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Interaction of the pitavastatin with model membranes

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ABSTRACT

Pitavastatin is a statin drug that, by competitively inhibiting 3-hydroxy-3-methylglutaryl-coenzyme A reductase, can lower serum cholesterol levels of low-density lipoprotein (LDL) accompanied by side effects due to pleiotropic effects leading to statin intolerance. These effects can be explained by the lipophilicity of statins, which creates membrane affinity and causes statin localization in cellular membranes. In the current report, the interaction of pitavastatin with POPC model membranes and its influence on the membrane structure were investigated using H, H and P solid-state NMR spectroscopy. Our experiments show the average localization of pitavastatin at the lipid/water interface of the membrane, which is biased towards the hydrocarbon core in comparison to other statin molecules. The membrane binding of pitavastatin also introduced an isotropic component into the ^{31}P NMR powder spectra, suggesting that some of the lamellar POPC molecules are converted into highly curved structures.

1. Introduction

Pitavastatin (NK-104, formerly known as Itavastatin or Nizvastatin, Kowa Company Ltd., Tokyo) is, along with other statins, an inhibitor of the enzyme HMG-CoA reductase. It is a synthetic drug [1,2] that significantly lowers serum total cholesterol, LDL cholesterol, and triglycerides and has several pharmacodynamic and pharmacokinetic properties that are significantly different from other statins [3,4]. Recent studies indicate that pitavastatin could represent a new and potentially better therapeutic choice for lipid lowering therapy than other currently available statins [4,5]. The ability of statins to inhibit HMG-CoA reductase is largely independent of the physicochemical properties of the active ingredient [6], while the pleiotropic effects are strongly dependent on it. Several clinical studies have shown that there are important molecular differences that are responsible for the broader pharmacological effects of statins [7,8]. In addition to lowering cholesterol, statins have other pleiotropic effects, including restoring endothelial function and reducing oxidative stress and inflammation [9–12].

The available data suggest that the lipophilicity and pleiotropy of the statins are linked, confirming the hypothesis that the secondary effects of statins depend on their binding and distribution in biological membranes [13–16]. It is known that the binding of amphiphilic molecules to lipid bilayers influences membrane parameters such as permeability or bilayer thickness [17–20]. The different lipophilicity of each statin influences their binding to the membrane and the depth of penetration into the lipid bilayer and thus their effect on the properties of membranes [21]. Such interactions can also be a source of unwanted side effects of drug molecules, since lipid membrane are an important contact side for such molecules [19]. Several studies are available on the interaction of various statins with model membranes [22–30]; however, the intermolecular interactions of pitavastatin with lipids are poorly represented. Some studies show a possible direct effect of pitavastatin on cell membranes, and it was suggested that pitavastatin may play an important role in modulating the fatty acid content in the erythrocyte membrane or have a phototoxic effect, mainly in cell membranes [31]. It is therefore worth investigating the molecular interactions between pitavastatin and membrane systems in more detail.

This article examines the interaction of pitavastatin and phospholipid membranes and their influence on the structure and dynamics of the membrane using NMR spectroscopy. The use of POPC as a model membrane provides the physiologically relevant liquid crystalline phase state of the membrane and represents a common lipid head group in native membranes along with a physiologically relevant unsaturation of

