PCCP

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

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Position and orientational preferences of drug-like compounds in lipid membranes: A computational and NMR approach.

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Running Title: Drug-membrane interactions.

Abstract

Permeation of drugs across lipid bilayers is a key factor in dictating how effective they will be. *In vivo*, the issue is compounded by the presence of drug-exporter proteins such as P-glycoprotein. However, despite intense effort, exactly what controls permeation and susceptibility to export is still poorly understood. In this work we examine two well-studied drugs for which interaction with P-glycoprotein has been studied before: amitriptyline, a known substrate and clozapine, which is not a substrate. Extensive MD simulations, including potential of mean force (PMF) profiles of the compounds in all possible protonation states, reveal that the preferred location of the compounds in different bilayers in different protonation states is remarkably similar. For both molecules in charged states, there is a substantial barrier to crossing the bilayer. Clozapine however, shows an energetic barrier to movement across the bilayer even in a protonation state that results in an uncharged molecule. For amitriptyline there is only a very small barrier of approximately 1.3 kcal/mol. Further analysis revealed that the conformational and orientational behavior of the two compounds was also similar, with the sidechain interacting with the lipid headgroups. This effect was much stronger if the sidechain was charged (protonated). These interactions with lipid bilayers were confirmed by NMR ROESY experiments. The results are discussed in terms of their potential interactions with export proteins like P-glycoprotein.

Introduction

The discovery and development of novel drugs that target proteins within the brain or central nervous system (CNS) is hampered by the complexity of the blood-brain barrier (BBB). The paracellular route (between cells at the barrier between the blood and the CNS) is blocked by the so-called "tight-junctions",[1] and therefore the access into the CNS is more limited. The most commonly presumed route, is one of simple diffusion through the endothelial cell membranes and thus much effort has gone into investigating the relationship between efficacy and lipid solubility since the pioneering work of Overton.[2]

A further complication is provided by the fact that many compounds, even if they are lipid soluble, are extruded back into the blood by the efflux transporters, the prime example being P-glycoprotein or Pgp.[3, 4] In the popular "vacuum cleaner model", drugs are proposed to reside in the non-polar core of the lipid bilayer, before they diffuse laterally into the binding site of Pgp, where ATP-binding and hydrolysis drives the export of the drug back into the blood-stream. Though it has been possible to build regression-based quantitative structure activity relationship (QSAR) models that can be used in a reasonably predictive fashion in the drug-discovery process to help remove Pgp susceptibility,[5] exactly what determines whether a compound will be susceptible to Pgp export remains unclear, despite intense effort.

The broad acceptance of the vacuum cleaner model implies that the initial interactions of a compound with Pgp would be influenced by knowledge of where, and in what orientation, a compound will adopt when absorbed into the lipid bilayer. Thus, we decided to explore this in more detail via two compounds, both of which are known to act at CNS targets and whose interaction with Pgp has been studied before. Amitriptyline (Fig. 1A) is a Pgp substrate[6-8], and is widely used as a serotonin-norepinephrine reuptake inhibitor.[9] Clozapine (Fig. 1B) is not a Pgp substrate[10-12] and is an atypical antipsychotic medication used in the treatment of schizophrenia. It binds to serotonergic as well as dopamine receptors.[13]

The interaction of various tricyclic drugs with lipid bilayers has previously been reported using various methods. Carfagna *et al.* reported electrostatic interactions between the protonated amino group of tricyclic drugs and the membrane surface and that these interactions were crucial to function.[14] Harder *et al*. reported a fluidizing effect of amitriptyline on lipid bilayers[15] whilst McIntosh used X-ray diffraction to show that a similar compound, chlorpromazine, induces inter-digitation of phosphatidylcholine tails.[16]

Casarotto and Craik[17] found that tricyclic antidepressants adopt an extended conformation in sodium dodecyl sulphate (SDS) micelles, with the tricyclic moiety occupying the hydrophobic region of the micelle and the dimethylammonium moiety residing at the micelle/water interface. The same group found amitriptyline adopts a folded conformation with the di-methylammonium terminal group in close proximity to the tricyclic moiety[18] and have also examined the ring flexibility of tricyclic antidepressants.[19] It has also been shown that clozapine forms extensive hydrogen-bond interactions with

neutral egg phosphatidylcholine monolayers and increases the average area per molecule in 1,2-palmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC)/brain phosphatidylserine mixtures in compression isotherms.[20]

In this paper we examine the orientational, conformational and positional preferences for two tricyclic compounds using a combination of molecular dynamics simulations and NMR experiments and consider the results with respect to their potential interaction with Pgp. This knowledge could potentially be exploited in the rational design of future CNS drugs to help avoid Pgp susceptibility.

Materials and Methods

Computational

Each simulation system consists of one tricyclic compound (amitriptyline or clozapine, see Fig. 1); 200 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC), 200 1,2-palmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC) or 189 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC) lipids in the initial setup, 8000 TIP4P water molecules[21] and zero to two chloride ions (depending on the protonation state of the drug). Amitriptyline has one protonatable nitrogen and clozapine has two (Fig. 1). We consider all protonatable states as individual systems and hereafter refer to the unprotonated states as neutral (as they have no charge).

The one-dimensional (1D) PMF was calculated using umbrella sampling with the reaction coordinate along the *z*-axis of the system, which corresponds to the bilayer normal. The umbrella potential acts on the center of mass of the drug molecule, with a harmonic potential of force constant of 1000 kJ $mol^{-1}Å^{-1}$, which was found to be the minimum value required in a previous systematic test of force constants for similar sized compounds.[22] The starting configuration for each umbrella window was obtained by placing the drug molecule independently at different z-coordinates resulting in 61 windows separated by a width (along *z*) of 1.0 Å.

To introduce the drug molecules into the system at each window, a slow-growth approach (similar to that typically employed in free energy perturbation calculations) was used to transform a non-interacting molecule to a fully-interacting molecule over a period of 0.5 ns. During this process the drug molecule was held fixed and the rest of the system was free to move. Each window was then simulated for 20 ns with the first 4 ns considered an equilibration period (under identical simulation conditions, thus leading to 16 ns of production data for each window). Production data derived in this way has been shown previously to be statistically uncorrelated to the equilibration portion of the trajectories.[22] The harmonic force was applied for the entire duration of the simulation.

