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ORIGINAL PAPER

Effect of sodium bicarbonate as a pharmaceutical formulation excipient on the interaction of fluvastatin with membrane phospholipids

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Abstract Excipients in the pharmaceutical formulation of oral drugs are notably employed to improve drug stability. However, they can affect drug absorption and bioavailability. Passive transport through intestinal cell walls is the main absorption mechanism of drugs and, thus, involves an interaction with the membrane lipids. Therefore in this work, the effect of the excipient $NaHCO_3$ on the interaction of the anticholesterolemic drug fluvastatin sodium (FS) with membrane phospholipids was investigated by ^1H NMR and FTIR spectroscopy. Sodium bicarbonate is often combined with fluvastatin for oral delivery to prevent its degradation. We have used model DMPC/DMPS membranes to mimic the phospholipid content of gut cell membranes. The results presented in this work show a 100% affinity of FS for the membrane phospholipids that is not modified by the presence of the excipient. However, $NaHCO_3$ is shown to change the interaction mechanism of the drug. According to our data, FS enters the DMPC/DMPS bilayer interface by interacting with the lipids' polar headgroups and burying its aromatic moieties into the apolar core. Moreover, lipid segregation takes place between the anionic and zwitterionic lipids in the membranes due to a preferential interaction of FS with phosphatidylserines. The excipient counteracts this favored interaction without affecting the drug affinity and location in the bilayer. This work illustrates that preferential interactions with lipids can be involved in passive drug permeation mechanisms and gives evidence of a possible nonpassive role of certain excipients in the interaction of drugs with membrane lipids.

Keywords Statin · Lipid segregation · Drug-membrane interaction · Nuclear magnetic resonance · Infrared spectroscopy · Drug bioavailability

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Introduction

Fluvastatin sodium (FS) is a synthetic inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase. The conversion of HMG-CoA into mevalonate by this enzyme is the first step leading to the biosynthesis of cholesterol (Sarr et al. 2008). This drug belongs to the statins, a class of medicines amongst the most extensively sold on the international market (Davidson and Toth 2004). The structure of FS is presented in Fig. 1. This molecule is amphiphilic, and its great permeability through jejunum cell walls has been related to its favorable octanol/water partition coefficient (logP) of 3.24 (Davidson and Toth 2004; Lindahl et al. 1996). HMG-CoA reductase inhibitors such as fluvastatin are susceptible to degradation at pH below 8 and may convert from their active hydroxy acid to their inactive lactone form (Papageorgiou et al. 2009). As the pH in the gastrointestinal (GI) tract varies from 1–3.5 in the stomach to 7–8 in the intestine, sodium bicarbonate is often combined with FS for oral delivery to maintain an alkaline pH that prevents drug degradation and favors the bioactive structure (Jackson et al. 2000; Kabati and Vivilecchia 1994; Papageorgiou et al. 2009). In general, excipients such as $NaHCO_3$ are added to pharmaceutical formulations to facilitate the administration, manufacturing,

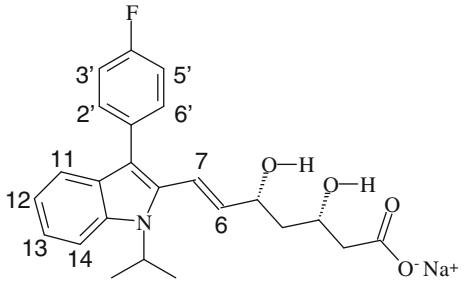

Fig. 1 Chemical structure of fluvastatin sodium (FS) with ^{1}H labels as used in Fig. 2

or identification of the drug, or to improve its stability or aesthetic aspect (Jackson et al. 2000). Although they are supposed to be inert, it has been shown that excipients can influence the absorption and subsequent bioavailability of a drug (Jackson et al. 2000 and references therein).

Because passive membrane permeation is the principal route of fluvastatin through the intestinal cells, we have studied the effect of the excipient $NaHCO_3$ on the membrane penetration mechanism. In particular, we were interested in the interaction of the drug with the phospholipids. Biological membranes are natural lipid-rich bilayers that preserve the intracellular contents from the external milieu. These membranes include the epithelial barrier of the small intestine where most of the nutrient and drug absorption takes place, mainly via a passive permeation mechanism (Camenisch et al. 1996; Seo et al. 2006). The lipid composition of biological membranes is different from one tissue to another, but glycerophospholipids represent the most common and frequently encountered structural lipids in eukaryotic membranes (Seydel 2002). Phosphatidylcholines account for more than 50% of these phospholipids (van Meer et al. 2008) and must be considered in the composition of a model membrane. Moreover, negatively charged phosphatidylserine (PS) is invariably present in intestinal cell membranes with a proportion of 13% on average (Avdeef 2003). To better understand the effect of sodium bicarbonate on the interaction of FS with the most abundant phospholipid constituents in the gut cell walls, model membranes composed of dimyristoyl-phosphatidylcholine (DMPC) and dimyristoyl-phosphatidylserine (DMPS) were thus employed. Results are compared to pure DMPC model membranes to verify a potential particular role of anionic lipids on the drug/membrane interaction.