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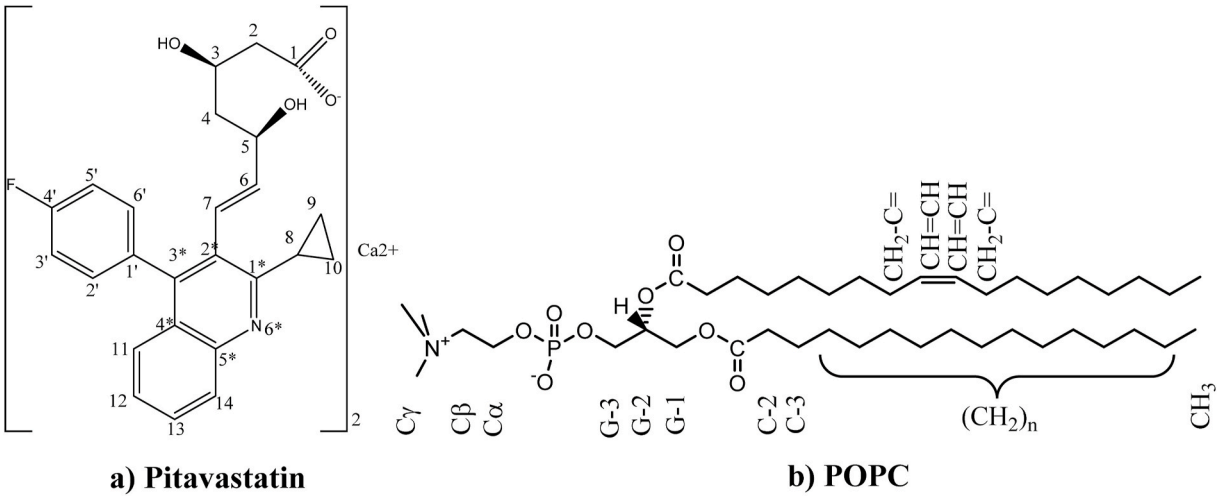

Fig. 1. Chemical structure of a) pitavastatin and b) POPC. Capital letters (for POPC) and numbers (for pitavastatin) refer to the corresponding peaks assigned in the ^{1}H MAS NMR spectra are shown in Fig. S2.

the *sn*-2 chain. Nuclear magnetic resonance (NMR) spectroscopy is a technique widely used to investigate the molecular order, dynamics and structure of lipid bilayers and the interaction of various drug molecules with lipid molecules [32–36]. Nuclear Overhauser enhancement spectroscopy (NOESY) allows to determine the localization of small molecules in the phospholipid membrane. By analyzing the intermolecular cross-relaxation rates between the protons of the compounds under study and the corresponding segments of membrane phospholipids, this method enables the localization of the drug in lipid membranes to be determined [32,34,37,38].

The chemical structure of pitavastatin is shown in Fig. 1. It contains a trisubstituted quinoline core and an unsaturated heptenic acid side chain. It differs from other synthetic statins by the inclusion of a cyclic propyl unit in place of the more typical isopropyl ring substituent. Its hydrophilic properties were evaluated using the octanol-water partition coefficient (logP), which was calculated according to Molinspiration (http://www.molinspiration.com). A logP value of 3.91 classifies pitavastatin as a fairly hydrophobic molecule compared to other statins. Because of these structural properties, pitavastatin is likely to be incorporated into the membrane.

2. Materials and methods

2.1. Sample preparation

The phospholipids 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and its per-deuterated *sn*-1 chain analog POPC-d_{31} were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL); pitavastatin (chemical structure is shown in Fig. 1) was purchased from Sigma-Aldrich (Darmstadt, Germany). All chemicals were used without further purification. To prepare samples for NMR experiments, pitavastatin and the lipids (POPC or *sn*-1 chain perdeuterated POPC-d_{31}) were dissolved in chloroform and mixed in the desired molar ratio. After evaporation of the solvent, samples were re-dissolved in cyclohexane and converted into a fluffy powder by lyophilization overnight at high vacuum. The samples were hydrated with 50 wt% D_2O buffer for ^{1}H NMR or H_2O buffer for ^{2}H NMR measurements (100 mM NaCl, 10 mM HEPES, pH 7.4). After hydration of the sample, 10 cycles of freezing-thawing and centrifugation were applied, and the sample was finally placed into a 4 mm MAS rotor.

2.2. Qualitative assessment of pitavastatin binding to POPC membranes

UV spectroscopy was used to obtain the absorbance spectra of pitavastatin. Large unilamellar vesicles (LUVs) of POPC were prepared by the extrusion method as reported in Ref. [39]. Samples were prepared in a ratio of 1:20 pitavastatin/POPC. Pitavastatin was coincubated with 100 μM POPC LUVs for 20 h, after which the sample was subjected to ultracentrifugation (208,000×*g*). The supernatant and a control sample were examined with the UV–vis spectrometer to assess the amount of LUV bound pitavastatin. Spectra (200–450 nm) were acquired using UV permeable cuvettes on a Cary 60 UV–vis spectrophotometer (Agilent).

