NOTE

Combined Quantitative and Mechanistic Study of Drug–Membrane Interactions Using a Novel ^{2}H NMR Approach

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ABSTRACT: Several analytical methods are available for determining the partition coefficients of drug compounds in model phospholipid membranes, but such methods provide little information at the molecular level about how the membrane affinity of drugs relates to their interactions with the lipid molecules. A new ^{2}H nuclear magnetic resonance (NMR) approach has been developed here that quantifies the affinity of ^{2}H-labeled small molecules for different phospholipid membranes and, simultaneously, provides information on the mechanism of the drug–membrane interaction. In the example given, ^{2}H NMR analysis of a weakly basic ion pump inhibitor found that the drug partitioned preferentially into membranes of predominantly unsaturated or short-chain phospholipids. The ^{2}H NMR analysis also suggested that the membrane specificity of the drug was directly correlated to the ability of its phenyl moiety to penetrate into the interior of the lipid bilayer. The ^{2}H NMR approach could be of value in guiding medicinal chemistry toward or away from structures promoting interactions with specific types of biological membranes. J Pharm Sci 93:507–514, 2004

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INTRODUCTION

The physical association between pharmaceutical compounds and cellular membranes is crucial for the absorption and distribution of the drug, for its delivery to the pharmacological target, and for its passage across the blood–brain barrier.[1] In some cases, the drug may act directly on the plasma membrane; for example, the pharmacology of the anticonvulsant valproic acid[2] and the anticancer agent adriamycin[3] is thought to involve interactions with lipid bilayers. In other cases, the accumulation of a drug at the membrane surface may counter pharmacological efficacy by lowering the effective concentration of the drug and reducing its bioavailability.[4]

Drug lipophilicity can be described by a simple partition equilibrium model and quantified as a partition coefficient (K_p) representing the molecular distribution in a binary isotropic solvent

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system such as n-octanol/water.[5] Partition coefficients of drugs in solvent mixtures are not always reliable predictors of lipophilicity, however. Basic aromatic amines, for example, tend to partition into phospholipid membranes more readily than is suggested by their octanol/water partition coefficients.[6] Methods that examine drug partitioning into model membranes such as phospholipid vesicles are generally more reliable predictors of lipophilicity. Of these, the most accurate are nonperturbing spectroscopic[7] or chromatographic methods[8] that measure drug distributions at equilibrium without physically separating the membrane and aqueous fractions.

Current analytical methods fail to establish the connection between the membrane affinity of drugs and how they associate with the lipid bilayer. The aim of this work was to investigate the utility of ^{2}H nuclear magnetic resonance (NMR) spectroscopy as a new method for elucidating the relationship between the membrane affinity of amphiphilic drugs and how they interact with the membrane at the molecular level. It is well known that the ^{2}H NMR spectra of ^{2}H-labeled amphiphiles associated with lipid bilayers may exhibit powder line shapes characterized by a quadrupole splitting $\Delta\nu_q$ (Fig. 1B), which contains information on the molecular order and dynamics of the membrane-bound amphiphile.[9] Another application of ^{2}H NMR has been to measure the affinity of amphiphiles for aqueous membrane and cellular suspensions in terms of an equilibrium membrane K_p value.[10] This work combines these two applications of ^{2}H NMR for the first time to determine simultaneously (a) the affinity of the drug for model membranes containing different phospholipids, and (b) the relationship between membrane affinity and the order and stability of the drug when associated with the lipid bilayers. An aromatic amine compound was selected for the study (Fig. 1A) because of its amphiphilic characteristics, weak basicity, and pharmaceutical relevance as a gastric H^+/K^+-adenosine triphosphatase (ATPase) inhibitor.

