

MINIREVIEW—COMPUTATIONAL ANALYSIS OF GPCRS

Does the Lipid Bilayer Orchestrate Access and Binding of Ligands to Transmembrane Orthosteric/Allosteric Sites of G Protein-Coupled Receptors?

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ABSTRACT

The ligand-binding sites of many G protein-coupled receptors (GPCRs) are situated around and deeply embedded within the central pocket formed by their seven transmembrane-spanning α -helical domains. Generally, these binding sites are assumed accessible to endogenous ligands from the aqueous phase. Recent advances in the structural biology of GPCRs, along with biophysical and computational studies, suggest that amphiphilic and lipophilic molecules may gain access to these receptors by first partitioning into the membrane and then reaching the binding site via lateral diffusion through the lipid bilayer. In addition, several crystal structures of class A and class B GPCRs bound to their ligands offer unprecedented details on the existence of lipid-facing allosteric binding sites outside the transmembrane helices that can only be reached via lipid pathways. The highly organized structure of the lipid bilayer may direct lipophilic or amphiphilic drugs to a specific depth within the bilayer, changing local concentration of the drug near the binding site and affecting its binding kinetics. Additionally, the

constraints of the lipid bilayer, including its composition and biophysical properties, may play a critical role in “pre-organizing” ligand molecules in an optimal orientation and conformation to facilitate receptor binding. Despite its clear involvement in molecular recognition processes, the critical role of the membrane in binding ligands to lipid-exposed transmembrane binding sites remains poorly understood and warrants comprehensive investigation. Understanding the mechanistic basis of the structure-membrane interaction relationship of drugs will not only provide useful insights about receptor binding kinetics but will also enhance our ability to take advantage of the apparent membrane contributions when designing drugs that target transmembrane proteins with improved efficacy and safety. In this minireview, we summarize recent structural and computational studies on membrane contributions to binding processes, elucidating both lipid pathways of ligand access and binding mechanisms for several orthosteric and allosteric ligands of class A and class B GPCRs.

Introduction

The G protein-coupled receptor (GPCR) family represents one of the largest and most diverse families of proteins; proteins in this family are encoded by 1%–3% of the genes in our genome. These integral membrane proteins are involved in many physiologic and pathophysiological processes and continue to be the most privileged targets for approximately

33% of all small-molecule drugs currently on the market (Overington et al., 2006; Santos et al., 2017). GPCRs, also known as seven-transmembrane (7TM) receptors are highly dynamic (Kobilka, 2007; Kobilka and Deupi, 2007; Manglik and Kobilka, 2014; Manglik et al., 2015; Hilger et al., 2018) and exist in an equilibrium between multiple, functionally distinct conformational states. A broad spectrum of extracellular signals (e.g., photons, neurotransmitters, hormones, lipids, ions, and odorants) interact with GPCRs through their primary “orthosteric” binding site and transduce the signals by conformational rearrangement of the membrane-spanning

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ABBREVIATIONS: BPTU, 1-(2-(2-(tert-butyl) phenoxy) pyridine-3-yl)-3-(4-(trifluoromethoxy) phenyl) urea; BSA, buried surface area; GCGR, G protein-coupled glucagon receptor; GPCR, G protein-coupled receptor; K_d , intrinsic binding affinity; K_d' , observed binding affinity; K_{mem} , membrane partition coefficient; k_{on} , measured association rate; $\log K_{IAM}$, partition coefficient measured using immobilized artificial membrane; MESA, membrane-exposed surface area; MK-0893, 3-((4-((1S)-1-[3-(3,5-dichlorophenyl)-5-(6-methoxynaphthalen-2-yl)-1H-pyrazol-1-yl]ethyl)-phenyl)formamido)propanoic acid; NAM, negative allosteric modulator; NNC0640, 4-[[[4-(cyclohexylphenyl)-[(3-methylsulfonylphenyl)-carbamoyl]amino]methyl]-N-(2H-tetrazol-5-yl)benzamide; P2Y₁R, P2Y₁ receptor; PAR, protease-activated receptor; PF-06372222, 3-[[4-[(R)-(3,3-dimethylcyclobutyl)-[[6-[4-(trifluoromethyl)imidazol-1-yl]pyridin-3-yl]amino]methyl]benzoyl]amino]propanoic acid; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; PAM, positive allosteric modulator; SASA, solvent-accessible surface area; 7TM, seven-transmembrane; TMH, transmembrane helix.

7TM region. This triggers intracellular signaling pathways through heterotrimeric G-proteins, β -arrestins, and other signaling proteins. Recent advances in the structural biology of GPCRs combined with biophysical, spectroscopic, and computational studies have provided new details (Latorraca et al., 2017) on the dynamics of the transmembrane domain critical for mediating downstream signals. Although the majority of the currently used drugs and probe compounds target endogenous agonist-binding orthosteric sites, substantial research efforts in recent years (Christopoulos, 2014) have been directed toward developing allosteric modulators that bind to sites that are topographically distinct, yet conformationally linked to the orthosteric sites. In principle, allosteric ligands can potentiate (positive allosteric modulators, PAMs) or inhibit (negative allosteric modulators, NAMs) signal transduction by altering the affinity for orthosteric ligands and by stabilizing active or inactive conformational states, respectively. They have also been found to modulate receptor interactions with intracellular signal-transducing molecules, or to have no net effect (neutral allosteric ligands, NALs) on orthosteric agonists. In some cases, the binding of allosteric ligands (Ago-PAMs) alone is adequate to induce receptor activation, though these compounds may increase the potency and/or efficacy of orthosteric ligands. The advantages of allosteric modulators (Gentry et al., 2015; Changeux and Christopoulos, 2017; Foster and Conn, 2017) include the prospect of greater receptor subtype selectivity, biased signaling (Rankovic et al., 2016; McCorvy et al., 2018), and the potential to “fine tune” endogenous agonism while maintaining the natural signaling patterns. Furthermore, allosteric modulators exhibit very slight amounts of positive or negative cooperativity that result in a “ceiling level” for the allosteric effect with a possibility of on-target safety in overdose situations (Keov et al., 2011).

All GPCRs share common structural features (Cherezov et al., 2007; Dawaliby et al., 2016) that include 1) an extracellular N terminus; 2) seven transmembrane helices (TMHs) forming a closed bundle; 3) three extracellular and three intracellular loops connecting the TMHs; and 4) an intracellular C terminus. Generally, endogenous ligands of GPCRs bind inside a crevice (the orthosteric binding site) formed by the TMH bundle. In case of class C GPCRs, the native ligands bind to an extracellular “Venus fly trap domain” connected to the N terminus, either in addition to or in place of the orthosteric site. However, recently solved experimental structures of GPCRs cocrystallized with their allosteric ligands revealed multiple (Christopoulos et al., 2014) spatially distinct binding sites at: 1) the extracellular vestibule of the receptor through which the orthosteric ligands enter; 2) the intracellular ends of the helices, partially overlapping with the binding surfaces of signaling molecules such as G proteins; and 3) the external surface of the receptor/lipid bilayer interface outside the 7TM helical bundle. A recent comprehensive review (Lu and Zhang, 2018) discusses the diverse locations and key receptor-ligand interactions of 18 crystal structures of classes A, B, C, and F GPCRs in complex with such small-molecule allosteric modulators. Crystal structures, in which the receptor-bound ligand molecules are significantly exposed to and buried within the membrane lipids, are extremely intriguing because of their binding-site locations that can be reached only via membrane pathways. Under these circumstances, partitioning of the

ligands to a discrete bilayer depth, in addition to their suitable orientation and conformation within the membrane, becomes essential for subsequent access and binding to the intrabilayer receptor. The phospholipid bilayer can effectively increase the efficiency of ligand binding to an intrabilayer receptor site, even at low concentrations, by reducing the degrees of freedom of the drug and limiting it to a specific region of the bilayer. Although membrane interactions can result in improved binding kinetics (Sykes et al., 2019), excessive membrane accumulation of ligands can lead to toxicity through off-target interactions. Therefore, simply increasing ligand lipophilicity to gain efficacy for membrane-associated targets is often unfavorable in terms of the overall drug profile. Knowledge of membrane partitioning characteristics such as quantitative bilayer distribution, preferred location (depth), orientation, and conformation of drug molecules within the bilayer is essential for understanding their target binding kinetics, onset and duration of action, and disposition.

Although it is outside the scope of this review, it is important to recognize the fundamental role of the plasma membrane in proper localization, structure, and function of GPCRs (Ostrom and Insel, 2004; Phillips et al., 2009; Genheden et al., 2017). The lipid composition (including the presence of “lipid rafts”) and the biophysical properties of the membrane (such as thickness, curvature, fluidity, and surface tension) have been shown to affect structure, function, and agonist/antagonist binding and activation of GPCRs as well as the corresponding signaling proteins (Yang et al., 2005; Escribá et al., 2007; Periole et al., 2007; Dawaliby et al., 2016; Bruzzese et al., 2018). Most importantly, cholesterol and phospholipids can act as allosteric modulators of GPCRs by binding directly to receptor sites and/or by changing the membrane properties (Oates and Watts, 2011; Sengupta and Chattopadhyay, 2015; Gimpl, 2016; Guixà-González et al., 2017). In this review, we will focus mainly on allosteric binding sites that exist at the receptor/lipid bilayer interface, emphasizing the critical role of the membrane for ligand access and binding to these novel allosteric sites. We will also summarize some recent structural and computational studies that offer unprecedented insights into the atomistic details of plausible lipid pathways for orthosteric ligands.