The (OPLS) forcefield[23, 24] was used with OPLS compatible lipid parameters provided by M. Ulmschneider and as previously described[25]. Drug topologies

were obtained using the topolbuild (obtained from www.gromacs.org/@api/deki/files/93/=topolbuild1_3.tgz) and charges for the drug molecules were manually assigned according to OPLS[23, 24] atom types. Parameters for Cl^- were also taken from OPLS.[23, 24] The Berendsen algorithm[26] was used to couple the temperature of the system with a coupling constant of 1 ps at 318 K. The system pressure was coupled in semi-isotropic fashion (x and y, independent of z) at 1 bar, using the Berendsen algorithm with a compressibility of $13x10^5$ bar and a coupling constant of 1 ps. Electrostatic interactions were accounted for by a particle-mesh Ewald method[27] with a real-space cutoff of 10 Å and a grid spacing of 0.015 Å. The van der Waals interactions were computed with a cutoff of 10 Å, and fourth-order interpolation. The timestep was 2 fs and the LINCS algorithm[28] was used to constrain bonds that contained hydrogen atoms.

All simulations were performed using the GROMACS (www.gromacs.org) simulation package.[29, 30] VMD was used for visualization and orientation analysis.[31] The PMF profile was constructed from the biased distributions of the centers of mass of the drugs, using the weighted-histogram analysis method implemented via the g_wham analysis program in the GROMACS simulation package.

Experimental

1,2-Dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC) and perdeuterated DHPC (d-DHPC) were purchased from Avanti Polar Lipids and used as received. Amitriptyline and clozapine were obtained from Sigma. Bicelle samples containing lipid and detergent were prepared in 50 mM phosphate buffer, pH 7.0, 99.9 % D_2O, and 5 % azide for ROESY experiments and in a multiple-buffer solution of 20 mM potassium citrate, 20 mM potassium phosphate and 20 mM potassium carbonate and 99.9% D_2O for the pH titration experiments. Bicelle samples were made by first preparing a solution of DHPC in buffer. Then an appropriate amount of this stock solution was added to a weighed amount of drug (in powder form) and then DMPC (in powder form). The sample was subjected to cycles of freezing followed by sonication until the solution was clear.

Alternatively bicelle samples were made using a thin film preparation method as follows. DMPC (and drug) were dissolved in chloroform in a glass vial; the chloroform was evaporated under an N_2 stream while rotating the vial; the thin film was further dried under a high vacuum overnight. The thin film was then rehydrated with D_2O on rollers for up to 10 hours. Subsequently d-DHPC was added to form bicelles. In both cases, the bicelle mixture was subsequently titrated to the appropriate pH.

ROESY experiments

Rotational nuclear Overhauser effect spectroscopy (ROESY) experiments[32-34] were recorded at 500 MHz (^1H) with a 300 ms mixing time on drug and bicelle

samples containing d-DHPC and protonated DMPC. The magnitude of the NOEs between drug and lipid protons were taken to be proportional to the crosspeak volume determined using NMRPipe.[35]

Results

Simulation of Spontaneous Partitioning

We first performed 6 x 20 ns of unrestrained MD simulations with amitriptyline (Fig. 1A) initially positioned in bulk solution 16 Å from either edge of a pure POPC bilayer to investigate spontaneous partitioning of a tricyclic compound into bilayers. Amitriptyline contains a protonatable choline group with a pKa of 8.6 (calculated using Marvin, MarvinSketch 5.9.4, 2012 from ChemAxon) and simulations were run on both the protonated and neutral forms of amitriptyline. We observed spontaneous partitioning into POPC bilayers (Fig. 2) for both repeat simulations of protonated amitriptyline and a single simulation of neutral amitriptyline.

Neutral amitriptyline appeared to partition with no preferred orientation with both the ring and sidechain moieties interacting with POPC simultaneously (10.1 - 11.1 ns) and with its long axis perpendicular to the bilayer normal. In contrast protonated amitriptyline appeared to partition with its ring moiety first and its long axis parallel to the bilayer normal. Once inside the head-group region of the lipid bilayer, neutral amitriptyline was able to freely rotate and penetrate further into the bilayer interior. Protonated amitriptyline on the other hand, remained in a fixed orientation with its sidechain pointing towards the headgroups and the ring moiety pointing towards the bilayer interior. This orientation appeared to be maintained by interactions between the dimethyl ammonium group of amitriptyline and the phosphate groups of the POPC lipids.

Unrestrained Partitioned Simulations

To explore sampling of the interactions of amitriptyline and clozapine with lipids more extensively, subsequent simulations were started with the compounds placed at 1 of 5 locations within POPC bilayers; at the middle of the bilayer (z = 0 Å) or at either of 2 locations within each leaflet (z = ±8, ±17 Å) with the long axis of the ring moiety roughly perpendicular to the bilayer normal (to enhance sampling of what was assumed to be a potentially unfavourable orientation). The singly protonated and neutral forms of both amitriptyline and clozapine were simulated.

In order to assess the preferred location of the compounds, we analysed the density profiles of the centers-of-mass of both compounds. Partial densities of the compound centers-of-mass were histogrammed as a function of the z-coordinate (i.e. lipid bilayer depth). The results for the neutral and singly protonated species in POPC are shown in Fig. 3. Neutral forms of amitriptyline exhibited a greater preference for the bilayer relative to bulk solution than the neutral form of clozapine. Furthermore, clozapine exited the bilayer in all

simulations (at t = 3.1, 7.1, 10.3, 11.7 and 19.1 ns). These results suggested that protonated forms of both drugs preferred to reside in the interface, but there was a difference in localization preference for the neutral forms.

Potential of Mean Force Profiles

While a clear difference in localization between amitriptyline and clozapine was observed in the unrestrained simulations, particularly of their neutral forms, the asymmetry of the density profile plots indicated incomplete sampling and we thus turned to umbrella sampling to calculate potential of mean force (PMF) profiles of the permeation process for these molecules. PMF profiles were calculated for all possible protonation states of the two compounds in DPPC and POPC bilayers. We also performed the calculations in DMPC bilayers to match NMR experiments. The profiles calculated for amitriptyline and clozapine in POPC, DPPC and DMPC lipids were broadly similar with no considerable differences in the location of the profile minima across lipids (SI Fig. 1). Given the similarity of observations in all simulated lipid bilayer systems, and for brevity, all analyses reported subsequently are for the DMPC system only.