2.3. Solid-state NMR spectroscopy

For the ^{1}H MAS NMR measurements, a Bruker Avance III 600 MHz NMR spectrometer equipped with a 4 mm double resonance HR MAS probe with a ^{2}H lock was used. Samples were spun at a MAS frequency of 6 kHz. The π/2 pulse length was 4 μs. To calibrate the chemical shift, the CH_3 group of the acyl chains of POPC (in the absence of pitavastatin) was set to 0.885 ppm (relative to TMS) [40].

Two-dimensional ^{1}H MAS NOESY spectra were acquired with mixing times of $\tau_m = 0.1$, 100, 200, 300, and 500 ms and a relaxation delay of 3 s. Spectra were recorded with 256 data points in the indirect dimension. The values of the diagonal and cross peaks were obtained by integration in the Bruker Topspin 3.6.3 software. The spectra were analyzed using the spin-pair-model as described in Refs. [34,41] with a program written in Python 3.7.

In the stationary ^{31}P NMR experiments, performed on a Bruker Avance III 600 MHz NMR spectrometer (Larmor frequency for ^{31}P at 243.00 MHz), a Hahn echo sequence with ^{1}H decoupling, a relaxation delay of 3 s, and a 10.0 μs 90° pulse length was used. ^{31}P NMR line shapes were simulated with a program written in Python 3.7.

Stationary ^{2}H NMR measurements were acquired on a Bruker DRX300 NMR spectrometer at a resonance frequency of 46.1 MHz for ^{2}H using a high-power probe equipped with a 5-mm solenoid sample coil. A quadrupole echo pulse sequence was used [42] at a spectral width of 500 kHz. The π/2 pulses with a duration of 3.3 μs were separated by a 50 μs delay; the recycle delay was 1 s. About 55,000 scans were accumulated for a decent signal-to-noise ratio. The order parameter profiles were determined by fitting a simulation to the symmetrized ^{2}H NMR spectrum with the help of a script written in Python 3.7.

All measurements were carried out at a temperature of 30 °C.

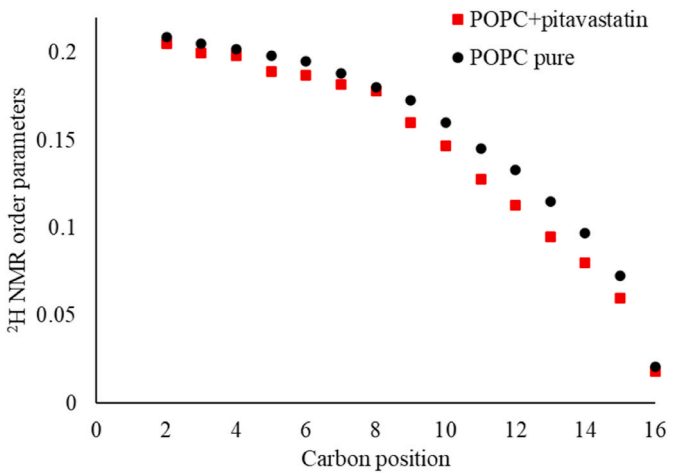

Fig. 2. ^{2}H NMR order parameters of pure POPC-d_{31} membranes and POPC- d_{31} membranes in the presence of pitavastatin. The error of measurement is smaller than symbol size.

3. Results and discussion

3.1. UV data on the binding of pitavastatin to POPC membranes

To evaluate the binding of pitavastatin to lipid membranes, we used UV absorption spectroscopy to analyze the supernatant of an ultra-centrifuged POPC LUV/statin mixture (20:1 M ratio) and a control sample without LUV. About 27% of pitavastatin molecules bind to lipid vesicles, which is comparable to other hydrophobic statins such as atorva-, lova- and ceristatin [29]. In the following experiments, the molar concentration of pitavastatin was 20 mol% (relative to POPC) in order to ensure a suitable intensity of the cross peak between the statin and the POPC bilayer in the ^{1}H MAS NOESY experiments.

