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# The interaction of cannabinoid receptor agonists, CP55940 and WIN55212-2 with membranes using solid state $^2$H NMR

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ABSTRACT

Two key commonly used cannabinergic agonists, CP55940 and WIN55212-2, are investigated for their effects on the lipid membrane bilayer using $^2$H solid state NMR, and the results are compared with our earlier work with delta-9-tetrahydrocannabinol ($\Delta^9$-THC). To study the effects of these ligands we used hydrated bilayers of dipalmitoylphosphatidylcholine (DPPC) deuterated at the 2′ and 16′ positions of both acyl chains with deuterium atoms serving as probes for the dynamic and phase changes at the membrane interface and at the bilayer center respectively. All three cannabinergic ligands lower the phospholipid membrane phase transition temperature, increase the lipid *sn-2* chain order parameter at the membrane interface and decrease the order at the center of the bilayer.

Our studies show that the cannabinoid ligands induce lateral phase separation in the lipid membrane at physiological temperatures. During the lipid membrane phase transition, the cooperative dynamic process whereby the C-$^2$H segments at the interface and center of the bilayer spontaneously reach the fast exchange regime ($^2$H NMR timescale) is distinctively modulated by the two cannabinoids. Specifically, CP55940 is slightly more efficient at inducing liquid crystalline-type $^2$H NMR spectral features at the membrane interface compared to WIN55212-2. In contrast, WIN55212-2 has a far superior ability to induce liquid crystalline-type spectral features at the center of the bilayer, and it increases the order parameter of the *sn-1* chain in addition to the *sn-2* chain of the lipids. These observations suggest the cannabinoid ligands may influence lipid membrane domain formations and there may be contributions to their cannabinergic activities through lipid membrane microdomain related mechanisms. Our work demonstrates that experimental design strategies utilizing specifically deuterium labeled lipids yield more detailed insights concerning the properties of lipid bilayers.



## 1. Introduction

It is generally accepted that the phospholipid microenvironment can influence membrane protein function, and indeed recent pharmacological studies indicate that lipid membrane integrity has a profound effect on the functions of cannabinoid receptors [1–3]. Thus, it is beneficial to carefully evaluate the perturbation effects of widely used cannabinergic ligands on membrane systems in order to obtain a more complete understanding of their pharmacological profiles. Our earlier studies on the ability of classical cannabinoids ($\Delta^8$-THC/$\Delta^9$-THC) to perturb membrane bilayers have suggested that cannabinoid biological activities may be exerted in part by modulating the thermodynamic properties of the cellular membrane bilayer [4–7].

A series of NMR studies showed that the site and geometry of hydroxyl groups on classical cannabinoids can affect the orientation of the ligand in a membrane environment [6–13]. In $\Delta^9$-THC the phenolic hydroxy group associates with the hydrophilic head groups of the phospholipids within the bilayer. This causes the long axis of the molecule to orient perpendicular to the phospholipid chains, conversely, the O-methyl, biologically inactive analog orients with its long axis parallel to the phospholipid acyl chains. Cannabinergic ligands carrying more than one polar hydroxyl group orient in a manner that allows all hydroxyl groups to orient towards the corresponding polar membrane interface [6–14].

Amphipathic conformational characteristics were defined in solution studies of the conformationaly flexible non-classic CP55940 compound (Fig. 1). In a chloroform solution, all three hydroxyl groups were oriented on the same face of the bicyclic rings, most likely stabilized by hydrogen bonding between them [15]. The dimethylheptyl side chain adopted one of four preferred conformations, in all of which the chain is almost perpendicular to the phenol ring. A study of a two conformationaly restrained, diastereomeric nonclassical cannabinoids, CP55,244 and CP97,587, showed that hydrogen bonding between hydroxyl groups is indeed responsible for an increase in the conformational population in which all hydroxyl groups are located on the same face of the ring system [16].

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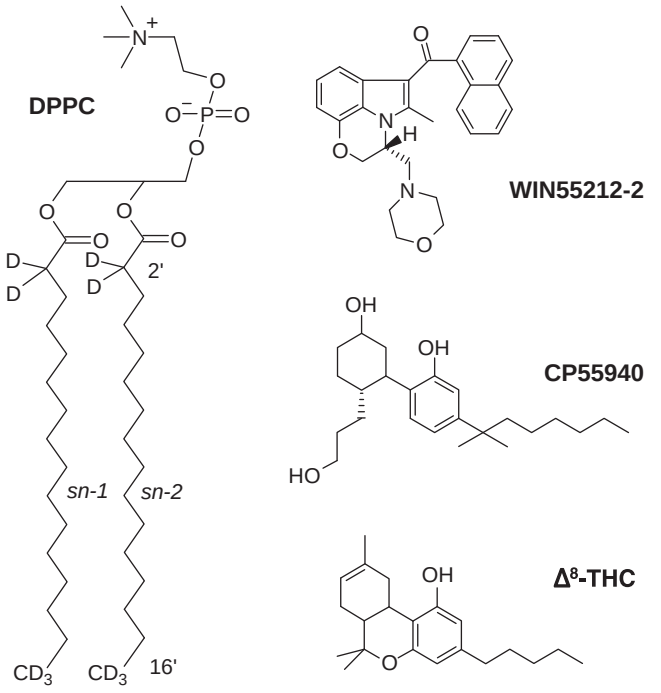


**Fig. 1.** Structure of CP55940, WIN55212-2, $\Delta^8$-THC and dipalmitoyl phosphotidylcholine (DPPC) with the labeled 2′ and 16′ positions highlighted.