The Structure-Membrane Interaction Relationship Affects Ligand Action and Disposition

The partitioning of drugs within the plasma membrane or the membranes of subcellular organelles is known to affect drug interactions with membrane-associated proteins, including G protein-coupled receptors (Sykes and Charlton, 2012; Sykes et al., 2014; Dickson et al., 2016), ion channels (Schmidt et al., 2012; Huang et al., 2017; Laverty et al., 2017; Poveda et al., 2017), cytochrome P450 enzymes (Berka et al., 2011; Paloncýová et al., 2014; Di Meo et al., 2016), and transporters (Melchior et al., 2012; Clay and Sharom, 2013), influencing their efficacy and fate in the body. In addition, evidence is accumulating for micropharmacokinetic-pharmacodynamic mechanisms (Swinney, 2006; Schoop and Dey, 2015; Swinney et al., 2015; Vauquelin, 2015) in which the so-called “nonspecific” membrane interactions of drugs can influence their receptor binding kinetics and affect affinity as

well as residence time. According to one “microkinetic” model (Vauquelin and Packeu, 2009; Vauquelin, 2015, 2016), the surrounding lipid bilayer can influence the molecular recognition process (i.e., ligand molecules binding to transmembrane proteins) through the following mechanisms: 1) by acting as a reservoir for the drug, 2) by “preorganizing” the drug molecule in its appropriate orientation and conformation for binding, and 3) by altering the time the drug molecule needs to reach its receptor (see Fig. 2).

In a membrane-facilitated ligand binding process, the ligand molecule must first partition to a specific, energetically favorable location and adopt an appropriate orientation and conformation in the membrane bilayer before laterally diffusing to an intrabilayer receptor-binding site. The specific makeup and organization of the lipid bilayer may restrict lipophilic or amphiphilic drugs to a particular depth of penetration in the bilayer. Furthermore, the orientation of a drug relative to its binding site might also be optimized by the lipid bilayer structure, which may limit the drug’s degrees of rotational freedom (Sargent and Schwyzer, 1986; Schwyzer, 1991, 1995a,b). Drugs may exhibit entirely different conformational equilibria in membrane compared with those in aqueous bulk; a conformation fitting to the receptor binding site may exist only as a metastable state. Presumably, the drug enters the membrane, undergoes a conformational change, and then binds to a protein with a precise and metastable conformation. In addition, drugs that enter (wash in) rapidly into membranes attain relatively low concentrations in the membrane and leave rapidly, compared with slowly concentrating drugs that “load” into the membrane at greater concentrations, and then slowly wash out again. Thus, slowly accumulating drugs are available to interact with membrane-associated receptors over a longer period. For drug molecules targeting membrane-associated receptors, even subtle differences in structural features whose basis is the structure-membrane interaction relationship can result in altered drug-lipid interactions. This will affect the preferred location, orientation, conformation, and local concentration of the drug molecules in the immediate vicinity of the receptor binding site, and therefore directly influence observed pharmacology (Vauquelin and Packeu, 2009; Swinney et al., 2015; Vauquelin, 2015, 2016; Gherbi et al., 2018). Structural modifications designed to optimize drug interactions with membrane lipids can produce desired changes in the clinical half-life of drugs; for example, a short-acting parent compound can be rationally modified into a long-acting structural analog by the addition of a hydrophobic functional group at the proper location.

For compounds binding to a membrane-associated receptor, high receptor affinity may result from high intrinsic affinity for the receptor site (low value of dissociation rate, k_{off}) or a high membrane partition coefficient (K_{mem}) that produces a change in the value of the measured association rate (k_{on}), or a combination of these two (see Fig. 1). Particularly, a compound with a high K_{mem} value, and thus higher local concentrations surrounding the receptor, may contribute to a longer duration of action (Vauquelin and Charlton, 2010), in part owing to altered k_{on} , which directly affects receptor rebinding (Vauquelin, 2010). Increasing a drug’s lipophilicity to boost drug efficacy would not only increase the local concentration around the target but would also increase the likelihood of off-target pharmacology resulting from to nonspecific binding to other membrane-associated proteins,

which in turn may result in an increased risk of toxicity. However, large increases in affinity obtained by changes in k_{on} may be beneficial for highly selective ligands (Huggins et al., 2012) and/or where topical delivery is anticipated, such as β_2 -adrenergic receptor (β_2 -AR) agonists that are delivered to the lungs by inhalation. Agonists of β_2 -AR (Cazzola et al., 2011, 2005) are primarily used to treat asthma and other pulmonary disorders. They cause smooth muscle relaxation, dilation, and opening of the lung airways by β_2 -AR activation. These agents exhibit a wide range of clinical action duration (short, long, and ultra-long), arguably owing to their altered interactions with the membrane compartment surrounding the receptors. In a series of studies (Sykes and Charlton, 2012; Sykes et al., 2014; Dickson et al., 2016), researchers from Novartis demonstrated that for several clinically relevant β_2 -AR agonists and antagonists, the degree of phospholipid interaction affects the drug’s binding kinetics profile and thus the observed affinity. For drugs possessing a high membrane affinity, the high local concentration of the drug in the membrane surrounding the receptor (calculated from the membrane partition coefficient, $\log K_{\text{IAM}}$, measured using immobilized artificial membranes) was shown to affect its association rate (k_{on}) and thus its observed binding affinity (K_{d}). For example, despite sharing the same pharmacophore (the adrenaline-mimicking saligenin pharmacophore), the measured affinity of salmeterol is three orders of magnitude higher than salbutamol (Sykes and Charlton, 2012), driven by the large difference in their association rates, attributable solely to membrane binding (Szczuka et al., 2009) (see Fig. 2). Upon correction of this increased membrane affinity (Mason et al., 1991; Vauquelin and Packeu, 2009), both agonists have comparable intrinsic binding affinity (K_{d}) values for the inactive form of β_2 -AR. Oftentimes, for membrane-embedded targets, the role of the membrane is neglected when the affinity of a drug is measured; however, this membrane contribution can affect the “observed” or “apparent” binding affinity (K_{d}), making the results difficult to interpret. The intrinsic or “true” affinity (K_{d}) is the affinity of the drug for the receptor, excluding the contribution from the membrane. It has been shown that for salmeterol, the membrane contribution affects the overall experimental binding affinity, and after uncoupling the membrane’s contribution, the intrinsic dissociation affinity constant (K_{d}) is much more comparable to the affinities of other structurally analogous β_2 -AR agonists (Dickson et al., 2016). The lipid bilayer may serve as a reservoir for lipophilic/amphiphilic ligands (such as salmeterol), which in turn helps to concentrate the ligands near the binding site of the receptor, thereby affecting the observed association rate without necessarily changing interactions with binding site residues. The difference in affinity between salmeterol and salbutamol has been attributed (Rhodes et al., 1985; Green et al., 1996) to a possible interaction of the lipophilic phenylalkoxyalkyl tail of salmeterol with phospholipids in the bilayer. As this review was being prepared, an X-ray crystal structure (Masureel et al., 2018) of β_2 -AR bound to salmeterol with a resolution of 2.9 Å was published, revealing an exosite that is close to the orthosteric binding site wherein the lipophilic phenylalkoxyalkyl chain interacts with several hydrophobic residues. It may be possible that the receptor association and dissociation of salmeterol occur through lipid pathways via the transmembrane helices; but this possibility warrants further investigation.

Lipophilic and Amphiphilic Ligands May Access Their Membrane-Associated Receptors through Lipid Pathways

For the majority of the G protein-coupled receptors, the orthosteric binding site is deeply embedded within and around the central pocket formed by the 7TM-spanning α -helical domains. Generally, the hydrophilic endogenous ligands are assumed to enter the pocket directly from the aqueous phase (Dror et al., 2011). Recent evidence for the β_2 -AR (Sykes and Charlton, 2012; Sykes et al., 2014), cannabinoid (Hurst et al., 2010), sphingosine-1-phosphate (Hanson et al., 2012), and opsin receptors (Hildebrand et al., 2009), along with ion channel ligands (Rhodes et al., 1985; Mason et al., 1991), suggests that amphiphilic and lipophilic molecules partitioned into the membrane gain access to these binding sites via lateral diffusion between the α -helical transmembrane domains of the receptors. The concentrating effects of membrane-associated drugs and lateral diffusion of drugs across the two-dimensional surface rather than along three-dimensional pathways in the aqueous bulk alone could significantly increase receptor association rates and the corresponding affinity values by the “reduction of dimensionality” rate enhancement (McCloskey and Poo, 1986). In this section, we discuss some recent structural and computational studies that offer details on ligand access/exit pathways and binding to transmembrane domains of GPCRs.