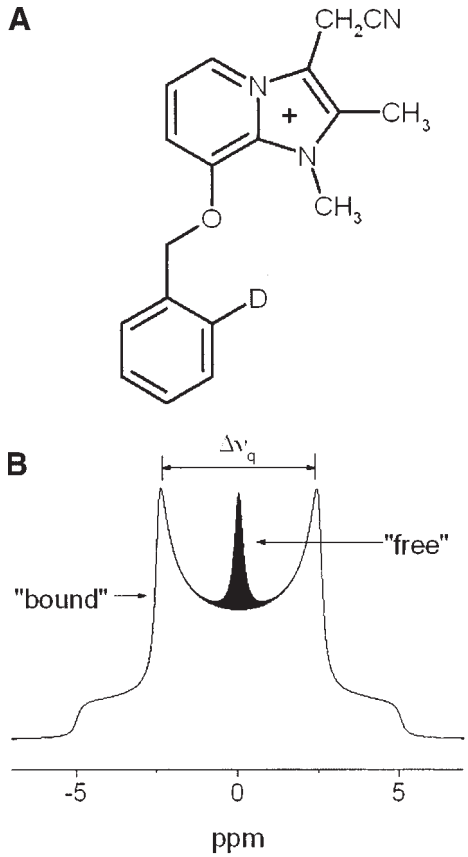

Figure 1. The chemical structure of [*o*-^{2}H]DCMPIP (A) and a simulated ^{2}H spectrum of [*o*-^{2}H]DCMPIP in a lipid bilayer suspension (B). The shaded area of the spectrum (A_i) represents "free" drug not associated with the lipid bilayer. The unshaded area (A_p) is the broad powder spectrum from "bound" drug associated with the membrane. The quadrupole splitting $\Delta\nu_q$ is measured as shown.

EXPERIMENTAL

Synthetic Methods

The deuterated drug compound 1,2-dimethyl-3-cyanomethyl-8-[*o*-^{2}H]phenylmethoxyimidazo[1,2-*a*]pyridinium ([*o*-^{2}H]DMCPIP) was prepared according to the methods of Kaminski et al.[11] by reaction of 2-dimethyl-3-cyanomethyl-8-hydroxyimidazo[1,2-*a*]pyridine with [*o*-^{2}H]benzyl chloride, followed by reflux of the product with iodomethane.

Preparation of Large Multilamellar Phospholipid Vesicles

Large unilamellar vesicles were prepared containing either pure dilauroylphosphatidylcholine (DLPC), dimyristoylphosphatidylcholine (DMPC), dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), or distearoylphosphatidylcholine (DSPC). Mixed vesicles of DMPC and DOPC were also prepared. The phospholipids (10–50 mg) were dissolved in 1:1 chloroform/methanol and the solvent was evaporated to form a thin lipid film. The film was

suspended in 1 mL of deuterium depleted water and centrifuged at 50,000*g*. The lipid pellets were retained and lyophilized overnight. [*o*-^{2}H]DMCPIP was dissolved to 1.25–10 mM in 10 mM Tris-HCl (pH 7.4 in deuterium depleted (d.d.) water) and 10–20 μL of the solution was added to the lipid.

NMR Spectroscopy

Deuterium NMR spectra were recorded at an operating frequency of 55.12 MHz. Samples were contained within sealed 5-mm-diameter quartz tubes positioned transverse to the magnetic field and maintained at temperatures of 4° to 60°C (±0.1°). Spectra were obtained using either a π/2 pulse-acquisition sequence with quadrature phase cycling or the quadrupole echo sequence[12] with π/2 pulse lengths of 3–8 μs and a recycle delay of 1 s.

Data Analysis

Simulations of two-component ^{2}H NMR spectra were performed using a computer program that generated Lorentzian line shapes convoluted with axially symmetrical quadrupolar powder patterns with intensity profiles calculated according to Seelig.[13] The computer algorithm took as variables $\Delta\nu_q$, K_p, and the Lorenztian line width at half height ($\Delta\nu_{1/2}$). The simulations providing the closest fit to the experimental spectra were determined by χ-square analysis.

THEORETICAL

Calculation of Partition Coefficients

If the exchange rate (ν_{ex}) of a ^{2}H-labeled amphiphile between the membrane and aqueous phase is slow with respect to the residual quadrupolar interaction (i.e., $\nu_{ex} \ll \Delta\nu_q/2$), a ^{2}H NMR spectrum is observed that consists of a broad "powder" component from the membrane-associated drug and a narrow central component from the free drug (Fig. 1B). The areas of the two components are proportional to the concentration of the drug in the two environments and K_p can be calculated according to[10]:

$$K_P = \frac{A_p V_a}{A_i V_m} \tag{1}$$

where A_i and A_p are the areas of the narrow inner component and broad powder component, respectively, obtained by spectral simulations. V_a and V_m are the volumes of the aqueous and membrane fractions.

