A Mechanistic Model Explaining Ligand Affinity for, and Partial Agonism of, Cannabinoid Receptor 1

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Abstract

Cannabinoid receptor 1 (CB1) is thought to be the primary receptor involved in determining the activity of the phytocannabinoids, including tetrahydrocannabinol (THC) and cannabidiol (CBD). However, it may also interact with many of the >120 other minor cannabinoids, and there has been significant effort in preparing synthetic cannabinoids with either enhanced agonistic or antagonistic activity. This design process is aided if there is a reliable computational model that can quantitatively predict binding; unfortunately this is challenging and hasn't previously existed as the receptor, a G-protein coupled receptor, is highly dynamic. Furthermore, although the general idea of mechanism of agonism is understood, some compounds are partial agonists; the molecular mechanism of partial agonism is not clear. In this report we provide a highly effective model to predict a ligand's affinity for the orthosteric site of CB1, use this model to register the predicted affinity (high and low) of two homologous prophetic cannabinoids, and also discuss how a mechanism for THC partial agonism arises natively from the model, consistent with experimental data. To be successful, the model accounts not only for molecular interaction, but crucially, the partition of ligands into lipid membranes; we believe that this is the first computational small molecule-protein binding model to incorporate this partition into the equation. This model highlights both the capacity and need to include quantitative physico-chemical properties alongside calculated affinities in predictive tools for protein binding.

Keywords: partition coefficient, GPCR, partial agonist, predictive model, cannabis, THC

Introduction

Cannabinoids, alkyl resorcinol-functionalized diterpenes produced by *C. sativa*, are thought to act primarily through agonism and antagonism of human G-protein-coupled receptors (GPCRs).[1] Δ^9-Tetrahydrocannabinol (**THC**) is a well-established partial agonist of cannabinoid receptor 1 (CB_1) where it occupies the orthosteric site, the primary binding site for endogenous ligands located in the extracellular-facing well that is formed by the transmembrane helices. The majority of drugs that target GPCRs act at the orthosteric site.[2,3] Full agonists sitting in the orthosteric site open the G-protein binding domain on the cytosolic side of the protein, while antagonists prevent G-protein binding by inducing a conformational change that closes the site. Partial agonists have intermediate activity. Since the initial discovery of **THC** and other related cannabinoids, numerous modifications and analogs have been synthesized to define the structure–activity relationship (SAR) of **THC** with both CB_1 and CB_2. THC analogues differ primarily at two major sites: the ring system and the alkyl chain (**Figure 1A**). According to Bow and co-workers, the length of the alkyl chain is the key parameter for determining CB_1 receptor activity; a minimum of three carbons

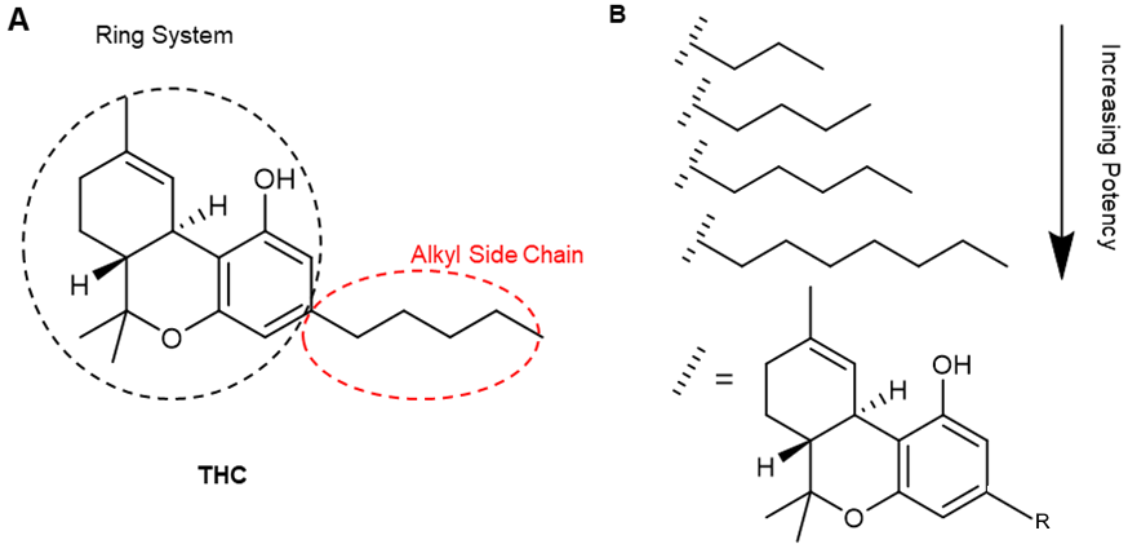

Figure 1 Overview of **THC** and its ring system, highlighting the various side chain lengths discussed in the article.

is necessary to bind the receptor, with activity peaking at eight-carbons and falling off as length increases past that point (**Figure 1B**).[4]

Recently, Citti *et al.* isolated a pair of novel phytocannabinoids: tetrahydrocannabiphorol (**THCP**) with a seven-carbon alkyl chain,[5] and tetrahydrocannabutol (**THCB**) with a four-carbon alkyl chain.[6] Both had higher *in vitro* binding activity (k_i= 1.2 and 15 nM, respectively) than that reported for five-carbon **THC** (k_i= 40 nM).[4,6] Citti proposes that this differential activity arises because the orthosteric binding site of CB_1 has three hydrophobic pockets[7]: The main hydrophobic pocket (M-pocket) which houses the ring system of **THC** homologs; the long hydrophobic pocket (L-pocket) formed by TMs III, V, and VI, which can accommodate the long heptyl chain of THCP and the pentyl chain of THC; and the hydrophobic sub-pocket (S-pocket) formed by F170, F200, L387, M363, L359, and C386 that lies towards the toggle switch residues needed to activate the receptor. This last is located at the intersection between the M-pocket and the L-pocket (Error! Reference source not found. and **Video S1**).). As they are too short to benefit from the hydrophobic L-pocket, the propyl and butyl chains of **THCV** and **THCB** instead sit in the S-pocket. Citti argues that this is the reason for **THCB**'s higher affinity for CB_1 than the longer **THC**.[5,6]

However these new findings contradict the literature: first, **THC** analogues with alkyl chains shorter than 5 carbons—or longer than 8 carbons—have decreased binding affinity compared to those with lengths in that range with affinity peaking at 8 carbons as noted.[4] However, they report that four-carbon **THCB** (K_i=15 nM) has higher binding affinity than five-carbon **THC** (K_i=40 nM). Second, the binding affinity of **THCP** and **THCB** were compared to the **THC** and **THCV** affinity values reported by Bow and Rimoldi which are the least generous available.[4] Several binding affinities have been reported for **THC** (K_i= 40.7 ± 1.7[4], 35.64 ± 12.4[8], 25.1 ± 5.54[9], 5.05[10] and 2.9 ± 0.3[11]) and **THCV** (K_i = 75.4[4], 46.6[12] and 22 ± 5[13]). There is a large variation in the

experimental data available in the literature which could arise from different testing conditions and protocols and great caution must be taken in overreliance and overinterpretation of small differences that arise from any sort of concentration-dependent non-thermodynamic technique. Finally, interaction of a ligand with the centrally located W356$^{6.48}$ and F200$^{3.36}$ toggle switch differentiates whether a given ligand is an agonist, "triggering" the switch and inducing the conformational change on the cytoplasmic side of the protein allowing for G-protein interaction; or an antagonist, occupying the pocket and preventing the switch being triggered.[1]

This result intersects with our own interest in this receptor and defining precisely how the ligands interact to design new cannabinoid-based therapeutics. The community understands the mechanism of action of agonists, but it is less clear whether there is a consensus mechanism of action for partial agonists. In particular, what makes them *partial* agonists? Lacking any crystallographic data of the receptor with any bound phytocannabinoid, this question remains outstanding. Furthermore, it highlights that we have an imprecise understanding of the experimental binding affinity, even for these well studied major cannabinoids, with reasonable estimates of the K_i varying by over an order of magnitude. We wished to develop a theoretical model for determining binding affinity for use in screening of new compounds and correct for factors that may not be normally accounted for in methods such as docking. Generally, this is done using an all-atomic molecular modelling study, but this provided inconsistent results: affinity for the receptor was not sufficient, in and of itself, to describe the observed *Ki.* This however can partially be explained by the different mode of entry of ligands into CB_1 compared to many G-protein-coupled receptors: it enters from the lipid membrane, not the solvent. This is a factor not considered by methods such as docking, which only considers binding to the receptor and assumes the ligand is solvated in water. With this information, and using a library of 21 **THC** homologues

with experimental data (**Figure 2**), we propose a mathematical model to predict the affinity of a ligand for CB_1, and a conceptual model to determine whether a ligand is likely to be agonist, antagonist, or partial agonist, and propose a mechanism by which partial agonists function as such. During the preparation of this article, Shukla and colleagues published on the mechanism of action of THC as a partial agonist using complementary techniques to our own, and in close agreement with our proposed mechanism, providing further confidence in the reliability of our hypothesis.[14]

2.0 Methods

Information on how proteins and ligands were prepared, docking, MD and MM-GBSA protocols are available in the supplementary information.