The Lipid Pathway for Retinal: 11-*cis* Retinal and All-*trans*-Retinal Access and Leave the Opsin-Binding Pocket through a Ligand Channel That Has Openings into the Membrane. Rhodopsins are class A GPCRs (class A GPCRs form the largest subfamily, constituting approx. 90% of all GPCRs). Rhodopsin is composed of an apoprotein opsin that is covalently linked to a chromophoric ligand, 11-*cis*-retinal, that acts as an inverse agonist and keeps the receptor in its inactive state. Upon activation by light (a photon), 11-*cis*-retinal undergoes isomerization to all-*trans*-retinal, an agonist that triggers conformational changes in the protein and turns on the signaling pathway, which eventually leads to vision. Retinal epithelial cells are responsible for the synthesis of 11-*cis*-retinal; rhodopsin regeneration occurs when the all-*trans*-retinal on activated rhodopsin is replaced by a newly synthesized 11-*cis*-retinal. Structural, mutational, and computational studies support the existence of and provide extensive details on the dynamics of a continuous retinal channel (approx. 70-Å length) in the active state of the protein. This channel traverses parallel to the membrane plane and opens into the membrane layer through openings A (between TM1 and TM7) and B (between TM5 and TM6), respectively. Hildebrand et al. (2009) investigated the channel properties with respect to the passage and uptake of 11-*cis*-retinal and the release of all-*trans*-retinal using two active forms of the crystal structures of opsin, namely the ligand-free apoprotein opsin (PDB ID 3CAP) (Park et al., 2008) and opsin bound to a high-affinity peptide derived from the C terminus of the α -subunit of the G protein (PDB 3DQB) (Scheerer et al., 2008). Both membrane-facing openings A and B are lined with hydrophobic aromatic residues, and the central retinal binding pocket is polar. Using a skeleton search algorithm and molecular docking, the study by Hildebrand et al. found that the inner width of the channel ranges from 11.6 to 3.2 Å. Throughout the channel, there are four constriction points, including a 90° elbow-like kink near the polar residue

Lys296. These constriction sites are narrow and require significant stretching to allow passage of the retinal β -ionone-ring. The absence of electron density for Lys296 in the studied crystal structures indicates that the sidechain of this residue could be highly flexible. The smallest constriction site—just after opening A, with the 90° kink at Lys296—seems to be selective for 11-*cis*-retinal with a possible bent conformation instead of for extended all-*trans*-retinal. The probability of retinal entering the binding site through opening A as 11-*cis*-retinal (with the β -ionone ring making the first contact with hydrophobic residues at the entrance) and exiting through opening B as all-*trans*-retinal (again with the β -ionone ring interacting first with the aromatic residues lining the opening) seems very high. Additional docking simulations, in which side-chain flexibility of the binding site residues was allowed, explored multiple low-energy binding poses within the channel; analysis of these docked orientations and conformations provides convincing evidence for ligand entry and exit through lipid pathways.

The Lipid Pathway for Sphingosine 1-Phosphate Receptor Ligands. The sphingosine-1-phosphate receptor family comprises five members (S1P₁₋₅) and the activation of these receptors by exogenous ligands can result in modulation of lymphocyte trafficking, endothelial development, heart rate, and vascular tone. A nonselective agonist prodrug, fingolimod, has been approved by the FDA for the treatment of relapsing-remitting multiple sclerosis (Brinkmann et al., 2010). The crystal structure of the S1P₁ receptor in complex (Hanson et al., 2012) with a selective antagonist (*R*)-3-amino-(3-hexylphenylamino)-4-oxobutylphosphonic acid (ML056) shows an N-terminal domain that folds over the top of the receptor binding site and essentially prevents ligands from entering from the extracellular side. This structural constraint, coupled with the fact that many of the S1P₁ ligands exhibit delayed saturation of receptor binding in the presence of excess ligand (Mandala et al., 2002), leads to the possibility that the ligands gain access to the binding site through the lipid bilayer, specifically between a gap in helices I and VII. Utilizing the crystal structure of S1P₁R (PDB 3V2Y) embedded in a membrane composed of POPC and cholesterol, Stanley et al., (2016) investigated the potential lipid binding pathway for the antagonist, ML056, using unbiased all-atom molecular dynamics simulations totaling approx. 800 microseconds. The series of simulations revealed a clear binding pathway through the lipid bilayer with four distinct steps: 1) ML056 moves through the membrane and comes in contact with a transmembrane-facing binding pocket formed by amino acids located mainly on TMH 1 and 7; 2) the phosphonate group of ML056 interacts with R292^{7,34} and S38, and the protonated primary amine interacts with E294^{7,36} (the lipophilic tail of the ligand fits into a cavity formed by hydrophobic residues V51^{1,38}, F52^{1,39}, I55^{1,42}, V298^{7,40}, and L302^{7,44}); 3) the tail of the ligand inserts itself into the entrance channel to interact with L102^{2,61} and L297^{7,39}; and 4) the hydrophobic tail of the ligand enters the orthosteric binding site first, followed by the polar head group. During the binding process, the N-terminal helix unfolds and there is an analogous increase in the size of the channel, which is thought to accommodate the ligand as it enters the binding site. It should be noted that although ML056 was placed in the bulk membrane around the receptor, it did not enter the binding site in every simulation.

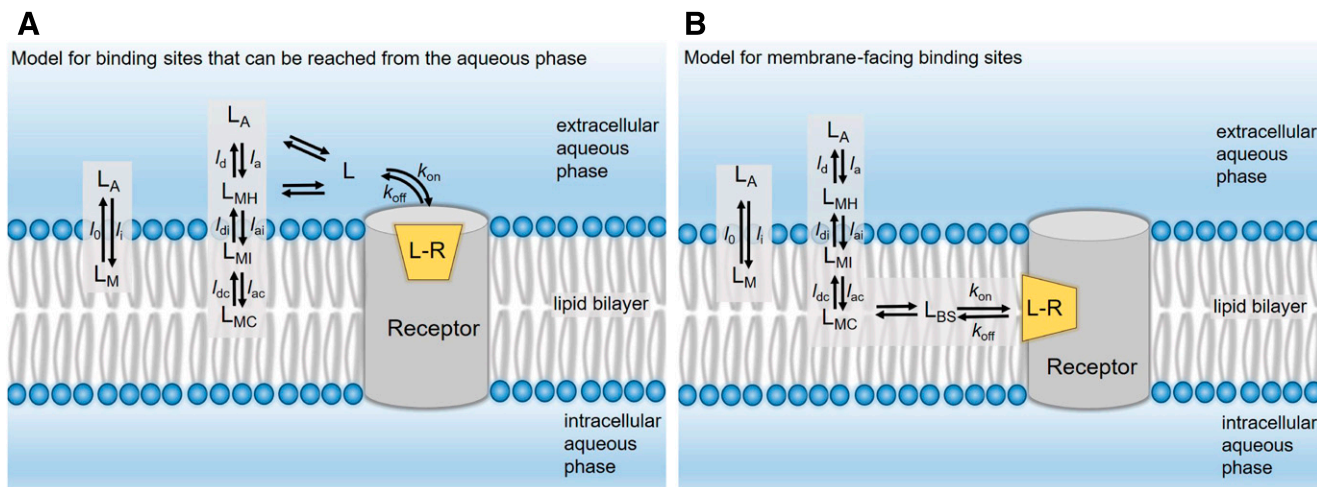
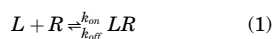


Fig. 1. Models that account for membrane partitioning of lipophilic/amphiphilic ligands binding to solvent-exposed binding site (A) and transmembrane lipid-exposed binding site (B). The fundamental prerequisite for a drug (ligand) to elicit its biologic effect is the molecular recognition process in which the ligand molecule (L) selectively binds to its target receptor (R) to form a receptor-ligand complex (LR). For a simple one-to-one reversible bimolecular reaction (eq. 1), the binding affinity of the receptor-ligand complex at equilibrium can be characterized by the dissociation constant, K_d . The equilibrium dissociation constant (typically measured in moles per liter) is the ratio between the rates of the backward (k_{off}) and forward (k_{on}) reactions, which in turn, are directly proportional to the concentrations of free ligand, unoccupied receptor, and receptor-ligand complexes, respectively (eq. 2).



$$K_d = \frac{k_{off}}{k_{on}} = \frac{[L] \cdot [R]}{[LR]} \quad (2)$$

The association rate (forward reaction) is dependent on the k_{on} value as well as the ligand concentration in the target compartment. Typically, the endogenous ligands of GPCRs are polar and are assumed to reach their orthosteric binding sites directly from the extracellular aqueous compartment (L_A). The equations used to calculate the overall affinity constant and kinetic rates assume that the interacting molecules are homogeneously distributed and concentration of ligand available to bind target is equal to that in the bulk aqueous phase. However, it is important to recognize that highly lipophilic and/or amphiphilic ligands may partition into the surrounding membrane driven by their equilibrium membrane partition coefficient, K_{mem} and alter the observed pharmacology. K_{mem} is the ratio between the equilibrium concentrations of ligand molecules in the membrane compartment (c_{mem}) and the aqueous phase (c_{aq}); K_{mem} can also be calculated from the transfer rates l_i and l_o (see eq. 3; part A).

$$K_{mem} = \frac{c_{mem}}{c_{aq}} = \frac{l_i}{l_o} \quad (3)$$

where l_i and l_o are the membrane wash-in and washout rates of the ligand, respectively. In this scenario, the ligand concentration near the receptor is different from its concentration in the aqueous bulk. On the basis of their lipophilicity and amphiphilicity profiles and their ionization states, ligand molecules may reside at discrete locations within the membrane compartment: 1) at the surface, interacting with hydrated polar head groups (L_{MH}); 2) at the interface between the polar head groups and the hydrophobic tails, interacting with both strata (L_{MI}); or 3) at the hydrophobic lipid core (L_{MC}). For compounds with high lipophilicity and/or amphiphilicity, the equilibrium concentration near the binding site can be affected by the magnitude of individual transfer rates (see part A) representing adsorption and desorption (subscripts “a” and “d” respectively) at the headgroup, interface, and core regions of the membrane.