Extraction of Motional and Orientational Information

Amphiphiles within fluid lipid bilayers undergo rotations about a principal axis of motion, usually the bilayer normal. The measured quadrupole splitting $\Delta\nu_q$ for a ^{2}H-labeled amphiphile (Fig. 1B) is related to an orientational order parameter S_{CD} of the carbon-deuterium bond[13]:

$$\Delta\nu_Q = \frac{3}{4} A_Q S_{CD} \tag{2}$$

where A_Q is the static deuterium quadrupolar coupling constant (175 kHz for a C–H bond) and S_{CD} can take values from 0 (highly disordered) to 1 (highly ordered). For axially symmetric motions, S_{CD} can be expressed as[14]:

$$S_{CD} = \left|\frac{3\cos^2\theta - 1}{2}\right| S_{mol} = S_\theta S_{mol} \tag{3}$$

where S_θ reflects the average orientational angle θ between the carbon-deuterium bond in the labeled moiety and the main axis of motional averaging. S_{mol} is an order parameter for the angular fluctuations of the C–D bond relative to the main axis of motion. Hence, $\Delta\nu_q$ contains information about the average molecular orientation of a C–D bond in lipid bilayers and on the amplitude of its motions, but it is not possible to determine S_θ and S_{mol} independently from the single deuterated site.

RESULTS AND DISCUSSION

The physiochemical properties of cell membranes can have a profound influence on how they interact with drugs,[15] and a series of experiments was designed to examine systematically the interactions of [*o*-^{2}H]DMCPIP with model phospholipid membranes varying in their composition. The drug was synthesized with ^{2}H incorporated into the phenyl ring adjoined to the imidazopyridine system by a flexible ether linkage (Fig. 1A). The spectrum of the deuteron therefore reports on the membrane affinity of the drug and on the changes in the motional and orientational behavior of the phenyl ring within the different membrane environments. The line shapes of all

the ^{2}H NMR spectra recorded were consistent with slow exchange of [*o*-^{2}H]DMCPIP on the residual quadrupolar (millisecond) time scale[10] and with axially symmetrical motions of the membrane-associated drug. Control experiments confirmed that no signal was present from residual D_2O.

The first series of experiments examined the effect of membrane fluidity on the membrane affinity and dynamics of [*o*-^{2}H]DMCPIP. The fluidity of the lipid bilayers was controlled by preparing mixed membranes of the unsaturated hydrocarbon chain phospholipid DOPC and saturated DMPC in varying proportions. Membrane fluidity increases with increasing unsaturation so that, at a constant temperature above the chain melting temperature T_m, the only experimental variables are the physical properties of the hydrophic membrane interior. If associations between [*o*-^{2}H]DMCPIP and the membrane involve only superficial electrostatic interactions with the choline headgroups, it is expected that the line shape of the ^{2}H spectra will change very little in response to the proportions of DMPC and DOPC. If the drug penetrates deeper into the lipid bilayer, the proportions of DOPC and DMPC may exert greater influence over the ^{2}H NMR line shape.

Figure 2A shows the spectra of [*o*-^{2}H]DMCPIP added to membrane suspensions of DMPC and DOPC. The values of K_p obtained from ^{2}H NMR spectra of [*o*-^{2}H]DMCPIP in membranes containing different proportions of lipids are shown in Figure 2B. K_p for the drug in pure DMPC membranes was close to a value of 12 (Fig. 2B). This value increased only slightly when the drug was added to membranes containing up to 50% DOPC. However, a substantial increase in K_p was observed when the DOPC content of the membrane was raised to more than 50% of total lipid, reaching a maximum value of 96 in 100% DOPC membranes (Fig. 2B). Hence, [*o*-^{2}H]DMCPIP has a high affinity for membranes containing a dominant proportion of unsaturated lipids but a much lower affinity for membranes of predominantly saturated lipids. Unsaturated lipid membranes are loosely packed and disordered by the presence of the double bonds, which may allow the phenyl group of the drug to extend into the hydrophobic interior where it is stabilized in an energetically favorable state by van der Waals forces.