3.0 Results and Discussion

To predict the binding affinity between ligands and receptors as well as to characterize the different binding modes, an *in silico* study was conducted on a total of 21 **THC** homologues with experimentally measured binding affinity towards CB_1, including the antagonist **THCV**,[7] weak agonists **THCA**,[15] partial agonists **THCB** and **THC**,[6] and agonists **THCP**, **AM11542**, **AM841**, **AM12033**, **AM4030**, **HU-210**, ajulemic acid (**AJA**) and **Nabilone**, which have various potency and selectivity (**Figure 2**).

We investigated several parameters to develop a model capable of providing a reliable and accurate correlation between experimental binding affinity and *in silico* docking results. We want to highlight that this is a hard problem: correlation of computational prediction to experiment when the experimental data was all collected in parallel using a single methodology by a single user is still challenging; however, this rich data set does not exist for the CB1 receptor. Instead, we need

to compare data collected by multiple research groups using similar (but not identical workflows) with various ligands. This will introduce variance as experimental *Ki*s measured are highly dependent on protein expression levels and the precise conditions of the data collection. However, although this increases the difficulty in generating the model, it also makes any successful model far more robust, and inherently more useful. To tackle this challenge, we first examined rigid-receptor docking (RRD) with scaled van der Waals radii of non-polar atoms (1.0, 0.8, and 0.6) to represent some of the flexibility present within the receptor, an approach well precedented to provide good correlation to experiment.[16] It generally works best when the initial protein structure best reflects the binding mode of the specific class of ligands, a reasonable expectation seeing the superficial similarity of the ligand library. The docking was followed by further analysis to better determine the free energy of the complex (and consequently the binding energy) using Prime/MM–GBSA calculations.[17] These analyses began with the lowest energy docked conformer in each case, once this pose was visually confirmed to be a reasonable conformer. The experimental K_i values, rigid docking scores (RRD) and Prime/MM-GBSA predicted binding free energies are listed in **Table 1**.

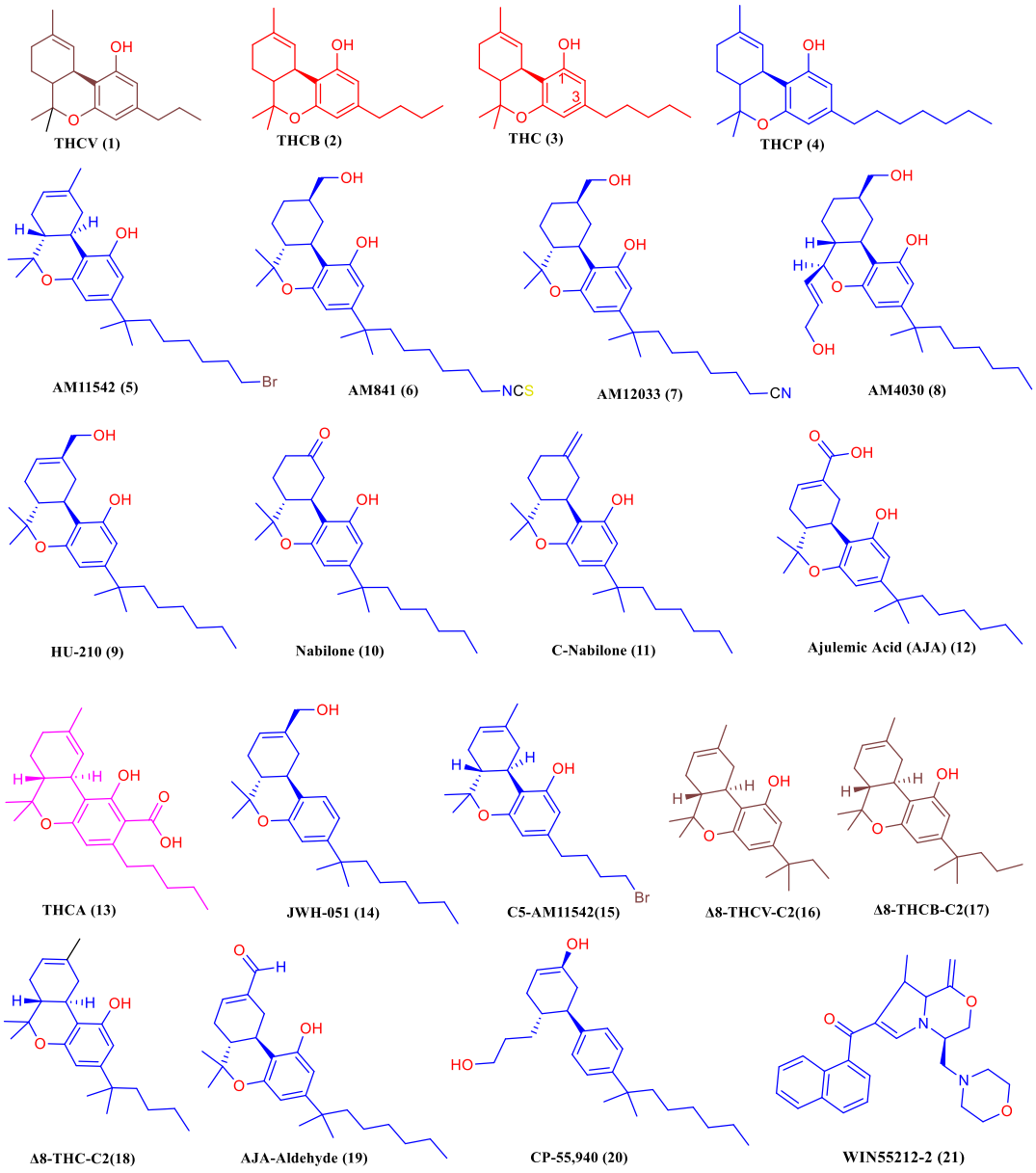

Figure 2 Structures of the **THC** analogues with known experimental binding affinities used in this study. Those in red are partial agonists, in purple weak agonists, in blue potent agonists, in brown antagonists.

Table 1 RRD scores, IFD docking scores, LogP and predicted binding-free energies (kcal/mol) obtained by Prime/MM–GBSA and Md/MMBGSA of the CB_1 ligands.

Ligand	K_i (nm)[a]	r_w scaling factor						IFD docking score (kcal/mol)	LogP	MD/MM-GBSA ΔG_{bind} (kcal/mol)
		Rigid Docking Score (kcal/mol)			Prime/MM-GBSA ΔG_{bind} (kcal/mol)					
		1	0.8	0.6	1	0.8	0.6			
THCV (1)	22[13]	-9.12	-8.63	-7.99	-62.09	-54.83	-51.64	-10.81	4.91	-39.09
THCB (2)	15[6]	-9.92	-8.99	-8.24	-62.78	-56.16	-54.25	-10.98	5.3	-43.71
THC (3)	2.9[11,18]	-10.13	-9.07	-8.52	-61.93	-63.9	-55.76	-11.51	5.66	-44.92
THCP (4)	1.2[5]	-5.07	-8.65	-8.02	-57.09	-55.65	-67.06	-11.81	6.44	-47.71
AM11542 (5)	0.11[19]	-8.51	-8.7	-8.05	-55.01	-68.11	-67.9	-12.65	7.58	-57.48
AM841 (6)	1.14[20]	-3.03	-11.36	-8.89	-47.7	-77.74	-73.36	-11.73	5.98	-65.25
AM12033 (7)	0.51[20]	-9.34	-10.03	-9.47	-70.63	-70.08	-73.55	-13.46	4.3	-61.36
AM4030 (8)	0.7[21,22]	-5.44	-8.68	-9.07	-49.09	-64.51	-62.58	-12.28	5.34	-58.92
HU-210	0.73[23]	-8.68	-9.425	-8.25	-81.01	-68.02	-76.49	-12.18	5.83	-63.78
Nabilone (10)	2.19[24]	-9.75	-8.53	-7.78	-70.71	-69.33	-68	-11.91	5.5	-53.71
C-Nabilone (11)	1.82[24]	-5.45	-8.97	-7.51	-64.13	-58.11	-37.19	-11.6	6.72	-63.49
AJA (12)	32.2[25]	---[b]	-9.45	-7.61	---	-55.43	-43.35	-10.62	5.83	-43.94
THCA (13)	23.51[8]	---	-5.98	-5.07	---	-34.99	-34.16	-10.89	5.59	-37.53
JWH-051 (14)	1.2[26]	-9.25	-8.61	-6.92	-68.3	-71.1	-71.67	-12.1	6.56	-63.83
C5-AM11542 (15)	10.8[27]	-9.88	-9.59	-7.85	-61.45	-65.45	-66.44	-10.88	5.9	-49.51
Δ^8-THCV-C2 (16)	14[28]	-8.87	-9.27	-7.9	-62.07	-59.49	-61.29	-10.5	5.99	-40.22
Δ^8-THCB-C2 (17)	10.9[28]	-8.48	-8.79	-8.15	-66.37	-55.06	-65.55	-10.74	5.59	-43.87
Δ^8-THC-C2 (18)	3.9[28]	-8.51	-8.83	-7.8	-63.39	-64.84	-50.54	-11.27	5.79	-52.35
AJA-Aldehyde (19)	2.24[24]	-7.39	-9.27	-8.03	-64.5	-68.4	-64.81	-12.03	5.70	-54.56
CP55940 (20)	0.58[23]	-9.45	-8.71	-7.31	-67.49	-58.55	-74.27	-12.35	5.10	-60.67
Win55212-2 (21)	1.9[29]	---	---	-6.25	---	---	-49.21	-12.35	4.15	-54.35