For lipophilic and/or amphiphilic compounds binding to extracellular orthosteric sites, the contributions of membrane partitioning and interactions with various bilayer strata (and thus the local concentration) to the intrinsic k_{on} and K_d values can be accounted for by the following equations (eqs. 4–6):

$$k_{on} = \frac{k_{on}'}{K_{mem}} \quad (4)$$

where k_{on}' is the measured association rate constant. It is important to note that this relationship (eq. 4) is true only if the membrane equilibration time for the ligand is fast relative to its receptor association and dissociation rates.

$$K_d = \frac{k_{off}}{k_{on}} = \frac{k_{off}}{k_{on}'} \times K_{mem} \quad (5)$$

$$\bar{K}_d = K_d' \times K_{mem} \quad (6)$$

Here, K_d' and K_d represent the “observed” or “apparent” dissociation affinity constant (determined in bulk experimental studies) and “intrinsic” dissociation affinity constant of the ligand calculated after correcting for the membrane contribution, respectively. The most challenging scenario arises when the binding site is deeply embedded within the membrane and ligands can reach their binding sites only through lipid pathways (see part B). In such cases, the drug concentration near the binding site (L_{BS}) is dependent on the location of the binding site within the membrane compartment relative to the drug’s preferred membrane location—for example, at the membrane core (L_{MC}), interface (L_{MI}) or hydrated headgroup (L_{MH})—as well as the diffusion coefficient of the ligand in question. It is important to recognize that K_{mem} -based adjustments may often underestimate the effect of the membrane partitioning of ligands, because the ligand concentration in the vicinity of the receptor may be different from the concentration in the bulk membrane owing to discrete localization effects. Also, the actual volume of the membrane occupied by the drug is substantially less than the total membrane volume.

In contrast to the association rate (k_{on}), the dissociation rate (k_{off}) is independent of the ligand concentration and depends solely on the specific molecular interactions of the ligands with the binding site residues. For ligands targeting membrane-facing binding sites, the participation of lipid molecules in the binding process (as one of the components of the binding site) needs different treatment, as these interactions would affect k_{off} as well. The extent to which the membrane lipids are capable of engaging in the binding process can be appreciated by noting the significant membrane-exposed surface area of the ligands in the analyzed crystal structures (see Fig. 5; Table 1).

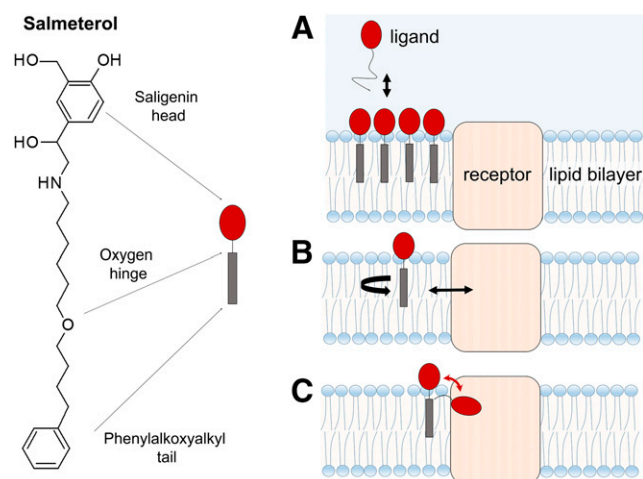


Fig. 2. Multiple lipid bilayer roles in molecular recognition of trans-membrane proteins. According to the “microkinetic” model, the cell membrane can govern drug-binding processes by: (A) acting as a local reservoir for the drug and thus prolonging the drug exposure of the target; (B) modulating the orientation and conformation of the drug for favorable receptor binding; and (C) reducing the time the drug needs to reach its receptor, thus facilitating the approach. The long-lasting bronchodilatory effect of salmeterol (a β_2 -adrenergic receptor partial agonist with a receptor-binding pharmacophore saligenin head, a hydrophobic phenylalkoxyalkyl tail, and an oxygen hinge), may be attributed to a combination of these effects. Adapted from Vauquelin (2016).

The Lipid Pathway for Cannabinoid Ligands. By using isothiocyanate covalent labeling studies, Makriyannis and coworkers (Pei et al., 2008) have shown that a cannabinoid, (–)-7'-isothiocyano-11-hydroxy-1',1'-dimethylheptyl-hexahydrocannabinol (AM841), enters the cannabinoid CB2 receptor through the lipid bilayer. Inspired by this study, Hurst et al. (2010) investigated a potential lipid pathway entry and interaction for endogenous cannabinoid sn-2-arachidonoylglycerol (2-AG) with the CB2 receptor in a POPC bilayer using microsecond-timescale all-atom molecular dynamic simulations. This study demonstrated that 2-AG first partitioned out from the bulk lipid at the interface between transmembrane helices (TMH) 6 and 7 before entering into the binding site by crossing between TMH6 and TMH7. This ligand entry results in the breaking of an ionic lock between TMH3 and TMH6 at the intracellular end and causes the displacement of TMH6 away from TMH3. Subsequent to manually applied protonation at the D3.49/D6.30 positions, further access by 2-AG deep into the binding pocket results in a conformational change in the W6.48 “toggle switch” and a significant flow of water. Several biophysical studies indicate that the listed observations are similar to the signature events (Farrens et al., 1996; Lin and Sakmar, 1996; Javitch et al., 1997; Ghanouni et al., 2000, 2001; Ballesteros et al., 2001; Jensen et al., 2001; Nakanishi et al., 2006; Kobilka and Deupi, 2007; Grossfield et al., 2008) that occur following ligand-induced activation of rhodopsin and β_2 -adrenergic receptors. It should be noted that although 2-AG was placed around the receptor in various positions in the bilayer, the ligand did not enter the receptor in every simulation. However, it was observed to enter only through TMH6 and TMH7, suggesting a specific lipid access pathway for this endogenous ligand. The negative cases were used as controls to compare the conformational changes (in W6.48) upon 2-AG entry into the receptor.

The Lipid Pathway for Protease-Activated Receptor-1 Ligands. A recent study (Bokoch et al., 2018) combining MD simulations and kinetic cell signaling experiments suggests a possible lipid bilayer pathway for vorapaxar, a small molecule antagonist of protease-activated receptor-1 (PAR1). As previously reported, an unbiased long timescale (≤ 10 microseconds) molecular dynamic study (Zhang et al., 2012) did not result in spontaneous dissociation of vorapaxar owing to the slow experimental off rate (approx. 20 hours) of vorapaxar. Hence, the dissociation of the ligand from the binding pocket was investigated by temperature-accelerated molecular dynamics, an enhanced sampling method used to accelerate the dissociation process. In 8 out of 13 simulations, vorapaxar dissociates and exits the binding site via the lipid bilayer, passing between TMH4 and TMH5 (three times) and between TMH6 and TMH7 (five times), with multiple “metastable states” along the dissociation pathway forming reproducible sets of receptor and lipid interactions. Most remarkably, a qualitative analysis of contacts between the nonhydrogen atoms of vorapaxar and the receptor-membrane system in all metastable states revealed that POPC lipid molecules interacted with the ligand more frequently than any other residues from the receptor. In the PAR1-vorapaxar crystal structure (Zhang et al., 2012), the ethyl carbamate group of the ligand protrudes through a tunnel between TMH6 and TMH7 and points toward the lipid bilayer. In all of the trajectories involving the TMH6-TMH7 tunnel, the same ethyl carbamate group was found to leave the pocket first and interact with the outer leaflet of the lipid bilayer on the way out of the binding pocket. Further chemical biology experiments with alkyl derivatives of vorapaxar (hexyl vorapaxar and N-boc-hexyl vorapaxar) using functional calcium flux assays demonstrated that the addition of a bulky lipophilic group to vorapaxar did not decrease its antagonistic efficacy and had a relatively small effect on the inhibition kinetics. This is possible only if vorapaxar and its derivatives can enter and exit PAR1 via the TMH6-TMH7 tunnel; therefore, these experiments provide convincing evidence that these hexyl and N-boc-hexyl tails can extend through the TMH6-TMH7 tunnel, adopting the same binding pose as the parent molecule in the crystal structure. This study suggests that the contribution of membrane lipids can be exploited to selectively alter on and off rates by modifying ligand-lipid interactions.

Membrane-Facilitated Ligand Access and Binding to Extrahelical Allosteric Binding Site of P2Y1 Receptor.

A recent study (Yuan et al., 2018) investigated the plausible membrane-facilitated molecular mechanism underlying the binding of allosteric antagonist 1-(2-(2-(tert-butyl) phenoxy) pyridine-3-yl)-3-(4-(trifluoromethoxy) phenyl) urea (BPTU) to the transmembrane extrahelical allosteric site of the P2Y1 receptor (P2Y1R), a class A GPCR that is a promising target for antithrombotic drugs. This computational study used a combination of conventional molecular dynamics simulations and multiple enhanced-sampling methods (Gervasio et al., 2005; Provasi et al., 2009; Limongelli et al., 2010; Saladino et al., 2012; Jämbeck and Lyubartsev, 2013; Schneider et al., 2015), including umbrella sampling (Kästner, 2011; Bochicchio et al., 2015; Lee et al., 2016), well-tempered metadynamics (Barducci et al., 2008; Yuan et al., 2018), and funnel-metadynamics (Troussicot et al., 2015; Comitani et al.,

2016; Shang et al., 2016). The results obtained included: 1) the preferred location of BPTU in the POPC bilayer without the receptor; 2) the dynamics of BPTU as it transitions from the aqueous bulk phase into the lipid bilayer and subsequently binds to the receptor site by two-dimensional lateral diffusion; 3) a quantitative, well defined free energy surface; and 4) an accurate estimation of the BPTU-P2Y1R binding free energy. The free energy profile for the partitioning of BPTU in the POPC bilayer indicated a preferred (energetically favorable) location, which is approximately 12 Å from the center of the bilayer, above its receptor binding site (approx. 5 Å from the center of the bilayer). The analysis of the angle between the long axis of BPTU and the bilayer normal (z -axis) as a function of the distance of BPTU from the center of bilayer revealed that the interaction with lipids at this bilayer depth favored BPTU adoption of a preferential orientation and conformation to facilitate reaching its binding site (see Fig. 3). The strong H-bond interactions of BPTU's urea group with the polar head groups of lipids, and the subsequent π - π stacking and hydrophobic interactions with I118 and F119 of extracellular loop 2 as first contacts with the extra-helical site, enabled BPTU to move from three-dimensional diffusion in the aqueous phase to two-dimensional diffusion in the membrane. The lowest energy conformation of BPTU from the free energy surface, obtained through well tempered metadynamics, was approximately equivalent to the conformation obtained in the experimental crystal structure (Zhang et al., 2015) (PDB ID 4XNV). It is critical to note that in this bound position, the ligand molecule is only partially bound to the shallow binding pocket, while the rest of it is buried in the lipid hydrophobic region. Though polar headgroups of lipids play a critical role in the dynamics of the binding process, only hydrophobic tails seem to be necessary for the final bound pose, at least in this case. The excellent agreement between the experimental binding free energies and the values calculated using these state-of-the-art simulation techniques appears to support the described binding mechanism.