The effect of the excipient on the binding affinity of FS to the model membranes is studied by ^{1}H nuclear magnetic resonance (NMR) using longitudinal diffusion experiments. Fourier transform infrared (FTIR) spectroscopy is exploited to verify the impact of $NaHCO_3$ on the interaction of FS with the phospholipids in the model membranes. This method allows the exploration of the mechanism by which the drug and its excipient interact with both the polar and apolar regions of the phospholipid bilayer, and verification of the occurrence of preferential drug-lipid interactions in phospholipid mixtures. Our results show a strong affinity of fluvastatin for the membrane, which is unaltered by the excipient. The drug is shown to induce phase separation in the DMPC/DMPS mixture by preferentially interacting with anionic phospholipids. Upon addition of the excipient $NaHCO_3$, membrane homogeneity is re-established without affecting the affinity of the drug for the membrane. A penetration mechanism of fluvastatin in model intestinal cell membranes is proposed, and the effect of the excipient $NaHCO_3$ on the drug/lipid interaction is discussed.

Materials and methods

Materials

Protonated DMPS (sodium salt) and DHPC (dihexanoylphosphatidylcholine) as well as DMPC with either protonated or deuterated (d_{54}) acyl chains were purchased from Avanti Polar Lipids (Alabaster, AL, USA) and used without further purification. Fluvastatin sodium was provided by Corealis Pharma (Laval, QC, Canada). Deuterium oxide (D_2O) was obtained from CDN Isotopes (Pointe-Claire, QC, Canada), while the excipient $NaHCO_3$ and hexamethyldisilane were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Sample preparation

Multilamellar vesicles (MLVs) were prepared by mixing 36 mg of phospholipids in 120 μL of water or the aqueous solution of drug and/or excipient, giving a total proportion of 23% (w/w) lipids in water. The drug and excipient were diluted in water then added to the lipid mixture to obtain a lipid-to-drug molar ratio of 25:1 and a lipid-to-excipient (L/E) molar ratio of 7.5:1 if not otherwise specified. This lipid/fluvastatin ratio was selected to better identify any effect of the drug on the membrane. All samples underwent at least three series of freeze (liquid N_2)/thaw (50°C)/vortex shaking cycles and were stored at −20°C. The pH was 6 in all FS-containing samples, and ~8 in the presence of the excipient. To study the impact of FS and $NaHCO_3$ on model membranes containing negatively charged lipids, samples were prepared with DMPC and DMPS at a molar ratio of 10:1 using DMPC-d_{54}. Interference of water with the FTIR spectra was eliminated by utilization of D_2O. ^{1}H NMR experiments were performed using fast-tumbling

bilayered micelles (bicelles) prepared as described above for the MLVs by mixing DMPC with DHPC at a long-to-short chain (q) ratio of 0.5 in D_2O. Negatively charged bicelles (Bic/PS) were made by replacing 10 mol% of DMPC with DMPS (Marcotte et al. 2003; Struppe et al. 2000).

^{1}H NMR lateral diffusion experiments

The measurement of the lateral diffusion of molecules in solution by ^{1}H NMR was performed at 37°C in D_2O on a Varian Inova 600 MHz NMR spectrometer using a double-resonance z-gradient probe (Varian, Walnut Creek, CA, USA). A bipolar pair longitudinal eddy current delay [BPP (LED)] pulse sequence was used as described by Wu et al. (1995). The chemical shifts were referenced relative to an external solution of 2,2-dimethyl-2-silapentane-5-sulfonic acid (DSS) set to 0.0 ppm. The ^{1}H 90° pulse length was 3.6 µs. The gradient pulse duration δ was 25 ms. The diffusion times (Δ), i.e., the time between the PFG pulses, ranged between 0.175 and 0.250 s to ensure an attenuation of the echo intensities by at least 80%. A total of 24-K data points were obtained, and four scans were acquired for each selected gradient strength (G, in G/cm) with a recycle delay of 3 s. A complete attenuation curve was obtained by measuring 25 gradient strengths linearly incremented between 1.98 and 49.55 G/cm. The diffusion (D_S) was calculated using the following equation (Altieri et al. 1995):

$$A(G) = A(0)\exp\left[-(\gamma\delta G)^2(\Delta - \delta/3)D_S\right] \quad (1)$$

where $A(G)$ is the echo amplitude and γ is the ^{1}H gyromagnetic ratio. The gradient strength was calibrated using back calculation of the coil constant from a D_2O diffusion experiment using $D_S = 1.9 \times 10^{-5}$ cm^2/s at 25°C (Holz 2000).

FTIR experiments

Infrared spectra were recorded with a Nicolet Nexus 670 Fourier transform spectrometer (Thermo-Nicolet, Madison, WI, USA) equipped with a narrow-band mercury-cadmium-telluride (MCT) detector and a germanium-coated KBr beam splitter. Twenty microliters of the sample was placed between CaF_2 windows separated by a 6 µm Mylar spacer. A total of 100 interferograms were acquired with a resolution of 2 cm^{-1} in the spectral range of 4,000–650 cm^{-1} at various temperatures ranging from 15 to 50°C. The spectra were corrected for water vapor and CaF_2 contribution by subtraction of a reference spectrum using an empty cell. The data were processed with the software Grams/AI version 7.02 (Galactic Industries, Waltham, MA, USA). The spectral region corresponding to the carbon-hydrogen and carbon-deuterium stretching vibrations was baseline-corrected using a linear function, while a cubic function was used for the carbonyl and carboxylate stretching vibration regions.