[a] For ligands where multiple K_i values have been reported in the literature, the lowest reported value was selected; with the differences in reported values ranging to an order of magnitude and dependent on the tool used to measure the value, there is error built into our model. The lone exception is for **HU-210**, where the employed reported value of 0.73 nM is higher than the lowest value, 0.25 nM. This provides better correlation with our model, suggesting that the higher value may prove more correct should the value be redetermined by a third measurement.

[b] --- indicates that the ligand does not dock to the orthosteric binding site of CB_1.

There is only weak correlation between the experimental values (log K_i(nM)) and the RRD score (kcal/mol; **Figure 3A**, **Error! Reference source not found.**). The Pearson correlation coefficient (R^2) is 0.081, 0.065, and 0.110 for r_w scaling factors 1.0. 0.8 and 0.6, respectively. This is an extremely poor correlation. An MM-GBSA refinement does little to improve the correlations, and although it does become statistically significant with r_w scaling factors of 0.8 or 0.6, this remains

a poor tool for predicting binding affinity (Error! Reference source not found.**B, Figure S1**). This suggests that there might be more adjustments occurring in the receptor depending on very fine details of the ligand than one would necessarily expect based on their similarity by inspection. This both implies that induced docking might prove more useful, and that mechanism might be dependent on minor adjustments to the binding pocket.

Induced fit docking (IFD) is a far more computationally expensive than RRD, but it allows for considerable flexibility in the binding site residues which works well for systems with moderate flexibility and differences in the binding mode of various ligands.[30] This can be important if the initial pocket in a given conformation is too restrictive or permissive to accommodate a ligand (meaning the RRD will be artificially poor), and both the pocket and ligand must mutually adapt to each other when forming a complex.[31] However, IFD can introduce additional errors in measurement if the pocket is too flexible, and can be less useful for prediction than RRD if the ligand classes are all similar to one another. IFD generally shows better results in reproducing the native conformations of complexes,[32] and this was used with all 21 ligands (**Table 2**).

Even by inspection, these results seem to reflect what we know form experimental science: increasing the number of side chain carbon atoms in the series from **THCV** to **THCP** leads to improved docking scores. Overall, the correlation between the experimental values (log K_i(nM)) and IFD (kcal/mol) has dramatically improved compared to the RRD. The Pearson correlation coefficient (R^2) is 0.807 with a ρ-value $<1 \times 10^{-5}$ (Error! Reference source not found.**C**). However, there are several ligands whose behaviour is not consistent with the model, such as **AM12033** and **AM11542**. This could be simply that no model is perfect, and that we should be satisfied with a good correlation, or it could be that free energy of binding alone does not model the system correctly.

Let us consider the assumptions of the system. Efficacy depends on several factors beyond simply the affinity of a drug for its target, including the ability of the drug to enter the cell, the stability of the drug over the lifetime of the experiment, and whether it is sequestered through some competing biochemical mechanism. All of these essentially affect the localized concentration of the drug. Generally, for drugs with a similar scaffold, many of these features would be expected to be largely equivalent. Furthermore, most GPCRs, indeed most membrane proteins, interact with their ligand in the bulk extracellular fluid, so many of these mechanisms are not relevant. However, Class A GPCRs can have their orthosteric site opening into the lipid bilayer, and CB_1 is one such protein.[1] Consequently, the relevant concentration is not the concentration of the drug in solution, but rather the concentration of the drug in the lipid bilayer and these are not the same. Consider two drugs with the same binding affinity for a type A GPCR like CB_1 that differ only in their water solubility: hydrophobic **A,** and hydrophilic **B**. For the same bulk concentration, **A** would be expected to partition into the lipid bilayer to a greater degree than **B**. This would give **A** a higher localized concentration to bind with the GPCR. Cannabinoids enter the cannabinoid receptors *via* the lipid bilayer.[33-35] Recently, Hurst and *at el.* demonstrated *via* molecular dynamics that ligands access the binding pockets of other class A GPCRs *via* the lipid bilayer.[36] This is consistent with our models where during all MD simulations, the orthosteric site's opening never left the lipid bilayer.

Lipophilicity can be expressed as the logarithm of the partition coefficient (logP) between 1-octanol and water.[37-39] The prediction of this parameter is a key tool in modern drug design.[40] We utilized the log(P) for all 21 ligands, obtained using QikProp (**Table 1**).[41] We then used the imperialist competitive algorithm (ICA), as implemented in MATLAB,[42] to generate a series of best fit equations to the data set with different exponential forms, constants, and relationships between the binding term, derived from the IFD binding, and the hydrophobicity partition term,

derived from logP.[43] The best fit equation improved the Pearson correlation coefficient square (R^2) from 0.81 to 0.92 (Error! Reference source not found.**D**), and correctly shifted the "outlier" ligands towards the trend; partitionability into the lipid bilayer explains the discrepancy between **AM12033** and **AM11542** binding affinity and efficacy. The equation of our fit is as follows:

Optimized Fit: $K_i = (X + 13.166)/1.1755$

Where K_i is measured in nM and $X = IFD\ Score - 0.03(\text{logP})^2$. The values of the constants are, of course, empirically derived. To the best of our knowledge, this is the first theoretical framework for predicting efficacy based on combining binding affinity calculated through all-atomistic modelling, and hydrophobicity, not only for the cannabinoids but for any class of ligand; we see no reason why this same methodology could not be applied to any other system where ligands need to partition into compartments, although permeability functions might prove a more useful parameter if the ligand simply needs to passively pass through a bilayer rather than act from the bilayer as in this case. An enzymatic stability term could similarly be employed for ligands that enter a cell through the lysosome and must survive processing to engage with their target, although this is admittedly a bit more challenging to estimate.