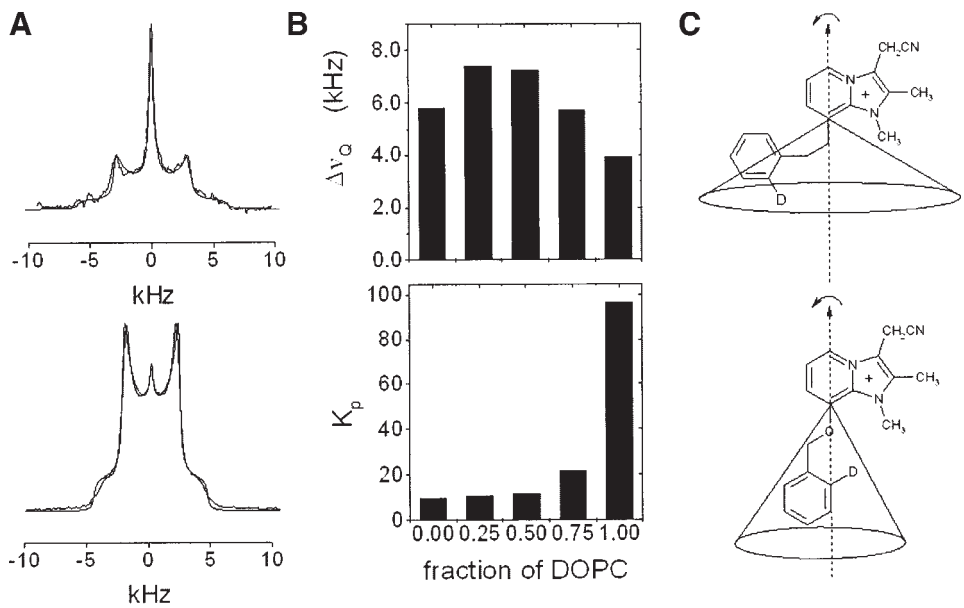

Figure 2. A study of the effect of lipid unsaturation on the membrane partitioning of [*o*-^{2}H]DCMPIP. Values of K_p and $\Delta\nu_q$ were measured from ^{2}H NMR spectra of the drug in membranes containing DMPC and DOPC. The spectra of the drug in pure DMPC (top) and DOPC (bottom) are shown (A). The values of K_P and $\Delta\nu_Q$ for [*o*-^{2}H]DCMPIP in pure DMPC and pure DOPC membranes illustrate the differences in the distribution and dynamics of the drug in the different bilayer suspensions (B). The spectral observations are interpreted as different orientations and motional amplitudes of the phenyl ring about the linker group in saturated and unsaturated lipid membranes (C). The experimental temperature was 37°C.

Quadrupole splittings measured from the spectra of [*o*-^{2}H]DMCPIP in the mixed DMPC/DOPC membranes (Fig. 2A, bottom) offer insight into why the drug has a higher affinity for unsaturated lipid membranes than for saturated membranes. The splitting of 5.9 kHz measured from the spectra of [*o*-^{2}H]DMCPIP in pure DMPC membranes (Fig. 2B) indicates that the molecular motions of the drug, which appear to be axially symmetrical, give rise to a 30-fold reduction of the static quadrupolar interaction (~175 kHz). From previous studies on related compounds, it is assumed that the principal rotational axis of the drug is coincident with the C–O bond and parallel with the bilayer normal.[16] Although the conformation of the molecule in DMPC membranes cannot be determined unambiguously, it was assumed as a starting point for the analysis that the drug adopts a planar, extended structure in which the angle between C–D bond and the rotational axis is 60°. However, substitution of $\theta = 60^\circ$ into eq. 3 indicates that rapid rotation of the entire drug molecule about the single, principal axis would be insufficient to scale the quadrupolar interaction to 5.9 kHz. Hence, the drug does not undergo rotational diffusion as a rigid body and additional internal motions must be present, which scale $\Delta\nu_q$ further. Such internal motions probably arise from excursions of the ^{2}H-phenyl group about the ether linkage, which give rise to large angular fluctuations of the C–D bond around the principal axis of molecular rotation (i.e., $S_{mol} \ll 1$). In membranes containing 25% DOPC and 75% DMPC, $\Delta\nu_q$ increased to 7.8 kHz indicating that the presence of the small proportion of unsaturated lipid altered the mean orientation of the ^{2}H-phenyl group (i.e., a change in θ) or reduced the amplitude of motion (i.e., an increase in S_{mol}), or both. However, further increases in the proportion of DOPC in the membrane led to a decrease in $\Delta\nu_q$ until a value of 3.9 kHz was reached for pure DOPC membranes (Fig. 2B). Hence, the order of the phenyl group within the membrane may again decrease as the DOPC content is raised from 25 to 100%. No evidence for phase separation of the DOPC/DMPC mixtures was found, either in the presence or absence of the drug. Such a possibility, although unlikely for this binary lipid mixture, cannot be ruled out entirely.