Results

Effect of the excipient $NaHCO_3$ on the affinity of fluvastatin for the membrane phospholipids

Fluvastatin is known to traverse jejunum cell walls mainly by a passive diffusion mechanism, but the affinity of the drug towards the biomembranes remains to be quantified, especially in the presence of an excipient. We have thus measured the percentage of association of FS with the model membranes using ^{1}H NMR lateral diffusion experiments. Because liquid-state NMR studies fast-tumbling molecules or objects in solution, isotropic bilayered micelles (bicelles) were used in lieu of lipid MLVs classically employed in FTIR analysis. Bicelles are made when long- and short-chain phospholipids assemble in an aqueous solution. The long-chain DMPCs (±DMPS in this work) form the planar section of bicelles, the edges of which are stabilized by the short-chain dihexanoylphosphatidylcholines (DHPC), thus leading to disc-shaped fast-tumbling bilayers at low long-to-short chain lipid molar ratios (typically $q \leq 1$) (Marcotte and Auger 2005; Sanders and Schwonek 1992; Triba et al. 2005). A q ratio of 0.5 was employed in this work. To verify any specific affinity of the drug for phosphatidylserines or phosphatidylcholines, the effect of FS ± $NaHCO_3$ on PC bicelles was compared with bicelles made of 10 mol% DMPS (Bic/PS).

The ^{1}H NMR spectra of FS in water, bicelles, and Bic/PS with and without $NaHCO_3$ are shown in Fig. 2. The aromatic region of FS is shown for better clarity as lipid resonances obscure most of those of the drugs at lower chemical shifts. The resonance assignment, obtained by peak integration and multiplicity analysis, is in agreement with Cermola et al. (2007) in the case of FS in water. The interaction of FS with the model membranes is readily evidenced by a broadening as well as a shift of the aromatic proton resonances of the drug when in a membrane environment. No changes of the ^{1}H resonances assigned to the rest of the molecule were observed. This result, thus, indicates that the aromatic moieties of FS would be inserted into the bilayer, a location unmodified by the presence of $NaHCO_3$. The comparable changes in the ^{1}H chemical shifts of the drug in all membrane systems suggest a similar environment upon insertion into the bilayer.

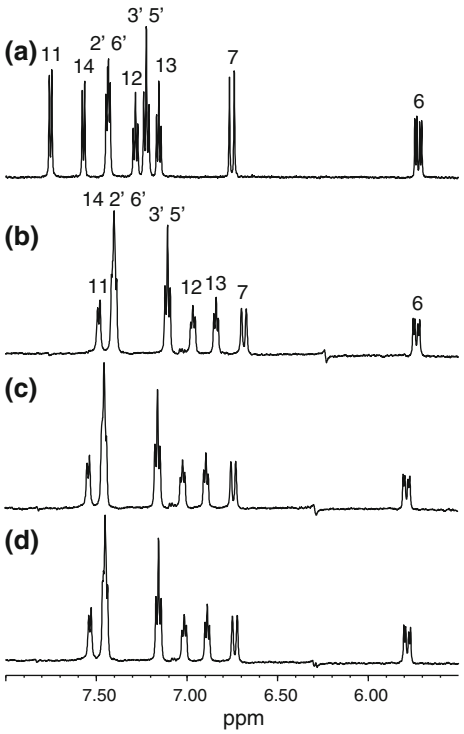

Fig. 2 Aromatic region with peak assignment of the ^{1}H NMR spectrum of fluvastatin obtained in **a** water, **b** bicelles, **c** Bic/PS, and **d** Bic/PS and $NaHCO_3$ at a lipid-to-excipient molar ratio of 7.5:1, at 37°C

The association of FS to bicelles—or to Bic/PS—evidenced by the ^{1}H NMR spectra can be quantified using a pulsed field gradient stimulated echo (PFGSTE) ^{1}H NMR experiment (Chartrand et al. 2010; Gao and Wong 1998; Marcotte et al. 2004a). It allows for the measurement of the self-diffusion coefficients of free fluvastatin molecules in water (D_{free}) as compared to their diffusion observed (D_{obs}) in the presence of model membranes. If FS is in rapid exchange on the NMR time scale between the free and bound states, as proposed by Stilbs (1987), D_{obs} will be an average of the diffusion coefficients for the free and bound (D_{bound}) molecules, where D_{bound} corresponds to the diffusion coefficient of bicelles. Following this two-site model, the percentage of FS bound to the membrane can be calculated as follows:

$$\frac{D_{obs} - D_{free}^{\phi}}{D_{bound} - D_{free}^{\phi}} \times 100 \qquad (2)$$

A corrected free diffusion coefficient $D_{free}^{\phi} = A \times D_{free}$ is employed to take into account the possibility of diffusion hindrance by the presence of bicellar obstacles in the solution. As is well described in the literature, an obstruction factor $A = 1/(1 + 0.5\phi) = 0.935$ can be calculated using a spherical model, which is well suited for bicelles with a q ratio of 0.5 (Chartrand et al. 2010; Gaemers and Bax 2001). Because phospholipids are rapidly exchanged between the solution and bicelles, the diffusion coefficient of bicelles was more accurately determined using hexamethyldisilane (HMDS) resonances, a hydrophobic probe that inserts into the apolar core of the model bilayers (Chartrand et al. 2010; Stilbs 1987).

Table 1 presents the diffusion coefficients of FS, bicelles, and Bic/PS, as well as the percentage of association of the drug to the membranes, with or without use of its excipient. A lipid-to-drug molar ratio of 25:1 was used to better monitor the effect of the drug on the membrane. Because the excipient is the major constituent of a drug formulation, a high concentration of $NaHCO_3$ was employed, leading to a lipid/FS/$NaHCO_3$ molar ratio of 7.5/0.3/1. As can be observed, FS has a strong affinity for both model membranes, with a 100% binding of the drug being calculated in zwitterionic and anionic bicelles, with an estimated experimental error of 4%. Interestingly, the excipient does not hinder the association of FS to the lipid membranes even when used at high concentration.

