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REVIEW

# The interaction of steroids with phospholipid bilayers and membranes

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## Abstract

Steroids are critical for various physiological processes and used to treat inflammatory conditions. Steroids act by two distinct pathways. The genomic pathway is initiated by the steroid binding to nuclear receptors while the non-genomic pathway involves plasma membrane receptors. It has been proposed that steroids might also act in a more indirect mechanism by altering biophysical properties of membranes. Yet, little is known about the effect of steroids on membranes, and steroid-membrane interactions are complex and challenging to characterise. The focus of this review is to outline what is currently known about the interactions of steroids with phospholipid bilayers and illustrate the complexity of these systems using cortisone and progesterone as the main examples. The combined findings from current work demonstrate that the hydrophobicity and planarity of the steroid core does not provide a consensus for steroid-membrane interactions. Even small differences in the substituents on the steroid core can result in significant changes in steroid-membrane interactions. Furthermore, steroid-induced changes in phospholipid bilayer properties are often dependent on steroid concentration and lipid composition. This complexity means that currently there is insufficient information to establish a reliable structure–activity relationship to describe the effect of steroids on membrane properties. Future work should address the challenge of connecting the findings from studying the effect of steroids on phospholipid bilayers to cell membranes. Insights from steroid-membrane interactions will benefit our understanding of normal physiology and assist drug development.

**Keywords** Cortisone · Progesterone · Steroids · Phospholipid bilayers · Membranes · Molecular interactions · Non-genomic effect

## Introduction

Steroids are polycyclic compounds found ubiquitously in nature. In prokaryotes and eukaryotes, they are components of the cell membrane and act as signalling molecules. Steroids are crucial in a wide range of physiological functions including energy metabolism, growth and reproduction, inflammation and immunosuppression and circadian rhythms. Steroids are also widely used as pharmaceuticals to treat inflammatory conditions such as eczema, lung diseases including asthma and chronic obstructive pulmonary disease and autoimmune conditions such as rheumatoid arthritis or lupus (Williams 2018; Strehl et al. 2019).

The signalling mechanism of steroids is generally classified into two distinct pathways: the genomic and non-genomic pathway. The genomic pathway is transcription-dependent, and physiological effects usually take place over hours or days. In contrast, the non-genomic pathway bypasses gene transcription (i.e. is transcription-independent) and is characterised by rapid onset and shorter duration of effects (Falkenstein et al. 2000; Alangari 2010; Colciago et al. 2020). The two mechanisms also differ in the type of receptors involved. In the genomic pathway, the steroid binds to a cytoplasmic nuclear receptor. Subsequently, the receptor translocates to the nucleus where it binds to specific DNA sequences, which then alters the transcription of selected genes. In the non-genomic pathway, the steroid binds to a plasma membrane receptor that causes a signalling cascade in the cytoplasm (e.g. kinase pathways). The genomic and non-genomic pathways are not mutually exclusive and influence each other (Hammes and Davis 2015, Wilkenfeld et al.

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2018), and both mechanisms of actions have implications for understanding normal physiology (Trochoutsou et al. 2015, Colciago et al. 2020, Bollen and Atherton 2021) and treatment of diseases (Alangari 2010, Hammes and Davis 2015, Mitre-Aguilar et al. 2015).

As the non-genomic pathway involves receptors in the plasma membranes, the steroid-membrane interactions can influence receptor binding. For example, the steroid binding sites on the NMDA/glutamate receptors (Kostakis et al. 2011; Borovska et al. 2012), GLIC (Cheng et al. 2018) and $GABA_A$ (Laverty et al. 2017) receptors are located in the transmembrane helices. Thus, receptor binding requires the steroid to bind to the membrane surface. Depending on the location of the binding site, the steroid might have to partition into the hydrophobic core of the membrane. These steroid-membrane interactions are not just part of the mechanism but have drug design implications. For example, the $IC_{50}$ values of pregnanolone sulphate analogues for NMDA receptors positively correlate with the lipophilicity of the steroid (Borovska et al. 2012). Similar contributions of lipophilicity to potency were reported for anaesthetic steroids acting on $GABA_A$ receptors (Chisari et al. 2009).

Besides the role of steroid-membrane interaction in receptor binding, it has been proposed that these interactions could be part of a more indirect mechanism of non-genomic pathways (Fig. 1). This proposed indirect mechanism is based on the effect of steroids on the biophysical properties of the phospholipid bilayer in plasma membranes. Cholesterol is known to alter the fluidity, lipid order and thickness of phospholipid bilayers. Given the similar structure and physico-chemical properties of cholesterol and steroids, it is not surprising that steroids can alter phospholipid bilayer properties. In 1961, (Willmer 1961) proposed that steroids interdigitate into membranes and alter membrane fluidity, which then leads to downstream effects either via a receptor or other mechanisms. In an early review of genomic vs non-genomic effects of steroids, McEwen suggested that steroid-induced changes in membrane properties are too non-specific for a non-genomic mechanism of action (McEwen 1991). This is based on the argument that steroids would act on all membranes exposed to them rather than just membranes with steroid receptors.

Since McEwen's review in 1991, we have accumulated a large body of evidence showing that the membrane is more than an inert, homogenous, hydrophobic slab. The spatial organisation of lipids is neither static nor homogenous, and this dynamic nature is associated with a wide range of physiological and pathophysiological functions, many of which involve lipid-receptor interactions (Phillips et al. 2009; Corradi et al. 2018). Furthermore, membrane thickness (Cybulski and de Mendoza 2011), lipid asymmetry (Doktorova et al. 2020) or other physico-chemical properties of the phospholipid bilayer component in cell membranes can alter the function of membrane proteins (Lundbæk 2008; Marsh 2008; Barrera et al. 2012). Consequently, small molecules that perturb membranes can directly affect the function of membrane-embedded proteins (Lundbæk 2008, Ingólfsson et al. 2014, Sarin 2015, Mayne et al. 2016, Sághy et al. 2018, Srivatsav et al. 2018, Cox and Gottlieb 2019). (Whiting et al. 2000) studied the effect of cholesterol and various steroids on the fluidity of phosphatidylcholine liposomes, synaptosomal plasma membranes and sarcoplasmic reticulum membranes, as well as the mobility of the integral membrane protein $Ca^{2+}$ ATPase in

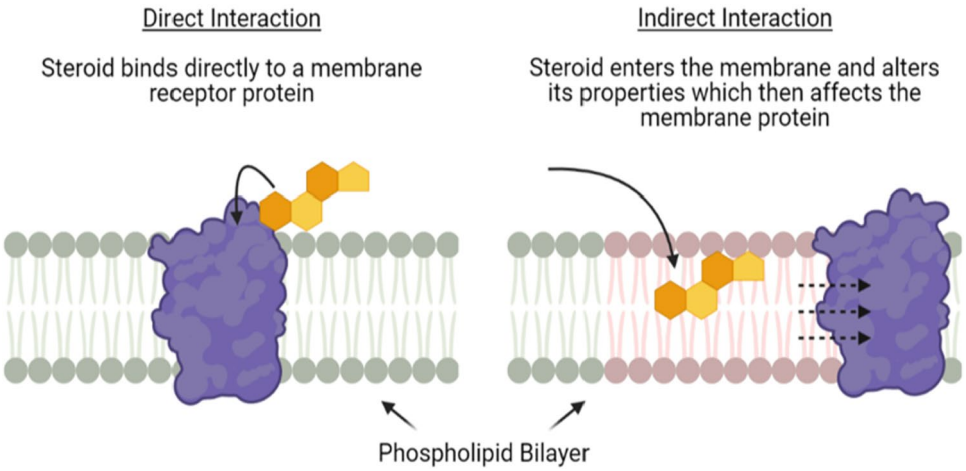


**Fig. 1** Direct and indirect mechanism of steroids associated with their non-genomic pathways. Direct interaction involves the steroid binding to a receptor in the plasma cell membrane. Depending on the location of the receptor binding site, this mechanism may or may not involve interactions with the phospholipid bilayer part of the membrane. The indirect mechanism involves the steroid binding to the phospholipid bilayer and, at sufficiently high concentration, altering the structure or fluidity of the membrane, which subsequently affects the receptor

sarcoplasmic reticulum membranes. Cholesterol decreased enzyme activity, whereas 17 β-estradiol, progesterone and testosterone increased activity. The authors related these effects back to changes in membrane fluidity and protein mobility induced by the steroid. Changes in membrane fluidity and their cellular effects can also have clinical and drug design implications. Clarke et al. (1990) showed that the steroid 17 β-estradiol and the breast cancer drug tamoxifen significantly decrease the fluidity of breast cancer cell membranes (MCF7, MDA-MB-436). Changes in membrane fluidity were accompanied by cytotoxicity independent of the oestrogen receptor that is the target of tamoxifen. The authors suggested that this steroid- and tamoxifen-induced change in membrane fluidity might have contributed to the cytotoxicity of high-dose endocrine breast cancer therapy. In a more recent study, Schultz et al. (2018) also discussed the potential for changes in membrane fluidity to have cellular effects. The authors showed that the steroid-like compound carbenoxolone, a gap junction inhibitor, corrects the abnormal growth of defective astrocytes in a mouse model of Batten disease (a fatal, neurological disease affecting children and adolescents). Based on previous work by (Tovar et al. 2009) on the effect of carbenoxolone on neuronal membrane properties, (Schultz et al. 2018) suggested that carbenoxolone alters the fluidity of membrane microdomains at the location of astrocyte communication. In addition, other cellular abnormalities related to membrane fluidity, such as decreased fluid endocytosis, were reversed by carbenoxolone as well as other steroids such as 7-ketocholesterol.