Structural Evidence for Lipid-Facilitated Binding to Transmembrane Allosteric Sites of Class A and Class B GPCRs

Recent progress in crystallography and protein engineering has resulted in an exponential increase in the number of membrane protein structures, especially GPCRs (Lu and Zhang, 2018), available in multiple conformations (active, inactive, and intermediate metastable states), which in turn has provided remarkable details of structure-activation-function relationships (Granier and Kobilka, 2012; Shoichet and Kobilka, 2012; Kobilka, 2013; Manglik and Kobilka, 2014; Hilger et al., 2018). Here, we summarize several X-ray structures of class A and B GPCRs crystallized with their allosteric modulators (PAMs, agoPAMs, and NAMs), specifically bound at the extrahelical transmembrane binding sites that are embedded deep within the lipid bilayer (see Fig. 4; Table 1). Remarkably, these structures reveal that functionally important allosteric binding sites are spread across the entire bilayer depth, which can be reached only through lipid pathways. These allosteric ligands most probably partition into the membrane and concentrate at the vicinity of these binding sites before binding (Blanton et al., 1988). All but one of the ligands (TAK-875)

have a calculated 1-octanol/water log P value above 3, which means they prefer a lipid phase 1000 \times more than an aqueous phase and probably first partition into the membrane, after which they can diffuse to their respective binding sites at the lipid bilayer/receptor interface (Table 1). The calculated distances between the center of mass (COM) of the bound ligands and COM of phosphorus atoms of the upper leaflet of the membrane lipids (POPC) indicate the diversity in bilayer depth of these lipid-facing sites (Table 1).

Desolvation of Ligands While Binding to Membrane-Exposed Receptor Sites. Binding of a ligand to its target receptor site involves changes in the solvent-accessible surface area (SASA) for both species. The extent to which the ligand and its binding site are desolvated and the amount of buried SASA provide direct insights into the contributions of solvents and hydrophobic interactions in the molecular recognition process. A strong correlation between the experimental binding affinities of several protein-ligand complexes and their SASA quantities has been reported (Li et al., 2018). Interestingly, for membrane-exposed binding sites, the binding of ligands often does not involve the removal of any water molecules from the binding sites. In contrast, the lipid molecules surrounding these sites are displaced during the ligand-binding event. Nevertheless, a significant portion of these ligands remains exposed to the lipid molecules surrounding the binding sites. Although it is well known that the membrane partitioning of ligand molecules significantly affects their local concentrations and subsequently may contribute to their k_{on} rates, the extent of lipid-ligand interactions at the receptor site may also influence their k_{off} rates. It is reasonable to consider these lipid molecules as part of the binding site given the magnitude of their interactions that contribute to the overall binding affinity as well as residence time of these ligands. This unconventional but logical treatment of the membrane as part of the receptor in membrane-exposed binding sites needs a careful, revised treatment of the k_{off} rates, thus incorporating the

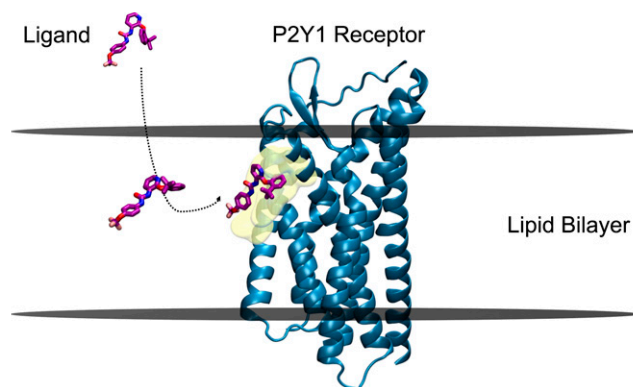


Fig. 3. Membrane-facilitated ligand access and binding. BPTU, a small-molecule antagonist (negative allosteric modulator) partitions within the membrane first, and reaches the transmembrane extrahelical binding site of the P2Y1 receptor through a lipid pathway. The preferred orientation and conformation of BPTU within the POPC bilayer near the binding site, obtained by funnel metadynamics simulations, is strikingly similar to that of the bound ligand in the crystal structure, indicating the plausible role of membrane lipids in “pre-organizing” the ligand for receptor binding. BPTU and P2Y1 receptor molecules are represented in stick and secondary structures, respectively. The approximate positions of the upper and lower leaflets of a hypothetical lipid bilayer are marked by two horizontal lines perpendicular to the bilayer normal. The binding site residues are depicted as transparent surface models in the color yellow. Picture adapted from Yuan et al. (2018).

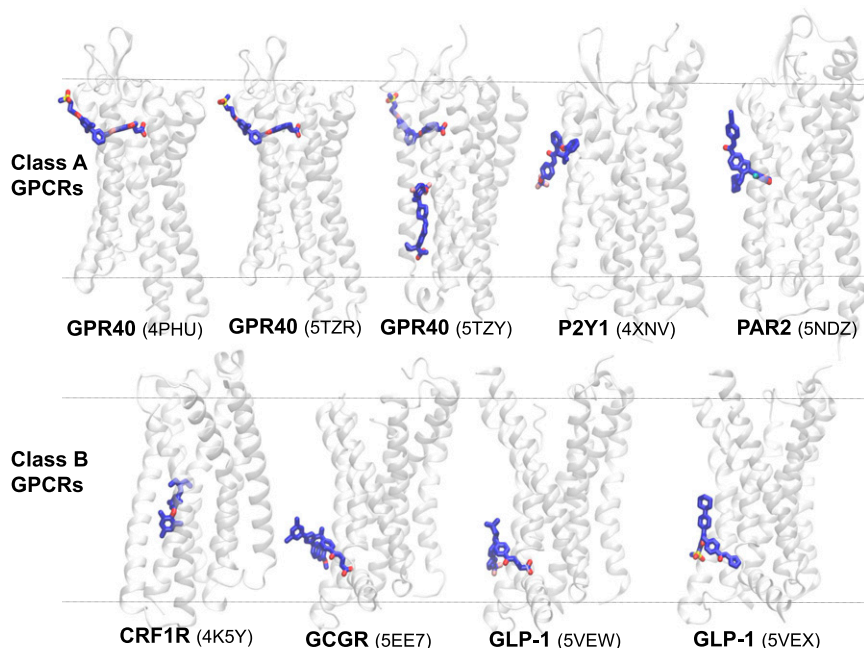


Fig. 4. Transmembrane allosteric sites in GPCRs span the entire bilayer normal (z -axis). X-ray structures (PDB IDs are given in the brackets) of class A and class B GPCRs and their ligands bound to transmembrane allosteric sites embedded within and facing the lipid bilayer. The receptors and bound ligands are present in secondary structure (gray) and stick (blue) representations, respectively. CRF1R, corticotropin-releasing factor receptor 1; GCGR, glucagon receptor; GLP-1, glucagon-like peptide-1 receptor; GPR40, free fatty acid receptor; P2Y1, P2Y purinoreceptor 1; PAR2, protease activated receptor 2. The approximate positions of the upper and lower leaflets of a hypothetical lipid bilayer are marked by two horizontal lines perpendicular to the bilayer normal. The Ballesteros and Weinstein amino acid numbering system (Ballesteros and Weinstein, 1995) used in the main text as a superscript of the absolute sequence number, begins with the TMH number and is followed by a locant (0.50 assigned to most highly conserved amino acid in a TMH and other residues numbered relative to it).

effects of the membrane-partitioning characteristics into the overall dissociation rate constants.