3.2. Effect of pitavastatin on membrane lipid packing and head group orientation

The effect of pitavastatin on the phase state of the membrane was investigated using stationary ^{31}P and ^{2}H NMR spectroscopy. The ^{31}P NMR spectrum in the presence of pitavastatin (Supplementary Fig. S1) exhibit the typical powder NMR pattern of lamellar liquid-crystalline

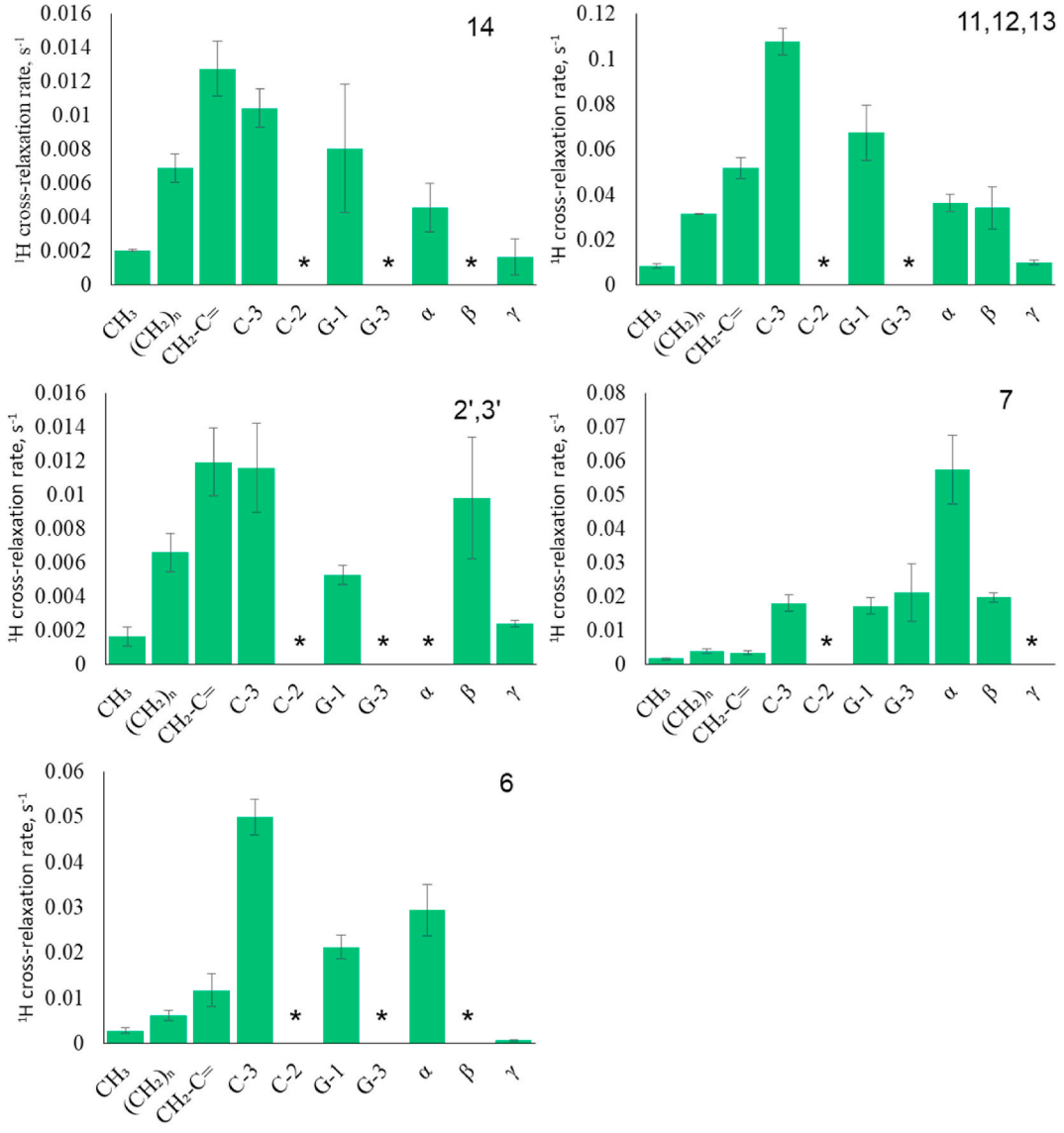

Fig. 3. Cross-relaxation rates (s^{-1}) between pitavastatin protons and lipid segments in POPC membranes at a concentration of 20 mol% and a temperature of 30 °C. Cross-peaks marked with * were not analyzed because the signal peaks of pitavastatin and POPC overlapped.

bilayer membranes, indicating that the membrane morphology was not disturbed in the presence of the active ingredient. However, the spectrum also contains an isotropic component, which accounts for about 17% of the spectral intensity and represents an indicator of lipids in highly curved structures such as micelles, cubic phases, or pores [41–43]. Such phases have not been observed in POPC samples containing other statins [29]. For the lamellar bilayer phase, there was a slight increase in chemical shift anisotropy ($\Delta\sigma$) to 47 ppm in the presence of pitavastatin compared to 45 ppm for pure POPC. This indicates that the incorporation of pitavastatin does not significantly affect the orientation and mobility of the head groups of phospholipids. A similar situation was observed for rosuvastatin ($\Delta\sigma = 47$ ppm), although the chemical structure is not very similar and rosuvastatin is more hydrophilic [29].

To investigate the influence of pitavastatin binding to the hydrocarbon chain region of the lipid membrane, smoothed chain order parameters in the profiles were calculated based on the ^{2}H NMR spectra of the *sn*-1 chain of deuterated POPC-d_{31}. The ^{2}H NMR spectrum (not shown) of POPC membranes in the presence of pitavastatin again shows a typical powder pattern, which is characterized by an overlay of Pake doublets with different residual quadrupole splitting and a certain isotropic contribution, which is also observed in the ^{31}P NMR spectrum. Pitavastatin lowers the POPC order parameters across the chain (as shown in Fig. 2), which corresponds to a slightly thinner membrane and more flexible segments of the methylene chain. Compared to other statins, however, the effects are rather moderate [29].