The PMFs in DMPC bilayers as a function of the center of mass of the compounds in protonated and neutral forms is shown in Fig. 4. Key data from the PMF calculations are summarized in Table 2. The singly protonated forms of both compounds exhibited similarly shaped profiles with an energetically favourable well of -4.6 to -4.9 kcal/mol compared to bulk centered around the interfacial region between the lipid tail and headgroup region (12-14 Å from the bilayer centre). To move from these wells in order to cross the middle of the bilayer involves the crossing of a barrier at the centre of the bilayer (see Fig. 4). The height of this barrier compared to the lowest point of the interfacial wells is considerable for both singly protonated forms (8.1 kcal/mol for amitriptyline and 9.3 kcal/mol for clozapine; see Table 2). An even larger barrier of 15.1 kcal/mol exists for the doubly protonated species of clozapine. For the neutral species, the energy barrier for amitriptyline is lowered more than it is for clozapine. Neutral clozapine exhibits a profile with a 3.7 kcal/mol barrier in the middle of the bilayer (with respect to the lowest point of the interfacial well), in contrast to the profile seen for neutral amitriptyline which exhibits a smaller barrier of ~1.3 kcal/mol.

Drug Conformations

We next assessed the conformational behaviour of the compounds. For amitriptyline, the distributions of distances from the dimethylallylamine sidechain N-atom to all three of the aromatic ring moieties were measured in order to capture any significant mobility of the side chain[18] (Fig. 5). All three distance-distributions spanned a range of 4 Å with the dimethylallylamine sidechain N to ring 1 distance having the broadest distribution. The distributions are broadly similar regardless of protonation state (Fig. 5). However, the protonated form tends to push the nitrogen of the dimethylallylamine group toward ring 1 evidenced by a shift of the probability

density maxima for the 'sidechain N to ring 1' distance to smaller values, as observed experimentally for amitriptyline[18] and also for the closely related compound imipramine[19, 36]. In both protonation states and at all locations within the bilayer and in bulk solution, an extended conformation predominated (Fig. 5B).

Clozapine is predicted to have reduced sidechain mobility. Distances were as defined in Fig 6. The distribution of the methyl piperazine sidechain to ring 1 distances (Fig. 6A) was more affected by protonation state than that of the methyl piperazine sidechain to ring 2 distances (Fig. 6B). Bilayer location appeared to effect both distance distributions with greater effects observed for the protonated compared to the neutral forms, which appeared relatively invariant to bilayer location. The most extended conformations were observed for the protonated forms of clozapine, while the maxima of both distance distributions moved towards smaller methyl-piperazine sidechain to ring distances in the neutral form (Fig. 6).

In contrast to amitriptyline, the primary maxima of the dimethylallylamine sidechain N to ring 1 distance distribution was observed to shift to smaller distances in the order singly protonated > doubly protonated > neutral (Fig. 6A). In addition, bilayer location appeared to have a strong effect on the protonated form of clozapine with a dramatic shift in the position of the primary maxima towards smaller methylpiperazine sidechain to ring 2 distances upon moving from bulk solution to the centre of the bilayer (Fig. 6B).

Drug Orientations

The effects of protonation state on the conformations of amitriptyline and clozapine may be explained by their interactions with the bilayer during the permeation process as illustrated in Figs 7 and 8. The dimethylallylamine sidechain of protonated amitriptyline had a much more restricted orientation compared to that of the neutral form due to a strong interaction between the protonated side chain and the phosphate head groups. This was particularly apparent at the centre of the bilayer where the sidechain-phosphate interactions resulted in an extended conformation in which the aromatic rings lay perpendicular to the bilayer normal while the sidechain was oriented parallel to the bilayer normal pointing towards the lipid phosphates. This may account for the decrease in the population of the secondary folded conformation as protonated amitriptyline moved from bulk to the bilayer centre (cf Fig. 5). In contrast, the dimethylallylamine sidechain of the neutral form of amitriptyline appeared much more unrestricted within the bilayer adopting orientations that ranged from 0 to 90 ° relative to the bilayer normal (Fig. 7).

For clozapine, a similar (to amitriptyline) effect on the restriction of orientation for the protonated forms was observed (Fig. 8). The combined interaction of the ring and sidechain protons of the doubly protonated form of clozapine with the lipid phosphate groups appeared to prevent rotation of the molecule about the bilayer normal as was observed for the neutral and singly protonated forms.

Localization of the compounds determined by NMR

Rotational nuclear Overhauser effects (ROE) between drug and lipid protons were measured to obtain additional information on the position of the amitriptyline and clozapine aromatic rings in the lipid bilayer (Fig. 9). The ROE data suggests that the ring moiety of amitriptyline is embedded within the lipid tail region of the DMPC bilayer consistent with the position of the PMF minima (Fig. 4), which coincides with the lipid tail methylenes. A high cross-peak volume with acyl chain methyl and methylene groups was observed for ring protons of both compounds (Fig 9. A-D). This suggests similarity in localization of ring systems, with both of them favouring the bilayer interior and the protonated "sidechains" pointing either towards lipid glycerol or bilayer centre. The data also shows slight skewing of ring systems with one side of the ring buried deeper in the acyl chains than the other. Some differences can be observed in the cross peak volumes - ring protons of clozapine show higher values when compared to amitriptyline. Taken together with the internal rigidity of clozapine (due to less rotatable bonds than amitriptyline), suggests that there are more interactions with the acyl chains and a less dynamic behaviour of clozapine in the lipid bilayer.

Discussion

In this paper we have examined the behavior of two tricyclic drugs and their interaction with lipids using NMR and computational methods. We were particularly interested in these compounds because they are part of a larger effort to better understand the determinants of what makes a compound more susceptible to Pgp transport. A persistent issue is that of the protonation state of the lipid-embedded species. Indeed, it has recently been argued that the rate of protonation can modulate the diffusion speed of anaesthetics into bilayers.[37]

One might expect from these results that the more bilayer permeant species is the neutral form of the compounds and hence would also be the species that would be more likely to interact with Pgp. The deeper location of the energetic minima of the neutral forms compared to the protonated forms agrees with Boulanger *et al* who observed the uncharged forms of anesthetics to penetrate deeper into the membrane.[38, 39]

It is interesting to note that there is only a very small energetic barrier to crossing the middle of the bilayer for the neutral form of amitriptyline (1.3 kcal/mol), but a much larger one (3.7 kcal/mol) for clozapine. Neutral valproic acid[40] (a non-substrate for Pgp) also exhibits a large barrier (15 kcal/mol) to crossing the middle of the bilayer.[41]