Despite these indications that steroid-membrane interactions might be part of their non-genomic pathways, little is known about the effect of steroids on the biophysical properties of the phospholipid bilayer. In his 1961 paper "steroids and cell surfaces", (Willmer 1961) summarised the findings of early work on the packing of steroids at the air–water and water-heptane interface. He noted "In interpreting these results, it must therefore be borne in mind that both the nature of the surface and of the molecules in question are of the utmost importance and that the problem of packing pure steroids into a heptane-water or air–water interface may be very different from packing them into an already strongly orientated structure such as a phospholipid monolayer or even into a mixed film of phospholipid and cholesterol together with other lipid materials". This clearly indicated that steroid-membrane interactions are much more complicated than simple hydrophobic effects that can be captured by lipophilicity measure (e.g. the water-octanol partition coefficient, logP). He also speculated that depending on the moiety on the C17, the steroid might show different interactions with lipids. He stated, "The actual position and orientation of these side-groups may well determine the stability of a packed system and therefore be very important in determining the physiological potency of a compound."

Since these early days of studying steroid-membrane interactions, techniques to characterise molecular interactions have come a long way. Yet like other small molecule-membrane interactions, studying the effect of steroids on membranes remains challenging (Li et al. 2018, Chan and Cheng 2019, Bagheri et al. 2020, Le-Deygen et al. 2020). Membranes are complex and dynamic supramolecular structures whose properties depend on lipid composition and environmental factors such as temperature, levels of hydration, pH and type and concentration of ions present in the surrounding aqueous environment. Small molecules interacting with the surface of the membrane or intercalating into the hydrophobic core add another layer of complexity. Furthermore, many effects of small molecule-induced changes in membrane properties are concentration-dependent. As will be outlined in this review, even small changes in the structure of the steroid can result in different effects.

The focus of this review is to outline what is currently known about the interactions of steroids with phospholipid bilayers and illustrate the complexity of these systems. We focus predominantly on cortisone and progesterone, as both compounds have been studied widely due to their importance in physiology and clinical applications. The effect of cholesterol on membranes has been studied extensively (Mannock et al. 2010, Róg and Vattulainen 2014, Yang et al. 2016, Subczynski et al. 2017). For most parts, we thus exclude cholesterol unless it is used as a reference for evaluating the effect of other steroids or to mimic the composition of mammalian cell membranes. This exclusion extends to other sterols that are components of cell membranes such as ergosterol, stigmasterol and hopanoids found in fungal, plant and bacterial cell membranes, respectively. Note that we use the terms phospholipid bilayer and membrane interchangeably and specifically indicated if we refer to cellular membranes.

Most biophysical experiments of steroid-membrane interactions are carried out at steroid concentrations ranging from 2 to 25 mol%, sometimes higher. While these concentrations help establish concentration-dependent effects on membrane properties, the mol% is much higher than what would be reached for physiological serum levels of steroid hormones such as progesterone or cortisone. Similarly, the $IC_{50}$ of steroids and derivatives thereof are usually in the nM range. However, as noted by (Alsop et al. 2016), the local concentration of steroids after injection can reach 20 mol%. Intramuscular injection of progesterone can cause rapid increase in serum levels of progesterone to concentrations 2–5 times the level in the luteal phase (the phase in the menstrual cycle where progesterone is the highest). Progesterone has a membrane/water partition coefficient of about 8000 (logP 3.9) meaning that the concentration of the steroid in the membrane will be even

higher. Thus, the steroid concentrations used in biophysical studies of steroid-membrane interactions can be relevant to the local and temporal steroid concentrations reached in pharmacological treatments.

### Structure and nomenclature of steroids

Steroids consist of a 17-carbon skeleton composed of four fused rings arranged in a characteristic manner. By convention, the rings are denoted A, B, C and D (Fig. 2). For unambiguous naming and identification of steroids, the carbons are numbered 1 to 17. Modifications to the substituents on the steroid core result in a vast diversity of compounds with different physico-chemical properties. Classes of steroids are often distinguished by their substituents at C17 or C3. Figure 2 shows the structure of the steroid core alongside cortisone and progesterone.

## Effect of steroids on membrane structure

The structure of phospholipid bilayers is generally described using a series of properties including area per lipid (APL), bilayer thickness and lipid order parameters.

APL is the average, cross-sectional area accessible by each lipid. Bilayer thickness is usually defined by the average distance between specified reference atoms in the two opposing leaflets in the bilayer (e.g. the phosphorous atoms). The lipid order is a measure of the intrinsic degree of orientational order in lipid tails. These properties are given by how densely the lipids in the bilayer are packed, and are thus closely linked to other properties such as permeability, flip-flop rates and the presence of microdomains. APL and bilayer thickness are often determined using X-ray or neutron scattering measurements (Pabst et al. 2010; Brun et al. 2013), while lipid order parameters are determined using electron paramagnetic (EPR) spectroscopy (Abboud et al. 2018) or nuclear magnetic resonance (NMR) spectroscopy (Seelig 1977, Seelig and Waespe-Sarcevic 1978, Gross et al. 1997, Leftin and Brown 2011). APL, bilayer thickness and lipid order parameters can also be obtained from molecular dynamics (MD) simulations (Leftin and Brown 2011, Bennett and Tieleman 2013, Enkavi et al. 2019).

Steroids are hydrophobic or amphipathic and can alter bilayer structure either through interactions at the water–lipid interface or through inserting into the hydrophobic core. Alsop et al. (2016) combined X-ray diffraction and MD simulations to investigate the concentration-dependent effect of cortisone on the structure of POPC bilayers that were exposed to 0 to 50 mol% cortisone. In the MD simulations, addition of cortisone caused a concentration-dependent decrease in bilayer thickness, as indicated by the density profiles (Fig. 3A). This bilayer thinning was accompanied by an increase in APL from 65 $Å^2$ in the absence of cortisone to 105 $Å^2$ when cortisone reaches 50% (Fig. 3B). Consistent with this bilayer thinning and expansion, the order parameter of the lipid tails decreased in simulations with cortisone, indicating that the membrane is more disordered in the presence of cortisone (Fig. 3C). These concentration-dependent changes of the bilayer observed in MD simulations were confirmed by X-ray diffraction experiments. The intensity of the Bragg peaks in the out-of-plane reflectivity data gradually decreases with increasing cortisone concentration (Fig. 3D). This reduced intensity indicates an increased disorder in the bilayer. The X-ray diffraction data also showed a significant decrease in the lamellar spacing ($d_z$) and membrane thickness ($d_{HH}$) (Fig. 3E/F).

In a follow-up study, Khondker et al. (Khondker et al. 2019) showed that these cortisone-induced changes in membrane structure are significantly reduced in the presence of cholesterol. The concentration-dependent decrease in Bragg peak intensity is much less pronounced in bilayers composed of POPC/cholesterol (7:3 mol/mol) compared to POPC-only bilayers (compare Fig. 3D and Fig. 4A). This difference indicates that the cortisone-induced increase in lipid disorder is less pronounced in cholesterol-containing bilayers. Similarly, the decrease in the lamellar spacing ($d_z$) and membrane thickness ($d_{HH}$) are reduced in POPC/cholesterol compared to POPC membranes. These findings

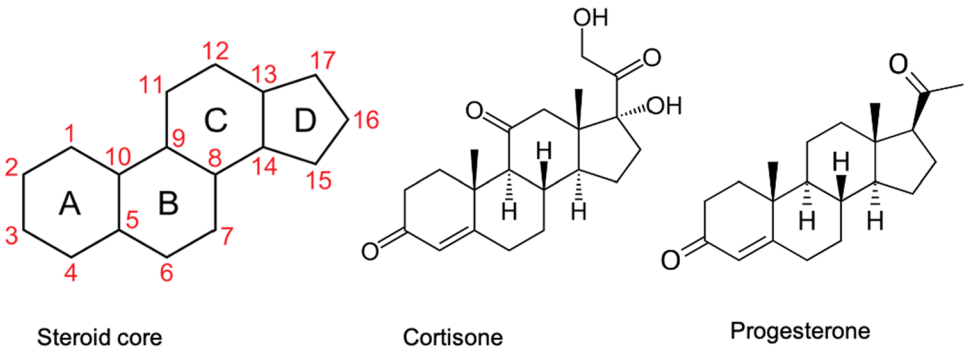


**Fig. 2** Structure and nomenclature of steroids. The rings in the steroid core are denoted by A, B, C and D and the carbons numbered 1 to 17