In this paper, we calculated the extent to which the SASA of the bound ligands (presented in the X-ray crystal structures discussed here, and summarized and represented in Figs. 4 and 5; Table 1, respectively) are either buried in the receptor sites, denoted by “buried surface area” (BSA), or exposed to the lipid molecules, denoted by “membrane-exposed surface area” (MESA). The SASA values for the bound receptor-ligand complexes, as well as for the unbound receptors and the unbound ligand molecules, were calculated using the NACCESS program (Hubbard and Thornton, 1993; <https://>

www.uclb.com/i/software/naccess.html?item=naccess) with a probe radius of 1.4 Å. The X-ray structures of the receptor-ligand complexes were treated in MOE (Molecular Operating Environment 2013.08, 2018) (all water, nonprotein, and nonligand molecules were removed; missing residues and atoms were added wherever necessary). In principle, the solvent-accessible area of a ligand in its receptor-bound form provides the MESA value, whereas the difference between the SASA of an unbound ligand and its MESA value provides the BSA value, the proportion of the ligand surface area that is buried in the receptor. The majority of these ligands are highly lipophilic and characterized by up to 90%

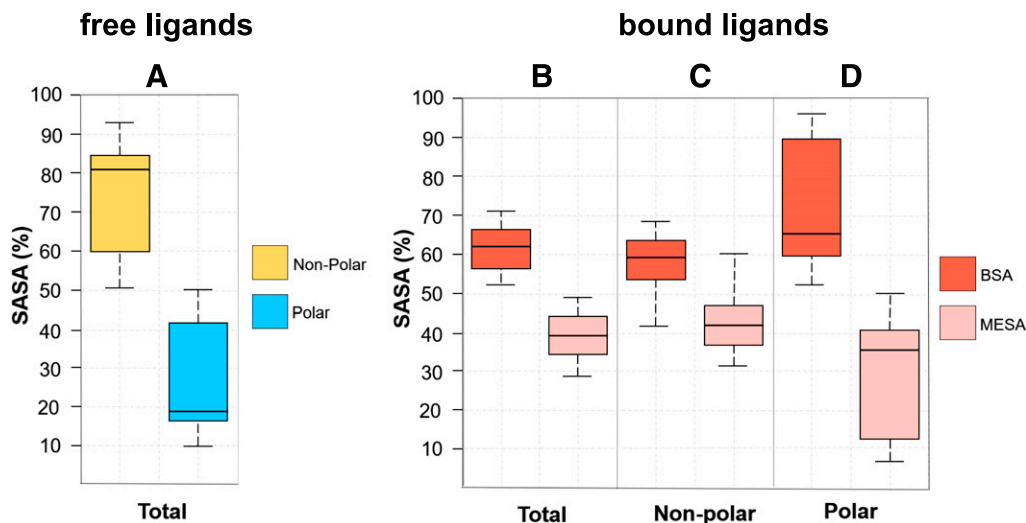


Fig. 5. Box and whisker plots of solvent-accessible surface area (in percent) characteristics of the allosteric GPCR ligands (discussed in Table 1) in free (A) and receptor-bound states (B–D). The majority of the ligands are highly lipophilic with larger nonpolar SASA (A). In their receptor-bound states, significant (approx. 40%) portions of their total SASA (B) remain exposed to membrane lipids, denoted as membrane-exposed surface area, whereas $\geq 60\%$ of their total SASA are buried within the receptor binding sites, denoted as buried surface area (BSA). Nonpolar SASA (C) of these ligands have BSA and MESA distributions very similar to those of their total SASA. However, the polar SASAs are mostly buried into the receptor sites (D). Lines inside the boxes denote medians, lower and upper ends of the boxes denote 25th and 75th percentiles respectively, and whiskers denote the lowest and highest observations.

TABLE 1

X-ray structures of class A and B GPCRs in which the allosteric ligands are bound to transmembrane lipid-facing binding sites, which can be accessed only through lipid pathways

GPCR Class	Receptor	Allosteric Ligand Name	PDB ID	Bilayer Depth (Å) ^d	Ligand Lipophilicity		Major Species at pH 7.4	Solvent-Accessible Surface Area (SASA) ^b				
					C log P	log D _{7.4}		Unbound Ligand			Bound Ligand	
								Total (Å ²)	Polar (%)	Nonpolar (%)	MESA (%)	BSA (%)
A	GPR40	TAK-875 ^c	4PHU	16.1	1.30	1.23	Negative	656.5	49.8	50.2	31	69
	GPR40	MK-8666 ^c	5TZR	16.4	3.75	1.62	Negative	913.4	16.8	83.2	34	66
	GPR40	AP8 ^d	5TZY	30.5	5.23	4.05	Zwitterionic	923.9	15.6	84.4	34	66
	P2Y ₁	BPTU ^e	4XNV	22.7	7.50	5.53	Neutral	739.8	34.8	65.2	40	60
	PAR2	AZ3451 ^e	5NDZ	18.0	7.96	6.37	Neutral	773.1	18.1	81.9	43	57
B	CRF1R	CP-376395 ^c	4K5Y	23.6	7.61	5.7	Positive	658.1	49.4	50.6	39	61
	GCCR	MK-0893 ^c	5EE7	31.6	4.88	3.42	Negative	971.6	9.9	90.1	44	56
	GLP1R	PF-06372222 ^f	5VEW	34.7	4.61	-0.53	Negative	908.3	13.3	86.7	30	70
	GLP1R	NNC0640 ^f	5VEX	31.0	4.08	1.28	Positive	976.2	41.5	58.5	45	55

CRF1R, corticotropin-releasing factor 1 receptor; GLP1R, glucagon-like peptide 1 receptor; GPR40, free fatty acid receptor 1; PAR2, protease-activated receptor 2.

^aBinding site location (bilayer depth) within the membrane, measured as the distance between center of mass (COM) of the ligand and that of the phosphorus atoms in the upper leaflet, measured along the bilayer normal.

^bCalculated using NACCESS (Hubbard and Thornton, 1993) with a probe of radius 1.4 Å; C log P: 1-octanol/water partition coefficients calculated using Bio-Loom for Windows version 1.5 (2006); log D_{7.4}: partition coefficient at pH 7.4 that was calculated using ACD/Percepta (Advanced Chemistry Development, Inc., Toronto, ON, 2015). For bilayer depth calculation, the membrane-embedded crystal structures were obtained from the OPM database (Lomize et al., 2012). The average bilayer thickness was 36.92 Å for all the studied receptors.

^cPartial agonist.

^dFull allosteric agonists, AgoPAMs.

^eAntagonist.

^fNegative allosteric modulator.

nonpolar SASA (see Fig. 5A; Table 1). Interestingly, for all these ligands, approximately 40%–55% of their nonpolar SASA remains exposed to the membrane lipids in their bound forms (see Fig. 5, B and C; Table 1). However, large quantities (50%–95%) of their polar SASA are still buried within the binding sites (see Fig. 5D).

Class A GPCRs

Although all GPCRs share common structural features (e.g., 7TM helices, along with the ability to interact with G-proteins), substantial differences between them allow them to be classified into various groups. The diversity among the GPCR superfamily has allowed for the A-F classification system developed by Kolakowski in 1994 (Lagerström and Schiöth, 2008). Class A (rhodopsin-like) GPCRs make up the largest member of the family, which are further divided into four groups (α , β , γ , δ). These different groups bind various ligands, including peptides, lipids, amines, and purines, and there does not seem to be a clear correlation between the phylogenetic location of the receptor and the endogenous ligand. Clusters exist, with the amine-binding ligands primarily in the α -group and the majority of peptide-binding ligands in the β -group. The nucleotide-binding receptors and the glycoproteins are mainly confined to the δ -group (Fredriksson et al., 2003). There is great diversity in the composition and size of the N terminus and the extracellular loops for these receptors (Venkatakrishnan et al., 2013).

Free Fatty-Acid Receptor 1 Bound to Partial Agonists TAK-875 and MK-8666, and to Ago-PAM AP8. GPR40, also known as the free fatty-acid receptor 1, has been shown to be a promising target for novel treatment of type-2 diabetes mellitus (McGarry and Dobbins, 1999; Flodgren et al., 2007). Natural ligands of the receptor include full agonists such as saturated C₁₂–C₁₆ and unsaturated C₁₈–C₂₀ free fatty acids, which mediate insulin secretion and glucagon-like peptide 1 secretion. The receptor was crystallized (Srivastava et al., 2014) with a small-molecule

partial agonist, TAK-875 (PDB ID 4PHU), that stimulates glucose-dependent insulin secretion. The structure revealed a unique binding mode for TAK-875 at the transmembrane extrahelical allosteric binding site located in between TMH3 and TMH5, at a bilayer depth of approx. 16.1 Å (see Fig. 4; Table 1). The most striking feature of the bound ligand, aside from the position of the binding site at the receptor-lipid interface, was that more than 58% of the nonpolar SASA was sticking out and exposed to the membrane, specifically toward the head group region of the bilayer. In contrast, more than 95% of the bound ligand's polar SASA was buried within the binding pocket, with the carboxyl group of TAK-875 forming H-bonds with multiple arginine (Arg183^{5,39} and Arg258^{7,35}) and tyrosine (Tyr91^{3,37} and Tyr240^{6,51}) residues of the receptor. The spatial and topological location of the binding site with respect to the bilayer and the 380-fold higher affinity of the ligand for the lipophilic phase over the aqueous phase together suggest the first step of preferential partitioning into the bilayer around the receptor, followed by a lateral movement in between TMH3 and TMH5.

Two additional crystal structures for GPR40 have been published (Lu et al., 2017). One structure features another partial agonist, MK-8666 (PDB ID 5TZR), bound at the same TAK-875 binding pocket; the other features an AgoPAM AP8 (PDB ID 5TZY) and MK-8666, which are both bound at the same time. MK-8666s BSA and MESA profiles are similar to that of TAK-875 (66% and 34%, respectively), except that it has a larger nonpolar SASA (83% vs. 50%) implying more lipophilic nature of MK-8666 (see Table 1). The binding site of the AgoPAM revealed that it occupied a well defined pocket at a bilayer depth of approx. 30.5 Å (see Fig. 4; Table 1); this pocket was formed by transmembrane helices 3–5 and intracellular loop 2 (ICL2) outside of the helical bundle and away from the orthosteric binding site. The lipid-facing binding site is unique and composed of three distinct regions: 1) toward the extracellular side, the top of the binding site is formed by the intersection of TMH3, TMH4, and TMH5 with residues Ile130^{4,49}, Leu133^{4,52}, Val134^{4,53}, and Leu190^{5,46}, which

collectively form a hydrophobic pocket to accommodate the terminal trifluoromethoxyphenyl moiety of AP8; 2) toward the intracellular side, the bottom portion of the binding site is formed by a polar cavity (Tyr44^{2,42} and Ser123^{4,42}) that interacts with the carboxylate group of AP8; 3) the middle region is formed by residues located on ICL2 and its surrounding area (Leu106^{3,52}, Phe110^{3,56}, Tyr114^{ICL2}, Phe117^{ICL2}, and Tyr122^{4,41}), form stabilizing interactions with the AP8 methyl and cyclopropyl moieties, sealing the AP8 pocket from the intracellular environment.