Effect of the excipient $NaHCO_3$ on the interaction of fluvastatin with the membrane phospholipids

The ^{1}H NMR results described above indicate that the excipient does not affect the affinity of FS for the membrane. They also reveal that the aromatic moieties of the drug would be inserted into the membrane. The subsequent aims of our work were thus (1) to gain a better insight on the interaction mechanism of FS with the membrane phospholipids and (2) to verify the potential involvement of the excipient with respect to this interaction. FTIR spectroscopy experiments were thus performed as it is a powerful method to probe the hydrophilic and hydrophobic regions of a phospholipid bilayer simultaneously. The impact of a drug on the membrane structure and organization can be readily assessed by monitoring key frequencies related to the stretching vibrations of several structural groups such as carbonyl in the headgroup, or methylenes within the apolar core (Mendelsohn and Mantsch 1986). Multilamellar vesicles (MLVs) made of DMPC and 10 mol% of DMPS were used as model intestinal cell membranes and compared to pure DMPC MLVs. Saturated DMPC and DMPS vesicles were employed for better comparison with bicelles. In addition, as will be seen in the following section, their melting temperature allows observing the gel-to-fluid phase transition by FTIR spectroscopy as opposed to unsaturated phospholipids. It also permits studying drug-lipid interactions in the fluid phase at a temperature around 37°C.

Table 1 Translational diffusion coefficients of bicelles and fluvastatin used to calculate the fraction of membrane-associated drug

System	Diffusion ($\times 10^{-10}$ m^2 s^{-1})[a]		Association of fluvastatin (%)[b]
	Bicelles	Fluvastatin	
Fluvastatin	–	5.02	–
Bic/FS	0.72	0.67	100
Bic/FS/$NaHCO_3$	0.70	0.64	100
Bic/PS/FS	0.68	0.68	100
Bic/PS/FS/$NaHCO_3$ (1)[c]	0.69	0.69	100
Bic/PS/FS/$NaHCO_3$ (3)[c]	0.63	0.69	98

[a] The diffusion coefficients are an average of three resonances

[b] A percentage of error of 4% is estimated (Marcotte et al. 2004a)

[c] Lipid-to-excipient molar ratios of (1) 15:1 and (3) 3.8:1

Apolar region of the membrane

As extensively demonstrated in the literature, the thermotropic phase behavior of phospholipid membranes can be exploited to assess the impact of molecules such as drugs, cholesterol, peptides, and proteins on the organization of the hydrophobic region of the lipid bilayers (Auger et al. 1987; Bensikaddour et al. 2008; Casal and Mantsch 1984; Marcotte et al. 2004b; McMullen et al. 1994; Mendelsohn and Mantsch 1986). This behavior can be established by plotting, as a function of temperature, the methylene symmetric (ν_s) or asymmetric (ν_a) stretching frequencies of the acyl chains found at 2,850 and 2,920 cm^{-1}, respectively (Mendelsohn and Mantsch 1986). These infrared stretching vibrations are sensitive to changes in the trans/gauche conformer ratio, thus providing information on structural rearrangements in lipid bilayers such as the gel ($L_{\beta'}$)-to-liquid crystal (L_α) phase transition (Casal and Mantsch 1984; Mendelsohn and Mantsch 1986). Moreover, the mass variation due to isotopic substitution such as deuteration will change the frequency of the absorption band without affecting the force constants (Mendelsohn and Mantsch 1986). This effect can be exploited to simultaneously verify the interaction of FS and $NaHCO_3$ with both the zwitterionic and negatively charged lipids. This is done by using membranes made of DMPC with perdeuterated acyl chains (DMPC-d_{54}) and protonated DMPS, and by monitoring the temperature dependence of both DMPC-d_{54} CD_2 and DMPS CH_2 stretching frequencies.

The changes induced by the addition of the drug and excipient were first verified on the apolar region of pure DMPC model membranes by plotting the thermotropic behavior of this lipid, as illustrated in Fig. 3. The CH_2 symmetric stretching vibration was selected because it does not overlap with the CH_3 asymmetric stretching mode at ~2,956 cm^{-1}. In addition, FS absorbs very poorly in this region of the infrared spectrum (Papageorgiou et al. 2009). The gel-to-liquid crystal phase (melting) transition

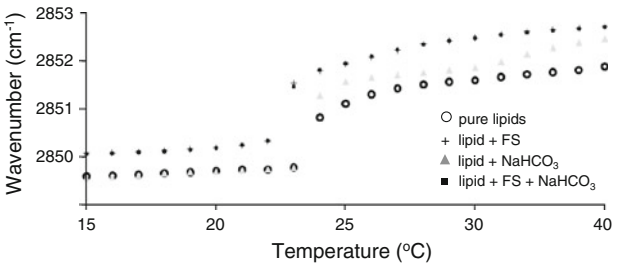

Fig. 3 Temperature dependence of the frequency of the CH_2 symmetric stretching vibration of DMPC vesicles in the absence or presence of FS and $NaHCO_3$ at a lipid-to-FS and lipid-to-$NaHCO_3$ molar ratios of 25:1 and 7.5:1, respectively

temperature (T_m) of DMPC is observed at ~23.5°C in Fig. 3, consistent with the theoretical value of 23°C (Marsh 1990). The small increase in the T_m value can be accounted for by the use of deuterated water (Wang and Chen 1993). The addition of $NaHCO_3$ to DMPC vesicles induces a minor reduction in the lipid melting temperature as well as a small augmentation in $\nu_s CH_2$ in the L_α phase, indicating some level of disordering. The presence of FS in the DMPC bilayer environment, however, increases the CH_2 symmetric stretching band frequency in both lipid phases and reduces the gel-to-fluid phase transition temperature by 1°C. The changes induced by fluvastatin are explained by an insertion of the drug in the bilayer with ensuing disordering in the acyl chain conformation. The thermotropic behavior of DMPC as detected by infrared spectroscopy is identical whether FS is added in combination with its excipient or not.