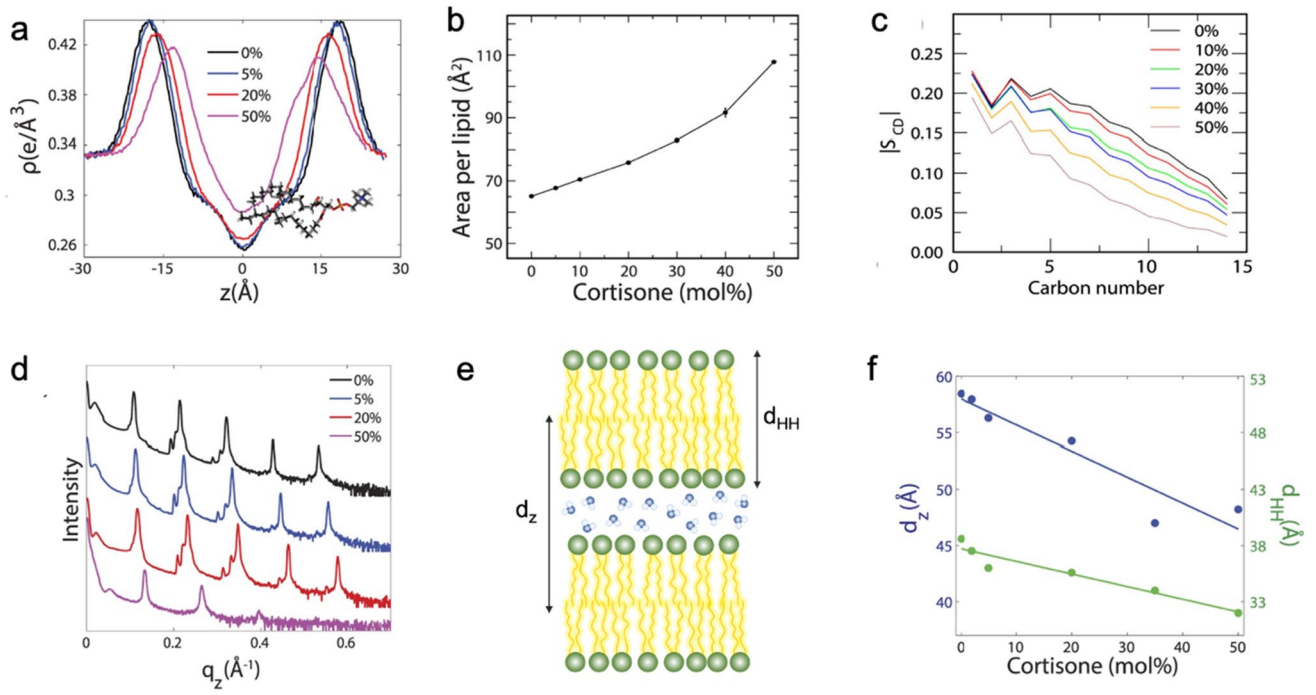


**Fig. 3** Effect of cortisone on the structure of POPC bilayers as determined by X-ray diffraction experiments and molecular dynamics simulations in a study by (Alsop et al. 2016). **A** Electron density profiles from MD simulations of POPC bilayers with increasing concentrations of cortisone. $z$(Å) refers to the bilayer normal where $z=0$ is the centre of the bilayer (where the POPC lipid tails meet). **B**, **C** Area per lipid (B) and lipid order parameter (C) as a function of cortisone concentrations obtained from MD simulations of a POPC bilayer in the presence of increasing cortisone concentrations. **D** X-ray diffraction pattern of POPC membranes containing increasing amounts of cortisone. **E** Lamellar spacing ($d_z$) and membrane thickness ($d_{HH}$) of stacked POPC membranes. **F** $d_z$ and $d_{HH}$ of POPC bilayers as a function of cortisone concentrations. A–D and F adapted from (Alsop et al. 2016)

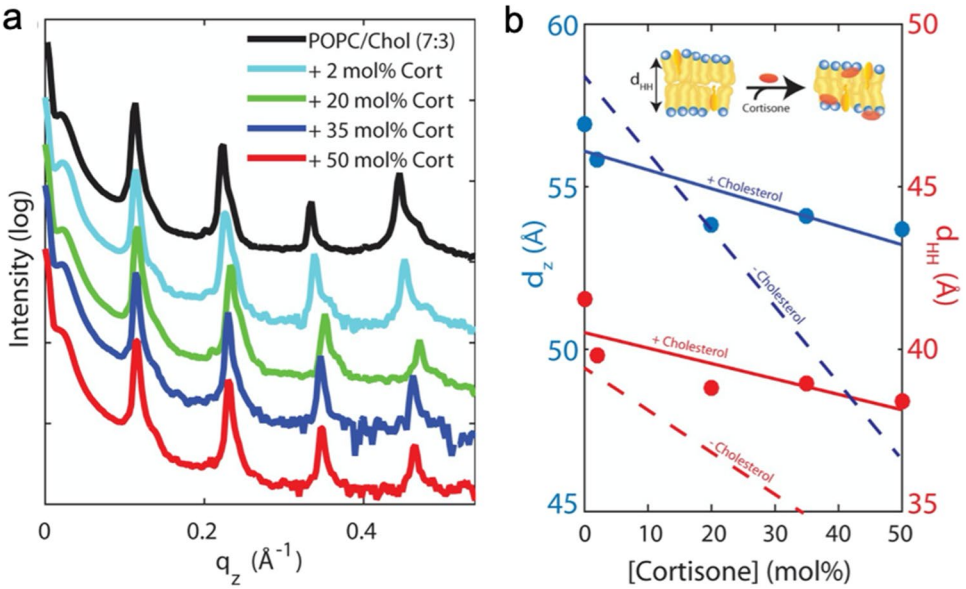


**Fig. 4** Effect of cortisone on the structure of POPC-cholesterol (7:3 mol%) bilayers as determined by X-ray diffraction experiments and molecular dynamics simulations by (Khondker et al. 2019). X-ray diffraction pattern (**A**) and $d_z$ and $d_{HH}$ (**B**) of POPC-chol membranes containing increasing amounts of cortisone. Figures adapted from (Khondker et al. 2019)

suggest that cholesterol suppresses cortisone-induced membrane thinning. To the best of our knowledge, at the time of writing this review, there were no studies reporting the effect of progesterone or other steroids on the structure of phospholipid bilayers.

## Location and dynamics of steroids in phospholipid bilayer

### Insertion depths and orientation

Rationalising the effect of steroids on the structural properties of membranes involves understanding how the steroids interact with the phospholipid bilayer, including information on the insertion depth and orientation (Fig. 5). Insertion depth is usually given as a distance between the steroid and the membrane, using either the membrane centre of mass (COM) or the lipid head groups as a reference. Orientation refers to the angle formed between vectors running along the steroid and the membrane normal. Both measures can be semi-quantitative to quantitative, depending on the resolution of the method. For example, the atomistic-level details in MD simulations mean the insertion depth for the head and tail of the steroid (usually defined by C3 and C17, respectively) can be differentiated. Similarly, the resolution of angle information is sufficiently high to distinguish between steroids that are parallel, perpendicular or at any other angle to the membrane surface. Such resolution can also be obtained from NMR experiments of membrane-bound compounds using isotropic bicelles or micelles (Matsumori and Murata 2010). To the best of our knowledge, this method has not been applied to steroid-membrane systems. Other wet-lab techniques used to determine insertion depth and orientation of small, membrane-bound molecules include Fourier transform infrared (FTIR) spectroscopy (Abboud et al. 2018) and X-ray diffraction (Pabst et al. 2010). In FTIR spectroscopy, the shift in specific bands is used to determine whether the steroid interacts predominantly with the lipid head groups at the interfacial region or inserts into the hydrophobic core. In X-ray diffraction, the electron densities computed from Bragg peaks are used to determine insertion depth. In both cases, the resolution is generally lower compared to MD simulations or NMR.

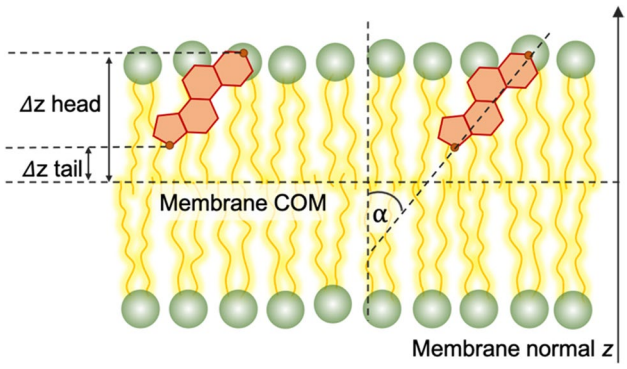


**Fig. 5** Insertion depth and orientation of a steroid molecule in the membrane. Insertion depth is usually defined by distances between the steroid and the membrane COM or the phosphate groups. If the resolution of the method allows it, insertion depth of the steroid head and tail can be defined separately. Orientation is defined by the angle formed between vectors running along the steroid and the membrane normal