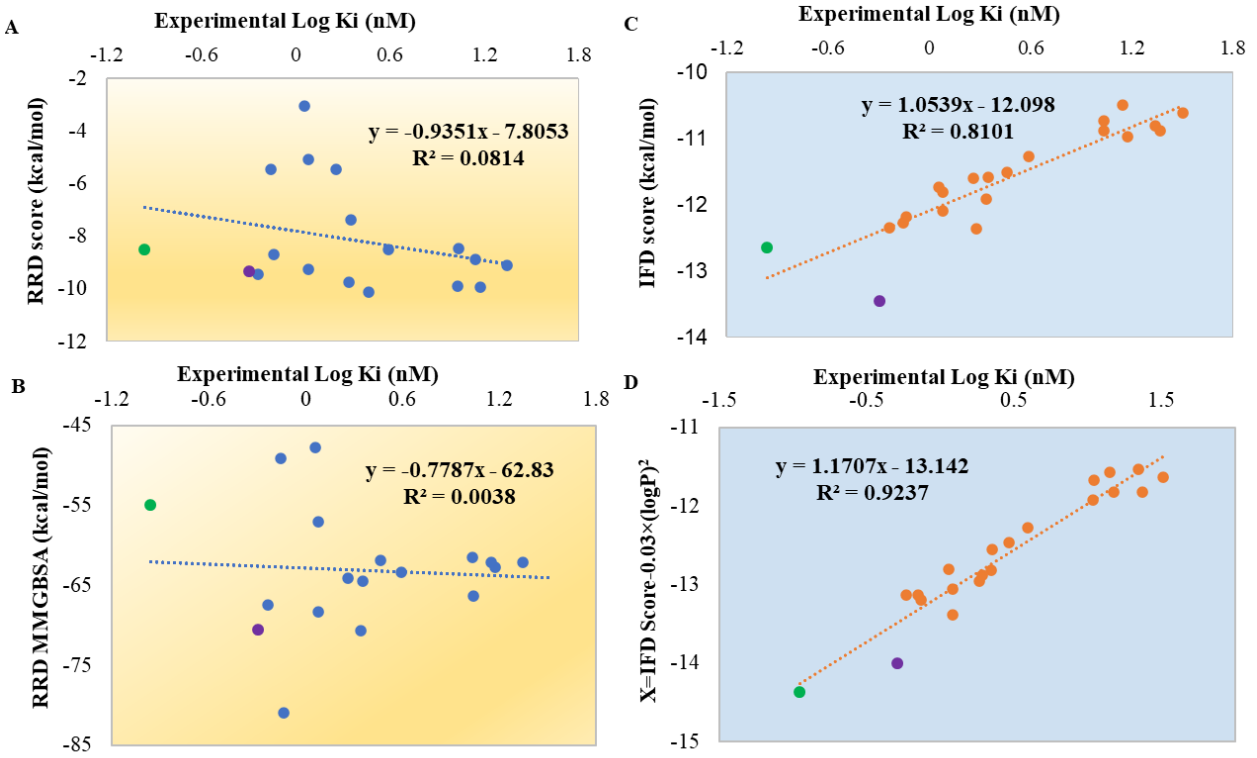

Figure 3 Correlation analysis between experimental values, (A) RRD, (B)Prime/MM-GBSA, (C) IFD scores and (D) IFD scores optimized with Lipophilicity. **AM11542** and **AM12033** are shown in Green and Purple colors, respectively.

We then looked at how well the model worked to predict the binding affinities of different analogues that were not included in the training data set. We compared the binding of **Δ^8-THC**, whose binding affinity for CB_1 has been variously reported as 44 nM[4,44] or 47 nM with binding predicted by our model.[45] **Δ^8-THC** differs from **THC** only by the location of the olefin in Ring C meaning we can expect similar lipophilicity and likely a similar binding mode; under this understanding the values do seem rather high compared to that of **THC** (2.9 nM). Docking the ligand using IFD provided a reasonable conformation, and a calculation of the lipophilicity and its use in our equation estimates a K_i of 11.02 nM (**Figure 4C** and **4D**). This is lower than **THC** and is within range of values reported in the literature but is on the lower end. This is important as one of the reports for **Δ^8-THC** also estimated the binding affinity of **Δ^9-THC** to be 40 nM,[4] which is an outlier compared to other measurements (see above). Based on our model, we propose that the

binding affinity of **Δ^8-THC** has been significantly underestimated in reports to date, and its value might benefit from re-measurement.

Using our model and our understanding of the structural features responsible for CB_1 binding, we prophesize two new related molecules of which we predict one will prove a very high affinity binder and CB_1 full agonist, while the other will be inactive. **THC** has been the subject of many structure activity relationship studies (**Figure 4A**). Gómez-Jeria and coworkers developed a pharmacophore model for classical cannabinoid-CB_1 interactions (**Figure 4B**).[46] The C1 phenol group is required for good selectivity for CB_1 over CB_2, and we have already discussed the importance of the alkyl chain. Binding affinity can also be enhanced by hydroxylation of the C11 methyl group as can be seen in the AM-series (**Figure 4A**).[45] Using this information, and aiming for synthetic simplicity, we propose two unknown compounds, both simple **Δ^8-THC** homologues, **THCN** with 9 methyl groups and **THCU** with 11 methyl groups. We conducted the IFD and calculated the lipophilicity and then predicted the binding affinity based on our model (**Figure 4C**). The alkyl side chain of **THCN** extends perfectly into S-pocket while **THCU** is too long and does not fit into the orthosteric site; it will not be able to fit in the receptor, and we expect it to be largely inactive. The predicted binding constant for **THCN** is 0.84 nM, which would make it the best binding phytocannabinoid-like molecule. The synthesis of these new compounds is currently underway for their evaluation but we wish to register the prediction in the literature in advance.

It is important to know which one of the reported binding affinities for **THC** and **THCV** correlates best with predicted values. We calculated the correlation of experimental binding affinity and IFD score for all ligands except **THCV** and **THC** (Error! Reference source not found.). The Pearson correlation coefficient (R^2) was 0.797 or 0.918 for IFD (kcal/mol) and IFD scores optimized with lipophilicity respectively. A calculation of the IFD score and the lipophilicity their processing

through our equation estimates a K_i for **THCV** and **THC** of 26.93 and 4.11 nM respectively, which is near the expected values.

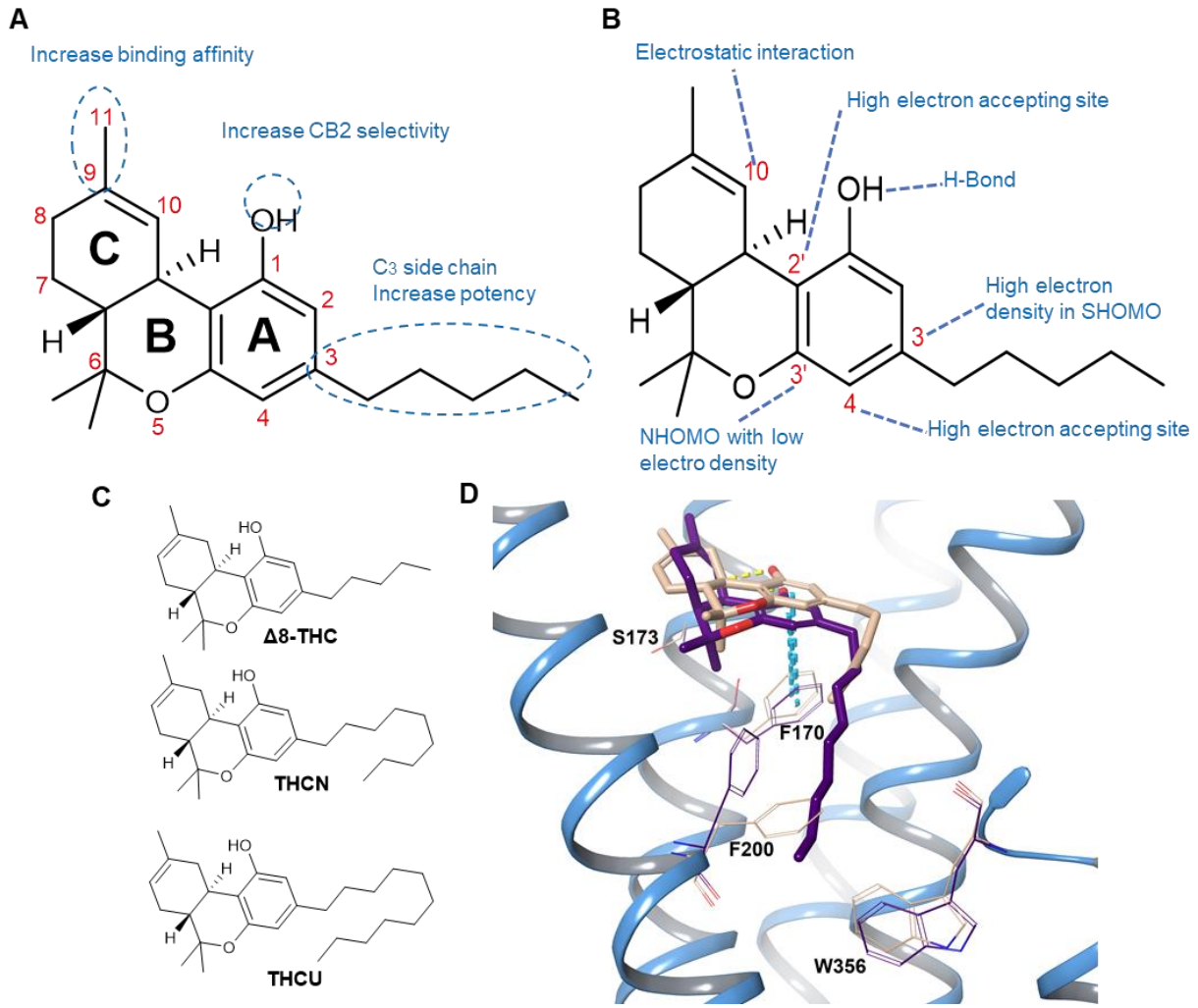

Figure 4 (A) common chemical modifications on **THC** skeleton and (B) Proposed pharmacophore for classical cannabinoids interacting with CB1 receptors. (C) structures of **Δ^8-THC**, **THCN** and **THCU**. (D) binding poses of **THCA** (bisque) and **THCN** (indigo) in complex with CB1. In all figures, oxygen is in red, and nitrogen is in blue. H-bonds are represented by yellow dotted lines, and π-π interactions by blue dotted lines. Key residues related to the ligands are highlighted in the same color as their present ligand.