To verify any preferential interaction of fluvastatin with the anionic or zwitterionic lipids in the model gut cell membranes, the thermotropic phase behavior of DMPC-d_{54} and DMPS was then evaluated (Fig. 4). The temperature dependence of the symmetric CD_2 stretching vibration (~2,090 cm^{-1}) for DMPC-d_{54} in the mixed MLVs is plotted in Fig. 4a. To better demonstrate the effect of $NaHCO_3$ on the drug/membrane interaction, three different

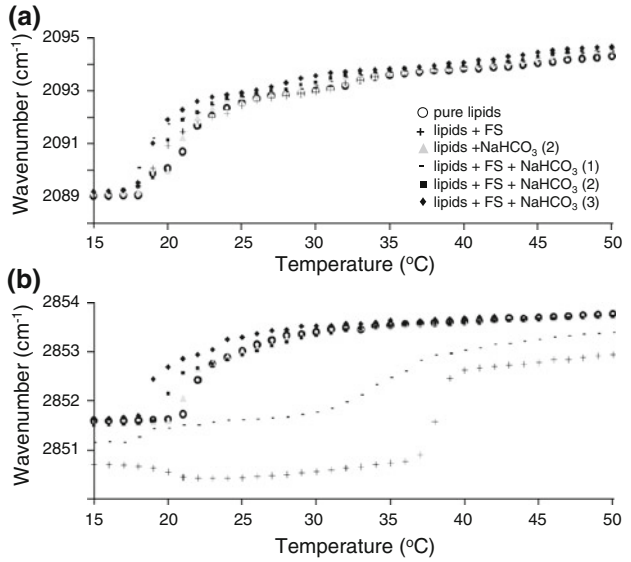

Fig. 4 Temperature dependence of the frequency of **a** the CD_2 symmetric stretching vibration of DMPC-d_{54} and **b** the CH_2 symmetric stretching vibration of DMPS in DMPC-d_{54}/DMPS model membranes in the absence or presence of fluvastatin and $NaHCO_3$. Lipid-to-FS molar ratio of 25:1 and lipid-to-$NaHCO_3$ molar ratios of (1) 15:1, (2) 7.5:1, and (3) 3.8:1 were employed

L/E molar ratios were employed: 15:1, 7.5:1, and 3.8:1. First, the $L_{\beta'}$-to-L_α phase transition of DMPC-d_{54} is observed at ~21°C in the pure binary lipid mixtures consistent with the values reported in the literature for deuterated lipids (Guard-Friar et al. 1985; Mendelsohn and Mantsch 1986; Moore et al. 1996). The addition of either FS or $NaHCO_3$ at an L/E molar ratio of 7.5:1 has virtually no effect on the thermotropism of this lipid. However, when both the drug and excipient are present in the bilayer environment, T_m is reduced by 2°C and $\nu_s CH_2$ shows a small increase in both lipid phases, especially at high excipient concentrations. This result suggests that the combination of sodium bicarbonate with FS induces some disordering in DMPC deuterated acyl chains.

Figure 4b presents the temperature response of DMPS $\nu_s CH_2$ in model DMPC-d_{54}/DMPS membranes. It shows that in the case of pure mixed vesicles, the melting temperature of DMPS is 22°C, i.e., close to the expected value (22.6°C) for a homogeneous miscible mixture (Silvius and Gagne 1984) considering the respective T_m and mole fractions of the lipids (Marsh 1990):

$$T_m = \chi_A \cdot (T_m)_A + \chi_B \cdot (T_m)_B \quad (3)$$

where A = DMPC-d_{54} (T_m = 21°C, χ = 0.9) and B = DMPS (T_m = 37°C, χ = 0.1). As was observed for DMPC-d_{54}, the thermotropic behavior of DMPS is not modified by pure $NaHCO_3$ at an L/E molar ratio of 7.5:1. On the contrary, a cooperative phase transition occurs at a higher temperature when pure fluvastatin is added to DMPC-d_{54}/DMPS membranes. The T_m of 38°C in this case is close to the $L_{\beta'}$–L_α transition of 37°C for pure DMPS (sodium salt) (Marsh 1990), indicating that a lipid segregation is induced by the presence of the drug in the membrane environment. The effect of FS on the melting temperature of DMPS is accompanied by a diminution (~1.5 cm^{-1}) of the CH_2 symmetric stretching frequency of DMPS acyl chains, indicative of a greater ordering of the negatively charged lipids in this PS-rich environment. This chain ordering could be due to the tighter packing of phosphatidylserines versus their PC analogs due to intermolecular H-bonds, as detailed by Petrarche et al. (2004). A small transition is observed in the gel phase at ~21°C, close to the T_m value of DMPC-d_{54}. This could be explained by the compartmentalization of DMPS molecules into more ordered DMPS gel domains.