Atkovska et al. (2018) used MD simulations to characterise the steroid-membrane interactions of 26 steroid compounds with a wide range of functional groups attached to the steroid core. For each steroid, a 500-ns simulation of a POPC bilayer exposed to 14 steroids of the same type was carried out. Insertion depth and orientation of the steroids were determined for the head and tail of the steroid, defined by the C3 and C17 atoms, respectively. An in-depth comparison of the 26 steroids showed that "the steroid core alone does not impose any consensus orientation in the membrane shared by all steroids". Depending on the functional groups at C3 and C17, the steroids adopt different orientations. While some steroids exhibit a clear preference for a specific orientation, many steroids sample a wide range of orientations. The authors reported that the main driver for orientation as well as insertion depth is the ability of functional groups to form hydrogen bonds (h-bonds) with the lipid head groups. On the steroid, hydroxyl groups can act as h-bond donors, thus forming stable interactions with the lipid head groups. A methyl or acetyl group or alkyl chains cannot form h-bonds. On the lipid, the phosphate oxygen and the carbonyl oxygen in the lipid head group can act as hydrogen bond acceptors, while the alkyl chain in the hydrophobic core lacks hydrogen bonding capacity. As a result of these distinct hydrogen bond capacities in the two membrane regions, only steroids with a relatively clear distinction between the hydrogen bonding capacities of the head and tail show a well-defined orientation. For example, the steroid pregnenolone has a hydroxyl on C3 (head) and an acetyl group on C17 (tail) meaning compared to the head, the tail has reduced h-bonding capacity (Fig. 6a). As a result, pregnenolone shows a strong preference for a vertical orientation with the tail inserted into the hydrophobic core and the head interacting with the head groups (Fig. 6b and 6c). In progesterone, the hydroxyl is replaced by a ketone group and the head and tail have similar h-bonding capacity (Fig. 6d). Compared to pregnenolone, progesterone loses its strong preference for a single orientation (Fig. 6e and 6f). Comparison of orientations for the 26 steroids shows the same effects for other structurally similar steroids (e.g. estrone and β-estradiol).

The insertion depth of the different steroids is another direct reflection of the h-bond capacities in functional groups of the steroid head and tail. For steroids with a strong preference for a vertical position, the insertion depths of the head and tail differ as one end of the steroid is inserted in the hydrophobic core while the other end of the steroid sits at

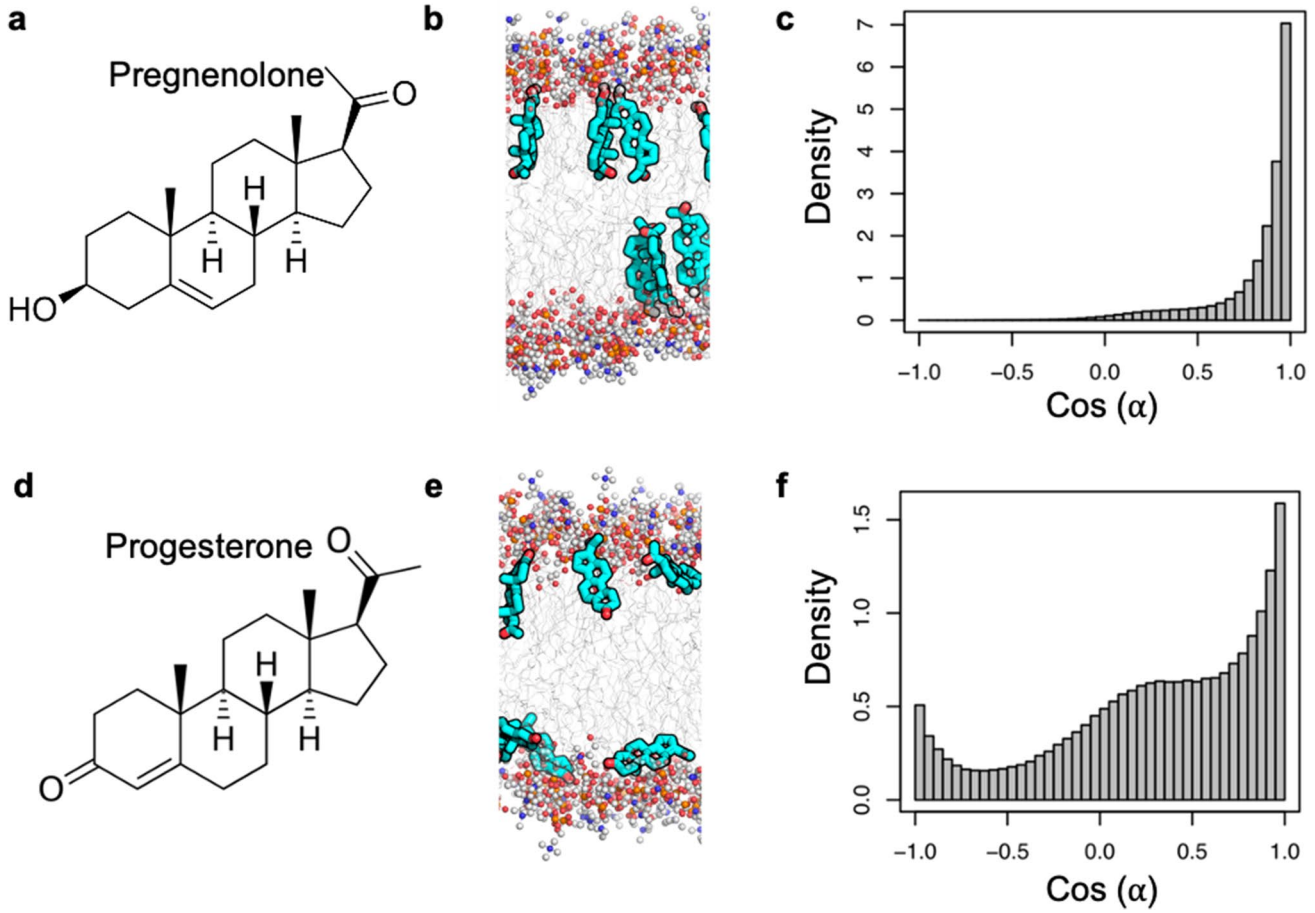


**Fig. 6** Orientation of pregnenolone and progesterone in POPC bilayers obtained from MD simulations by Atkovska et al. (2018). **a**, **d** Structure of pregnenolone and progesterone. **b**, **e** Snapshots from simulations. Head groups are shown as ball and stick representation, lipid tails as grey lines and pregnenolone and progesterone as sticks in cyan/red (hydrogen atoms not shown). **c**, **f** Density vs cos (α) showing the distribution of orientations of pregnenolone and progesterone in POPC bilayer. The tilt angle α is defined as the angle formed between vectors running along the steroid and the membrane normal (see Fig. 5). Figures adapted from Atkovska et al. (2018)

the level of the lipids head groups. For steroids with a more variable or horizontal orientation, the insertion depths of the head and tail are similar.

Abboud et al. (2015) used FTIR spectroscopy to investigate the interaction of progesterone derivatives (Fig. 7) with multilamellar DPPC liposomes. Changes in the absorbance bands of the C = O and $PO_{2-}$ in the lipid head group and the $CH_2$ in the lipid tail were used as indicators of steroid insertion depth. All compounds caused shifts in the C = O and $PO_{2-}$, which indicates interactions with the lipid head groups. In contrast, no significant changes in the $CH_2$ bands were observed for any of the steroids, which suggests that none of compounds enters the hydrophobic core of the membrane. All derivatives contain a ketone group at C3, and a C17 moiety that retains some polar character (Fig. 7). Based on Atkovska's analysis, all derivatives will likely exhibit variable orientations like progesterone such that both head and tail sit in the interfacial regions. None of the progesterone derivatives investigated by Abboud et al. (2015) contain an aliphatic tail that would promote insertion into the hydrophobic core, which is consistent with the absence of changes to the frequencies of the C-H stretching bonds. The findings from Abboud et al. (2015) are thus consistent with the results for progesterone and other steroids from Atkovska et al. (2018) discussed above.

The paper by Atkovska et al. (2018) and Abboud et al. (2015) demonstrates how even small changes in the functional group attached to the steroid core can affect the insertion depths and orientation of the steroid in the membrane. We however have little information on how these variations in insertion depths and orientations relate to the ability of the steroid to alter membrane properties. Such an understanding will require combining structural information on steroid-membrane interactions with data on membrane