P2Y₁-P2Y Purinoreceptor 1 Bound to Antagonist BPTU. The purinergic receptors are divided into two subfamilies, G_q-coupled P2Y₁R and G_i-coupled P2Y₁₂R. Both subfamilies are involved in thrombosis formation, and the blockade of either receptor significantly decreases ADP-induced platelet aggregation (Jin and Kunapuli, 1998; Jacobson et al., 2011). Though many currently available antithrombotic drugs act on P2Y₁₂R, P2Y₁R has been suggested as a promising alternative that offers the advantage of a reduced bleeding effect. Recently, Zhang et al. (2015) crystallized the human purinergic G_q-coupled P2Y₁ receptor, in complex (PDB ID 4XNV) with a novel antagonist, BPTU, that reduces platelet aggregation with a minimal bleeding effect, for the treatment of thrombosis (Chao et al., 2013). BPTU is highly lipophilic ($\log D_{7.4} = 5.53$) and binds at a relatively shallow binding pocket (located at a bilayer depth of approx. 27 Å; see Fig. 4; Table 1) that faces the lipid bilayer and is formed by several aromatic residues located on TMH1, TMH2, and TMH3 and extracellular loop 1. The interactions of BPTU with the binding site residues are predominantly hydrophobic in nature. At one end, the trifluoromethoxyphenyl group of BPTU interacts with F62^{1,43} and F66^{1,47}; at the other end, the phenoxy moiety attached to the *tert*-butyl group engages in nonpolar interactions with a hydrophobic cavity formed by the residues from TMH2 and TMH3, including T103^{2,56}, M123^{3,24}, L126^{3,27}, and Q127^{3,28}. Though the *tert*-butyl group seems to be in close contact with the alkyl side chain of L103^{2,55}, it clearly protrudes into the lipid bilayer. Two H-bonds are formed between the amido-NH groups of BPTU's urea group and the carbonyl oxygen of L102^{2,55}. Around 60% of the ligand is buried inside the protein, exposing the remaining approx. 40% (see Table 1) of the ligand to the membrane environment. A recent computational study (Yuan et al., 2018) investigated the plausible lipid pathway and the role of the membrane in "preorganizing" the molecule for receptor binding, the details of which are discussed elsewhere in this article.

Protease-Activated Receptor 2 in Complex with Antagonist AZ3451. The protease-activated receptors (PARs) are involved in a variety of diseases, including cancer and various types of inflammation (Adams et al., 2011; Ramachandran et al., 2012). The structure of PAR1 in complex with the orthosteric small-molecule antagonist vorapaxar has been reported before; however, despite the sequence homology between PAR1 and PAR2, attempts to identify the PAR2 antagonists have been less fruitful. Recently the PAR2 receptor was crystallized (Cheng et al., 2017) in complex with an allosteric modulator (NAM), AZ3451, at a resolution of 3.6 Å (PDB ID 5NDZ), and it was suggested that the binding of this ligand probably prevents the structural realignment necessary for receptor activation and signaling. AZ3451 is a highly lipophilic molecule, and the location of the allosteric binding site (at the bilayer depth of

18 Å; see Fig. 4; Table 1) differs from allosteric sites observed for the P2Y₁ and the glucagon receptors (Jazayeri et al., 2016). The 1,3-benzodioxole ring of AZ3451 fits into a hydrophobic pocket formed by residues Ala120^{2,49}, Leu123^{2,52}, Phe154^{3,31}, Ala157^{3,34} (Gly157^{3,34} in wild type), Cys161^{3,38}, Trp199^{4,50}, and Ile202^{4,53}. Tyr210^{4,61} forms a weak hydrogen bond (3.4 Å) to N1 of the benzimidazole. Mutations of Gly157^{3,34} into cysteine or of Tyr210^{4,61} into leucine resulted in the decreased efficacy of AZ3451, indicating their contribution to selectivity. The cyclohexyl ring of the ligand lies at the membrane interface of the receptor and engages with Leu123^{2,52}, and the benzonitrile moiety points toward the extracellular aqueous interface, making π -stacking interactions with Y210^{4,61}. The significant nonpolar interactions (MESA of approx. 43%, see Table 1) between the ligand and the surrounding lipid molecules in the receptor-bound state might be a critical factor in imparting the receptor binding affinity.

Class B GPCRs

Class B GPCRs (secretin-like receptors) all bind peptide hormones and contain an extracellular hormone binding domain. The majority of this family of receptors share conserved cysteine residues in the N-terminal domain that form a network of three cysteine bridges (Grauschopf et al., 2000; Hofmann et al., 2001; Bazarsuren et al., 2002). Class A and class B GPCRs differ in their transmembrane helices, featuring distinct patterns of conserved residues in each class respectively (Siu et al., 2013). In general, the class B GPCRs feature the same overall architecture as class A GPCRs, but the available crystal structures of some class B GPCRs have revealed differences between the orthosteric binding pockets of the two classes. Class B GPCRs have an orthosteric binding pocket that is deeper and wider than that of any class A GPCR (Hollenstein et al., 2013) and development of nonpeptide small-molecule ligands targeting these sites has proved extremely challenging. Thus, allosteric modulation of these receptors offers a potential direction for developing low-molecular-weight activators and inhibitors as therapeutic agents (Hoare, 2007).

Corticotropin-Releasing Factor Receptor Type 1 Bound to Antagonist CP-376395. The corticotropin-releasing factor receptor type 1 (CRF1R) is a class B GPCR that has been shown to mediate the stress response and has been considered as a target for managing depression and anxiety (Bale and Vale, 2004). The receptor binds corticotropin-releasing hormone, a 41 amino acid peptide that acts as a neurotransmitter and can influence a variety of responses, including appetite control, cardiovascular regulation, immune function, and behavior (Hemley et al., 2007). Antagonists of CRF1R have been evaluated for the potential to treat different stress-related indications, including depression, anxiety, and irritable bowel syndrome (Hemley et al., 2007). The X-ray crystal structure of CRF1R bound to antagonist CP-376395 (PDB ID 4K5Y) reveals an extrahelical allosteric binding site embedded within the transmembrane domain of the receptor (Zorrilla and Koob, 2010). This allosteric pocket is topologically distinct (18 Å away from the center of the orthosteric binding pocket) and is 13–23 Å away from the center of mass of the small-molecule ligands in the crystal structures of some class A GPCRs (Hollenstein et al., 2013). The binding pocket is formed mainly by the

hydrophobic residues from TMH3, TMH5, and TMH6 of the receptor, which seem to open laterally through rearrangements, allowing the entry of the lipophilic ligand from within the membrane, possibly through lateral diffusion. In the bound state, the ligand molecule aligns perpendicularly to the membrane bilayer normal at a bilayer depth of approx. 23.1 Å (see Fig. 4; Table 1). The ligand forms an H-bond with Asn283^{5,50} through its pyridine nitrogen atom and the aryloxy moiety fits into a hydrophobic pocket formed by Phe284^{5,51}, Leu287^{5,54}, Ile290^{5,57}, Tyr316^{6,42}, Leu319^{6,45}, and Leu320^{6,46}. The exocyclic alkylamino group interacts with Gly324^{6,50}, Phe203^{3,44}, Leu280^{5,47}, Leu323^{6,49}, and Tyr327^{6,53}. However, a significant part of the ligand (MESA of 39%, see Table 1) is sticking out into the membrane, exposed to the hydrophobic environment of the lipid tails.

Glucagon Receptor Bound to Antagonist MK-0893.

Glucagon is a 29 amino acid peptide that plays an integral role in glucose homeostasis; its action is caused by the G protein-coupled glucagon receptor (GCGR), a class B GPCR deemed a potential drug target in the treatment of diabetes. Small-molecule GCGR antagonists have shown promise in treating patients with type 2 diabetes, resulting in reduction of fasting and postprandial glucose concentrations (Bagger et al., 2011). The crystal structure of GCGR in complex with antagonist MK-0893 (PDB ID 5EE7) reveals an allosteric site at the interface between TMH6 and TMH7 that extends further into the membrane to a bilayer depth of approx. 31.6 Å proximal to the lower leaflet (Jazayeri et al., 2016) (see Fig. 4; Table 1). This lipophilic ligand (more than 90% of its total SASA is nonpolar) engages in several important hydrophobic interactions that include interactions of the methoxynaphthalene moiety of MK-0893 with residues Leu329^{5,61}, Phe345^{6,36}, Leu352^{6,43}, Thr353^{6,44}, and with the alkyl chain of Lys349^{6,40} at the TMH5-TMH6 interface. Within the TMH6-TMH7 cleft, MK-0893 is surrounded by several polar contacts: The amide group forms H-bonds with Lys349^{6,40} and Ser350^{6,41}; and the carboxyl group forms a salt bridge with Arg346^{6,37}, in addition to interactions with Asn404^{7,61} and the backbone of Lys405^{7,62}, plus water-mediated H-bonds with Ser350^{6,41} and Leu399^{7,56}. The phenylethylpyrazole core of the ligand is in close contact with the Lys349^{6,40} and Thr353^{6,44} located on TMH6. Strikingly, the lipophilic dichlorophenyl moiety attached to the core pyrazole ring is entirely sticking out into the membrane, engaging in hydrophobic interactions with the lipid tails. The ligand atoms in its receptor-bound state remain significantly exposed to the membrane (MESA value of approx. 44%) indicating the apparent contribution of the membrane to the binding affinity.