Most interestingly, the addition of the drug and $NaHCO_3$ at L/E molar ratios of 15:1, 7.5:1, and 3.8:1 shows an effect antagonistic to that of FS with increasing proportions of $NaHCO_3$. At an L/E molar ratio of 3.8:1, the T_m of DMPS (19°C) is identical to that of DMPC-d_{54} in the same mixture and conditions (Fig. 4a), indicating the reestablishment of a homogeneous phospholipids mixture.

Altogether, the FTIR results presented in Figs. 3 and 4 show that fluvastatin disturbs the acyl chain ordering of the phospholipids in the membrane. They also reveal a preferential interaction with phosphatidylserines that induces lipid segregation, but not in the presence of the excipient. Because DMPC and DMPS share a common chain length, the different interaction of the drug with zwitterionic and negatively charged lipids could possibly be ascribed to the actual nature of the hydrophilic headgroup.

Polar region of the membrane

To verify this hypothesis, changes in the polar region of the bilayer were inspected by examining the variations of the carbonyl asymmetric stretching band ($\nu_{C=O}$). Studies have shown that this broad band, centered at ~1,735 cm^{-1}, arises from the superposition of free and H-bonded carbonyl groups found at 1,742 and 1,728 cm^{-1}, respectively (Blume et al. 1988; Lewis et al. 1994). Useful information on the hydration and H-bonding of the carbonyl groups can thus be obtained by observing changes in the relative intensity of these bands. In general, the free carbonyl band intensity is higher in the more compact gel phase as compared to the liquid crystalline phase because the former allows less penetration of water molecules (Schechter 1990). This can be readily observed in Fig. 5 where a more pronounced edge is observed in the gel phase at ~1,740 cm^{-1} in both zwitterionic and negatively charged membranes.

Examination of the DMPC MLVs in Fig. 5a reveals no influence of the drug and/or its excipient on the carbonyl

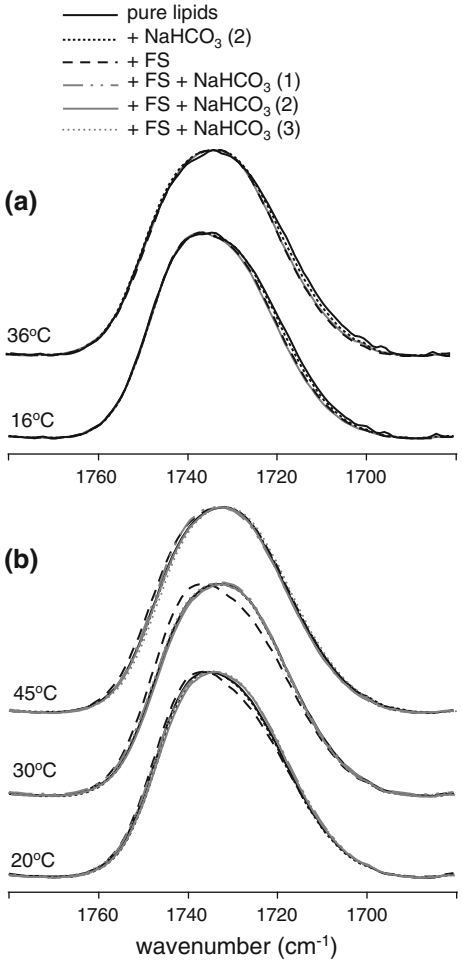

Fig. 5 Effect of fluvastatin and $NaHCO_3$ on the phospholipid C=O stretching band in **a** DMPC and **b** DMPC/DMPS model membranes, in the gel and liquid crystalline phases. Lipid-to-FS molar ratio of 25:1 and lipid-to-$NaHCO_3$ molar ratios of (1) 15:1, (2) 7.5:1, and (3) 3.8:1 were used

region of the bilayers in both the gel (16°C) and liquid crystal (36°C) phases. *A contrario*, Fig. 5b shows that fluvastatin raises the proportion of free carbonyl groups centered at $\nu_{C=O}$ ~1,740 cm^{-1} in model DMPC/DMPS membranes. This suggests the presence of more lipids in a more ordered phase or a reduced penetration of water molecules when FS is used free of excipient. When FS is mixed with its excipient even at low proportions (L/E = 15:1), the effect of the drug on the carbonyl region of the spectra is canceled. This effect is particularly evidenced at 30°C—i.e., between the T_m of DMPC and DMPS—at which the addition of $NaHCO_3$ to the drug reestablishes a liquid-crystal phase profile for this band as expected for a homogeneous DMPC/DMPS membrane.

It should be noted that the study of the carbonyl stretching band reveals changes occurring to both DMPC and DMPS in the model gut cell membranes. Therefore, to probe the interaction of fluvastatin and its excipient specifically on the phosphatidylserines, the stretching frequency of its carboxylate headgroup was studied. The carboxylate constitutes a useful probe of hydrogen and metal ion binding to the serine group. In particular, the asymmetric stretching band on the infrared spectrum can be readily studied (Mantsch and McElhaney 1991). It is located between 1,620 and 1,640 cm^{-1} depending on the extent of hydration. The spectrum of FS shows no interference with this lipid band (Papageorgiou et al. 2009).