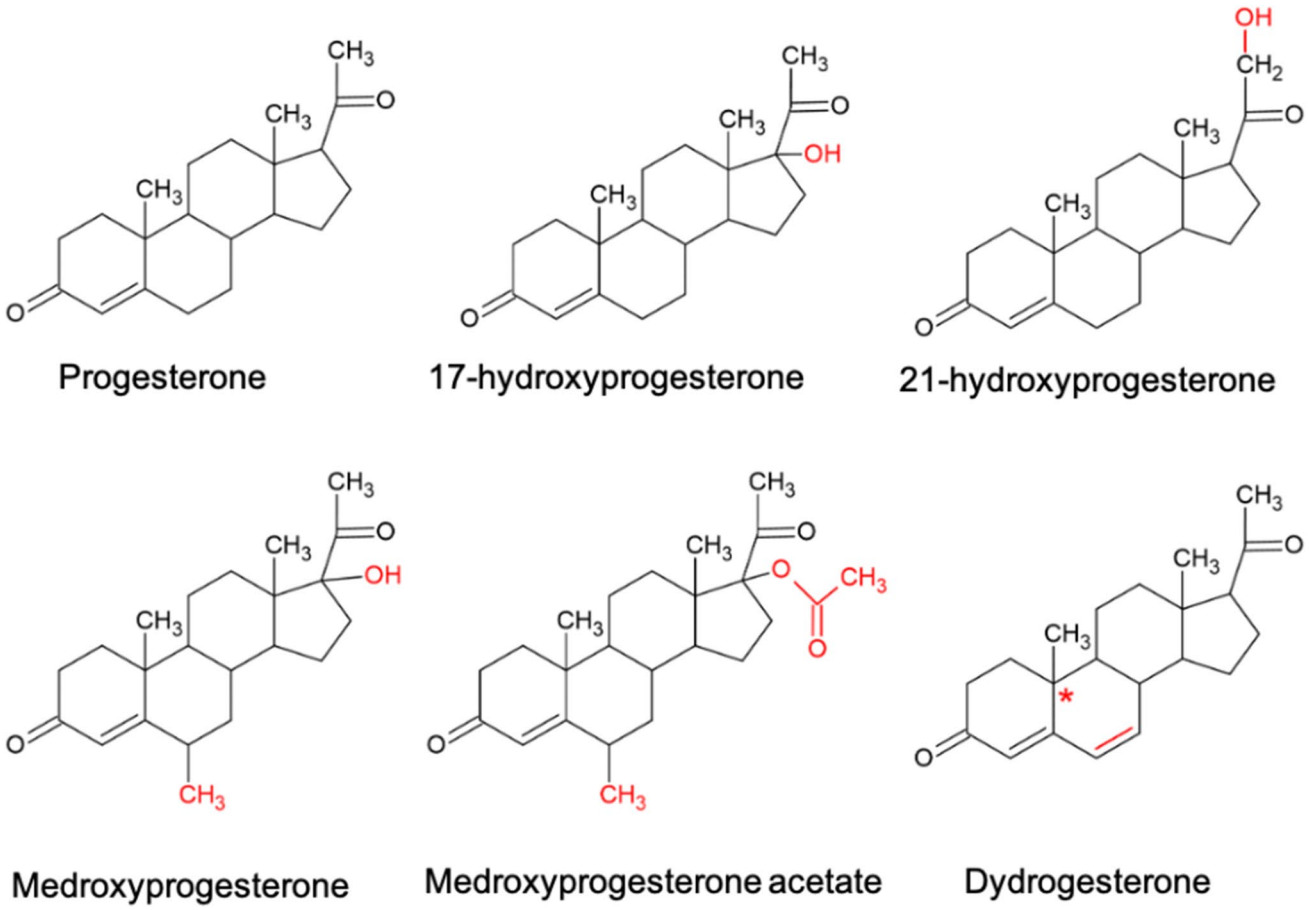


**Fig. 7** Structure of progesterone derivatives studied by Abboud et al. (2015)

properties. For example, a recent study by Hossain et al. (2021) combined MD simulation with tethered bilayer lipid membranes and electrical impedance spectroscopy to compare the effect of phenolic compounds on the permeability of POPC bilayers. The study showed that compounds with very similar structures can have very different abilities to alter ion membrane permeability. The atomistic insights from this and a previous study (Deplazes et al. 2020) allowed for rationalising the membrane-altering effects of the phenolic compounds.

## Steroid flip-flop

Lipid flip-flop is defined as the transverse movement of a molecule between the monolayers of a lipid bilayer and is related to cells maintaining lipid asymmetry in various membranes (van Meer 2011). The kinetics of the protein-free flip-flop in phospholipids and fatty acids has been studied for decades, yet the debate about the flip-flop rate ($k_{ff}$) continues. Estimates range from $ms^{-1}$ to $s^{-1}$ or even $h^{-1}$ (van Meer 2011, Allhusen and Conboy 2017). Allhusen and Conboy have recently published an excellent review on this topic (Allhusen and Conboy 2017).

Cholesterol lacks an ionic head and consequently it is usually reported to have a faster $k_{ff}$ than phospholipids. In polyunsaturated bilayers, the rate of cholesterol is in the sub-microsecond range and increases to seconds in saturated bilayers or membranes with high cholesterol content (Bennett et al. 2009). Based on that, we would expect that steroids with a cholesterol-like structure (i.e. a hydroxyl at C3 and a long acyl chain at C17) would show $k_{ff}$ values like cholesterol. The data from (Atkovska et al. 2018) supports this notion. Within uncertainty, β-sitosterol and dehydro-ergosterol show the same $k_{ff}$ as cholesterol, which is in the range of $10^{-4}$ to $10^{-6}$ $s^{-1}$. As discussed in the "Insertion depths and orientation" section, comparable h-bonding capacities at C3 and C17 position cause the steroids to exhibit a wide range of insertion depth and orientations that differ from the ones observed for cholesterol-like steroids. Not surprisingly, this affects $k_{ff}$. The values predicted for the 26 steroids span nine orders of magnitude. Further analysis showed that *k*ff anti-correlates with the number of hydroxyl groups in the steroid. There is an additional but much less pronounced

effect for the number of carbonyl groups. This wide range of *k*ff corresponds to the wide range of water/cyclohexane partition coefficients calculated in by Atkovska et al. in the same study (Atkovska et al. 2018). This correlation with partition coefficients is not surprising given that steroid flip-flop requires the molecule to overcome the energy barrier associated with the transition through the hydrophobic core.

Atkovska et al. (2018) also calculated $k_{exit}$, the rate for the steroid to exit the membrane. Unlike $k_{ff}$, the structurally diverse steroids showed a narrow range of *k*exit with most values in the order of 104 $s^{-1}$. The exception are cholesterol-like steroids where the acyl chain causes $k_{exit}$ to be 7–9 order of magnitudes lower than the other steroids. $k_{exit}$ requires the steroid to overcome the energy barrier of the water–lipid interface, which is poorly captured by water/cyclohexane system. Thus, not surprisingly, $k_{exit}$ does not correlate with water/cyclohexane partition coefficients but with water/POPC partition coefficients. To the best of our knowledge, there has been no study on the effect of steroids on the flip-flop rate of phospholipids or cholesterol in membranes.

### Crystallite formation of steroids

Another effect that is a direct consequence of the insertion depth and orientation of steroids in the membrane is the formation of crystallites. It is also an example of how steroid-induced change in membrane properties can depend on steroid concentration. The studies by Alsop et al. (2016) and Khondker et al. (2019) described in the "Effect of steroids on membrane structure" section were motivated by understanding local accumulation of the cortisone in the membrane and subsequent crystallisation. These effects are related to steroid flares, a major complication after intra-articular injection of cortisone.

Alsop et al. (2016) reported the experimental solubility limit of cortisone in POPC bilayers to be 20 mol%. At higher concentrations, the X-ray diffraction data indicated the formation of cortisone crystallites. In the subsequent study, Khondker et al. (2019) showed that the presence of cholesterol increases cortisone solubility and no crystallites were observed, even at 50 mol% cortisone. The combined data from MD simulations and X-ray diffraction provided insight into potential mechanism of crystallite formation. In the absence of cholesterol and at concentrations below its solubility limit, cortisone sits at water–lipid interface. This preferred orientation agrees with data from (Atkovska et al. 2018). At higher concentration, the steroid starts to accumulate in the hydrophobic centre of the bilayer. Combined with the steroid-induced membrane thinning, this accumulation enables cortisone molecules in opposing leaflets to interact. The authors proposed that these interactions can act as nucleation sites for crystallite formation. In the presence of cholesterol, membrane thinning is significantly reduced, and cortisone does not insert into the hydrophobic bilayer. Consequently, trans-bilayer interactions and the formation of cortisone crystallites are inhibited. Given the ubiquitous nature of cholesterol in mammalian membranes, these findings by (Khondker et al. 2019) also demonstrate the importance of considering cholesterol when relating the effects seen in cell membranes to findings obtained from measurements in phospholipid bilayers.

## Effect of steroids on fluidity of phospholipid bilayers

Membrane fluidity is a property that describes the viscosity of the membrane, which is given by how freely the membrane components can move. In cell membranes, this includes both the movement of proteins and lipids. In phospholipid bilayers, fluidity usually only refers to the movement of the lipids, which includes both the lateral diffusion of the lipid trough the bilayer and the mobility (flexibility) of the lipid tails. Like many other membrane properties, fluidity relates to lipid packing and thus depends on lipid composition, hydration levels, temperature, pH and the concentration and types of ions in the surrounding bulk solution.

Phospholipid bilayers and cell membranes undergo a large change in fluidity during phase change. In the context of biological membranes, the most relevant phase change is the transition from the gel phase to the liquid crystalline (fluid) phase (Fig. 8). If the temperature, $T$, is below the phase transition (melting) temperature, $T_m$, the bilayer is in the gel phase. In this phase, the lipids have relatively low lateral mobility, and the tails are more ordered, which results in tighter packing of the lipids. If $T$ is above $T_m$, the bilayer is in the liquid crystalline phase where lipids are more mobile resulting in a less ordered bilayer. As a result of these changes in lipid packing, the bilayer is more fluid in the liquid crystalline phase than in the gel phase. Depending on the conditions and type of lipid, the bilayer will move through an intermediate phase called the ripple phase at the pre-transition temperature ($T_p$). The gel to liquid crystalline phase transition can also be induced by changes in hydration, but temperature gradients are generally used when studying the effect of small molecules on fluidity. Changes in the fluidity of phospholipid bilayers can be determined from measurements of phase transition temperatures or lipid order. The most commonly used technique to determine phase transition temperatures is differential scanning calorimetry (DSC) (Taylor and Morris 1995). Lipid order can be estimated using fluorescence anisotropy (Sklar 1984; Best et al. 1987) or electron paramagnetic resonance spectroscopy (Zimmer 1984; Windle 1988).