At physiological pH it is likely that the singly protonated forms of these drugs will be the dominant species (at least in solution) given that the predicted pKa of the protonatable sidechain groups of amitriptyline and clozapine are 8.6 and 7.8 respectively. The question of what the pKa is, inside a bilayer environment is

more difficult to answer because there is no "bulk solution" and instead we tend to think of it as a measure of how difficult it is to protonate or deprotonate. Regardless of protonation state, the PMF profile minima of all the species studied were located within the bilayer at or near the headgroup regions. This is in agreement with studies showing high partition coefficients for both drugs and a study showing that efficiency of drug incorporation into bilayers is independent of protonation state.[42] This is also in agreement with studies showing a general interfacial location of aromatic molecules both experimentally for tryptophan residues,[43] amantadine,[44] anesthetics[39] as well as in MD simulations for cortisone,[45] adamantanes,[22] doxorubicin,[46] NSAIDs[47] verapamil, colchicine, rhodamine 123 and daunorubicin[48] and valproate.[41]

All protonated forms of the drugs studied exhibited barriers of at least 8 kcal/mol at the centre of the bilayer. This may be explained by strong interactions observed between the drug sidechain protons and the lipid phosphates. At the bilayer centre, these interactions resulted in the drug molecules adopting an extended conformation with the ring moieties oriented perpendicular to the bilayer normal and the "sidechain" extended outwards towards the headgroups. This orientation within the bilayer is similar to what was observed for cortisone, which, like the compounds studied here, is amphipathic possessing a polar sidechain and a more hydrophobic fused ring system.[45] As has previously been stated,[45] such an orientation may serve to maximize favourable drug-lipid contacts within the heterogeneous bilayer environment.

The presence of a large barrier in the PMF profiles for the protonated forms clearly suggests that it is energetically unfavourable for the drugs to remain at the bilayer centre. Casarotto and Craik found long range NOEs to be consistent with a model of amitriptyline adopting a folded conformation in DPPC vesicles [18]. In this folded conformation, the sidechain was folded backwards bringing the dimethylammonium terminal group in close proximity to the tricyclic rings.[18] The data from our simulations here suggests that on average the distance is indeed quite compact, though some quite extended states are not precluded.

Solution NMR has shown that the antidepressant drugs nitroxazepine and imipramine also adopt folded conformations in lipid bilayers.[36] It has also been suggested that the Pgp substrate verapamil adopts a folded conformation by optimizing polar and nonpolar interactions when incorporated into phospholipid vesicle bilayers.[49] We note that this kind of interaction is only possible where there is enough flexibility in the molecule and is not possible in more conformationally restricted molecules like clozapine.

Clearly, drug conformation is affected by environment. The conformations of the propyl-amino side chains of imipramine and amitriptyline have previously been reported to be solvent dependent.[50] A more complete study would involve the use of different phospholipids including anionic lipids. Jutila et al have shown chlorpromazine and clozapine have strong interactions with acidic PS phospholipids.[20] This seems particularly pertinent given the observation that the affinities of drugs for Pgp seem to be affected by lipid composition.[51] In addition, the cooperative effects of aggregation are likely to effect behaviour in the bilayer.

The limited treatment of cooperativity effects in the current study is clearly inadequate especially taking into account the observations of dimerisation in solution of imipramine[36, 50] and nitroxazepine.[36] Another limitation of the current study is that the treatment of polarization is likely to have a significant effect on the partitioning of compounds into the bilayer.

What do the results here suggest in terms of how these types of compounds interact with export proteins? There are two main factors; the first is how readily compounds can move between the lipid leaflets, which in turn, are functions of the free energy profiles and combinations of those profiles depending on the charge state of the different species. Compounds that can move between leaflets readily might be expected to engage with Pgp quicker and be more readily exported. However, it could also be the case that they are able to transition so efficiently that they effectively overwhelm the export cycle of Pgp and thus appear as non-substrates. Indeed, this has been observed for valinomycin, quinidine and quinine (MDR modulators with sub-second lifetimes in the bilayer).[52] The second factor is the whether the preferred orientation of the compounds facilitates or hinders lateral movement into the interior binding site of the export proteins. This aspect is something we are currently exploring further. We recognize that these speculations are thus far based on a very small number of compounds. Either way, the relationship between lipid interactions and exporter uptake requires much more investigation both experimentally and computationally.

Conclusions

In conclusion, the free energy profiles for these two compounds are very similar, but the neutral form of amitriptyline has a profile that would facilitate movement between leaflets once absorbed to the interface. Clozapine on the other hand, retains a relatively high barrier to movement across the bilayer even in the neutral form. The orientations of both compounds when they are within the bilayer are also similar in terms of the overall orientation, with the ring systems interacting with the non-polar tails and the protonatable substituents interacting with the lipid headgroups. This observation is supported by the ROESY NMR data. Many factors are likely to control whether a drug is susceptible to Pgp export, but much more work is required to delineate them fully before they can be exploited in a drug-design environment.

Acknowledgements

LD thanks the NC3Rs for support. JM acknowledges the Wellcome Trust for support. We thank the Advanced Research Computing center for compute time. Jolyon K. Claridge is acknowledged for assistance with NMR data acquisition.

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Table 1. Summary of Unrestrained Simulations.

Drug	Pgp substrate?	Protonation state	Lipid type	Duration (ns)*
Spontaneous Partitioning				
Amitriptyline	Y	Neutral	POPC	6 x 20
		Protonated	POPC	6 x 20
Starting inside Bilayer				
Amitriptyline	Y	Neutral	POPC	5 x 20
		Protonated	POPC	5 x 20
Clozapine	N	Neutral	POPC	5 x 20
		Protonated	POPC	5 x 20

*Spontaneous simulations were 6 repeats of amitriptyline placed randomly in the solvent either side of the bilayer. For simulations starting in the bilayer, 5 single repeats were performed starting with the drug in the bilayer at z = 0, ±8, ±17 Å.

Table 2. Summary of properties derived from the PMF calculations for all systems in a DMPC lipid bilayer. Well energy corresponds to the PMF value at the minimum of the profile, while the barrier is the difference between the well energy (PMF minimum) and PMF energy at the bilayer centre (0 Å).

Drug	Protonation State	Well energy (kcal/mol)	Well coordinate (Å)	Barrier (kcal/mol)
Amitriptyline	Neutral	-7.2	8.2	1.3
	Protonated	-4.9	12.1	8.1
Clozapine	Neutral	-6.1	10.2	3.7
	Protonated	-4.6	13.0	9.3
	Doubly Protonated	-5.8	14.0	15.1

Figure Legends

Figure 1. Schematics of the four compounds examined in this study: (A) amitriptyline and (B) clozapine.