Figure 6 shows that the carboxylate band is centered at 1,625 cm^{-1} for DMPS in pure DMPC/DMPS bilayers, typical of hydrated phosphatidylserines (Auger et al. 1990). This band shifts to higher frequencies (~2 cm^{-1}) from the gel to the fluid phase as it senses the phase transition of phosphatidylserines as shown elsewhere (Dluhy et al. 1983). Interestingly, the presence of fluvastatin shifts the COO^- band by ~2 cm^{-1} to lower frequencies at all temperatures. This effect can be rationalized in terms of the involvement of carboxylate groups in H-bonds with water molecules or polar moieties of the drug (Casal et al. 1987). FS also broadens the carboxylate band by ~24% at 20 and 30°C, and by 15% in the liquid-crystal phase (45°C). This band broadening could be interpreted as a less rigid conformation of the serine moiety. Interestingly, increased proportions of $NaHCO_3$ counteract the effect of the drug on

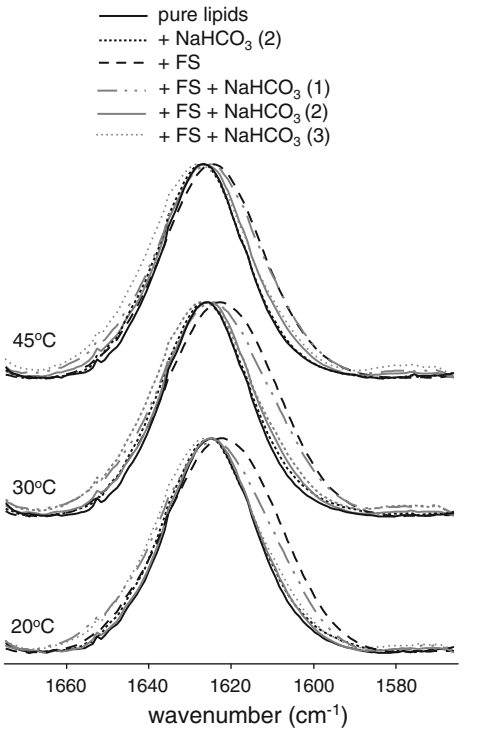

Fig. 6 Effect of fluvastatin and $NaHCO_3$ on the COO^- stretching vibration of DMPS headgroup in DMPC/DMPS model membranes, in the gel and liquid-crystal phases. Lipid-to-FS molar ratio of 25:1 and lipid-to-$NaHCO_3$ molar ratios of (1) 15:1, (2) 7.5:1, and (3) 3.8:1 were used

the frequency and width of the carboxylate band in good agreement with the re-establishment of membrane homogeneity.

Discussion

The aim of this work was to determine if the excipient $NaHCO_3$ often employed in fluvastatin oral formulation can affect the interaction of this drug with phospholipids in model intestinal cell membranes. Such a study is important because passive permeation is the main mechanism of penetration of this anti-cholesterol drug in intestinal cells whose membranes are principally composed of phospholipids (Brasitus and Schachter 1980). FS is known to be highly absorbed (98%) by patients (Papageorgiou et al. 2009). In this work, with the use of ^{1}H NMR and FTIR spectroscopy, we have determined whether (1) the affinity of FS for membrane phospholipids and (2) the mechanism of interaction are modified by the presence of a pharmaceutical excipient, $NaHCO_3$, using model DMPC and DMPC/DMPS membranes.

The ^{1}H diffusion experiments used in this work clearly show the high affinity of fluvastatin for the phospholipid membranes. Importantly, this affinity is unchanged when FS is used with $NaHCO_3$ as it was 100% in all mixtures (Table 1). This result is consistent with one of the desired characteristics of a pharmaceutical formulation, i.e., to not hinder the gastrointestinal absorption of a drug.

To demonstrate any potential involvement of the excipient in the drug/lipid interaction, we have investigated the effect of FS on model phospholipid membranes in the absence and presence of $NaHCO_3$. In a first step, we evaluated the interaction of the drug with zwitterionic lipids in PC membranes. As clearly evidenced by ^{1}H NMR spectra using bicelles, the aromatic moieties of FS would be inserted in the PC membrane as shown by the change in their chemical shifts and broadening of the resonances. The disordering of the lipid acyl chains observed by FTIR analysis with DMPC MLVs suggests that the bulky aromatic groups of FS molecules would be buried into the hydrophobic region of the bilayer. The polar groups of the drug are therefore expected to be found in the polar region of the membrane. Although no changes are seen in the carbonyl region of the bilayer when FS and/or $NaHCO_3$ are used, it is possible that H-bonds with water can be replaced by H-bonds with the hydroxyl and carboxyl groups of the drug, as such interaction was also observed for FS with chitosan (Papageorgiou et al. 2009). This hypothesis is supported by a previous study of membrane binding interactions of statins with biomembranes performed with immobilized artificial membranes (IAMs). Statins (including FS) were shown to interact with the PC polar head groups via van der Waals interactions and hydrogen bonds. The retention was enhanced by the statin lipophilicity, indicating that hydrophobic forces are important for the membrane association of these molecules (Sarr et al. 2008). Our data show in addition that the interaction of FS with phosphatidylcholines is independent of the presence of the excipient.