While the effect of cholesterol on the fluidity of cell membranes and phospholipid bilayers has been studied

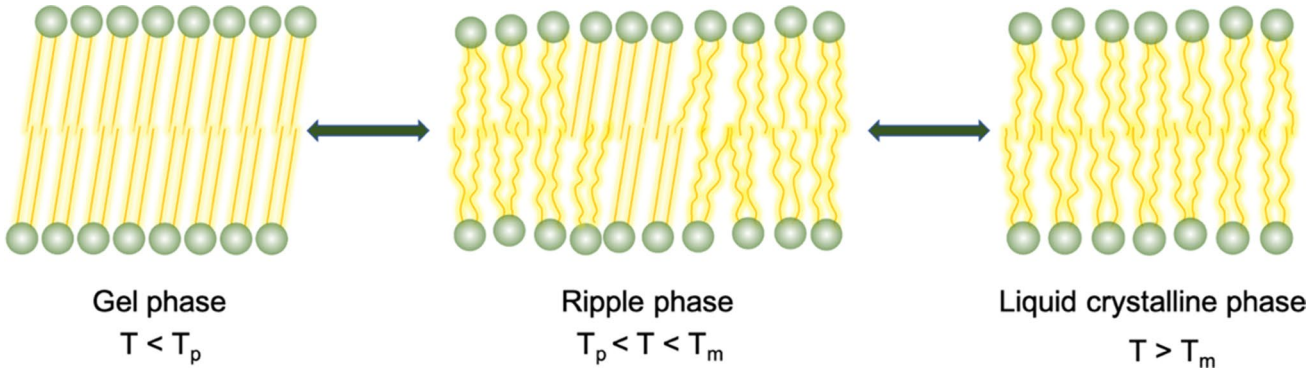


**Fig. 8** Temperature-dependent phase changes in phospholipid bilayers

extensively (Subczynski et al. 2017), a lot less is known about the effect of steroids. As outlined below, there are considerable differences and in some instances contradictions between findings. When comparing results from different studies, it is important to remember that small changes to the system or experimental conditions can affect the interactions between the lipids and thus alter lipid packing and fluidity. It is usually not possible to directly compare results between studies using cell membranes vs phospholipid bilayers. When comparing studies using lipid vesicles or supported lipid bilayers, the type of lipids used needs to be considered as lipid tail saturation or the charge in the head group can affect fluidity.

Table 1 summarises the findings from several studies investigating the effect of progesterone on membrane fluidity. Korkmaz and Severcan (2005) used multilamellar vesicles (MLVs) composed of the zwitterionic, saturated phospholipid DPPC and exposed them to progesterone at 3, 6, 12, 18 and 24 mol%. The DSC thermograms showed that $T_m$ for DPPC in the presence of progesterone is lower than for DPPC only. For 3% and 6% progesterone, $T_m$ drops from 41 to ~ 40.5° C and 39.7° C, respectively. At higher progesterone concentrations, the $T_m$ no longer changes. The thermograms for 6 and 12 mol% progesterone indicate the existence of phase separation. Combined with data form FTIR spectroscopy and turbidity, the authors suggest that progesterone increases fluidity of DPPC at lower concentrations (3 and 6%) but has the oppositive effect on fluidity at higher concentration.

Abboud et al. (2015) also used DSC to study the effect of progesterone on DPPC at DPPC:progesterone molar ratios of 100:0, 100:1, 100:2.5, 100:5, 100:10 and 100:25. This is equivalent to a range of 0 to 20 mol% and thus comparable to the concentrations used by (Korkmaz and Severcan 2005). The results from (Abboud et al. 2015) indicated that $T_m$ decreases in a non-linear but concentration-dependent manner from 41 in the absence of progesterone to 40.3° C at 100:5 (4.7 mol%), 38.9° C at 100:10 (9 mol%) and 30.9° C at 100:25 (20 mol%). The small drop in $T_m$ at low progesterone concentrations agrees with results from Korkmaz and Severcan ( 2005), but for higher concentrations, the results differ. While (Korkmaz and Severcan 2005) did not explicitly state the $T_m$ for the highest concentrations (24 mol%), based on the peak in the thermogram, $T_m$ appears to be around 39°

**Table 1** Findings from studies investigating the effect of progesterone on membrane fluidity

| Study | Method | Lipids | Progesterone concentrations* | Findings |
|---|---|---|---|---|
| (Korkmaz and Severcan 2005) | DSC, combined with FTIR and turbidity | DPPC vesicles | 3, 6, 12, 18 and 24 mol% | Increase in fluidity for lower concentrations (3% and 6%) then little to no change for higher concentrations (12, 18 and 24%) |
| (Abboud et al. 2015) | | DPPC vesicles | 1, 2.4, 4.7, 9 and 20 mol% | Increased in fluidity, concentration-dependent for all mol% |
| (Whiting et al. 2000) | Fluorescence anisotropy | Egg PC vesicles | 9, 16, 24, 28 and 32 mol% | Decrease in fluidity, concentration-dependent for all mol% |
| (Liang et al. 2001) | Fluorescence anisotropy | Egg PC vesicles | 0.2% | No effect on fluidity |

*for studies where progesterone concentration is reported in molar ratios or mass ratios, numbers were converted to mol% to facilitate comparison between studies

C. This is much higher than the 30.9° C for 100:25 (20 mol%) reported by (Abboud et al. 2015). Both studies used MLVs composed of DPPC, and vesicles were rehydrated with buffer at pH 7.4. The only apparent difference is the type of buffer used, Tris–HCl (0.1 M) in (Abboud et al. 2015) and phosphate buffer (0.01 M) in (Korkmaz and Severcan 2005). Given the $T_m$ agrees for lower progesterone concentrations, the difference in buffer is unlikely to be the source of the discrepancy in $T_m$ at higher concentrations.

In apparent contradiction to both (Korkmaz and Severcan 2005) and (Abboud et al. 2015), a study by (Whiting et al. 2000) reported that progesterone decreases fluidity in both lipid vesicles and cell membranes. The authors used fluorescence anisotropy to study the effect of progesterone, testosterone and 17 β-estradiol on phospholipid vesicles, synaptosomal plasma membranes and sarcoplasmic reticulum membranes. Fluidity was inferred based on changes in lipid order from the fluorescence anisotropy data. The results indicated that progesterone decreased fluidity, testosterone had no influence on lipid fluidity, whereas progesterone's aromatised metabolite, 17 β-estradiol, increased fluidity. All effects were concentration-dependent, with the mol% ranging from 9 to 32 mol%, which at least partially overlap with the concentrations used by the studies of (Korkmaz and Severcan 2005) and (Abboud et al. 2015) (Table 1). The progesterone-induced decrease in fluidity reported by (Whiting et al. 2000) thus appears to contradict the increased fluidity reported by (Korkmaz and Severcan 2005) and Abboud et al. (Abboud et al. 2015). The source of the disagreement might be related to the lipids used. Korkmaz and Severcan (2005) and Abboud et al. (2015) used vesicles composed of pure DPPC, which is a saturated lipid with 16 carbons in the tails (i.e. palmitic 16:0). In contrast, (Whiting et al. 2000) used egg phosphatidylcholine (PC), which is a mixture of both saturated and unsaturated PC lipids. Egg PC typically contains 33% saturated 16:0 (palmitic) PC, 13% saturated 18:0 (stearic) PC, 31% of the single saturated 18:1(oleic) PC and 15% doubly saturated 18:2 (linoleic) PC. Saturation of the tail has a strong effect on membrane fluidity. In addition to the disagreement of these three studies, (Liang et al. 2001) reported that progesterone, testosterone and 17 β-estradiol do not affect the fluidity of egg PC liposomes. Like (Whiting et al. 2000), Liang et al. (Liang et al. 2001) inferred fluidity from lipid order based on data from fluorescence anisotropy experiments. However, in Liang et al. (Liang et al. 2001), the concentration of progesterone used was 0.2 mol%, which is much lower than the concentration in the other studies (Table 1). Finally, progesterone-induced changes in membrane fluidity have also been demonstrated in cellular membranes. A study by Tsuda et al. (Tsuda et al. 2002) reported that progesterone increased membrane fluidity in erythrocytes membranes in concentration-dependent manner.

Studies also showed that changes in fluidity appear to be strongly affected by the substituents on the steroid core. In addition to progesterone, (Abboud et al. 2015) measured changes in $T_m$ for the steroids 17-hydroxyprogesterone (17-OHPG) and 21-hydroxyprogesterone (21-OHPG) (Fig. 7). The large drop in $T_m$ that was observed for the highest concentration of progesterone (20 mol%) was not present for 21-OHPG. 17-OHPG showed an increase in $T_m$, i.e. the opposite effect of progesterone. Similarly, (Liang et al. 2001) reported that 17 β-estradiol did not alter membrane fluidity, yet E3ol, which differs from 17 β-estradiol only by the addition of a hydroxyl at C17, significantly increased fluidity.