Although determining a model for predicting binding affinity of designer cannabinoids is critical to our current research program, affinity, as can be clearly seen, does not define the functional role of the ligand. Tight binders and weak binders can be either antagonists, partial-agonists, reverse agonists, or full agonists. The specifics are in how the ligand interacts with the receptor.[7] We have

not identified a clear theoretical literature model that differentiates between these roles. Consequently, we more closely investigated the binding mode of the homologous series of **THCV**, **THCB**, **THC** and **THCP** using IFD as differential receptor response to the ligands likely explains why the first is an antagonist, the middle two partial agonists, and the latter a full agonist (**Table 2**). Highly potent agonist **AM11542** was included as a control.

Table 2 IFD scores and predicted lipophilicity of THC homologues ligands.

Ligand	IFD Score (kcal/mol)		$\Delta E = E_S - E_L$ (kcal/mol)	Prime/MM-GBSA (kcal/mol)		$\Delta E = E_S - E_L$ (kcal/mol)
	S-pocket Pose	L-pocket Pose		S-pocket Pose	L-pocket Pose	
THCV (1)	-10.37	-10.81	0.44	-66.2	-63.96	2.24
THCB (2)	-10.98	-10.8	-0.18	-65.85	-62.24	-3.61
THC (3)	-11.51	-11.51	-0.07	-58.98	-57.87	-1.11
THCP (4)	-11.81	-10.75	-1.058	-69.61	-67.87	-1.74
AM11542 (5)	-12.65	-10.16	-2.49	-79.26	-74.25	-4.01

THCV, **THCB**, **THC** and **THCP** all adopt similar conformations in the orthosteric ligand-binding site. Their ring systems sit in the M-pocket in nearly superimposable geometries: they only differ in that the alkyl side chains of **THCB**, **THC** and **THCP** protrude into the smaller S-pocket towards the receptor-activating toggle switch (formed by F200 and W356), which does not occur for **THCV**, which instead extends into the L-pocket (**Figure A**). The phenolic C1-OH of all four cannabinoids forms a hydrogen bond with S173; in the case of **THCV** and **THCB**, it forms an additional H-bond with H178 (Error! Reference source not found.**A**). The ring systems, excepting that of **THCV**, participate in π-π interactions with the receptor's F170, which sits at the intersection of the three pockets. Hydrophobic interactions help retain the ligands affinity to the rest of the surface, and, as expected, these interactions increase in strength as the surface area increases due

to a lengthening alkyl chain with the IFD score rising from −10.81 to −11.81 moving through the series from **THCV** to **THCP** (**Table 2**).

Interested in mechanism, we focused in on the effects that cannabinoid binding has on the dynamics of the toggle switch formed by F200 and W356, respectively located on transmembrane α-helix 3 (TM3) and TM6. When an alkyl chain pushes between them, it forces open the two helices like chopsticks revealing the G-protein binding site on the cytoplasmic face, activating the receptor.[47,48] Their different positions are best described by comparing their form in the presence of **THC** and the highly potent inverse agonist Taranabant (**TNB**, **Figure C**; PDB ID: *5U09*)[49]. **TNB@CB$_1$** is akin to the empty inactivated receptor; but reduces its flexibility (hence inverse agonism), locking the two aromatic residues that make up the switch parallel to one another. This holds the transmembrane helices together. The ligand sits in the M-pocket, extending its side chain down the L-pocket with high affinity to prevent other ligands from binding. **THC** on the other hand, extending its tail into the S-pocket pushes the residues open, activating the receptor.

THC, along with its shorter homologues **THCV** and **THCB**, all have similar effects on the toggle switch with the key residues adopting the same conformation in the activated form (**Figure 5B** and **C**). **THCP** extends deeper into this pocket, forcing the residues even further apart, further opening up the G-protein binding site, facilitating activity, and helping to explain its full agonist role (**Figure 5D**). However, this does not explain why the shorter analogues are only partial agonists, or why **THCV** is an antagonist, as they interact the same way. The true story is more complicated.

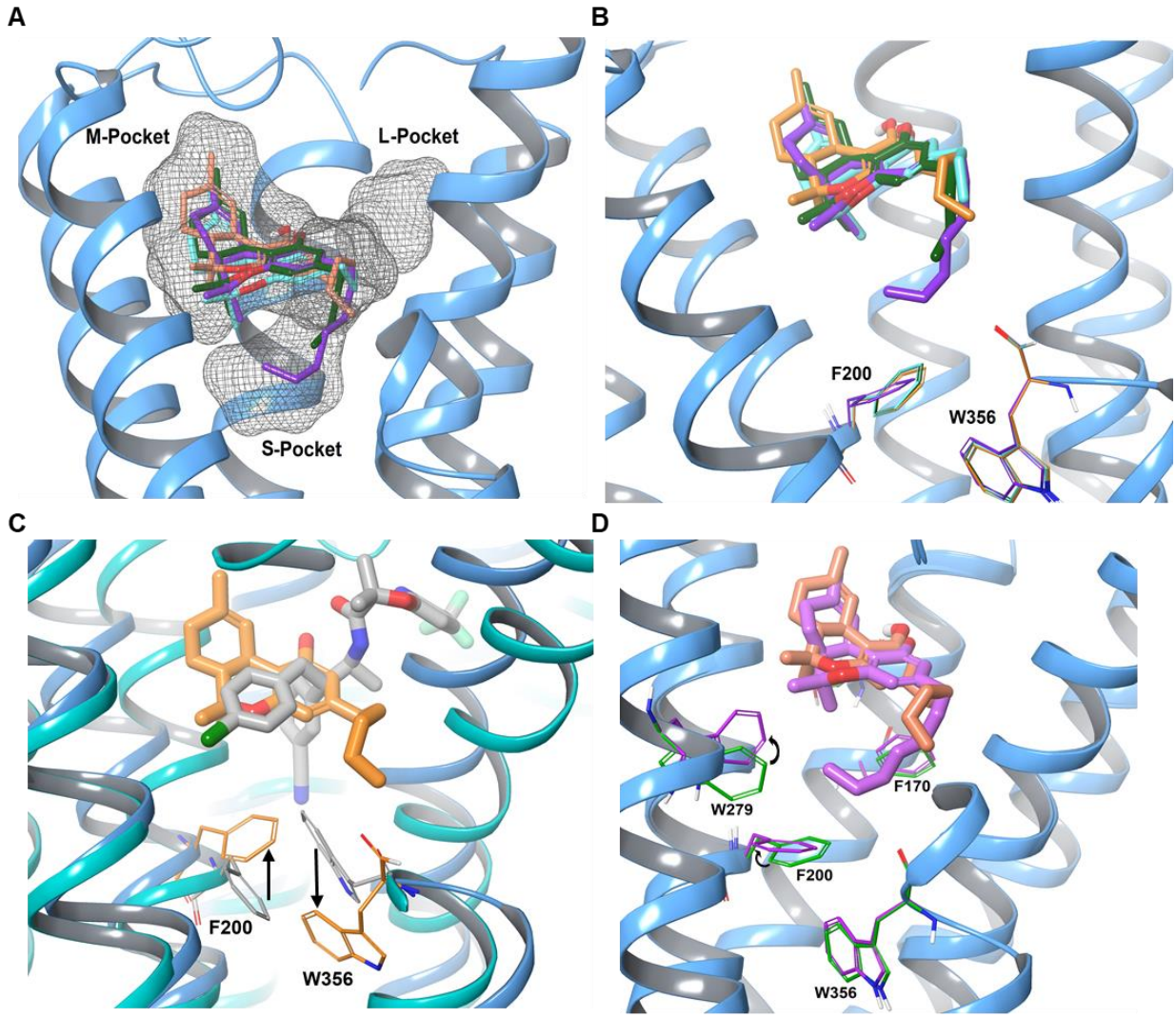

Figure 5 (A) binding poses of **THCV** (Cyan), **THCB** (dark green), **THC** (orange) and **THCP** (purple) in complex with CB1 (PDB ID: 6N4B). (B) binding poses of **THCV**, **THCB**, **THC** in complex with CB1. (C) superimposition of **THC@CB1** and **TNB@CB1** (gray) ligand-binding pockets; (D) binding poses of **THCP** and **THC** in complex with CB1. The oxygen atoms are in red, nitrogen in blue and sulfur in yellow, H-bonds in yellow dotted lines, π-π interactions in blue dotter lines and hydrophobic pocket is bordered in dash dark gray mesh. Key residues related to ligands have the same colors.