In the case of phosphatidylserine-containing membranes, major changes are observed if fluvastatin is used *without* $NaHCO_3$. Our results show that the drug selectively interacts with phosphatidylserines in DMPC-d_{54}/DMPS bilayers. This is evidenced by the FTIR monitoring of the CD_2/CH_2 stretching vibration frequencies as a function of temperature showing drug-induced lipid segregation. As a consequence, the DMPC-enriched parts created in the membranes undergo a gel-to-fluid phase transition at $\sim$21°C, while DMPS domains melt at DMPS's characteristic T_m of $\sim$38°C. Such a preferential interaction is also reported in the literature for glycophorin towards dipalmitoylPS in DPPC-d_{62}/DPPS bilayers (Mendelsohn et al. 1984). The DMPC-d_{54} acyl chain order is maintained in the PC-rich environment (Fig. 3b). It is however possible that the naturally tighter organization of DMPS acyl chains as compared to DMPC occludes the effect of the drug on the apolar region of the PS domains where, according to the ^{1}H NMR spectra, FS aromatic moieties would be buried. The increase in lipids in the gel phase is also revealed by the analysis of the carbonyl band where more free C=O groups are seen at temperatures below the T_m of DMPS (Fig. 4b). The shift of the IR carboxylate stretching band of the serine groups suggests that the polar portion of the drug would reside in the hydrophilic part of the bilayer, with the hydroxyl or acid groups most likely H-bonding with the COO^- groups of DMPS which are usually bound to nearby cations at physiological pH (Holloway and Mantsch 1988; Roux and Bloom 1990).

The preferential interaction of FS with phosphatidylserines can be rationalized in terms of the occurrence and availability of polar groups on the phospholipid headgroup. At the lipid-to-drug molar ratio of 25:1 used in this study, two phosphatidylserines are available per FS molecule. The various polar sites on the drug can thus interact with several DMPS molecules, explaining the drug-induced lipid separation. As compared to phosphatidylcholine headgroups, phosphatidylserines have more polar and charged moieties available for H-bonds or electrostatic interactions with the drug. The ability of phosphatidylserines to form hydrogen bonds was demonstrated by Lewis and McElhaney (Lewis and McElhaney 2000) and also supported by Pandit and Berkowitz (2002) from molecular dynamics simulations. Electrostatic interactions between the carboxylate group of FS and NH_3^+ group of DMPS are possible, but changes in the IR vibrational modes of the amine moiety are hardly seen because this group is known

to form strong H-bonds with water. The resulting broad IR bands are generally not useful in aqueous samples (Mendelsohn and Mantsch 1986).

Our results suggest that the interaction of pure fluvastatin with model gut cell membranes would benefit from preferential H-bonds and electrostatic interactions with the PS headgroup, in addition to an affinity of the aromatic moieties with the apolar chains of the lipids. However, this favored interaction with the negatively charged lipids is shown to be cancelled when the excipient $NaHCO_3$ is employed. More specifically, our FTIR data demonstrate that increasing the excipient concentration re-establishes the homogeneity in the bilayer, as well as the interaction of FS with the abundant DMPC molecules as seen by the perturbation of the acyl chains. The ^1H NMR experiments confirm that FS is still 100% associated with the DMPC/DMPS bilayer in which the aromatic groups are embedded, as revealed by both the shift and the broadening of the drug resonances on the ^1H NMR spectrum (Fig. 2d). The cancelling of the phase segregation is confirmed by the carbonyl and carboxylate bands recovering their original profiles (Fig. 4b).

The effect of $NaHCO_3$ on the interaction mechanism of FS with the model gut cell membranes can be justified by a competition, with FS, of Na^+ ions for the negative carboxylate sites on PS. When added in the aqueous DMPC/DMPS membrane milieu, $NaHCO_3$ dissociates and raises the pH value from 6 to 8, with the serine headgroup being totally ionized (Marsh 1990). The equilibrium of FS molecules is more displaced towards their negatively charged active state. There is also an increased amount of Na^+ in the solution, which represents 1.5, 2, and 3 times the total concentration of DMPS and drugs when the L/E molar ratio is 15:1, 7.5:1, and 3.8:1, respectively. As shown by Roux and Bloom (1990), Na^+ tends to remain above the PS headgroup, i.e., in the carboxylate region. The abundant Na^+ ions would thus occupy these sites that would no longer be available for hydrogen bonding with FS polar groups. This would explain the counter effect of $NaHCO_3$ on the carboxylate band frequency and width (Auger et al. 1990). Moreover, the increasing number of HCO_3^- counterions could also bind the NH_3^+ sites of the phosphatidylserines, dislodging FS molecules that were electrostatically bonded via their negative COO^- charge.

Conclusion

Fluvastatin penetrates through intestinal cell walls mostly via passive permeation enabled by its amphiphilicity and low molecular weight (Lindahl et al. 1996). Its interaction with cell membranes is thus important for its entry within the blood circulation and access to the liver, the target organ of HMG-CoA reductase inhibitors. By combining ^1H NMR and FTIR analyses, this work showed that the affinity of the drug for the membrane phospholipids is not modified by the use of the excipient $NaHCO_3$. It also demonstrated the interaction mechanism of FS in model gut cell membranes that naturally contain $\sim$10% of PS. A preferential interaction of the anticholesterolemic drug with negatively charged lipids was demonstrated and shown to induce segregation between the zwitterionic and anionic lipids. H-bonds and electrostatic interactions between FS and PS polar groups as well as hydrophobic interactions between the aromatic moieties and the bilayer apolar core would be important for the association of the drug to the membrane. The presence of the excipient $NaHCO_3$ reestablishes the lipid homogeneity in the bilayer without affecting the drug affinity and location in the bilayer interface, FS being able to interact with the abundant phosphatidylcholines. Excipients such as $NaHCO_3$ can change the pH and ion concentration of the drug microenvironment in the intestine (Jackson et al. 2000), triggering competition of the salt for FS binding sites on the negatively charged lipids. In conclusion, this work shows that preferential interactions with lipids can be involved in passive drug permeation mechanisms and demonstrates that excipients can play an important role in drug/membrane interactions.

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