Conformational studies of $\Delta^8$-THC and Me-$\Delta^8$-THC in bicelles [17] measured NOE's proving that, in a bilayer, $\Delta^8$-THC was indeed in the "amphipathic conformation". No such NOEs were observed for Me-$\Delta^8$-THC congruent with the notion that the long axis of this more lipophilic analog is aligned with lipid molecules so that the side chain group extends away from the ring system [18,19]. Anisotropic measurements further proved that $\Delta^8$-THC and Me-$\Delta^8$-THC are oriented perpendicular and parallel, respectively, in the bicelle environment consistent with their observed conformations.

In this study, we measure the interactions, within phospholipid model membranes, of CP55940 and WIN55212-2; two ligands that have been widely employed as pharmacological benchmark compounds [20,21] and whose conformational properties have been studied [15,22]. The biological effects of those ligands have been primarily attributed to their interactions with membrane associated cannabinoid receptors, members of the GPCR rhodopsin family [23–25]. Both compounds are potent cannabinoid receptor agonists, but have distinctly different structural features [26–28].

Membranes prepared from hydrated, fully deuterated phospholipids have been widely employed to study ligand effects on membranes, including, determination of order parameter profiles using solid state $^2$H NMR methods [29–31]. A recent study describes the effects of a series of cannabinoids on the order parameter along the length of the lipids in oriented bicelles. However, $^2$H NMR spectra and quadrupolar splittings obtained from fully deuterated bilayers are severely overlapped and prevent a detailed spectral analysis. Thus, differences between *sn-1* and *sn-2* chains of DPPC are not discernible [29]. Additionally the thermotropic spectral changes accompanying phase transitions within the membrane cannot be studied. For these reasons, the DPPC phospholipid molecule used in this study was synthesized with $^2$H specifically labeled only at the 2′ or 16′ positions on each of the DPPC acyl chains (Fig. 1). Thus we specifically monitor the ligand effect at the bilayer center and near its interface and are able to detect subtle, ligand induced, changes in the local dynamics of the specific lipid methylene segments through careful observation of spectral changes. Our results contribute to the understanding of the role of phospholipid membranes in the molecular mechanism of cannabinergic function.

## 2. Methods

### 2.1. Materials

The specifically deuterium labeled phospholipid molecules 1,2-[2′,2′-$^2H_2$]-DPPC and 1,2-[16′,16′,16′-$^2H_3$]-DPPC were synthesized in our laboratory according to literature procedures [32], unlabeled DPPC was purchased from Avanti Polar Lipids, Birmingham, AL. WIN55212-2 and CP55940 were obtained from the National Institute of Drug Abuse. Deuterium depleted water and chloroform were purchased from Aldrich.

### 2.2. Sample preparation

Appropriate amounts of each cannabinergic ligand and specifically deuterated DPPC phospholipid were dissolved in 2 ml of chloroform to produce a 0.1 drug/lipid molar ratio solution. The solvent was then evaporated by passing a stream of nitrogen over the solution at room temperature and the residue was placed under vacuum (0.1 mm Hg) for 12 h. The MLV phospholipid membrane with a 50:50 (w/w) lipid/water hydration level was produced by adding an appropriate amount of deuterium depleted water to the dried sample which was subjected to a freeze-thawing and vortexing procedure as described previously [5]. The membrane preparation was then introduced into a 5-mm glass tube and flame sealed under vacuum.

### 2.3. $^2$H-solid state spectroscopy

NMR experiments were performed on a Chemagnetics solid state spectrometer with a static wideline probe operating at a magnetic field of 6.9 T (46.6 MHz for $^2$H), using the quadrupole echo pulse sequence, $[(\pi/2)_x-\tau-(\pi/2)_y]$, with $\tau = 35$ μs and a recycle delay of 200 ms. Sample temperatures were regulated by the Chemagnetics temperature controller. Before recording the spectra, samples were held at 50 °C for 15 min to ensure complete equilibrium, and subsequently held at the designated temperature for another 10 min. $^2$H NMR experiments from each ligand containing sample were performed parallel with the control sample of hydrated DPPC bilayers.