Figure 2. Comparison of the spontaneous partitioning of protonated (A-D) and neutral (E-H) amitriptyline into a POPC bilayer at various time points during a simulation. For the protonated species, after the initial interaction with the membrane (A), there is a preferred position and orientation that amitriptyline adopts with respect to the lipid headgroups (B-D). In contrast the neutral species more readily explores positions deeper within the bilayer (F-H).

Figure 3. Partial density profiles of (A) amitriptyline and (B) clozapine along with the lipid headgroup phosphate of POPC bilayers. 20 ns simulations were set up with 1 drug molecule initially positioned at one of five locations in the bilayer (z = 0, ±8 and ±17 Å). The profiles represent the cumulative density from all 5 simulations. The blue lines are the results from singly protonated species and the green lines are from neutral species. The red lines represent the density of the lipid phosphate groups.

Figure 4. PMF profiles of amitriptyline (A) and clozapine (B) in DMPC bilayers. The center of bilayer is at z = 0 Å. For reference, partial densities of lipid groups are also shown: green: 900 kg/m^3 < lipid tail density < 1700 kg/m^3; pink: 350 kg/m^3 < lipid glycerol < 600 kg/m^3; orange:620 kg/m^3 < lipid phosphate < 700 kg/m^3 and blue:300 kg/m^3 < lipid choline < 400 kg/m^3.

Figure 5. Conformational distributions as defined by inset schematics for protonated amitriptyline (A) and neutral amitriptyline (B). Each distribution was calculated as a function of the *z*-coordinate defined by five lipid locations (as defined by inset DMPC lipid schematic on the right) or in bulk water. Dashed lines indicate the positions of the probability density maxima.

Figure 6. Clozapine conformational distributions as defined by inset schematics across all simulated protonation states for Ring 1 to sidechain distances (A) and the Ring 2 to sidechain distance (B). Each distribution was calculated as a function of the *z*-coordinate defined by five lipid locations (as defined by inset DMPC lipid schematic on the right) or in bulk water. Dashed lines indicate the positions of the probability density maxima.

Figure 7. Schematic representations of the directional preference of the dimethylammonium moeity of amitriptyline as shown be the green vectors. Red spheres correspond to the dimethylammonium proton in simulations of protonated amitriptyline (right panel). The tendency of the protonated dimethylammonium group to point towards the interface region of the bilayer is clearly visible.

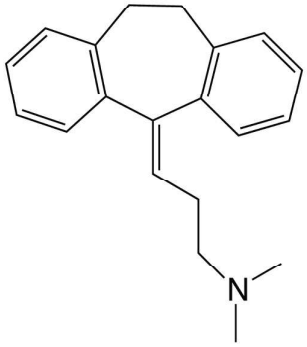
Figure 8. Schematic representations of the directional preference of the methyl

piperazine moiety of clozapine as shown by the green vectors. Red spheres correspond to the positions of the protons in simulations of both protonated forms. The orientational preferences are constrained in the order doubly protonated > singly protonated > neutral and is clearly visible in these plots.

Figure 9. Amitriptyline interactions with the (A) methylene and (B) methyl groups of DMPC acyl chains obtained from ROESY 1H-NMR. Clozapine interactions with the (C) methylene and (D) methyl groups of DMPC acyl chains. Small white spheres represent hydrogen atoms for which there was no crosspeak data. Nitrogen atoms are represented by blue spheres. The larger spheres on the ring system show the cross peak volume normalised against the diagonal peak volume and each other on a white to green scale varying between 0 and 84 (the maximum intensity).

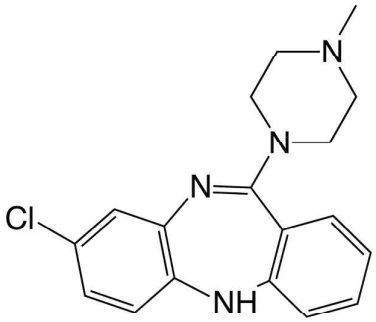
Figure 1

(A)



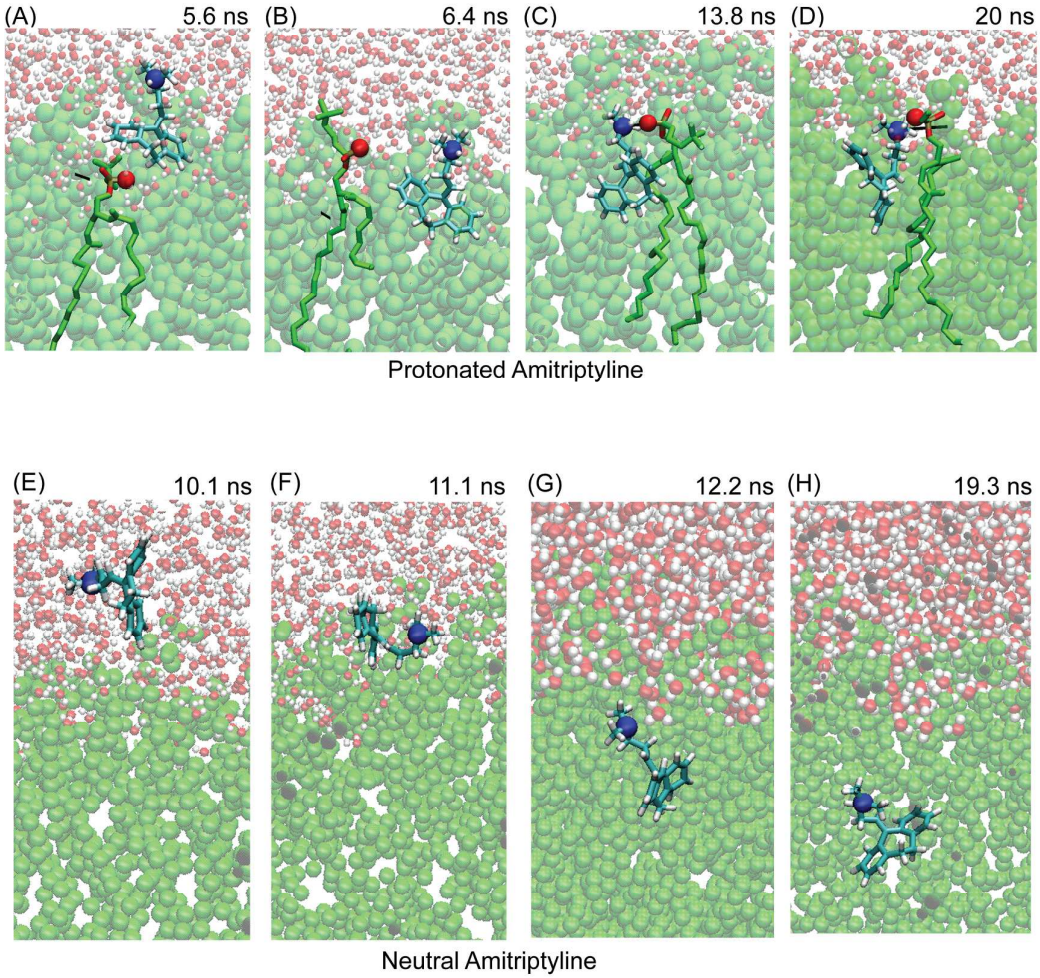
Amitriptyline

(B)