## Structure–activity relationship of steroids and their membrane-altering effects

There are not many structure–activity relationship studies on the membrane-altering properties of steroids. The available studies mostly focus on how cholesterol and structurally similar molecules affect fluidity, and the formation and stabilisation of ordered lipid domains (rafts) (Wenz and Barrantes 2003, Wang et al. 2004, Wenz 2012). To the best of our knowledge, there are no systematic SAR studies on the effect of steroids on other bilayer properties such as APL, membrane thickness, lipid order or the ion permeability of membranes.

Wenz and Barrantes (2003) compared the propensity of nine naturally occurring and synthetic steroids to promote or disrupt lipid domains in model membranes composed of saturated or unsaturated lipids. The steroids varied in their functional groups at C3, C11 and C17 and the number of double bonds in the steroid core (Fig. 9a). Cholesterol and 25-hydroxycholesterol were used as reference compounds. The aim of the study was to relate the structure and physico-chemical properties of the steroids to their activities on lipid domain formation. To determine the membrane activity, the authors used an approach developed by London and co-workers (London et al. 2000) based on the quenching of the fluorescent probe DPH by a nitroxide spin-labelled phosphatidylcholine (12-SLPC). The rationale behind the experiments is to use the difference in fluorescence quenching between a membrane where lipids are mostly randomly distributed and a membrane where lipids are segregate into domains (Fig. 9b). Multilamellar vesicles (MLVs) were composed of either the unsaturated DOPC and the fluorescent quenching lipid 12-SLPC or the saturated DPPC and 12-SLPC. In both cases, DPH was added. 12-SLPC has a phase behaviour like that of an unsaturated lipid such as DOPC (the $T_m$ of both lipids is < 0 °C). When mixing DOPC and 12-SLPC, the two components are randomly distributed and, on average, the majority of DPH molecules have a quencher nearby. This system shows high quenching and

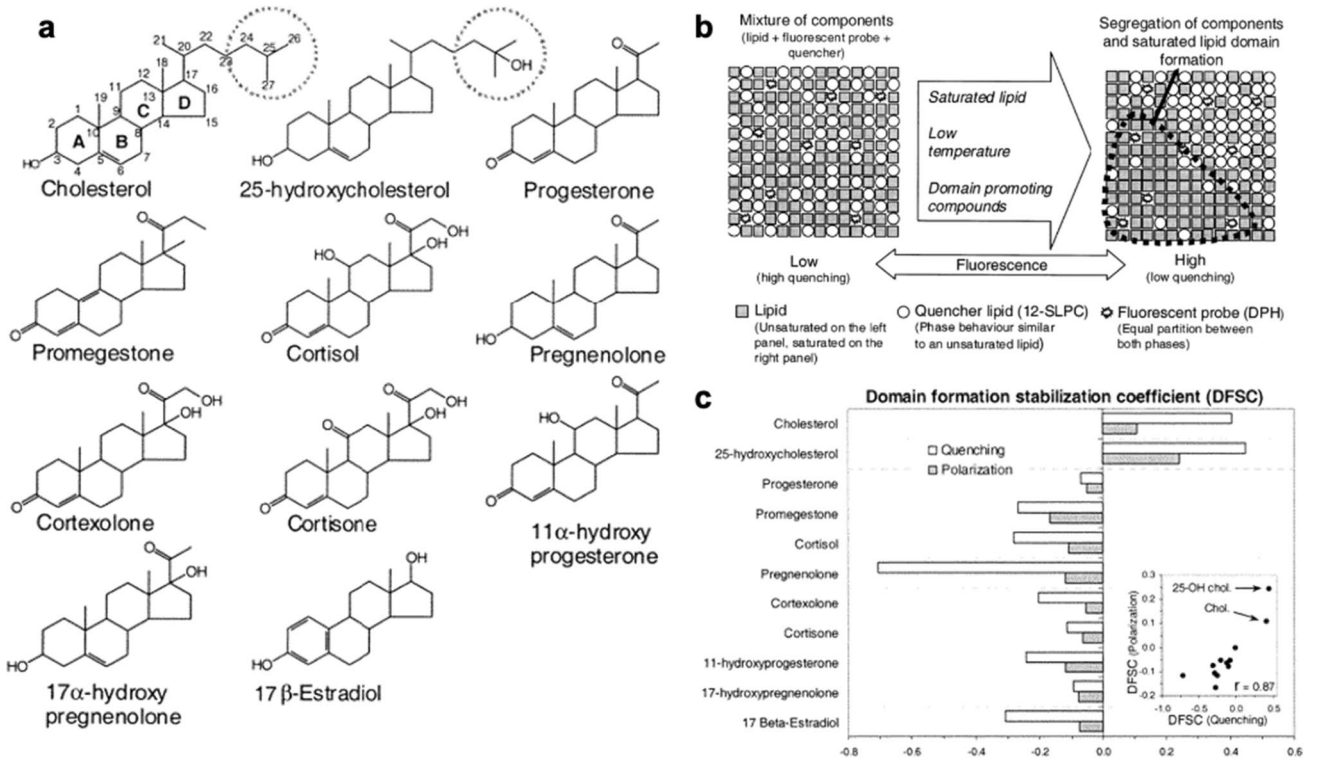


**Fig. 9** Structure–activity relationship studies on the propensity of steroids to promote or disrupt lipid domains by (Wenz and Barrantes 2003). **a** Structure of sterols and steroids investigated. The isooctyl side chains in the lipid domain-promoting steroids are circled. **b** Diagram illustrating the principle of quenching in membranes composed of saturated and unsaturated phospholipid, the fluorescent quenching lipid 12-SLPC and the fluorescent probe DPH. **c** Domain formation stabilisation coefficient (DSCF) for 11 sterols/steroids calculated based on fluorescence quenching and polarisation measurements. Positive and negative values indicate domain-promoting and domain-disrupting activity, respectively. Inset: scatterplot of DSCF from polarisation vs DSCF from quenching. Reprinted with permission from Wenz and Barrantes, Biochemistry, 2003. Copyright 2003 American Chemical Society

low fluorescence. In contrast, if 12-SLPC is mixed with the saturated DPPC, their differences in phase behaviour mean the two components will segregate. The unequal distribution of 12-SLPC and DPPC means the likelihood of DPH to have a quencher nearby differs between the phases and, on average, is lower in a segregate system compared to a homogenous system. Consequently, the system with lipid domains shows lower quenching and higher fluorescence than the system with evenly mixed lipids (Fig. 9b). In addition to this fluorescence quenching by 12-SLPC, the authors measured the fluorescence polarisation of DPH.

To this reference system, the sterols/steroids were added, and the fluorescence quenching and polarisation measurements were used to determine the effect of the 11 steroids on lipid domain formation. The underlying premise is that the steroids can affect lipid segregation by influencing the packing of the saturated lipid and thus promote or disrupt domain formation. Note that in this study, Wenz and Barrantes used the term "disruption" to refer to the de-stabilisation of lipid domain. This is different to disruption referred to in studies of the membrane permeabilising activity of detergents or antimicrobial peptides.

The data from the quenching and polarisation experiments were used to determine a domain formation stabilisation coefficient (DFSC), which reflects the domain-promoting and domain-disrupting activity of the compound (Fig. 9c). The relative DFSC values obtained from the two independent measurements agreed well with each other (Fig. 9c inset). The combined data showed that cholesterol and 25-hydroxycholesterol are domain-promoting (positive DFSC) while the other nine steroids were domain-disrupting (negative DFSC). The authors suggested that the following characteristics make steroids domain-promoting: "(i) the presence of an isooctyl side chain at C17; (ii) the absence of carbons attached to C23 (i.e., C24-C27) in any of the other (domain-disrupting) steroids; (iii) the presence of a small polar group at position C3; and (iv) the absence of polar groups in the fused rings, with the exception of substitutions at position C3 in the A ring".

The finding that an isooctyl chain at C17 combined with a small polar group is domain-promoting was confirmed in a similar study by (Wang et al. 2004). The authors used

the same fluorescence quenching experiments to study the effect of eight steroids on lipid domains in vesicles composed of DPPC/12SLPC or sphingomyelin (SM)/12SLPC. All eight steroids contained the same isooctyl chain as cholesterol. The variations between the steroids were the substituents on C3, C5 or C7 and the position of the double bond in the steroid core (Fig. 10). The data from the fluorescence quenching experiments and detergent insolubility experiments showed that lathosterol has an increased domain-promoting ability compared to cholesterol. 7-Ketocholesterol shows a domain-promoting ability similar to cholesterol. For all other steroids tested, the domain-ordering ability is weakened but not fully abolished. The results also indicated that the position of the double bond in the steroid core can alter domain formation or stabilisation. A C7-C8 double bond (e.g. in lathosterol) promotes domain formation compared to the C5-C6 double bond in cholesterol. In contrast, a C4-C5 double bond (e.g. in allocholesterol) reduced domain formation. The authors noted this effect of double bond position was "somewhat surprising, because any double bonds in the rings should help to make sterols more planar, a feature believed to be important for imparting the properties critical for allowing sterols to pack tightly with saturated lipids". The results however suggested that the position of the double bond matters independent of the planar nature of the steroid. The authors also noted that for compounds with two differences in substituents to cholesterol, the domain-promoting abilities were nearly additive.