## 3. Results

### 3.1. Temperature dependent $^2$H Spectra of 1,2-[16′,16′,16′-$^2H_3$]-DPPC phospholipid membranes

Fig. 2 depicts the temperature dependent static solid state, $^2$H NMR spectra obtained from DPPC phospholipid membranes into which CP55940 or WIN55212-2 where incorporated at 10 mol%. At temperatures above 41.5 °C, the control spectra are in the liquid crystalline phase (Fig. 2A). The six magnetically equivalent deuterons on each DPPC molecule give rise to a single well-defined "Pake Pattern" with a quadrupolar splitting of $\Delta\upsilon_Q = 3.2$ kHz. At temperatures below 41.5 °C, the control spectra undergo drastic broadening, an indication that the lipid membrane undergoes a sharp phase transition. This observation is consistent with the results from DSC experiments [5] and is attributable to a significant reduction of the motion of C-$^2H_2$ segments on the lipid acyl chains in the gel phase.

Fig. 2B and C depict the temperature dependent $^2$H NMR spectra of the 16′-labeled phospholipid bilayer acyl chains from 30 °C to 45 °C, obtained with the presence of 10 mol% of CP55940 and WIN55212-2. The results indicate that both ligands reduce the phase transition temperature as both CP55940 and WIN55212-2 exhibit liquid crystalline lineshapes at 39 °C. At 37 °C there is a clear spectral superposition of characteristic liquid crystal and gel lineshapes clearly indicating the co-existence of these two components. As the temperature is lowered, the gel spectral features become more prominent. However, there are also distinct differences between the

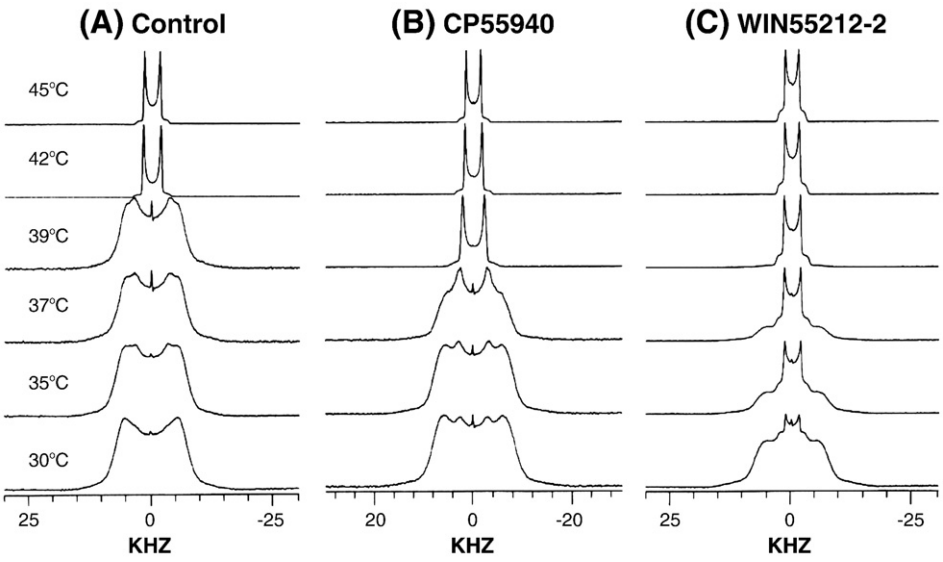


**Fig. 2.** Temperature dependent solid-state $^2$H NMR spectra obtained from fully hydrated multilamelar vesicles prepared from 1, 2[16′,16′,16′-$^2H_3$]DPPC. (A) Control, (B) with 10 mol% CP55940, and (C) with 10 mol% WIN55212-2.

two preparations. In the CP55940 spectra, the sharp liquid crystalline features persist until 39 °C; while at 37 °C, there is a broadening of the peaks and merging with the gel like spectral features which progressively dominate the lower temperature spectra. Conversely, in the WIN55212-2 spectra, the sharp 90° edges of the liquid crystal spectrum remain distinct down to 30 °C, while their spectral contribution gradually diminishes.

We have also compared the $^2$H splittings associated with the liquid crystal spectral component. At 45 °C the quadrupolar splittings are reduced from 3.2 kHz for the control, to 2.9 kHz and 2.7 kHz respectively for the CP55940 and WIN55212-2 preparations. This suggests that at the center of the bilayer, each of the ligands reduces the acyl chain order parameter and "enhanced fluidity" with WIN55212-2 having the more prominent effect.