The analysis of the measured splittings suggests a model for the interaction of [*o*-^{2}H]DMCPIP with the membranes. When the drug partitions into pure DMPC membranes, the ^{2}H-phenyl ring is unable to intercalate between the saturated lipid hydrocarbon chains. Instead, the phenyl ring undergoes high amplitude motions about its ether linkage and close to the membrane surface (Fig. 2C, top). Hence, the association of the drug with DMPC membranes is relatively superficial and the partition coefficient for [*o*-^{2}H]DMCPIP in predominantly saturated lipid bilayers is low. When small amounts of unsaturated lipids are present, the hydrocarbon chain packing is relaxed, which increases both the mean volume of the lipid molecules and the distance between them.[17] The phenyl group of the drug is now more readily accommodated within the stabilizing environment of the bilayer interior where its molecular motions are constrained (Fig. 2C, bottom). Further increases in membrane unsaturation lead to greater disorder in the bilayer, which allows for higher amplitude motional excursions of the now intercalated phenyl group, this time within the membrane interior rather than close to the surface. Hence, the ability of the phenyl group to penetrate the loosely packed lipid bilayers appears to underpin the higher affinity of [*o*-^{2}H]DMCPIP for unsaturated membranes.

The experiments described above were performed on liquid-crystalline membranes at temperatures well above the chain melting temperatures of the phospholipids, to provide a physiologically relevant environment for the drug. Interestingly, spectra of [*o*-^{2}H]DMCPIP in pure gel-phase DMPC membranes at 15°C exhibited only a single, narrow component (not shown), indicating that the drug interacted minimally with the membranes in the gel phase. This observation, although not physiologically relevant, provides further evidence that the drug requires fluid lipid membranes for partitioning.

Further experiments were performed to examine how interactions with lipid hydrocarbon chains affect the membrane affinity and molecular order of the drug in lipid bilayers. Lengthening the lipid hydrocarbon chains increases the van der Waals interactions between them, which in turn increases the melting temperature T_m of the lipids and decreases membrane fluidity at a given temperature.[18] A series of spectra were recorded of [*o*-^{2}H]DMCPIP in phosphatidylcholine membranes with saturated hydrocarbon chain lengths increasing as C12:0 (DLPC), C14:0 (DMPC), C16:0 (DPPC), and C18:0 (DSPC). The spectra were recorded at a constant reduced temperature T_{red} [where $T_{red} = (T - T_m)/T$] to ensure that the behavior of the drug was not modulated by reductions in membrane fluidity as the lipid chain

length increased. In practice, the actual temperature of the experiments varied between 4° and 60°C. Variable temperature measurements on the [*o*-^{2}H]DMCPIP in DLPC indicated that, provided the lipids were in the fully fluid phase, the membrane affinity of the drug was not significantly affected increasing the temperature from 5° to 60°C even though $\Delta\nu_q$ values indicated that the mobility of the phenyl ring increased significantly (Fig. 3A).

Values of K_p and $\Delta\nu_q$ were measured from the ^{2}H spectra of [*o*-^{2}H]DMCPIP in DLPC, DMPC, DPPC, and DSPC membranes (Fig. 3B). The value of both K_p and $\Delta\nu_q$ decreased as the lipid chains were lengthened two segments at a time from C14:0 to C18:0; indeed, a positive linear correlation was observed between K_p and $\Delta\nu_q$ in the four membrane systems (Fig. 3C). The spectrum of [*o*-^{2}H]DMCPIP in DSPC bilayers did not exhibit the broad component seen in all other spectra. Hence, either the drug does not associate with these membranes or its exchange rate onto and off the membrane is rapid on the millisecond time scale. The low affinity of the drug for long-chain membranes is probably because the phenyl ring is excluded from the highly ordered region of the long hydrocarbon chains close to the glycerol backbone. Surprisingly, the electrostatic interaction between the charged imidazopyridine ring of [*o*-^{2}H]DMCPIP and the phospholipid polar head groups is alone unable to stabilize the interaction of the drug with the membrane. As the length of the lipid chains is shortened, chain packing close to the glycerol backbone becomes more relaxed. The phenyl group is increasingly able to penetrate into the membrane interior where it is motionally restrained, as evidenced by the increasing value of $\Delta\nu_q$. The linear relationship between K_p and $\Delta\nu_q$ therefore suggests that the affinity of the drug for the different chain length lipid membranes is again driven entirely by the ability of the membrane interior to stabilize the phenyl ring.

