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Differential membrane fluidization by active and inactive cannabinoid analogues

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Abstract

The effects of the two cannabinomimetic drugs (−)-2-(6*a*,7,10,10*a*-tetrahydro-6,6,9-trimethyl-1-hydroxy-6*H*-dibenzo[*b*,*d*]-pyranyl-2-(hexyl)-1,3-dithiolane (AMG-3) and its pharmacologically less active 1-methoxy analogue (AMG-18) on the thermotropic and structural properties of dipalmitoyl-*sn*-glycero-3-phosphorylcholine (DPPC) liposomes have been studied by X-ray diffraction and differential scanning calorimetry (DSC). DSC data revealed that the incorporation of the drugs affect differently the thermotropic properties of DPPC. The presence of the more active drug distinctly broadened and attenuated both the pretransition and main phase transition of DPPC bilayers, while the inactive analogue had only minor effects. Small and wide angle X-ray diffraction data showed that the two cannabinoids have different effects on the lipid phase structures and on the hydrocarbon chain packing. The pharmacologically active analogue, AMG-3, was found to efficiently fluidize domains of the lipids in the L_{β}' gel phase, and to perturb the regular multibilayer lattice. In the liquid crystalline L_{α} phase, AMG-3 was also found to cause irregularities in packing, suggesting that the drug induces local curvature. At the same concentration, the inactive AMG-18 had only minor structural effects on the lipids. At about 10-fold or higher concentrations, AMG-18 was found to produce similar but still less pronounced effects in comparison to those observed by AMG-3. The dose-dependent, different thermotropic and structural effects by the two cannabinoid analogues suggest that these may be related to their biological activity.

Keywords: Cannabinoid; Drug; Phospholipid; Calorimetry; X-Ray

1. Introduction

The pharmacological activity of cannabinoids is believed to be controlled by two cannabinoid receptors (CB1 and CB2) [1,2]. However, certain experimental observations suggest that some physiological effects of cannabinoids can be attributed to their structural effects on cellular membranes [3,4]. In particular, cannabinoids were found to produce biochemical changes in a variety of membrane preparations, to affect membrane-related function, and to exert non-receptor-mediated effects on membrane-bound enzymes [5,6].

Cannabinoids are lipophilic molecules and may first interact with the lipid microenvironment that surrounds the membrane-associated enzymes [7,8]. These cannabinoid-phospholipid interactions trigger changes in the functions of a number of these proteins and thus alter a variety of physiological functions. A mechanism of action that involves cannabi-

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noid-phospholipid interactions has been suggested by the results of different physical methods [9–11]. It was argued in the literature that the possibility exists also for cannabinoids to interact first with phospholipids and then to diffuse to reach their specific receptor [12,13].

In an effort to obtain more detailed information on the regiochemical and stereochemical requirements for productive binding on the active site within a specific class of cannabinomimetic agents, we have sought to develop novel ligands possessing high affinity and selectivity for the cannabinoid receptors. Structure-activity relationship studies of classical cannabinoids have established that the alkyl chain at the C-3 position is an essential pharmacophore for cannabinoid activity. Our interest in exploring the role of side chain conformation on the activity of cannabinoids led us to design and synthesize analogues in which the side chain is conformationally more defined by adding substitution on the C-1′ position.

Among the analogues synthesized, compound 2-[6*a*,7,10,10*a*-tetrahydro-6,6,9-trimethyl-1-hydroxy-6*H*-dibenzo[*b*,*d*]pyranyl]-2-(hexyl)-1,3-dithiolane (AMG-3) (Fig. 1) was found to be a potent cannabinomimetic probe, with very high affinity to both cannabinoid receptors (K_i values of 0.32 and 0.52 nM for CB1 and CB2 receptors, respectively) [14]. The very high affinity of this analogue was attributed to two factors. First, its increased hydrophobicity at the benzylic side chain carbon may fit to a corresponding hydrophobic site of a receptor. Second, the side chain pharmacophore is conformationally more defined than the prototype cannabinoid (−)-Δ^8-THC. These structural characteristics may promote its proper integration into lipid bilayers and facilitate its interaction with the receptor when diffusing towards the active site, or may result in more favourable interactions with the phospholipid environment surrounding the enzymes or receptors. In parallel, its methoxy analogue (AMG-18) was also synthesized. This analogue lacks the phenolic hydroxyl group and possesses less biological activity.