### *3.2. Temperature dependent $^2$H spectra of 1,2-[2′,2′-$^2H_2$]-DPPC phospholipid membranes*

The $^2$H-labels at the 2′ positions of the *sn-1* and *sn-2* chains help to monitor the phase and the order parameter changes at the lipid membrane interface induced by each of the ligands as a function of temperature. Fig. 3 shows the temperature dependent spectra of the ligand free phospholipid membrane control and the 10 mol% CP55940 or WIN55212-2 membrane preparations. At temperatures above 41.5 °C, the control spectra exhibit complete liquid crystalline features (Fig. 3A) and give rise to three quadrupolar splittings of 12.0, 17.8 and 26.7 kHz, as opposed to a single quadrupolar splitting from 1,2-[16′,16′,16′-$^2H_3$]-DPPC (Fig. 2A). According to an earlier assignment at 45 °C, the 17.8 kHz and 12.0 kHz couplings arise from the stereochemically inequivalent 2′R-$^2$H and 2′S-$^2$H on the *sn-2* chain, and the 26.7 kHz coupling arises from the 2′-$^2H_2$ on the *sn-1* chain [5]. As the temperature is lowered, the control spectra undergo broadening, consistent with phase transformation (Fig. 2A). This progressive change of the deuterium NMR lineshape is attributable to a significant reduction of the rate of *trans–gauche* interconversion of deuterated methylene segments of the lipid acyl chains near the interface.

At 45 °C, the spectra from both CP55940 (Fig. 3B) and WIN55212 (Fig. 3C) membrane preparations show completed and well-resolved quadrupolar splittings, characteristic of a liquid crystal phase [31]. At 41.5 °C, shoulders arising from the gel-like phase become visible for

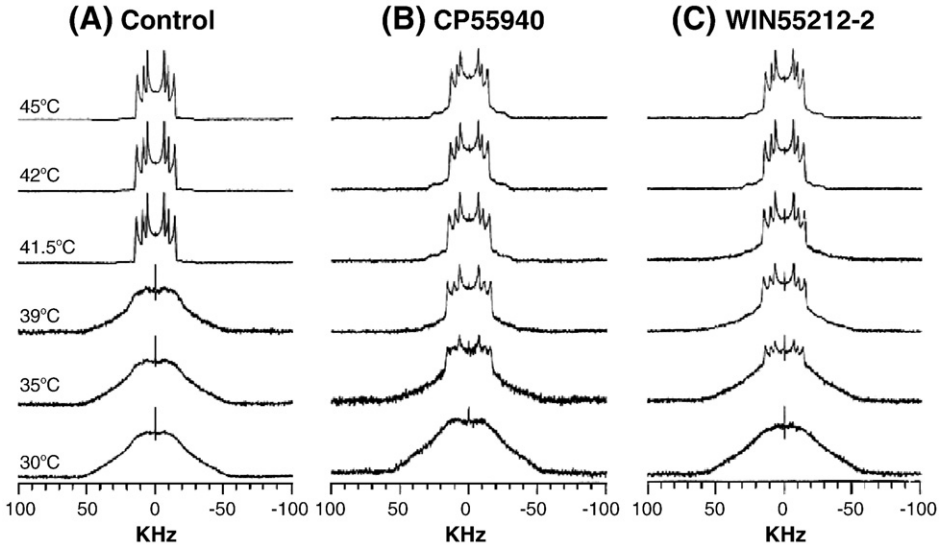


**Fig. 3.** Temperature dependent solid-state $^2$H NMR spectra obtained from fully hydrated multilamelar vesicles prepared from 1, 2[2′,2′-$^2H_2$]DPPC. (A) Control, (B) with 10 mol% CP55940, and (C) with 10 mol% WIN55212-2.

both ligand samples, suggesting that the ligands induce phase separation and restricted the rate of the 2′ segment trans–gauche interconversion near the phospholipid head group and interface. This effect gradually increases as the temperature is lowered but is more prominent in the WIN55212-2 spectra.

At 39 °C, the spectrum from the control membrane shows a flat top, conical shape, while the CP55940 and WIN55212-2 spectra show a sharp Pake pattern protruding from broadened rounded shoulders. This is attributable to a superimposition of spectral features characteristic of liquid crystalline and gel phases suggesting their individual contributions within the sample. The contribution from the gel phase components progressively increases for both compounds as the temperature is lowered however at lower temperatures there is a larger contribution from the liquid crystal phase in the CP55940 spectra (Fig. 3B&C). At 30 °C, for example, the spectra from the ligand free control membrane and the WIN55212-2 containing sample show typical gel phase spectral features with a conical shape and a rounded top. However, the CP55940 spectrum has a discernable flat top appearance suggesting higher mobility for a detectable population of DPPC molecules at this temperature. This suggests that CP55940 more effectively maintains liquid crystalline conditions at the membrane–water interface and may induce phase separation at these temperatures.