3.3. Membrane localization of pitavastatin

^{1}H MAS NMR spectroscopy was used to determine the location and distribution of pitavastatin in the POPC membrane. The ^{1}H NMR spectra of both pure POPC and POPC in the presence of 20 mol% pitavastatin are shown in Supplementary Fig. S2 together with the assignment of the NMR peaks. The NMR signals of pitavastatin are weaker than the POPC signals and are found mostly in the low-field region of the NMR spectrum. Most of the pitavastatin aromatic proton signals are well separated from the aliphatic lipid signals. Unfortunately, some signals from POPC and pitavastatin (e.g. C-2) overlap, making further analysis difficult. The assignments of ^{1}H signals are taken from the literature [46].

The pitavastatin molecule contains aromatic rings, in which, due to delocalized π-electrons, a ring current can be induced if the magnetic field is directed perpendicular to the plane of the aromatic system. This can lead to a shift in the chemical shifts of the POPC signals, either to stronger or weaker fields, depending on the orientation of the aromatic rings, and the magnitude of the shift will depend on the distance to the aromatic rings [47]. Thus, the average position of pitavastatin aromatic rings relative to POPC molecules can be estimated [37]. The induced chemical shifts for each phospholipid segment along the longitudinal axis of the lipid molecule are shown in Supplementary Fig. S3. The greatest change is observed in the CH_2–C = , C-2 and G-3 POPC segments. This suggests that the aromatic ring moieties of pitavastatin are located in the area of the main chain of glycerol and acyl chains and penetrate far into the hydrocarbon core. Broad distribution profiles indicate a high mobility of pitavastatin in the membrane, which is common for small molecules in liquid-crystalline membranes [37].