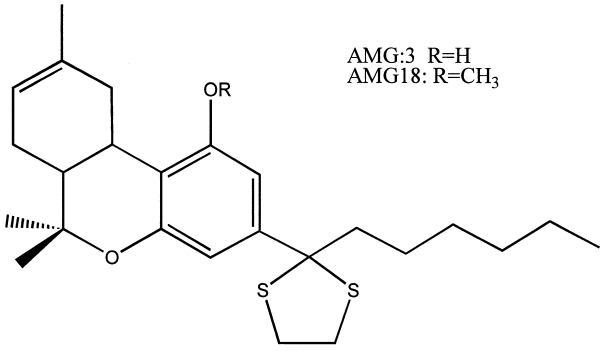

Fig. 1. Chemical structures of 2-[6*a*,7,10,10*a*-tetrahydro-6,6,9-trimethyl-6*H*-dibenzo[*b*,*d*]pyran-1-ol]-2-[hexyl]-1,3-dithiolane (AMG-3) and its methoxy analogue (AMG-18).

In the present study X-ray diffraction and differential scanning calorimetry (DSC) techniques were used to determine the effects of the interactions of AMG-3 and AMG-18 with the lipid bilayer structure in order to determine whether their differences in biological activity could be related to changes in the physical properties of membrane lipids. The compounds in this study have similarities to the (−)-Δ^8-THC and (−)-Me-Δ^8-THC cannabinoid pair, which has previously been studied using various biophysical techniques. Interestingly, these previous studies with the cannabinoid pair (−)-Δ^8-THC and (−)-Me-Δ^8-THC have shown differential effects [15,16]. These promising results led us to apply small and wide angle X-ray diffraction (SWAX) with fully hydrated phospholipid multibilayers to study the effects of AMG-3 and AMG-18. To our knowledge this is the first study in which SWAX is used to detect differential effects of drugs in the membrane bilayers.

2. Materials and methods

2.1. Synthesis of AMG-3 and AMG-18

The synthesis of the two cannabinoids was achieved according to published methods [14].

2.1.1. Structure identification of (−)-2-(6a,7,10,10a-Tetrahydro-6,6,9-trimethyl-1-hydroxy-6H-dibenzo[b,d]pyrany1)-2-hexyl-1,3-dithiolane (AMG-3)

^{1}H NMR (300 MHz, $CDCl_3$) d 6.8 (d, J = 1.98 Hz, 1H, H_4), 6.6 (d, J = 1.98 Hz, 1H, H_2), 5.45 (bs, 1H, H_8), 4.75 (s, 1H, OH), 3.4–3.2 (m, 4H, -S(CH_2)$_2$S-), 3.20 (m, 1H, $H_{10\cdot}$), 2.7 (br s, 1H, $H_{10\alpha}$), 2.3 (m, 2H, 2′ CH_2), 2.15 (m, 1H, H_7), 2.05–1.83 (m, 3H, $H_{6\alpha}$, H_7, $H_{10\beta,}$), 1.7 (s, 3H), 1.4 (s, 3H), 1.25 (br s, 8H,

-CH_2-), 1.1 (s, 3H), 0.85 (t, J = 7 Hz, 3H). Anal. ($C_{25}H_{36}O_2S_2$) C, H.

2.1.2. Structure identification of (−)-2-(6a,7,10,10a-Tetrahydro-6,6,9-trimethyl-1-methoxy-6H-dibenzo[b,d]pyrany1)-2-hexyl-1,3-dithiolane (AMG-18)

^{1}H NMR (300 MHz, $CDCl_3$) d 6.78 (d, J = 1.88 Hz, 1H, H_4), 6.7 (d, J = 1.88 Hz, 1H, H_2), 5.40 (bs, 1H, H_8), 3.80 (s, 3H, OCH_3), 3.33–3.26 (m, 4H, -$S(CH_2)_2$S-), 3.20 (m, 1H, $H_{10\alpha}$), 2.65 (br s, 1H, $H_{10\alpha}$), 2.3 (m, 2H, 2′ CH_2), 2.10 (m, 1H, H_7), 2.05–1.83 (m, 3H, $H_{6\alpha}$, H_7, $H_{10\beta,}$), 1.7 (s, 3H), 1.4 (s, 3H), 1.25 (br s, 8H, -CH_2-), 1.1 (s, 3H), 0.75 (t, J = 7 Hz, 3H). Anal. ($C_{26}H_{38}O_2S_2$) C, H.