The quadrupolar coupling $\Delta\upsilon_Q$ values due to the [2,2′-$^2H_2$] segment of DPPC as measured at 45 °C are also modulated by incorporation of cannabinoid ligands (Table 1). In the liquid crystalline phase, the lipid segment order parameter $S_{CD}$ may be directly derived from the experimental $^2$H NMR spectra. $S_{CD}$ is related to the quadrupolar splitting $\Delta\nu_Q$ between the 90° singularities by $\Delta\nu_Q = -3/4(e^2qQ/h)S_{CD}$, where $e^2qQ/h$ is the static quadrupolar coupling constant. Both CP55940 and WIN55212-2 increased the $\Delta\upsilon_Q$ values for both deuterons of the *sn-2* chain. These increases could result from an increase of the order parameter of the 2′-methylene segments of the *sn-2* chain. The increased order parameter may be due to inter-molecular hydrogen bonding between the lipid *sn-2* carbonyl group and the hydroxyl groups on the CP55940 or the morpholino group of WIN55212-2. The $\Delta\upsilon_Q$ value of the 2′-$^2H_2$ on the *sn-1* chain of DPPC is also sensitive to the presence of the cannabinoid ligands. WIN55212-2 increases the $\Delta\upsilon_Q$ value from 26.7 kHz to 27.3 kHz, while CP55940 has only a marginal effect on the *sn-1* dynamics at this region, with a slightly altered quadrupolar splitting of 26.6 kHz. Thus the order parameter changes in the *sn-1* chain are more sensitive to WIN55212-2.

## 4. Discussion

Lipid membrane preparations composed of specifically $^2$H labeled DPPC, hydrated multi-lamellar bilayers provide a relevant model for studying drug–membrane interactions. While full deuteration of phospholipids may reduce the phase transition temperature of the bilayer [33], we have found from DSC and $^2$H temperature dependent spectra (Figs. 2 & 3) that selective deuteration of a single phospholipid site maintains very similar thermotropic properties as the undeuterated lipid bilayers. Specifically deuterated phospholipids are, therefore, less invasive markers for studying phospholipid membrane phase behavior, structure, and dynamic properties using $^2$H NMR. As a result, more detailed and subtle ligand effects on the phospholipid membrane system may be detected at temperature ranges close to physiological conditions. Significantly, the control (ligand free lipid membrane) samples at the same condition demonstrate a close correspondence between the collective phase behavior and the synchronized evolution of the $^2$H NMR spectral lineshape from the two different sites along the lipid acyl-chains.

Our results show that both ligands lower the main phase transition temperature of the DPPC membrane bilayer, as indicated by the presence of liquid crystal features within the $^2$H spectra at temperatures below 41.5 °C. This effect, frequently found for many anesthetics [34–36], has also been observed with other cannabinoids [4,37]. Additionally, the phase transition of the phospholipid bilayer accompanying reduction in temperature is significantly ligand dependent. Incorporation of either of the ligands, leads to $^2$H-DPPC spectral inhomogeneity, with two slowly exchanging components within the NMR timescale as indicated by the superimposed sharp and broadened spectral lineshapes. The superimposed spectra indicate a possible ligand-induced lipid membrane lateral phase separation, which is often associated with domain formation as has been demonstrated for membranes with multiple lipid components [38]. We speculate that our observed superimposed spectral features may also be the result of cannabinoid-induced lipid membrane lateral phase separation and domain formation. However, the data may also be accounted for by a uniform distribution of ligand throughout the membrane, undergoing a slow or intermediate exchange between two distinct conformations on the NMR time scale.

### 4.1. Differential ligand effects at the center and interface of the lipid bilayer

A careful examination of the $^2$H-spectra in Figs. 2 and 3 reveals that the ligand-associated effects on the bilayer interface are distinctly different from those at the center of the bilayer. The spectra from the labeled lipid segments at the surface and the center of the bilayer demonstrate "out-of-sync" or un-coordinated $^2$H NMR spectroscopic features at temperatures that the lipid undergoes the "thermotropic phase transition". In contrast, the spectra obtained from the control, non ligand-containing lipid membrane samples show congruent spectra from each label at any given temperature. Thus, the presence of WIN55121-2 and CP55940 alters the timescale profile of the lipid chain trans–gauche interconversion dynamics or the local segment order parameter at different temperatures. Perturbations of the bilayer by the ligands may result in specific chain segments to fall in the fast exchange regime on the $^2$H NMR timescale ($10^{-5}$ s), that yields "liquid crystalline-type" spectral features in comparison to our control experiments. This suggests a ligand-induced timescale discontinuity between the interface and the central component of the bilayer acyl chains during the phase transition.

A close inspection of the spectra allows us to estimate the fraction of liquid crystalline-type spectral component within each spectrum. Based on the contribution of gel-type and liquid crystalline-type spectral components, our results indicate that at 39 °C, for the 1, 2-[2′,2′-$^2H_2$]-DPPC membranes (Figs. 3 & 4), approximately 90% of the total lineshape is dominated by liquid crystal features for the CP55940 sample as opposed to approximately 50% for the WIN55212-2 sample. Conversely, the data from the 1,2-[16′,16′,16′-$^2H_3$]-DPPC (Figs. 2 & 4), indicate that at 39 °C, the center of the phospholipid bilayer is entirely in fast exchange regime and gives almost completed liquid crystalline-type spectra for both cannabinoid ligands. This suggests that at 39 °C, the ligands may induce a lateral phase separation in DPPC lipid membrane such that a portion of the phospholipids remains in the gel phase with the majority of the lipids in the liquid crystalline phase. Interestingly, CP55940 is more effective compared to WIN55212-2 at