We then turned to the very potent AM-series analogues. Consistent with the literature and published crystal structures,[1] our model places the C ring system of all ligands into the M-pocket. Most of them extend their alkyl chain into the S-pocket, but that of **C5-AM11542** folds back over itself to extend into the L-pocket (Error! Reference source not found.**A**). They are all however,

highly effective at forcing open the toggle switch, with the distance between F200 and W356 starting higher than for **THC**, and increasing in the order of **C5-AM11452**, **AM4030**, **AM11542**, **AM12033** and **AM841** (Error! Reference source not found.**A**). **AM11542**, **C5-AM11542**, **AM841**, **AM12033** each have one π-π interaction with F170. **AM841**, a covalent inhibitor in its final form, has an extra H-bond with S173 and π-π interaction with F268 when it sits non-covalently in the pocket. The phenolic hydroxyl at C1 of **AM12033** forms a H-bond with H178 and the aliphatic OH group at C11 forms two H-bonds with D176 and S173. **AM4030** forms an extra π-π with F268 and the OH of the 6β-((E)-3-hydroxyprop-1-enyl) group form H-bond with F268.

Other agonists studied, like **HU-210**, **Nabilone**, **JWH-051**, **Δ^8-THCV-C2**, **Δ^8-THCB-C2**, and **Δ^8-THC-C2** behave very similarly (Error! Reference source not found.**B**). In all cases the S-pocket-occupying side chain forces open the toggle switch. Exceptions are **THCA** and **AJA**, which adopt a different conformation in the orthosteric site. The carboxyl group of **THCA** forms two H-bonds, one with S383, the other with H178, which induces a repositioning of the ring system, and consequently the alkyl chain remains in the atrium between the S- and L-pockets entering neither (Error! Reference source not found.**C**). Unusually, the ring system of **AJA** rotates 180˚C compared to all other ligands. The carboxyl and phenolic OH- groups form strong H-bonds with K192 and S173, respectively locking this unusual conformation (Error! Reference source not found.**C**, found for the lowest 10 energy docking poses) however this may be an outlier resulting from docking returning an incorrect pose and experiments would be required to determine if this pose is truly how it binds. All poses of **C-nabilone** show far different binding, with the ring sitting at the intersection of the S- and L-pockets and the alkyl chains sticking up into the M-pocket (Error! Reference source not found.**D**). The third lowest energy pose of **AJA-Aldehyde** is identical to the

activation state, and its alkyl chain extends to the S-pocket. Its docking score is also consistent with the equation, and it marginally improves the R-Squared value when added to the training set (≈0.03) (Error! Reference source not found.**D**).

These lowest energy docked conformers, however, fail to capture the complexity of the dynamic binding of the phytocannabinoids. **THC** is a CB_1 partial agonist, meaning that upon binding, it does not completely induce the conformational change associated with agonists. There are multiple mechanisms by which this could occur. One would be that in the docked conformation, **THC** simply does not induce enough pressure on the toggle switch to open the G-protein site. This is not supported by our model which predicts that it forces a similar conformation onto the protein as full agonists like **THCP** do. Alternatively, **THC** might drift away from the core of the orthosteric site and occupy a position higher in the cavity as **CBD** is predicted to do in the presence of **THC**. A third possibility is that the alkyl chain can flip from the S-pocket to the L-pocket. Shao *et al.* computationally docked **THC** to a relaxed receptor derived from the antagonist TNB-bound structure (PDB: *5TGZ*).[49] They predicted that the alkyl side chain of **THC** extends just towards toggle residue W356, and would likely activate it as an agonist. Similarly, when Hua and colleagues docked **THC** to the full-agonist bound structure (PDB: *5XRA*)[19], they predicted that **THC** would behave similarly to **AM11542** and that its alkyl side chain of **THC** extend towards F200 and W356. However, in the docking study accompanying their Cryo-EM structure of CB_1, Kumar *et al.*[50] proposed that **THC**'s alkyl chain is more flexible and potentially able occupy either the **L** or S-pockets. This has been further supported by Dutta and colleagues who, like us, proposed that this "switch hitting" behaviour explains the partial agonism of **THC**.[14] Evidence appears to support that **THCV** and **THCB** protrude into the S-pocket towards the toggle switch,[5-7] while **THCP** behaves similarly to **THC**, and occupies the L-pocket.[5]

As this might help mechanistically explain partial agonism, we analyzed the behavior of the alkyl chains of **THCV**, **THCB**, **THC**, **THCP** and **AM11542** in the orthosteric site. We employed IFD and MM-GBSA refinements of conformations of these ligands occupying both the S- and L-pockets and calculated the difference in preference for the two pockets ($\Delta E_{S/L}$, **Table 2**, **Figure**). Among these ligands only **THCV** has a positive $\Delta E_{S/L}$, meaning that it prefers to occupy the non-triggering L-pocket. This explains why it is an antagonist. However, the side chain is very short, and does not extend far into either pocket: even when it does insert into the S-pocket, it does not disrupt the toggle switch residues (**Figure 6A**). **THCV** forms similar hydrophobic interactions in both conformations, interacting with S383, C382, F379, I362, L359 and F170. For the slightly longer **THCB** and **THC**, the $\Delta E_{S/L}$ are −0.18 and −0.07, respectively. This is essentially 0, meaning that in both cases we would predict that the ligand fluctuates rapidly between occupying the two pockets. Unlike for **THCV**, the toggle switch residues do significantly change orientation depending on the location of the alkyl chain (**Figure B**). This arises because although both ligands form the same core interactions at the M-pocket with C382, F379, I362 and F170 (and, for **THC**, with M363, S383, L359) regardless of the orientation; they differ in their additional interactions when the alkyl chain enters one or the other pocket (**Figure C**). However, for **THCP** (**Figure D**) and **AM11542**, $\Delta E_{S/L}$ is high as their alkyl chains are effectively unable to occupy the L-pocket if the ring system is in a reasonable position within the M-pocket. This means they are locked into a conformation that forces open the toggle switch. They cannot move their alkyl chain into the non-activating L-pocket. This means that when bound, they must activate the toggle switch, explaining why they are full agonists. While we were working on this project, Dutta and co-workers employed MD simulations to show that THC's alkyl side chain plays a crucial role in determining its partial agonism.[14] Their research revealed that this side chain is essential for stabilizing the ligand in both

agonist and antagonist-like conformations within the receptor binding pocket.[14] Like us, they also showed that it can also fluctuate between the two pockets.