2.2. Materials used in biophysical studies

1,2-Dipalmitoyl-*sn*-glycero-3-phosphorylcholine (DPPC, purity > 99%) was obtained from Avanti Polar Lipids (AL, USA) and used without further purification. The sample preparation procedure was identical for DSC and X-ray diffraction. Appropriate amounts of phospholipid with or without cannabinoid were dissolved in chloroform/methanol (2:1, spectroscopic grade). The solvent was then evaporated by passing a stream of O_2-free nitrogen over the solution, and the residue was placed under vacuum (0.1 mmHg) for 12 h. For X-ray or DSC measurements, this dry residue was dispersed in appropriate amounts of bidistilled water to a concentration of 50% (w/w) by vortexing at 45°C, i.e. above the chain-melting transition of DPPC. Before temperature scan experiments, the samples were equilibrated at 4°C for 4 h.

2.3. X-Ray diffraction

Small and wide angle X-ray experiments were performed using an integrated SWAX system (Hecus M. Braun-Graz X-ray Systems, Graz, Austria), Kratky line collimation, equipped with a linear position-sensitive detector, a semitransparent beam stop for on-line measurement of the primary beam, and an automatic temperature/time programmer and data collection unit. The camera was operated at a rotating anode generator (RU-200B; Rigaku Denki, Japan), at 40 kV/70 mA, with Ni-filtered Cu-Kα radiation (wavelength 0.154 nm). Measurements were taken in the range between 4 and 45°C, with measuring times of 1000 s per temperature setting, and 300 s waiting time at each temperature before measurement. All scattering curves were background corrected and normalized to integral primary beam intensity [17].

2.4. DSC

After adding distilled water to the dry lipid/drug mixture (50% w/w), a portion of the sample (approx. 5 mg) was sealed in a stainless steel capsule. Thermograms were obtained on a Perkin-Elmer DSC-7 instrument. All samples were scanned at least twice until identical thermograms were obtained using a scanning rate of 2.5°C/min. The temperature scale

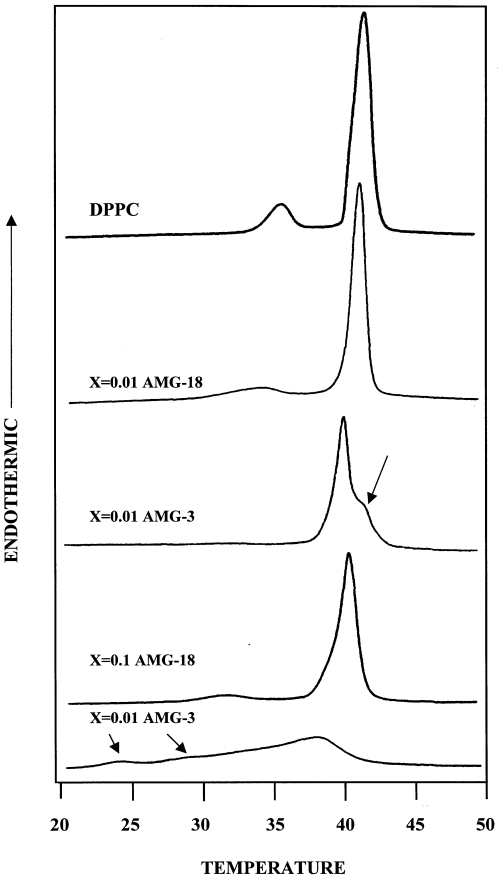

Fig. 2. Normalized differential scanning calorimetry scans of fully hydrated bilayers of DPPC with or without AMG-3 or AMG-18. The labelled scans with $x = 0.01$ (1 mol%) and $x = 0.1$ (10 mol%) refer to the corresponding added concentrations of AMG-18 or AMG-3.

of the calorimeter was calibrated using fully hydrated DPPC and indium as standard samples. We found that the thermograms from samples stored at freezer temperatures (−15°C) for several days were identical to those run immediately after sample preparation.