**Table 1**
Solid state $^2$H NMR quadrupolar splittings ($\Delta\upsilon_Q$) from $^2$H-labeled DPPC bilayers in the absence and presence of 10% CP55940 and 10%WIN55212-2, measured at 45 °C.

| $^2$H-label positions on DPPC | Quadrupolar splitting $\Delta\upsilon_Q$ (kHz) | | | |
|---|---|---|---|---|
| | Control | CP55940 | WIN55212-2 | THC[a] |
| 1,2-[16′,16′,16′-$^2H_3$] | 3.2 | 2.9 | 2.7 | 2.3 |
| 1-[2′,2′-$^2H_2$] | 26.7 | 26.6 | 27.3 | 27.1 |
| 2-[2′S-$^2$H] | 12.0 | 13.1 | 12.9 | 14.1 |
| 2-[2′R-$^2$H] | 17.8 | 18.1 | 18.7 | 18.9 |

[a] Data extracted from reference [5].

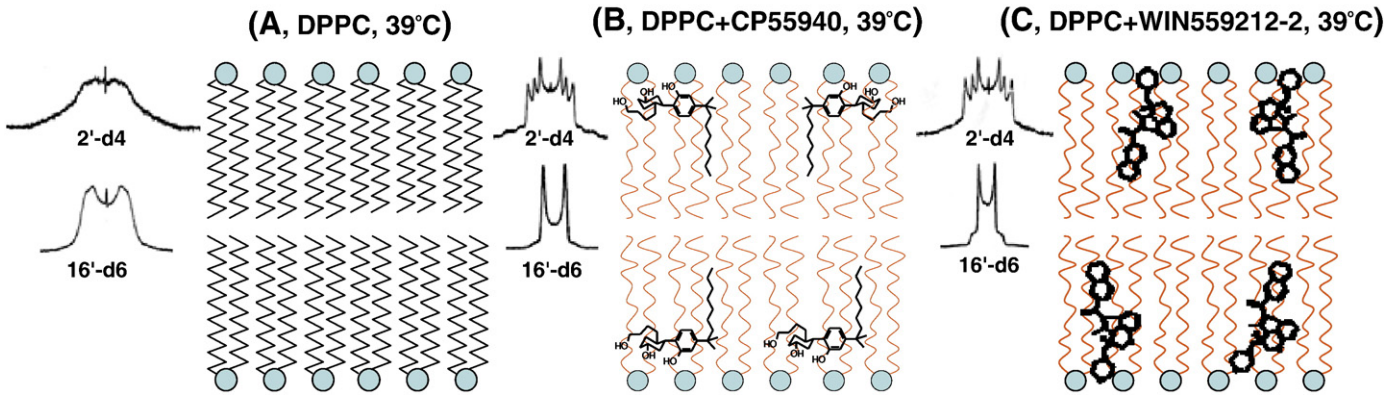


**Fig. 4.** Schematic representation of cannabinoid ligand location, orientation and effects on the lipid membrane phase at 39 °C. The $^2$H spectra measured from the bilayer interface (2′-$^2$H labels) and from the center of the bilayer (16′-$^2$H labels) are also shown. A) Pure DPPC lipid membrane is in the gel phase and both interface and center deuterium labels give broadened lineshapes. B) DPPC membranes with CP55940 are in a liquid crystalline phase. The likely position and orientation of CP55940 is shown. C) WIN55212-2 also lowers the lipid phase transition temperature. It anchors at the membrane interface most likely through its morpholine ring and extends its flexible hydrophobic aromatic rings deep into the bilayer center.

inducing the liquid crystalline-type of spectral features near the surface of the membrane.

At lower temperatures, the differential effect on the spectral features between the two sites within the ligand-containing membrane is more discernible and is clearly ligand dependant. For the CP55940 sample at 35 °C, the spectra from the 16′ deuterium label indicate that the membrane center is predominantly in a gel phase type of slow motion, while the well-resolved quadrupolar splitting fraction in the spectra from the labels at 2′ position indicates that a significant part of the phospholipid membrane at the interface is in a fast exchange regime. Conversely, for the WIN55212-2 containing membrane at 35 °C, the spectrum of the interface $^2$H-labels at the 2′ position exhibits a predominantly gel-type lineshape. The 16′ $^2$H labels at the bilayer center exhibit a superimposed spectrum with a substantial proportion of the liquid crystalline-type component with sharp 90° edges, which indicates that WIN55212-2 keeps a considerable fraction of the C-$^2$H segments at bilayer center in the fast exchange regime.