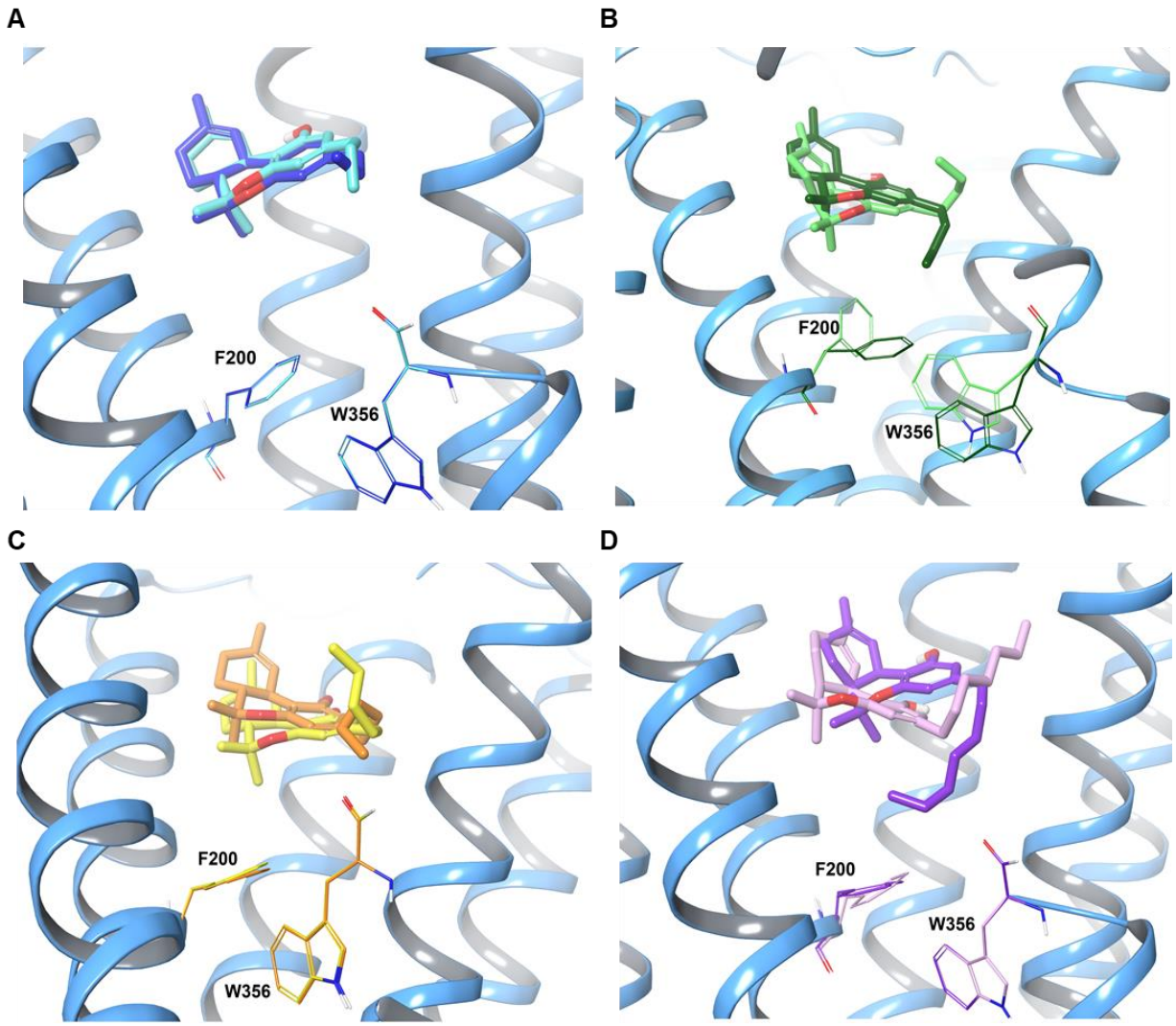

Figure 6 Superimposed docking poses of ligands (A) **THCV** in the **L-pocket** (cyan) and **S-pocket** (blue), (B) **THCB** in the **L-pocket** (dark green) and **S-pocket** (light green), (C) **THC** in the **L-pocke**t (yellow) and **S-pocket** (orange), (D) **THCP** in the **L-pocket** (purple) and **S-pocket** (rosepink). Ribbons are shown in light blue color and residues are colored as same as their related ligands.

We conducted molecular dynamics (MD) simulations for a diverse set of 21 ligands, embedded within a phosphatidylcholine (POPC) membrane and solvated with water and NaCl ions to achieve

a physiological concentration of 0.15 M. Extending over a 200 ns simulation period, our analysis employed MM–GBSA calculations to quantitatively estimate binding free energies within the orthosteric site. Notably, our findings revealed a strong correlation (R^2 = 0.66, **Figure S5A**) between calculated MM-GBSA values and experimentally determined *Ki* values, affirming the reliability of our computational approach for predicting ligand binding affinities. However, the addition of the hydrophobicity partition term to the training set did not significantly enhance the R-Squared value (0.03), suggesting its limited influence on binding free energy predictions in this context (**Figure S5B**). MD simulations of **THCV**, **THCB**, **THC** and **THCP** and **AM11542** in the L- or S-pocket were performed to investigate their effects on CB_1 activation *via* their interaction with toggle residues and conformational changes in the CB_1 transmembrane helices. CB_1 activation is characterized by the outward movement of TM5, TM6 and TM7 after the ligand interacts with toggle residues F200 and W356, which opens the G-protein binding pocket. This provides better correlation between MM-GBSA and *Ki* value (R^2= 0.94, **Figure S5C**) showing the importance of the alkyl chain towards right pocket. GPCR activation and conformational change can take a long time but occurred rather quickly in our simulations with changes observable within the first 200 ns of simulations. MD simulations were also extended up to 1500 ns, however this only resulted in the eventual movement of ligands out of the long hydrophobic pocket and once ligands (agonists or partial agonists) were no longer interacting with the toggle residues the receptor quickly converted to the inactive conformation, thus the analysis focused on the time frame were ligands remained within the pockets and interacting with the toggle residues to compare differences in receptor activation in these states.

In the case of **AM11542**, an agonist, clear activation and helix movement is observed when compared to the inactive receptor (**Figure A**). For **THCV**, very little movement is observed in the

helices (**Figure B**). This agrees with the experimental observations that it is an antagonist as receptor activation is not observed. In the cases of **THCB**, **THC** and **THCP**, some helical movement is observed and a partial opening of the g-protein binding site (**Figure C**, **D**, and **E**). The most notable change was observed in **THCP** which showed the largest movement of TM5 and TM6, though changes were not as pronounced as in **AM11542**, as **THCP** had begun migrating out of the binding pocket. In all cases besides **THCV**, the ligands bound with the side chain in the L-pocket exhibited greater movement in the helices than those in the S-pocket. This partial opening of the G-protein binding site could be the reason that some ligands act as partial agonists or antagonists. It could open just enough for the G-protein to be able to bind, however as it is not fully open binding is decreased and overall, a lower response is observed.

An examination of the distances between the helices, specifically helix 5 and helix 6 shows an interesting trend (**Table 3**). These helices move the most during receptor activation to open the G-protein binding site. **AM11542**, a strong agonist showed the greatest movement of the helices, consistent with full activation. In nearly all cases the ligand with the alkyl chain in the L-pocket resulted in greater receptor activation than when placed in the S-pocket. The exception being **THCV**, however both L and S conformations showed minimal movement and is consistent with an antagonist.

Taking a closer look at the toggle switches following MD simulations, the reason for the partial loop movement can be observed. The alkyl chain of **AM11542** extends deep into the CB_1 pocket, hitting both F200 and W356 toggle switches and significant movement is observed in both (**Figure 8A**). In the case of **THCV**, little movement is observed in the toggle residues, with a slight shift in F200 but not enough to trigger activation (**Figure 88B**). **THCB** sits deeper in the pocket and as a result in addition to this slight shift in F200, W356 also experience a slight shift downwards

(**Figure 8C**). **THC** interacts effectively with F200, and a significant rotation is observed (**Figure 88D**). Lastly, **THCP** sits significantly deeper in the pocket and can interact with both toggle

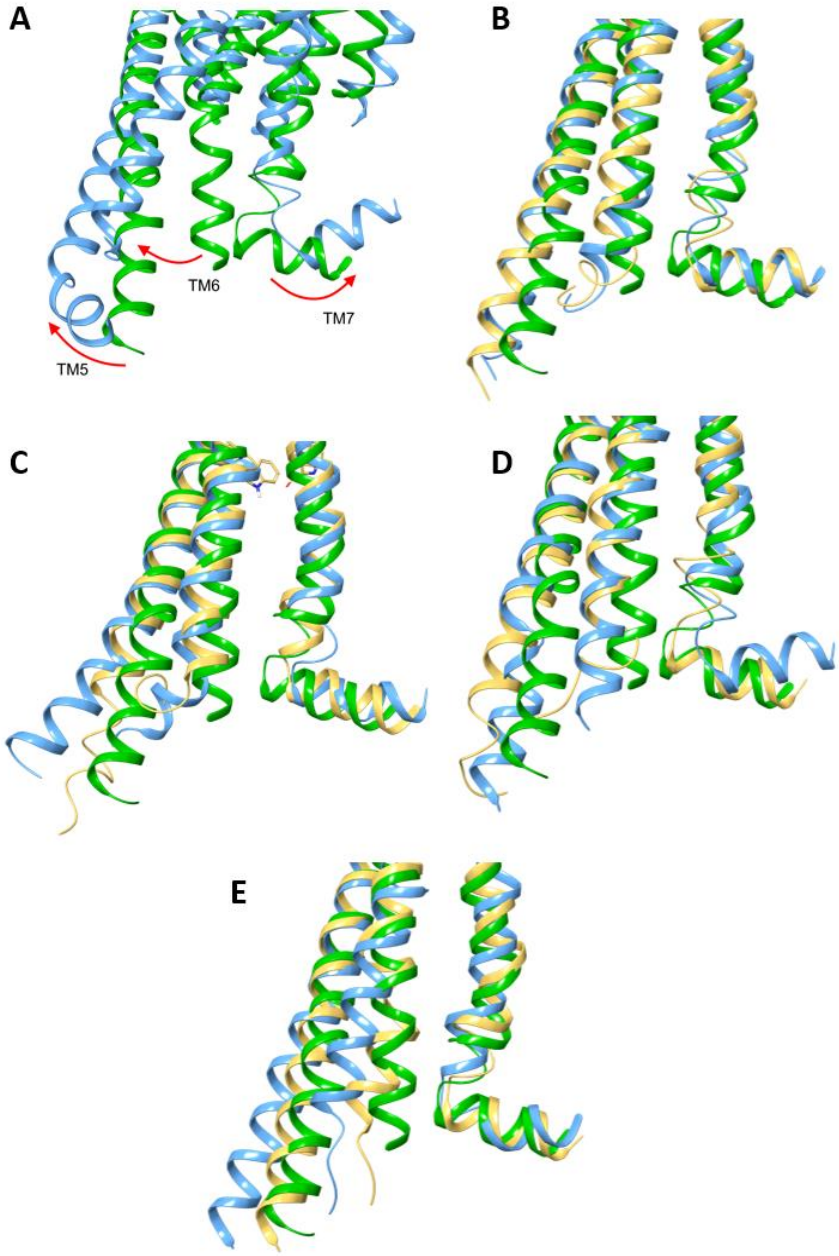

Figure 7 Superimposed structures of CB1 post MD simulation showing helix movement and receptor activation. Receptor with no ligand is represented in green, receptor with the ligand L-pocket in blue and receptor with the ligand originating in the **S-pocket** in orange. (A) **AM11542** (B) **THCV** (C) **THB** (D) **THC** (E) **THCP**.

residues in a manner like **AM11542** (**Figure 8E**). In all cases, the ligands in the L-pocket resulted in a more significant movement of toggle residues compared to those in the S-pocket.