3. Results

3.1. Differential scanning calorimetry

The effects of the two cannabinoids on the thermal behaviour of DPPC [18] in aqueous dispersion are shown in Fig. 2, and the relevant calorimetric parameters are summarized in Table 1. The DPPC bilayer exists in the gel phase (L_β') for temperatures lower than 33°C, and in the liquid crystalline phase for temperatures higher than 42°C (L_α'). In between 33 and 42°C the phospholipid bilayer exists in P_β' or ripple phase. The obtained DSC scan of fully hydrated DPPC multibilayers shows a pretransition centred at 35°C and a peak maximum at 41.2°C. The main phase transition is accompanied by several structural changes in the lipid molecules as well as systematic alterations in the bilayer geometry, but the most prominent feature is the *trans-gauche* isomerization taking place in the acyl chain conformation. The average number of *gauche* conformers indicates the effective fluidity, which depends not only on the temperature, but also on perturbations due to the presence of a drug molecule intercalating between the lipids.

While the control DPPC sample showed the well known features of pre- and main transitions, the presence of the cannabinoids had significant effects on both mesomorphic phase transitions of DPPC, however, with different calorimetric features for the two compounds. The presence of 1 mol% inactive analogue AMG-18 has only minor effects on the transition thermograms. Using the same concentration of the active analogue AMG-3 induces a strong broadening and decrease in temperature of the pretransition. The main transition is also broadened and the peak temperature is decreased by about 2°C. A shoulder is observed (labelled in Fig. 2) at the high temperature tail of the transition suggesting phase separation. The integral transition enthalpy is decreased by about 25% relative to the control DPPC system. At 10 mol%, the presence of the inactive analogue causes broadening of the transition thermograms. Again, at the same concentration, AMG-3

Table 1
Pretransition and transition temperatures (T_c), and enthalpy (ΔH) of the studied preparations

Sample	T_c (°C)	ΔH (cal/g)
DPPC	35.2, 41.2	1.6, 12.5
DPPC/(x = 0.01) AMG-18	35.6, 42.6	1.1, 10.7
DPPC/(x = 0.01) AMG-3	30.7, 39.1	0.3, 9.4
DPPC/(x = 0.1) AMG-18	33.4, 42.2	0.7, 10.1
DPPC/(x = 0.1) AMG-3	–, 37.3[a]	7.8

The first values in each column correspond to the pretransition, whereas the second values correspond to the main phase transition.
[a]Represents the highest peak of the DSC scan.

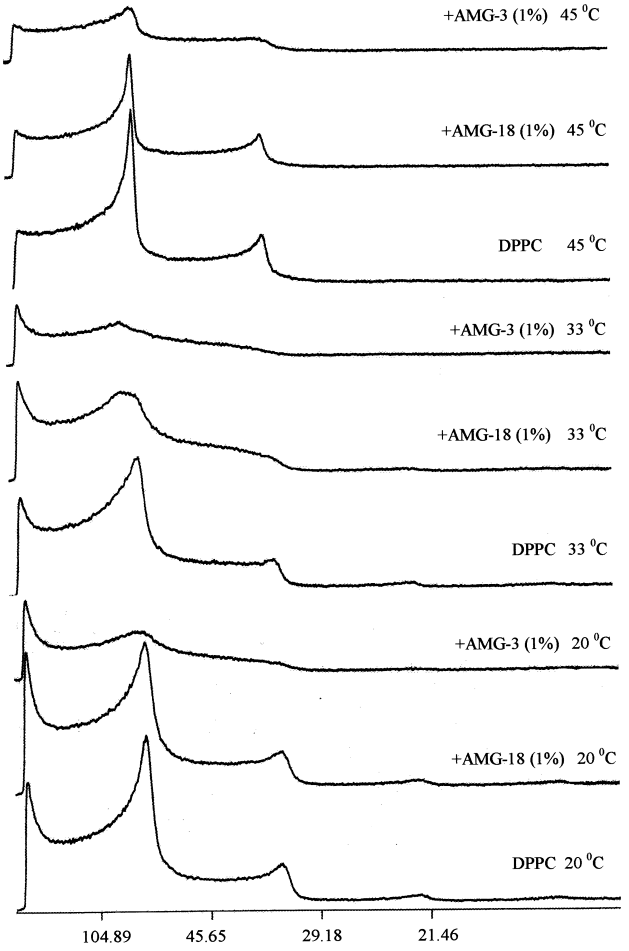

Fig. 3. Small angle X-ray diffraction patterns of (a) DPPC; (b) DPPC+1 mol% AMG-3 and (c) DPPC and 1 mol% AMG-18 at 20, 33 and 45°C.

exerted more significant broadening of the phase transition in DPPC multibilayers. Three different maxima are clearly visible and labelled in the same figure.