Glucagon-Like Peptide Receptor Bound to Negative Allosteric Modulators PF-06372222 and NNC0640. Glucagon-like peptide-1 receptor (GLP-1R) is a class B GPCR and is activated by endogenous GLP-1 peptides. Activation of the receptor leads to the inhibition of glucagon secretion and the stimulation of insulin secretion in a glucose-dependent manner (Cho et al., 2012); thus, GLP-1R agonists can be used for the treatment of type 2 diabetes mellitus. Antagonism of GLP-1R was also shown to be clinically useful in the treatment of acute and chronic stress as well as anxiety. Two X-ray structures of GLP-1R (Song et al., 2017), crystallized with negative allosteric modulators PF-06372222 (PDB ID 5VEW) and NNC0640 (PDB ID 5VEX), reveal that both NAMs bind at the same extrahelical site, located outside the TMH6 and

TMH7 helices, facing the lipid membrane. Interestingly, these are also NAMs of GCGR, and the binding modes of these ligands are remarkably like that of MK-0893 bound to GCGR. In addition, the trifluoromethylpyrazole end of PF-06372222 has extensive nonpolar interactions with the hydrophobic pocket formed by residues I328^{5,58}, V331^{5,61}, V332^{5,62}, L335^{5,65}, and F347^{6,36}, and the pyridine and phenyl rings of the ligand make hydrophobic interactions with the alkyl side chains of K351^{6,40} and L401^{7,56}. Most interestingly, the dimethylcyclobutane ring of the ligand sticks out into the lipid bilayer while making nonpolar contacts with L401^{6,38} and M397^{6,42}. The polar anionic carboxylic acid and amide groups of PF-06372222 make H-bonds with the side chains of S352^{6,41}, N406^{7,61}, and K351^{6,40} residues, respectively, and the aminopyridine moiety forms an H-bond with the side chain of the S355^{6,44} residue.

The binding mode and residue interactions of NNC0640 are notably similar to those of PF-06372222. The polar tetrazole group of the ligand makes H-bond interactions with the side chains of S352^{6,41} and N406^{7,61}, whereas the amide group forms H-bonds with the side chains of S352^{6,41} and K351^{6,40} and the urea amino group makes an H-bond with S355^{6,44}. The methylsulfone phenyl end of the ligand molecule occupies the same hydrophobic binding pocket occupied by the trifluoromethylpyrazole moiety of PF-06372222, engaging in nonpolar interactions with F324^{5,54}, I328^{5,58}, F347^{6,36}, and L351^{6,40} and the alkyl chain of K351^{6,40}. As with the dimethylcyclobutane ring of PF-06372222, the phenylcyclohexyl group distinctly sticks out into the lipid bilayer while making extensive nonpolar interactions with the S355^{6,44}, L359^{6,48}, F390^{6,31}, M397^{6,31}, and L401^{6,42} residues. In these unique binding modes, the NAMs restrain the movement of TMH6 away from TMH7 and thus prevent G protein coupling. MESA values for PF-06372222 and NNC0640 are 30% and 45%, respectively. However, it should be noted that the nonpolar SASA is much higher (86.7%) for PF-06372222 than it is for NNC0640 (which is only 58.5%). Interestingly for PF-06372222, more than 90% of its polar SASA is buried in the protein (see Table 1), whereas for NNC0640, 60% of its polar SASA is buried inside the receptor binding pocket.

Conclusions and Future Perspectives

Recent advances in GPCR structural biology and growing interest in allosteric modulation and biased signaling as novel therapeutic strategies have created tremendous opportunities as well as numerous challenges for rational drug discovery (May et al., 2007; Burford et al., 2011; Conn et al., 2014; Langmead and Christopoulos, 2014). The discovery of multiple spatially distinct yet conformationally linked allosteric binding sites located at the transmembrane extrahelical-lipid interfaces presents an enriched yet challenging landscape for small-molecule therapeutics. Many challenges must be overcome before the actions of allosteric modulators can be reliably detected, validated, and quantified, and the fact that transmembrane extra-helical membrane-facing binding sites can only be reached via lipid pathways adds further complexity. The tissue-to-tissue variations in lipid composition and the biophysical properties of the plasma membrane necessitate a thorough understanding of drug-membrane interactions in order for full advantage to be taken of the multiple mechanisms (Sargent and Schwyzer, 1986; Vauquelin

and Packeu, 2009) by which the membrane can facilitate protein-ligand recognition processes (Strasser et al., 2017). In addition, the remarkable extent to which ligands are exposed to the membrane environment while bound to these shallow, transmembrane binding pockets poses additional challenges for researchers in the molecular docking community. The challenge is how to effectively include membranes as one of the receptor components when making predictions about ligand binding poses and scoring the bound receptor-ligand complexes. In addition, the heterogeneity of the phospholipid headgroup, the presence of cholesterol, and the locations of binding sites with respect to the bilayer normal will need thorough consideration.

The ability to predict the quantitative bilayer distribution and the energetically favorable location (depth), orientation, and conformation of a given chemical within the lipid bilayer is a key to understanding the molecular recognition processes of ligands binding to membrane-facing sites of transmembrane proteins. Partition coefficients, either measured or calculated as $\log P$ using 1-octanol/buffer systems, have been shown to correlate well with a drug's therapeutic efficacy, intestinal absorption, plasma protein binding, metabolism rate, non-specific binding to off-targets, and toxicity (Hansch, 1969; Leo et al., 1971; Hansch and Zhang, 1993; Debnath et al., 1994; Hansch et al., 1995; Tarcsay and Keserú, 2013). Though $\log P$ values provide information about ligand lipophilicity, they do not account for the ability of the ligand to interact with the polar head groups of lipid membranes. Differences in drug partitioning into octanol/buffer versus membranes has been observed for a wide variety of drugs (Herbette, 1994; Sykes and Charlton, 2012; Sykes et al., 2014; Dickson et al., 2016). Most importantly, for ionized ligand molecules, the strong interactions of ionized species (bases and acids) with the zwitterionic phospholipid molecules are better captured by anisotropic lipid membranes than by the 1-octanol/water system. The anisotropic structure of the lipid bilayer has very different physical and chemical characteristics across the bilayer normal. Partitioning of drugs within the membrane and drug interactions with lipid molecules appear to exploit these differences to achieve an energetically favorable location, orientation, and conformation. The membrane-drug interactions mentioned above can also be quantified as K_{IAM} , a partition coefficient that essentially describes the degree to which a ligand concentrates from aqueous bulk phase into the phospholipid membrane (Taillardat-Ertschinger et al., 2002; Droge et al., 2017). K_{IAM} quantifies a ligand's degree of phospholipophilicity using column chromatography, consisting of immobilized artificial membrane (monolayers of phospholipid immobilized on a silica surface), to determine the extent of intermolecular interaction between ligand and lipid. Phospholipophilicity is a term used to describe membrane retention of chemicals and comprises hydrophobicity, polarity, and ionic (electrostatic) interactions (Tsopelas et al., 2017). However, the probability of utilizing this quantity to develop a meaningful structural optimization strategy seems slim. Furthermore, an in silico continuum solvent model that quantifies the percentage distribution of chemicals in various bilayer strata was developed using a novel surrogate system containing hexadecane and hydrated diacetylphosphatidylcholine as the core and headgroup, respectively (Natesan et al., 2014). This model mimics realistic membrane-drug interactions and provides approximate orientations of chemicals within the

bilayer. Experimental approaches (such as X-ray, NMR, surface plasmon resonance, and fluorescence quenching) for obtaining "native" drug structures in biologic membranes are extremely time-consuming and not adaptable to high-throughput screening in early-stage drug discovery. Increases in computing speed, owing to the combination of graphical processing units, improved algorithms, multiscale modeling methods, and more accurate force field developments (Bassolino-Klimas et al., 1993, 1995; Alper and Stouch, 1995; Park et al., 2003; Bemporad et al., 2005; Pastor and MacKerell, 2011; Tejwani et al., 2011a,b; Jämbeck and Lyubartsev, 2012a,b; Dickson et al., 2014; Lee et al., 2016), have made molecular dynamic simulations an essential and complementary tool for investigating the partitioning characteristics of small molecules within the membrane compartment and their subsequent recognition by transmembrane proteins at the atomic level.

The current practice of structure-affinity relationship analysis in drug design, which is mainly based on the protein-ligand interactions, needs modification for drugs targeting membrane-associated proteins such as GPCRs (Congreve et al., 2017). This is critical, especially when the ligand is expected to take a lipid pathway to reach its binding site and remains significantly exposed to membrane lipids in its receptor-bound form. Deconvolution of membrane- and protein-ligand contributions to the overall binding affinity will certainly be helpful in optimizing drug structures for better receptor affinity. Nevertheless, knowledge of the structure-membrane interaction relationships of drugs, which captures the "specifics" of the so-called nonspecific membrane interactions, will undoubtedly enable us to optimize rationally the structural features of drugs to exploit the many unique advantages of a membrane environment, resulting in improved receptor affinity, binding kinetics, and selectivity.

Authorship Contributions

Wrote or contributed to the writing of the manuscript: Szlenk, GC, Natesan.

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