptor in Hepes-buffered saline at 22 °C, a slight membrane binding was observed, with the majority of fluorescence occurring from cytosolic accumulation, presumably as a consequence of the high lipophilicity of the compound and therefore rapid cellular uptake.

In conclusion, in silico studies have suggested that the 3-naphthoyl position of the hCB_2 receptor agonist JWH-015 **1** could potentially tolerate conjugation to a bulky fluorescent dye. Photophysical requirements dictated the insertion of an additional 3-atom spacer between JWH-015 and NBD to ensure that the ultimate molecule would indeed be fluorescent. It has been previously established that non-fluorescent species are formed when NBD is conjugated directly to an aromatic amine. The mechanism of this fluorescence quenching has been investigated and attributed to Photoinduced Electron Transfer (PET).[19,22] Importantly, it was observed that an amide group placed between the aromatic amine and NBD prevented PET from occurring, even when the chain length was reduced to an acetyl (two carbon) spacer.[22] If a simple alkyl linker was used, the chain length required to prevent PET was found to be longer (nine carbon atoms). We therefore decided to install a short amine functionalised acetyl linker to separate the aromatic amine and NBD moieties and to keep the overall size of the molecule as close to **3** as possible. Chemistry, that incorporated substitution at the 3-position, was utilised to afford the NBD conjugate **12** in 7 steps. Unfortunately, this compound showed a >250-fold loss in affinity to the hCB_2 receptor and could not be used successfully in its intended application of fluorescence-based pharmacology assays. The decreased binding affinity of the glycyl derivative **11** may indicate that the hydrophobic binding pocket, which was to accommodate the NBD moiety, may not be as accessible (or as large) as originally predicted by our homology model. An alternative hypothesis could argue that the additional linker atoms produced potential conformations of the final molecule that create unfavourable steric clashes with the receptor structure.

However, it was rewarding to observe that the non-fluorescent 3-substituted naphthoyl precursor molecules **8**, **9** and **11** did show that affinity to the hCB_2 receptor could still be retained as long as the 3-substituent was kept small. In addition, modifications using nitro **8** and amino **9** groups led to compounds that demonstrated agonist behaviour when used at 1000 nM. To the best of our knowledge, this is the first reported modification to the 3-naphthoyl position of JWH-015 and is therefore an important addition to the established SAR of the indole-based cannabinoid ligands. It is also interesting to note that the recently reported indole-based hCB_2 agonist AM1241[10b,23] has a 5-iodo-3-nitro benzoyl substituent at the 3-position of an aminoalkylindole, which adds to the suggestion that the 3-aryl position within the 3-arylindoles may be an important extra binding pocket within the hCB_2 receptor.

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18. mp 250–252 °C (lit.[17b] mp 255–257 °C). ^{1}H NMR (DMSO-d_6) δ 13.89 (1H, br s, COOH), 9.22 (1H, d, *J* 2.4 Hz, 4-H), 8.93 (1H, d, *J* 8.6 Hz, 5-H), 8.75 (1H, d, *J* 2.4 Hz, 2-H), 8.43 (1H, d, *J* 8.6 Hz, 8-H), 7.97–7.89 (2H, m, aromatic). ^{13}C NMR (DMSO-d_6) δ 166.1 (4°, C═O acid), 144.0 (4°), 135.1 (4°), 133.6 (4°), 132.8 (4°), 131.5 (CH), 130.6 (CH), 128.8 (CH), 128.3 (CH), 126.3 (CH), 123.2 (CH). *m*/*z* (ESMS-) 217.0 (M^-). Calcd for $C_{11}H_7NO_4$ 217.04 (M^-). ν_{max} (KBr) cm^{-1}: 3500 (O─H), 3068, 2641, 1701 (C═O), 1597, 1529, 1453, 1412, 1336, 1287, 1253, 1203, 1102, 915, 796, 760, 687, 500.
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20. **12** ^{1}H NMR (DMSO-d_6) δ 10.54 (1H, br s), 9.45 (1H, br s), 8.55 (1H, d, *J* 8.9 Hz) 8.41 (1H, d, *J* 2.0 Hz) 7.95 (1H, d, *J* 8.2 Hz), 7.79 (1H, d, *J* 8.3 Hz), 7.66 (1H, d, *J* 2.0 Hz), 7.60–7.48 (2H, m), 7.41–7.32 (1H, m), 7.20–7.10 (1H, m), 7.01–6.92 (2H, m), 6.47 (1H, m), 4.45 (2H, br s, amide-CH_2), 4.21 (2H, t, *J* 7.3 Hz, indole *N*-CH_2), 2.45 (3H, s, CH_3-indole). 1.81 (2H, sextet, *J* 7.2 Hz, CH_2-propyl), 0.91 (3H, t, *J* 7.3 Hz, CH_3-propyl). *m*/*z* (ESMS$^-$) 561.1 (M^-), Calcd for $C_{31}H_{26}N_6O_5$ 562.2; (TOFES$^-$) 562.1962 (M^-), Calcd for $C_{31}H_{26}N_6O_5$ 562.1965 (M). Analytical RP-HPLC (Vydac reverse phase C8 column (150 × 4.6 mm), flow rate of 1 mL/min and UV detection at 254 nm); gradient 35% → 100% $MeCN_{(aq)}$ over 30 min. One major peak at R_t = 13.64 min.
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