3.2. X-Ray diffraction

3.2.1. Small angle X-ray diffraction

The small angle diffraction patterns for DPPC bilayers without and with 1 mol% cannabinoid are shown in Fig. 3 for the three representative temperatures 20°C, 33°C and 45°C which cover all mesomorphic states of the phospholipid bilayers.

DPPC bilayers in the gel phase show four distinct sharp diffraction orders indicating a high stacking regularity of the membrane phospholipids. At temperatures between the pretransition and main transition the structure is formed by corrugated bilayers with a periodic ripple and hexagonal chain packing. This is seen as a broadening of the reflections. At the liquid crystalline phase (L_α'), a sharpening of the peaks and an increase in intensity as well as diffuse scattering towards zero angle is observed [19]. At the gel and liquid crystalline phases 1 mol% AMG-18 causes only marginal changes in the diffraction pattern of DPPC. In the pretransition state the presence of AMG-18 causes a broadening effect on the diffraction peaks. The presence of active cannabinoid AMG-3 at 1 mol% and at the gel phase causes broadening of the diffraction peaks and the pattern has strong similarities to the P_β' 'ripple phase' or at the pretransition state shown in the diffraction patterns at 33°C of DPPC bilayers alone. These results indicate that the planar multibilayer gel structure becomes disordered by the presence of AMG-3. A clear sign for a periodic corrugation, however, cannot be found; it appears that the lattice correlation between the bilayers is lost through non-correlated domains of local curvature induced by AMG-3. The broadening effect of AMG-3 both in pretransition and liquid crystalline phases is more pronounced than that of DPPC bilayers and bilayers containing AMG-18.

The same trend is emerging using higher concentrations of cannabinoids (data not shown). For example at concentrations of 10 mol% and 20 mol% the two cannabinoids enhance their effects with the active one still to exert more significant effects.

3.2.2. d-Spacing versus temperature measurements

The total period repeat distances (d-spacing) measured for the three preparations at the concentrations of 1 mol% and 10 mol% are shown in Fig. 4. At gel phase and using 1 mol% concentration of drug only the active one causes an increase of d-spacing. The

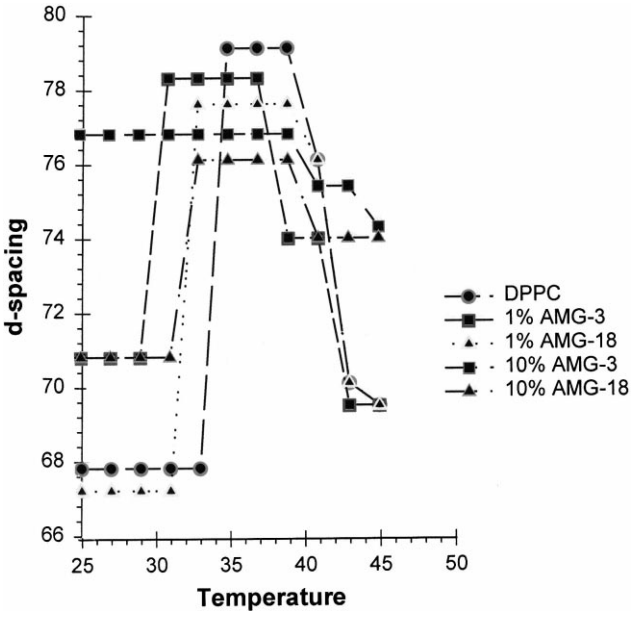

Fig. 4. Lamellar repeat d-spacing versus temperature of (a) DPPC (●); (b) DPPC+1 mol% AMG-3 (—■—); (c) DPPC+1 mol% AMG-18 (···▲···) (d) DPPC+10 mol% AMG-3 (–·–■–·–); (e) DPPC+10 mol% AMG-18 (—▲—).

pretransition is seen by the shift of the dominant small angle peak to larger spacings (i.e. smaller angles), by about the same amount as in the reference DPPC system at temperatures between 33 and 35°C. The appearance of distinct shoulders of the dominant small angle peak towards larger angles indicates that a rippled P_{β}'-like structure also prevails in the presence of AMG-3. Therefore, the P_{β}' structure appears to be the one which is least affected by AMG-3.