2D ^{1}H–^{1}H NOESY NMR experiments were carried out to obtain further insight into the orientation and localization of the statin in the lipid membrane (Supplementary Fig. S4). The determination of the intermolecular cross-relaxation rate enables the investigation of membrane binding, membrane organization and the localization and orientation of small molecules that are embedded in the membrane [34]. Due to the known high mobility and molecular disorder in liquid-crystalline membranes [48], the obtained cross-relaxation rates provide a quantitative measure of the probability of contact between interacting molecular groups [41,49]. Because of the strong dependence of the cross-relaxation rates on the interproton distance, a high contact frequency leads to high cross-relaxation rates, while a low contact frequency leads to low cross-relaxation rates [34].

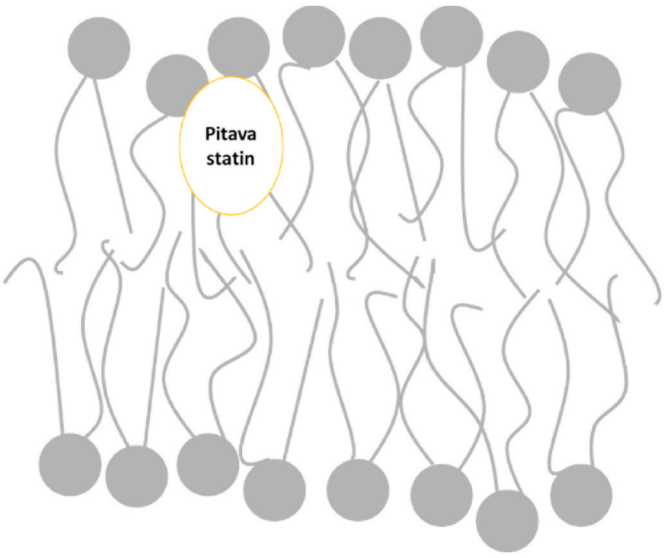

Fig. 4. Schematic representation of the average location of pitavastatin in POPC bilayer membranes.

The 2D NOESY spectra showed well-resolved cross-peaks between the various aromatic protons of pitavastatin (see Fig. 1 for definition) and the ^{1}H NMR signals of the various POPC molecular segments (Supplementary Fig. S4). Quantitative determination of cross-relaxation rates using the spin pair model showed that the aromatic rings of pitavastatin in the lipid bilayer in the upper chain/glycerol region (protons 11, 12, 13, as well as 14 and 2′, 3′) have a maximum for CH_2–C = or G-1 (Fig. 3). As seen in Fig. 3, the maximum of the distribution of protons 6 and 7 is shifted towards the head group (α); therefore, it can be assumed that this more polar part of the molecules is oriented towards the membrane-water interface. These data are consistent with the induced chemical shifts discussed above (Supplementary Fig. S3). Compared to other statins, pitavastatin is more deeply embedded in the lipid membrane, similar to the relatively hydrophobic statin cerivastatin (logP = 1.74).

4. Conclusions

The interaction of pitavastatin with a POPC model membrane was investigated along with its effect on lipid packing. It was found that the average localization of pitavastatin in the lipid bilayer is in the region of the upper POPC chain. Based on these results, Fig. 4 shows a schematic visualization of the localization of pitavastatin in the upper chain region of the POPC bilayer. This localization causes a small decrease in lipid chain order. The reduced ^{2}H NMR order parameters correspond to a thinner membrane, which is also more flexible and possibly more prone to penetration of other small molecules. Isotropic membrane phases are also observed in the presence of pitavastatin. Knowledge of the interaction of pitavastatin with cell membranes and its effect on lipid packaging can provide information on the pharmacological specificities and potential side effects of pitavastatin. With regard to the side effects of pitavastatin, lipophilicity is especially important because of its association with hepatoselectivity and its effect on the association of statins with extrahepatic tissue such as skeletal muscle, which is believed to cause muscle symptoms of statins [21]. Special attention to the relationship between statin structure and pleiotropic effects provides new insights into this clinically important drug.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2021.101143.

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