This differential effect is particularly strong in the WIN55212-2 spectra at 30 °C, where the bilayer interface yields an entirely gel-type lineshape, while the spectrum from the bilayer center gives a considerable fraction of liquid crystalline-type spectral component. This indicates a significant portion of the lipid segments at the center is in a fast exchange regime. We attribute the above observations to cannabinoid ligand-induced microscopic defects at the ligand-specific site(s) within lipid bilayer, which may cause a fraction of the lipid chain segments to fall in a fast exchange regime in the $^2$H NMR timescale and result in liquid-crystalline-like spectral features. Fig. 5 illustrates our proposed interpretation for the case of WIN55212-2. Depending on the distribution properties of the ligand in the lipid membrane, these ligand-induced membrane defects could be either clustered (Fig. 5B) or dispersed (Fig. 5C), within the lipid bilayer.

### *4.2. WIN55212-2 and CP55940 alter the lipid bilayer order parameter differently*

The quadrupolar splitting ($\Delta\upsilon_Q$) data obtained from the phospholipid membrane in the liquid crystalline phase reflects the local order parameter induced by the cannabinergic ligands. Table 1 summarizes the $\Delta\upsilon_Q$ values from the $^2$H spectra of two sets of specific $^2$H-labeled DPPC preparations in the absence and presence of 10% CP55940 and WIN55212-2 at 45 °C. $\Delta\upsilon_Q$ data obtained from the earlier studies of $\Delta^8$-THC/DPPC system are also included in the table for comparison.

The presence of either CP55940 or WIN55212-2 increases the $\Delta\upsilon_Q$ values of the 1,2-[2′,2′-$^2H_2$] segments of DPPC lipid membrane, as shown in Table 1. This is interpreted as an increase of order parameter of the 2′-methylene segments imposed by the presence of the ligand [5]. As the increase in $\Delta\upsilon_Q$ is more significant for the *sn-2* chain, we conclude that both ligand molecules produce ordering effect on the *sn-2* chain at this site. $\Delta^8$-THC was also found to possess a capability to increase the order parameter on the *sn-2* chain and the effect is more profound when compared with CP55940 and WIN22512-2, as demonstrated by the larger corresponding increase in $\Delta\upsilon_Q$ values (Table 1). In the case of *sn-1* chain, CP55940 shows a negligible effect on its order parameter at the

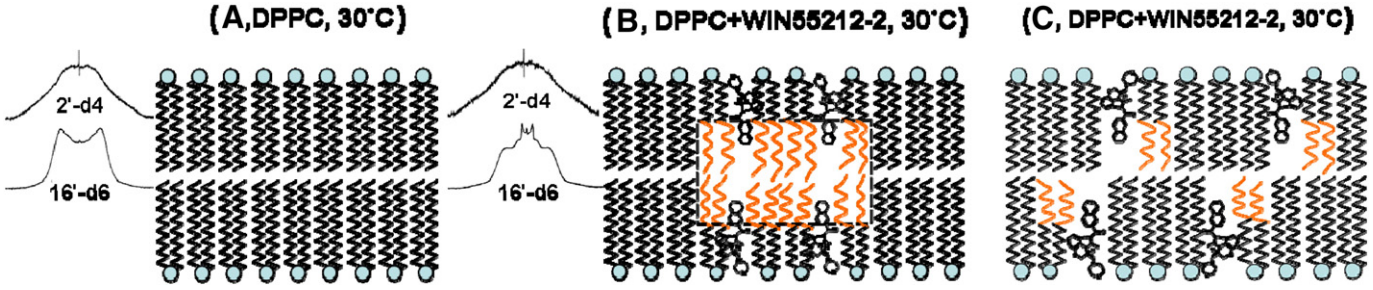


**Fig. 5.** The $^2$H NMR spectra, and illustration of the interpreted effects of WIN55212-2 on the DPPC lipid membrane at 30 °C. The ligand-free DPPC membrane bilayer in the gel phase gives broadened gel-type spectral lineshapes from the deuterium labels at the interface and the center of the lipid bilayer (A). With 10 mol% WIN55212-2, a broadened $^2$H-NMR spectral lineshape from the lipid bilayer interface, and a superimposed gel/liquid crystalline-type lineshape from the bilayer center are shown. Cannabinoid ligand-induced microscopic defects at the lipid bilayer center may cause a fraction of the lipid chain segments to fall in a fast exchange regime (orange color) on the $^2$H NMR timescale resulting in liquid-crystalline-like spectral features. Depending on the distribution property of the ligand in the lipid membrane, these lipid defects could be either clustered (B) or dispersed (C), within the lipid bilayer.

membrane interface, whereas, WIN55212-2 demonstrates a discernable ordering effect on 2′-methylene of the *sn-1* chain similar to that observed previously with $\Delta^8$-THC(Table 1).