The d-spacing versus temperature profile of DPPC bilayers containing 1 mol% AMG-3 is distinctly broader than that of DPPC and DPPC+1 mol% AMG-18 preparations, indicating an increase in bilayer stacking disorder caused by the active cannabinoid. This broadening is in agreement with DSC results. AMG-18 exerts much weaker effects on the bilayer stacking order and dimensions. The only noticeable effect is its cause of the lowering of the pretransition by about 2°C. The temperature dependence of the d-spacing at 10 mol% is also shown in Fig. 4. The results demonstrate that the presence of the active analogue in the DPPC bilayer phase still causes more broadening in the phase transition temperature than the inactive one, in agreement with DSC data. The intensity and d-spacing with the active AMG-3 differ significantly from those of DPPC, but to a lesser degree from those of the inactive analogue AMG-18.

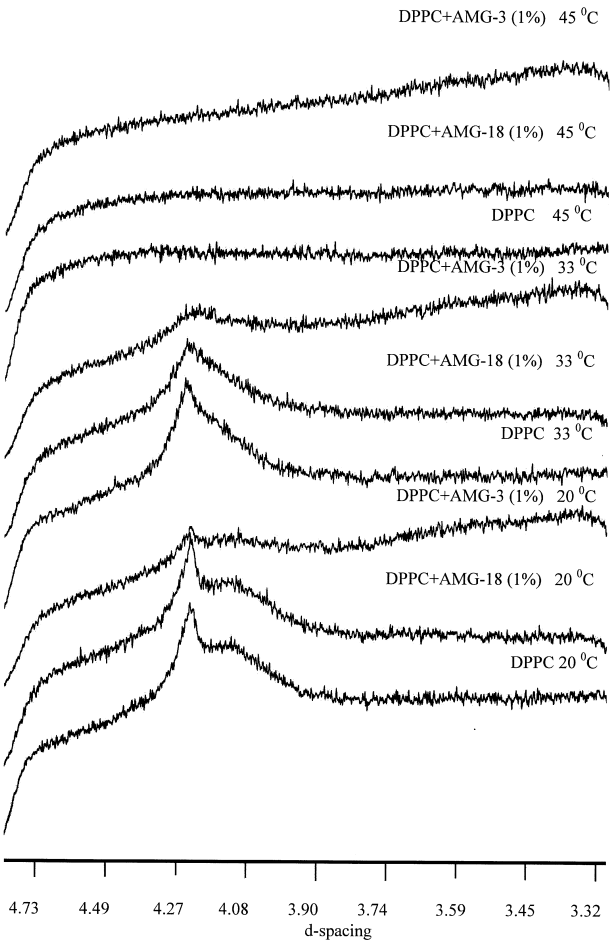

Fig. 5. Wide angle X-ray diffraction patterns of (a) DPPC; (b) DPPC+1 mol% AMG-3 at 20, 33 and 45°C.

3.2.3. Wide angle X-ray diffraction

Fig. 5 shows the wide angle X-ray diffraction patterns of DPPC bilayers without and with 1 mol% of cannabinoid. The wide angle pattern of DPPC bilayers at the gel phase where there is a tilted hydrocarbon chain packing, belonging to the orthorhombic subcell symmetry [20], is reflected by a prominent (2/0) peak around 4.22 Å ($s = 1/d = 0.24$ Å^{-1}) and a shoulder at 4.12 Å (1/1). Above the pretransition, there is a merge of the two peaks into one symmetric peak around 4.21 Å and the intensity is significantly reduced, suggesting the coexistence of fluid, disordered domains with ordered, hexagonally packed hydrocarbon chains, in agreement with NMR and recent X-ray data [17–20]. At the liquid crystalline phase a total disappearance of sharp wide angle peaks is observed. The wide angle patterns of DPPC bilayers containing 1 mol% AMG-18 resemble those of DPPC bilayers. Thus, the 4.22/4.11 peaks/shoulder pattern changes into a 4.21 Å symmetrical peak. The wide angle diffraction patterns of bilayers containing 1 mol% active analogue show one weak peak around 4.21 Å already from 20°C. This indicates a substantial loss in chain packing order. The peak asymmetry resembles that of the L_{β}' pattern, and it is therefore suggested that this relates to a reduced population of ordered, tilted chain domains within an increased proportion of disordered chains. When higher concentrations are used the active analogue still has stronger effects.