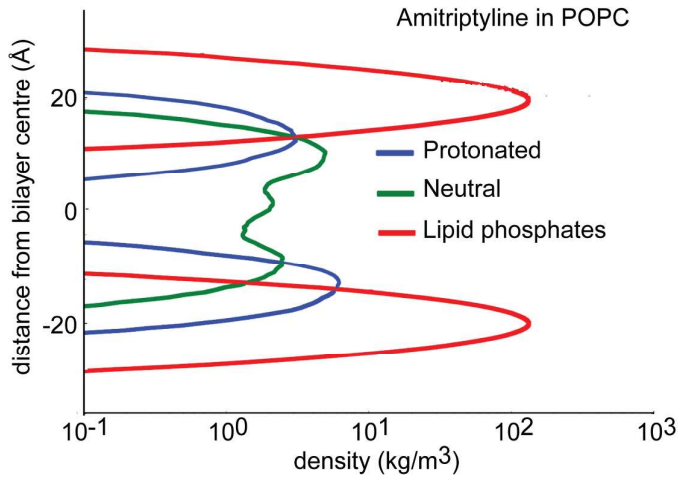
Clozapine

Figure 2



(A)

Figure 3



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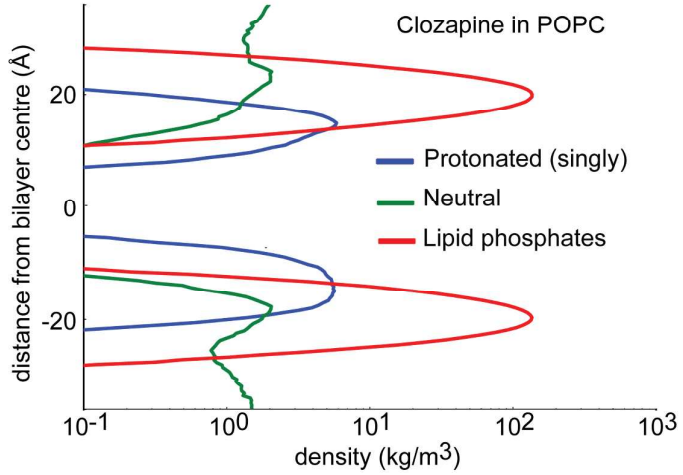


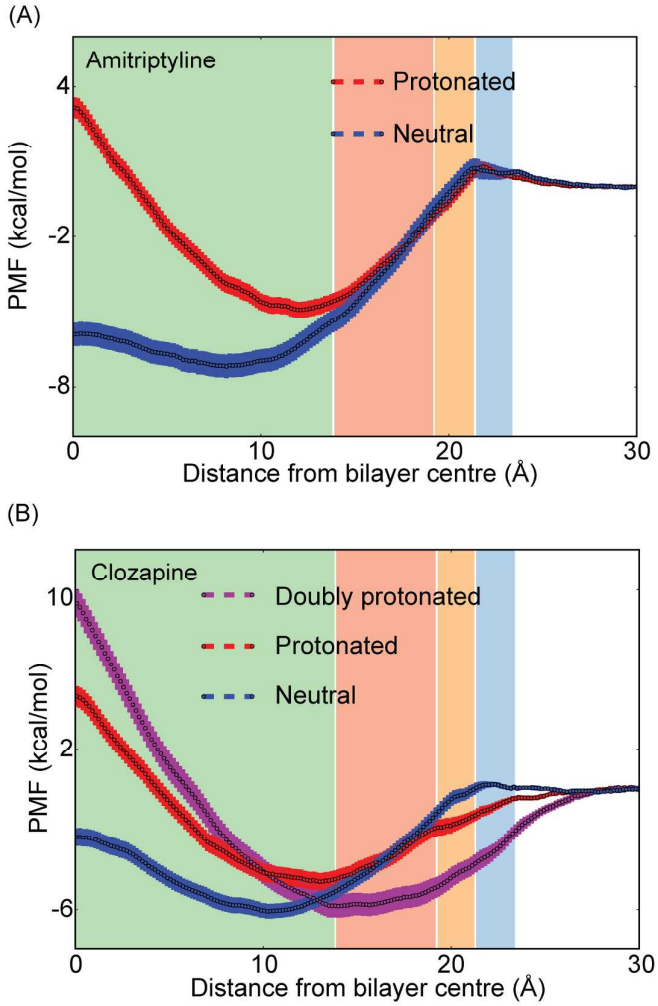
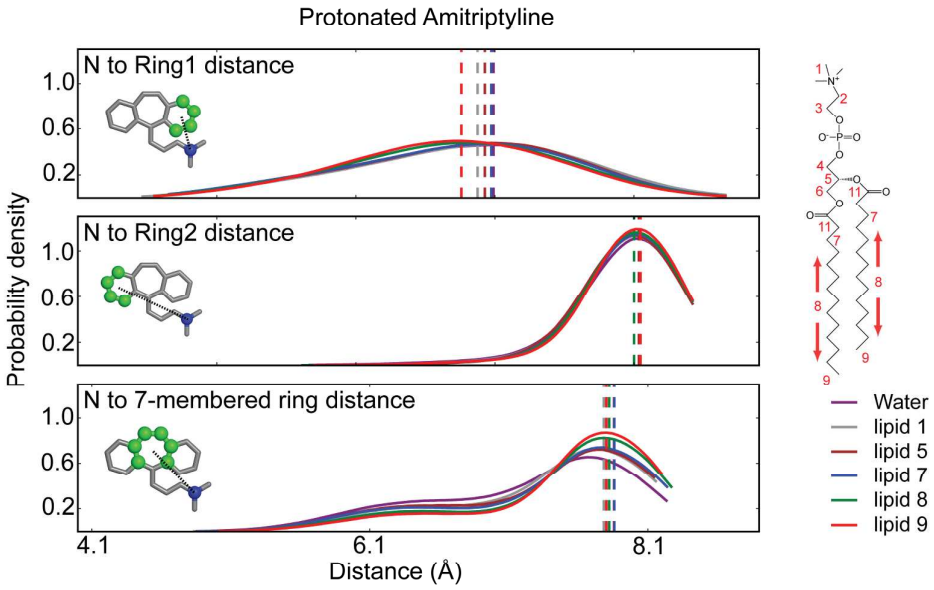