The $^2$H-quadrupolar splitting data demonstrate that in the liquid crystalline phase both CP55940 and WIN55212-2 increase the order parameter at the lipid interface and suggest that both ligands are localized toward the membrane surface near the interface (Fig. 4). In an earlier publication involving the conformational analysis of CP55940, all three hydroxyl groups of CP55940 were found to orient toward the upper face of the molecule that allows for a preferred orientation within the membrane [5,9,15,18,19]. This orientation favors a close interaction between both the cyclohexane and phenolic rings of CP55940 with the phospholipid membrane interface (Fig. 4B). This orientation resembles that observed for $\Delta^8$-THC that localizes the tricyclic terpene structure near the bilayer surface with the long axis of its tricyclic structure perpendicular to the phospholipid chains. The tricyclic structure of $\Delta^8$-THC results in greater increase of order parameter in the liquid crystal phase than the bicyclic structures of CP55940 and WIN55212-2, both of which have greater conformational flexibility.

We hypothesize that the interaction with the more flexible aminoalkylindole WIN55212-2 occurs through association of the morpholino group with polar components of the phospholipid head groups (Fig. 4C). This would allow the bulky hydrophobic rings to extend into the membrane interior where they may be accommodated within the hydrophobic acyl chains [22]. Both CP55940 and WIN55212-2 are located near the bilayer interface where they assume distinct orientations and exercise acyl chain ordering effects at the level of the methylene groups near the interface. The differential effect of CP55940 and WIN55212-2 on the phospholipid membrane, as reflected by the characteristics of the corresponding $^2$H NMR spectra, may be attributed to their differences in the specific ligand location, conformation and orientation within the lipid bilayer.

The $\Delta\upsilon_Q$ values from 1,2-[16′,16′,16′-$^2H_3$] DPPC lipid membrane terminal methyl groups of both of the acyl chains decreases by 0.3 kHz and 0.5 kHz with the presence of CP55940 and WIN55212-2, respectively. This spectral narrowing is an indication of a decrease of order parameter at the bilayer center resulting from the effects of ligand modulation and shows that WIN55212-2 exerts a greater measurable disordering effect. The change of order parameter induced by CP55940 and WIN55212-2 at the bilayer center are more modest compared to the effects induced by $\Delta^8$-THC [5], which decreases the $\Delta\upsilon_Q$ by 0.9 kHz. This is consistent with our model according to which all three amphipathic ligands are anchored at the bilayer interface and away from the bilayer center (Fig. 4). At that location, the ligands induce a reduction of the order parameter at the deeper levels of the bilayer. Because of its more rigid structure, $\Delta^8$-THC is anchored more effectively and hence its higher ability to perturb the bilayer [4,9]. CP55940 and WIN55212-2 are anchored less effectively. Additionally, their greater degree of conformational freedom allows them to be better accommodated among the acyl chains resulting in less perturbation to the lipid membrane. Hence the decreases observed in the quadrupolar splitting are more modest compared to those of $\Delta^8$-THC. By contrast, if the ligands were partitioned at the center of the bilayer, we would expect a local increase of acyl chain order parameter and an increase in the quadrupolar splittings, as was observed at the bilayer surface.

In conclusion, our results show that while these cannabinoid ligands share similar attributes with respect to their effects on DPPC multilammelar membranes bilayer, there are interesting differences. The ligands lower the phospholipid membrane phase transition temperature, increase the lipid *sn-2* chain order parameter at the membrane interface and decrease the order at the center of the bilayer. The ligands may induce lipid membrane lateral phase separation at physiological temperatures. During the lipid membrane phase transition, the collective mobility that allows the interface and the center of the bilayer to reach the fast exchange regime simultaneously ($^2$H NMR timescale) is affected. While CP55940 is more effective at inducing a fast exchange regime at the membrane interface, WIN55212-2 exerts a greater effect at the center of the bilayer. In addition, in the liquid crystalline condition, WIN55212-2 increases the order parameter of both *sn-1* and *sn-2* phospholipid acyl chains whereas CP55940 only increases the order parameter of 2′-methylene segment of the *sn-2* acyl chain. The changes of the 2′-methylene order parameter of *sn-1* and *sn-2*, as reflected by the change of corresponding $\Delta\nu_Q$ values, is mostly triggered by the orientation change of the DPPC glycerol backbone [39]. Our results suggest that the two compounds may have different interaction modes with the lipid interface. Each ligand may induce a specific orientation of the DPPC glycerol backbone, which bestows the characteristic set of 2′-methylene $\Delta\nu_Q$ values on the lipid acyl chains. These observations suggest that the cannabinoid ligand effects may involve domain formations within the bilayer and contributions to their cannabinergic activities are possible through a lipid microdomain related mechanism.

The biological effects of cannabinoids are exerted through membrane proteins that include family A G-protein coupled receptors, specifically, cannabinoid receptor 1 and 2[26]. A comprehensive molecular mechanism of how these protein targets interact with their respective ligands to exert their complex biological functions has yet to be fully explained. The unique membrane effects of the two cannabinoid agonists observed in this study may contribute to the understanding of the activation and functions of these membrane proteins.

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