CONCLUSIONS

Here, ^{2}H NMR has been shown to be a useful nonperturbing method for quantifying the membrane affinities of a drug or family of drugs. Unlike other spectroscopic methods for probing membrane partitioning, the ^{2}H NMR method can provide additional information to elucidate the mechanism by which the drug interacts with the membrane. Although isotope labeling is required,

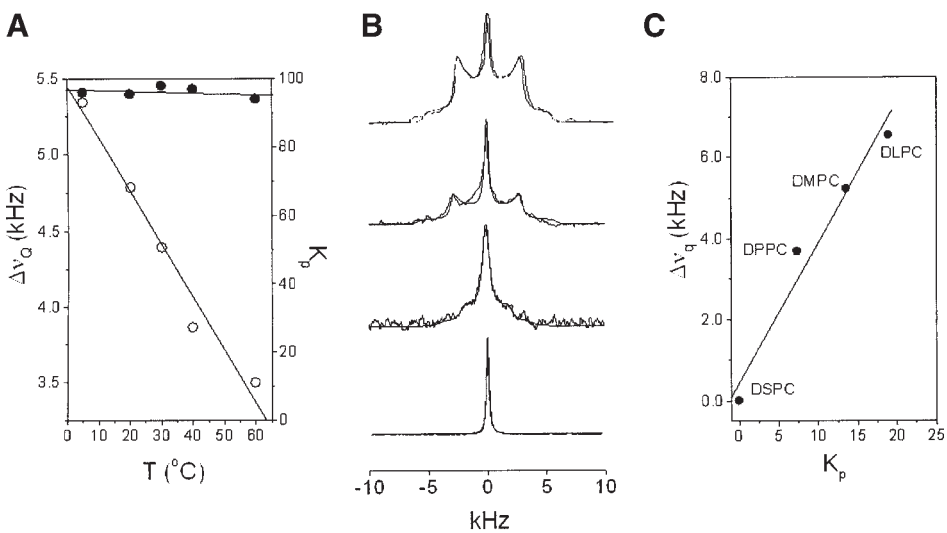

Figure 3. A study of the effect of lipid chain length on the membrane partitioning of [*o*-^{2}H]DCMPIP. A plot of K_p (filled circles) and $\Delta\nu_q$ (open circles) values for [*o*-^{2}H]DCMPIP in DLPC (C12:0) membranes at 4°–60°C (A) shows that there is little change in the partition distribution of the drug over the temperature range. The ^{2}H NMR spectra for [*o*-^{2}H]DCMPIP in membranes of phosphatidylcholine with different hydrocarbon chain lengths (from top: DLPC, DMPC, DPPC, DSPC) exhibited line shapes that were sensitive to the membrane composition (B). A plot of K_p against $\Delta\nu_q$ measured from the different spectra showed a positive linear correlation (C). Experiments were performed on fluid phase lipids at a reduced temperature of 0.09, which corresponds to an absolute temperature of 4°C for DLPC and 60°C for DSPC.

many small molecules can be ^{2}H-labeled at low expense and without major adaptation of the synthetic routes.

In the given example, ^{2}H NMR has revealed that the affinity of the drug [*o*-^{2}H]DMCPIP for unsaturated and short-chain lipid membranes is critically dependent on the accessibility of the membrane interior to the phenyl group of the drug. Interestingly, electrostatic interactions between the cationic charge of the drug and the membrane surface have little influence on partitioning behavior. Further insight into the mechanism of the drug–membrane interaction would be gained by knowing the orientation of the drug molecules within the lipid bilayer. Such information could not be determined unambiguously by the experiments described here, but may be obtained from ^{2}H NMR experiments on uniaxially aligned membranes as described by Opella et al.[19] If the angle between the lipid bilayer normal and the external magnetic field is known, analysis of ^{2}H spectra of [*o*-^{2}H]DMCPIP within the bilayer could, in principle, yield the molecular orientation of the drug relative to the bilayer normal. Such experiments have been very successful for hydrophobic, membrane-embedded peptides, but here the diffusion of the drug in and out of the membrane at equilibrium is expected to complicate the spectral analysis.

The drug investigated here is an H^+/K^+-ATPase inhibitor, which accesses its membrane embedded target from the extracellular face;[20] hence, interactions with the membrane would reduce the effective concentration of the inhibitor. The information provided by ^{2}H NMR might in this case help to guide the medicinal chemistry toward structures in which the phenyl group is replaced with a less lipophilic yet pharmacologically active moiety.

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