4. Discussion

The present investigation on cannabinoid drug-lip-

id membrane interaction was aimed at the following question: do drugs with different biological activity perturb lipid bilayers in the same manner? A positive answer to this question may substantially enhance our understanding of the molecular mechanism of drug action.

The two cannabinoid analogues investigated in this study have only a minor structural difference. AMG-3 possesses a phenolic hydroxyl group while in AMG-18 this is replaced by a methoxy group. Such a small structural difference in relatively complex organic compounds could be expected to be distinguishable only by a specific receptor protein. However, the present results show detectable differences in their interactions with DPPC bilayer membranes. There appears to be a complementary role for cannabinoid-lipid interaction in specific receptor recognition. In fact, it is argued in the literature that lipophilic drugs first are incorporated into the lipid matrix and then diffuse laterally towards the receptor site. Therefore, the topography and orientation of a drug molecule in the membrane bilayer may govern its approach towards the receptor site [12,13]. Consequently, both topography and orientation of drug molecules in membrane bilayers result from the physicochemical drug-membrane interactions.

It is convenient for the present discussion to first treat preparations that contain 1 mol% drug since these may be more relevant pharmacologically. The use of preparations that contain 10 mol% drug serves to describe the common trends of action by both analogues. The most noticeable effect of the active analogue, AMG-3, is fluidization, or disordering, of the hydrocarbon chains in the gel phase. This is accompanied by a disjoining and progressive randomization of the bilayer leaflets in the multilamellar stacks. The observation of a larger average repeat spacing, by about 2–9 Å, in the presence of AMG-3, can be taken as an indication for an increased thickness of the interbilayer water space which can be plausibly explained by the steric repulsion of bilayers with enhanced random, local curvature. Interestingly, using 1 mol% of AMG-18, these effects are practically negligible, but at 10 mol% they are quite visible.

Nothing is presently known on the arrangement of either cannabinoid analogue in the lipid bilayer membrane. We can only speculate that AMG-3 partitions favourably into the hydrocarbon chain region, even in the L_{β}' phase, which it partially fluidizes according to the present results. The bilayer appears to consist of fluid, AMG-containing domains, coexisting with domains of ordered L_{β}' phase. One possible consequence is that the elastic deformability of the membranes, and hence also the dynamic undulation [20,21], increase to lead to enhanced disorder. This could possibly explain the effects observed in the L_{β}' gel phase, but it is not sufficient to interpret the disordering in the liquid crystalline L_{α}' phase, where the chains are already fluid. We propose that AMG-3 causes local, positive curvature formation at the site of its intercalation into one half of a bilayer. Such local and uncorrelated 'bumps', which are not to be confused with dynamic undulation, could account for the increase in lamellar packing disorder. The fact that the 'ripple' phase, P_{β}', is least affected by the interaction with AMG-3 could be interpreted by the notion that there the drug inserts into the already curved ridges of the sawtooth structure.

The intercalation of the active drug molecule into membrane bilayers leads to a local expansion which can only be accommodated if the bilayer curves at this site, which in turn leads to a local fluidization of the annulus of lipids around the site of interaction. The phenolic hydroxyl group of AMG-3 serves as an anchor within the polar head group region of the lipids. This molecular feature is lacking with AMG-18 and this may explain its lesser effects on the phospholipid bilayers. It remains to be established by future studies to what extent and how this molecule is inserting into the bilayer. At higher concentrations the effects of AMG-3 and AMG-18 become closely similar. This indicates that their effects are not progressing in a linear fashion and approach saturation at very high concentrations.

In terms of methodology the present study shows that X-ray small and wide angle diffraction is very sensitive to structural alterations of lipid bilayers upon incorporation of an additive. In addition, since SWAX is a fast technique, these results should stimulate further investigations by the researchers working in the field of drug-membrane interactions and investigating problems related to biology and medicinal chemistry.

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