Figure 4

(A)

Figure 5



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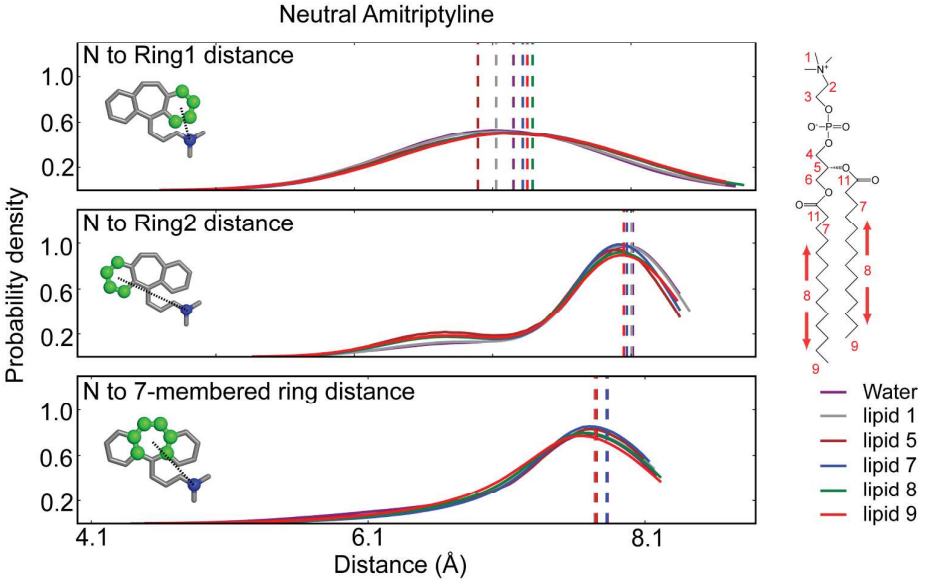
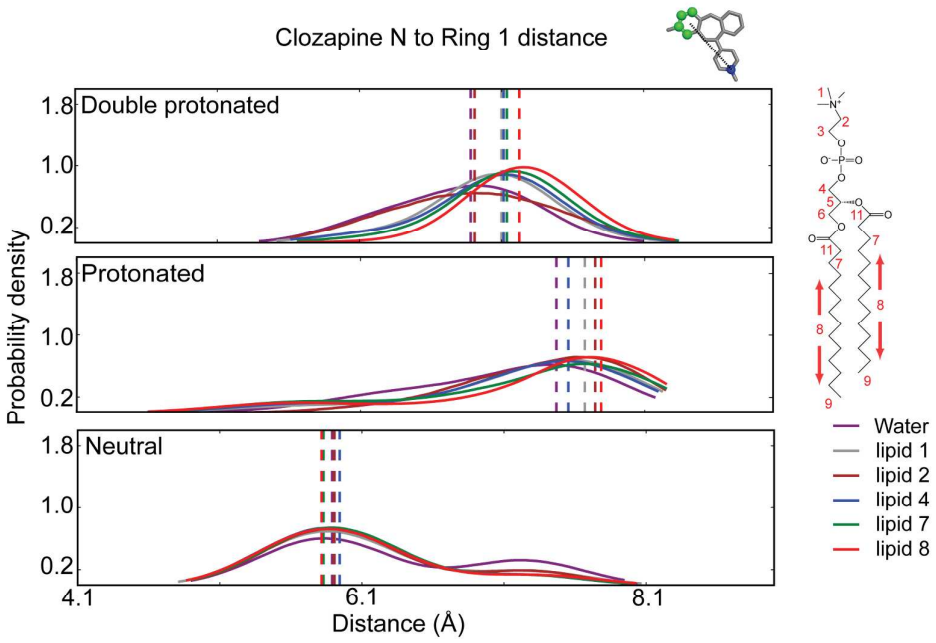