The findings by (Wenz and Barrantes 2003) and (Wang et al. 2004) can also be rationalised with the findings from (Atkovska et al. 2018) described in the "Insertion depths and orientation" section. The domain-promoting compounds cholesterol and 25-hydroycholesterol have an alkyl tail at C17 and an OH at C3, and thus show distinct hydrogen bonding capacity in the head and tail. Atkovska et al. (2018) reported that these compounds preferentially orient themselves with the tail inserted into the hydrophobic core and parallel to the phospholipid tails (i.e. perpendicular to the membrane surface). In contrast, the other nine steroids that are domain-disruptor have shorter tails on C17 (1 to 3 carbons) with hydroxyl and ketone groups at C3, resulting similar hydrogen bonding capacities in the head and tail. According to analysis by (Atkovska et al. 2018), these compounds are more likely to sit at the water–lipid interface and have variable orientations. In terms of altering lipid packing, compounds that insert into membrane core with an orientation parallel to the lipid tails are less likely to alter lipid packing (i.e. they promote ordering of lipid tails). This is consisting with cholesterol and 25-hydroycholesterol being domain-promoting compounds. In contrast, steroids with varying orientations that sit at the lipid interface are more likely to interfere with lipid packing, consistent with the other nine steroids being domain-disrupting.

Wenz later combined data from their 2003 study (Wenz and Barrantes 2003) with that of other studies, to perform principal coordinate analysis on the structural features of 83 sterols/steroids and their membrane activity (Wenz 2012). Independent variables were defined to describe the

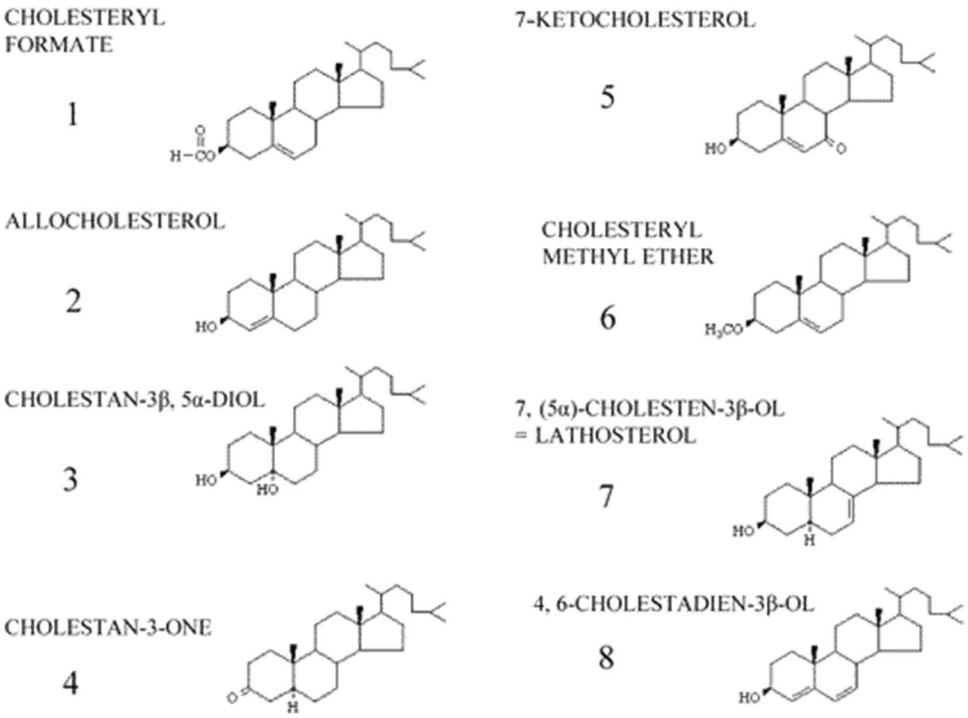


**Fig. 10** Structure of steroids and sterols studied by Wang et al. (Wang et al. 2004) for their ability to form and stabilise ordered lipid domains. Reprinted with permission from Wang et al., Biochemistry, 2004. Copyright 2004 American Chemical Society

molecular structure of the steroid. Specifically, 68 independent, binary variables were defined for the presence/absence of substituents in the steroid core (e.g. hydroxyl, methyl, keto, double bonds, etc. at a specific position in the ring system). The dependent variable was the activity of the steroid and defined as the "sum all the measured effects of sterols on membrane physical properties". Compounds were assigned as having either "rigidifying, molecular ordering, condensing effect, and/or raft promoting/stabilizing ability on membranes" or having "fluidifying, disordering, and/or raft disrupting/ destabilizing effect on membranes". The findings were very similar to Wenz's previous study and concluded that "the most important structural determinants influencing the physical properties of sterol-containing mixtures were the presence of an 8–10 carbon C17 isoalkyl side-chain, followed by a hydroxyl group at C3 and a C5–C6 double bond".

This study by (Wenz 2012) forms a good first approximation of structure–activity relationship for steroid-membrane interactions. Principal coordinate analysis is useful to explore similarities and differences of categorical data, but binary variables might not be sufficient to capture the complex effects of steroid-membrane interactions. Effects are often concentration-dependent and vary from gradual changes with increasing steroid concentration to sudden changes in properties once a critical concentration is reached. The analysis also neglects lipid composition. While difficult to capture, lipid composition is important because the same steroid can have different membrane-altering activities depending on the type of lipid (e.g. saturated or unsaturated tail) and bilayer composition (single lipid vs mixed lipids). Thus, defining a single variable that is the "sum all the measured effects of sterols on membrane physical properties" is likely not fine-grained enough to capture differences between effects caused by lipid composition. Given the importance of these differences to establishing reliable structure–activity relationship, a more nuanced approach is likely needed. In fact, Wenz (Wenz and Barrantes 2003) noted in his study that "findings and conclusions are averaged tendencies in the complex structure–activity relationship of sterols in membranes and may not agree with some reported cases".

## Summary and conclusions

In this review, we provide an overview of what is currently known about the interactions of steroids with phospholipid bilayers. The review outlined the findings of studies that investigated how the functional groups or substituent in the steroid affect their orientation and insertion depths in phospholipid bilayers. In addition, the review outlined what is known about the effect of steroids on membrane properties such APL, membrane thickness, lipid order and fluidity, and the ability of steroids to alter lipid domains.

It is clear from the combined findings of these studies that the hydrophobicity and planarity of the steroid core does not provide a consensus for steroid-membrane interactions. Even small differences in the substituents can alter the orientation and insertion depth of a steroid. To a large extent, these two characteristics determine the interaction of the steroid with the membrane. Specifically, the orientation and insertion depth relate to the interactions of the steroid with the lipid head groups and interfacial water at the membrane surface, and the interactions with the lipid tails in the membrane core. These interactions determine the capacity of the steroid to alter lipid packing.

Most membrane properties discussed in this review are related to lipid packing. Thus, if the hydrophobicity of the steroid is a poor predictor of how a steroid can alter lipid packing, it follows that water-hexane or water-octanol partition coefficients are not a reliable parameter to predict steroid-membrane interactions and by extension to predict the effect of steroids on membrane properties.

The fact that steroid-membrane interactions are more complex than the hydrophobicity of the steroid reflects our growing understanding that the water–lipid interface is not just a simple boundary between bulk water and the hydrophobic membrane core (Disalvo 2015). The interfacial water and the head groups and glycerol backbone of the lipids form a separate physico-chemical environment where the hydrogen bonding capacity of a steroid appears strongly influenced by steroid-lipid interactions.

The findings from studies discussed in this review also highlight the complexity of steroid-membrane interactions. Steroid-induced changes in membrane properties can depend on the concentration of the steroid and lipid composition. This complexity means that currently there is no reliable structure–activity relationship to describe the effect of steroids on membrane properties. It is unlikely that a single structure–activity relationship will be able to capture the effect of steroids on the different membrane properties. On the other hand, it might be possible that a set of descriptors can describe the orientation and insertion depth of the steroid. Based on this, it might be possible to predict the effect of the steroid on lipid packing and by extension on perpetrates such as APL, membrane thickness and fluidity.

Another challenge of steroid-membrane interactions lies in connecting the findings from phospholipid bilayers to cell membranes. Characterising the interaction of steroids with bilayers composed of single lipids is an important model system that will serve as reference system for studies on more complex membranes. By systematically varying lipid composition, the effect of different lipids can be evaluated. Finally, it is important to study steroid-membrane interactions in more complex membranes to relate the findings from

model systems to cellular membranes. Insights from characterising the effects of steroids on cell membranes will benefit our understanding of normal physiology and pathophysiology and assist drug development.

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## Declarations

**Ethics approval** Not applicable.

**Consent to participate** Not applicable.

**Consent for publication** Not applicable.

**Conflict of interest** The authors declare no competing interests.

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