Table 3 Distances between helix 6 and helix 5 from helix 1. Used as a measure for how open the G-protein binding site is.

	Distance between intracellular ends of H6 and H1	Difference from inactive receptor	Distance between intracellular ends of H5 and H1	Difference from inactive receptor2
Receptor	23.15	0.00	22.72	0.00
AM11542_L	29.94	6.78	29.34	6.63
AM12033_L	26.51	3.35	26.01	3.29
AM841_L	27.04	3.89	25.76	3.05
THCV_L	23.83	0.67	26.24	3.52
THCV_S	25.63	2.47	24.51	1.80
THCB_L	27.72	4.57	28.31	5.59
THCB_S	23.67	0.51	25.38	2.66
THC_L	26.85	3.70	26.98	4.27
THC_S	25.14	1.99	26.69	3.97
THCP_L	26.67	3.52	28.35	5.63
THCP_S	25.01	1.86	24.67	1.95

This indicates that the ability of the ligands to interact with these toggle residues is key to receptor activation and that smaller ligands with shorter chains fail to induce the structural changes required for full activation. Instead, what occurs is a partial activation, characterized by partial movement of TM5, TM6, and TM7, which correlates to the degree of how well the ligands can interact with either F200, W256 or both. This explains why some ligands such as **THC** behave as partial agonists, despite their high binding affinities and provides insight into the mechanism of partial agonists. This also highlights the importance of looking beyond the binding affinity when designing new ligands for receptors. The method through which they enter the binding pocket, in this case through the lipid membrane, is a key factor, along with the exact binding mode and residues that ligand interacts with. Depending on the active site residues that are interacted with, vastly different biological effects can be observed.

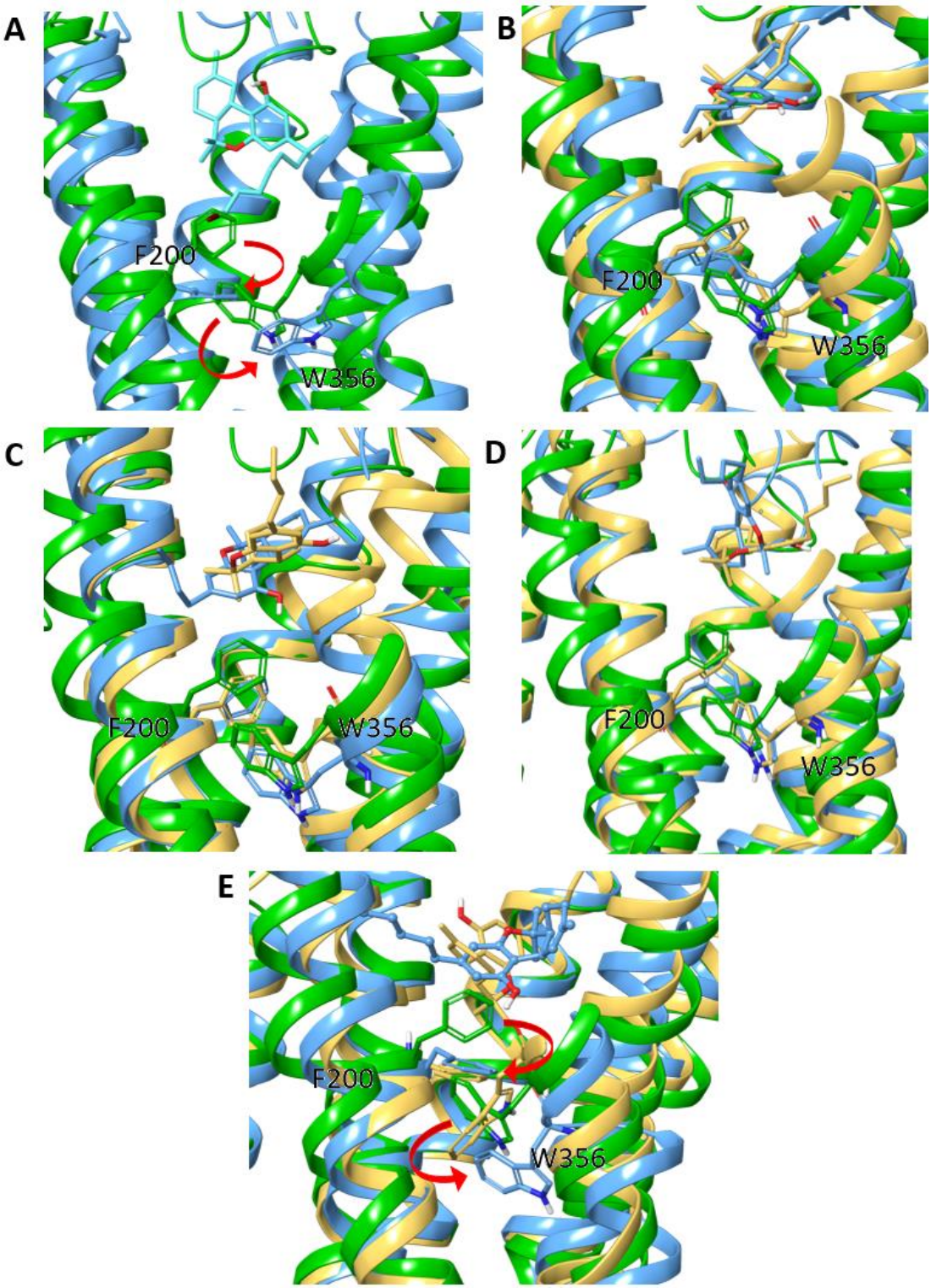

Figure 8 Superimposed structures of CB1 post MD simulation showing positions of toggle switches F200 and W356. Receptor with no ligand is represented in green, receptor with the ligand originating in the L-pocket in blue and receptor with the ligand originating in the S-pocket in orange. (A) **AM11542** (B) **THCV** (C) **THCB** (D) **THC** (E) **THCP**.

4.0 Conclusion

The hydrophobicity of the ligands was found to be essential for modelling and predicting binding affinity as the ligands enter CB_1 through the membrane. We developed a model for predicting binding affinity and activity of cannabinoids which can be used for further drug design efforts in the design of new cannabinoid-based ligands. We also determined that the binding pocket which the alkyl chain of cannabinoids occupy in the orthosteric site has a significant impact on their ability to activate the receptor and whether the ligands will act as agonists or antagonists. The ligands have to be able to interact with the toggle residues P200 and W356. How well they interact with the toggle residues also determines the degree of structural change in the receptor. Full agonists induct a larger conformation change in the toggle residues and subsequently helices 5, 6, and 7, move outwards to open the G-protein binding site. Partial agonists and antagonists were found to adopt an intermediate structure, where the binding site was neither fully open nor fully closed, which could be the cause of reduced activity, despite high binding affinity of ligands. This explanation is likely extendable to other GPCRs with partial agonist activity and a toggle switch. Combined this gives a more thorough understanding of how ligands interact with CB_1 and receptor activation, which in turn can be used to design and evaluate new cannabinoids.

Data Availability

All input and output files for the computational analyses can be obtained from the deposited data available from the Borealis Dataverse at http://doi.org/10.5683/SP3/3KJKR8.

Supporting Information

Full computational methodological details, additional tables and figures showing the binding mode of various ligands discussed are also provided. A video showing the comparison of the agonistic and non-agonistic binding modes of THC is also provided.

Competing Interests

JFT, DM and FS are all associated with Binary Star Research Services (BSRM). This generates an apparent conflict of interest. BSRM has no commercial interests in the subject of this manuscript and holds no intellectual property related to this manuscript. The interests of BSRM had no input into the methodology, research goals, or conclusions of this manuscript, and the company does not benefit from the publication of this manuscript nor did any of the authors receive any benefit from BSRM from its preparation or publication. BSRM provided no funding for this project.

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Author Contributions

Conceptualization, JFT and FS; Funding acquisition JFT; Investigation, DM, FS, SM; Methodology, All authors ; Visualization, DM, FS; Project administration, JFT; Graphical

abstract, SM; Supervision, JFT; Writing original draft, FS & DM; Writing – review and editing, All authors.

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