Figure 6



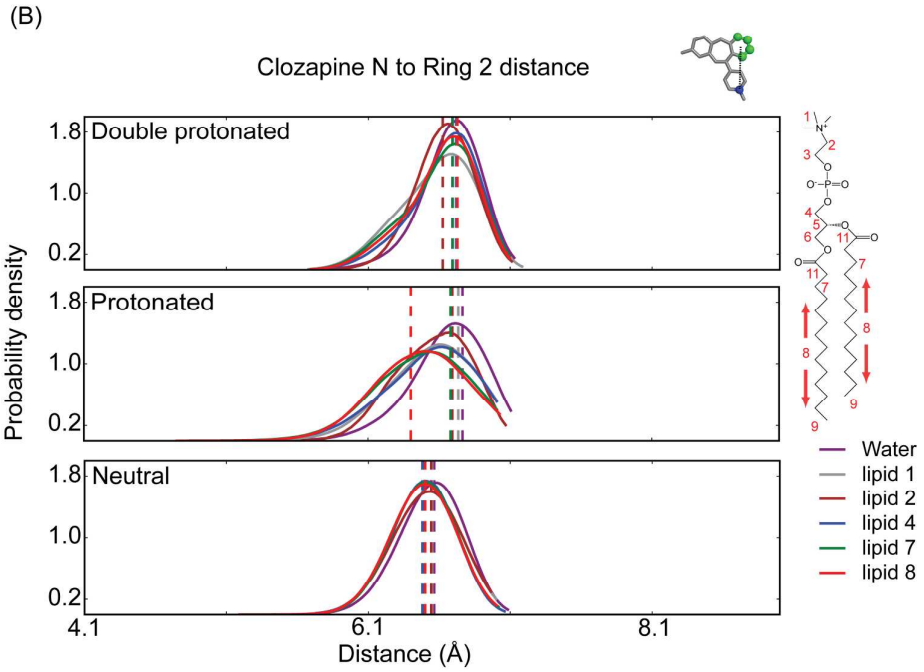


Figure 7

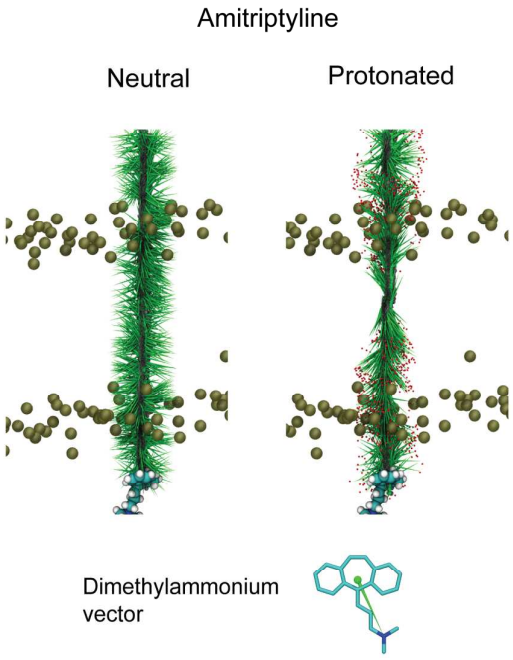


Figure 8

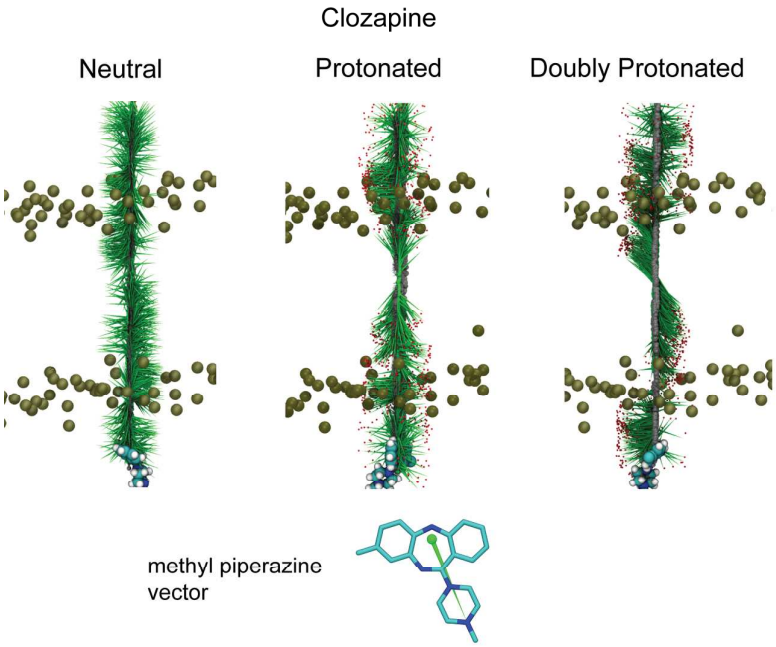
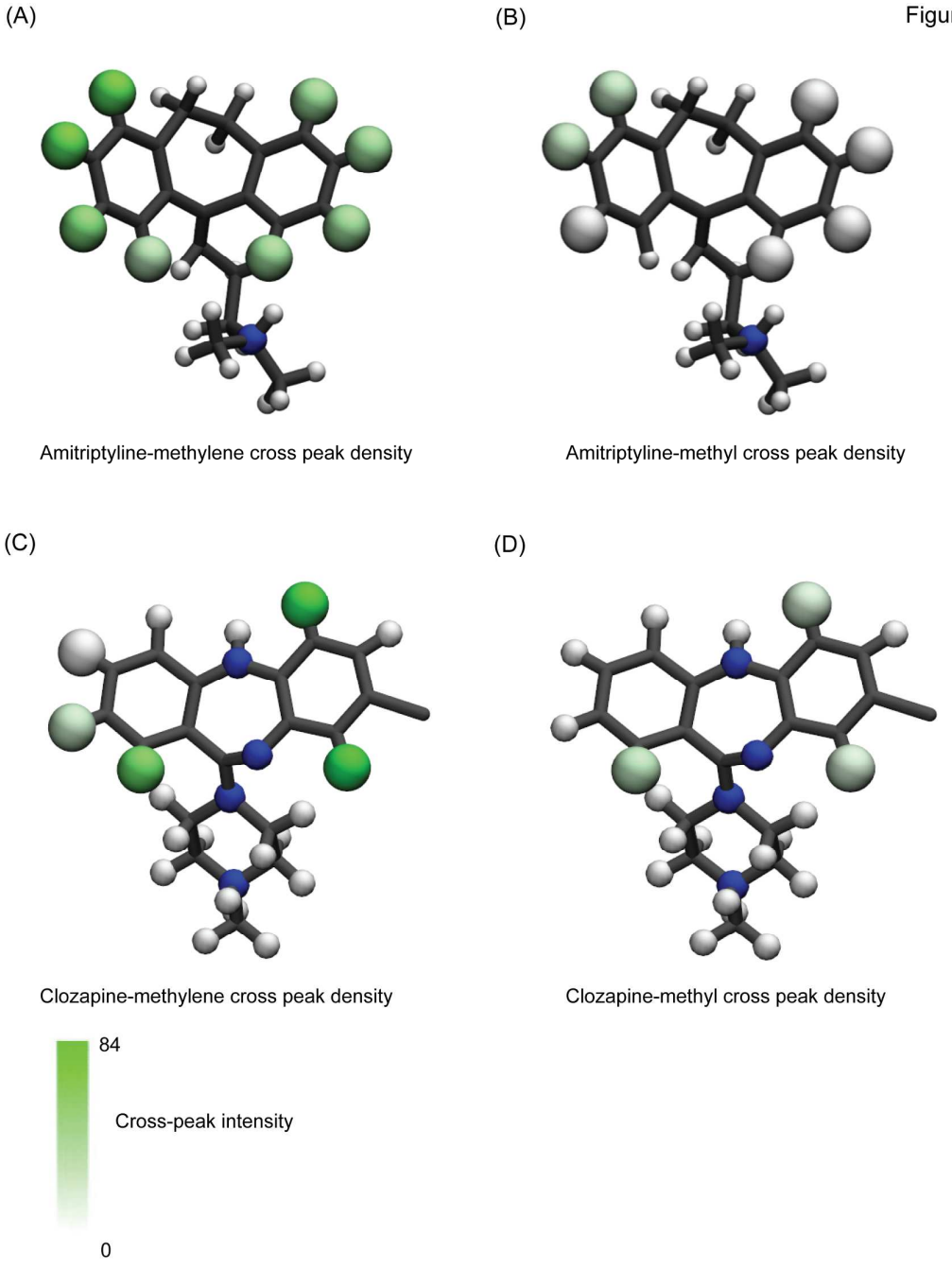


Figure 9



TOC Combining MD simulation with NMR to give a picture of drug-membrane interaction.