



Differential scanning calorimetry in drug-membrane interactions

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ABSTRACT

Differential Scanning Calorimetry (DSC) is a central technique in investigating drug - membrane interactions, a critical component of pharmaceutical research. DSC measures the heat difference between a sample of interest and a reference as a function of temperature or time, contributing essential knowledge on the thermally induced phase changes in lipid membranes and how these changes are affected by incorporating pharmacological substances. The manuscript discusses the use of phospholipid bilayers, which can form structures like unilamellar and multilamellar vesicles, providing a simplified yet representative membrane model to investigate the complex dynamics of how drugs interact with and penetrate cellular barriers. The manuscript consolidates data from various studies, providing a comprehensive understanding of the mechanisms underlying drug - membrane interactions, the determinants that influence these interactions, and the crucial role of DSC in elucidating these components. It further explores the interactions of specific classes of drugs with phospholipid membranes, including non-steroidal anti-inflammatory drugs, anticancer agents, natural products with antioxidant properties, and Alzheimer's disease therapeutics. The manuscript underscores the critical importance of DSC in this field and the need for continued research to improve our understanding of these interactions, acting as a valuable resource for researchers.

1. Introduction

One of the critical tools used in studying drug - membrane interactions is Differential Scanning Calorimetry (DSC). This is a thermoanalytical technique that measures the heat relating between a sample to a reference based on temperature or time. In the context of drug - membrane interactions, DSC is used to study the thermotropic phase behavior of lipid membranes and how this is affected by the presence of drugs [1]. Although DSC provides valuable information on drug - membrane interactions, the high complexity of biological membranes presents a significant challenge. Biological membranes are macromolecular structures composed of a diverse array of lipids, proteins, and carbohydrates [2]. The multifaceted complexity inherent to this subject matter presents significant challenges in the systematic

examination of drug-membrane interactions, whether conducted *in vivo* or *in vitro*. To overcome this challenge, researchers often use simplified models of biological membranes. These models, which can range from simple lipid bilayers to more complex structures that incorporate proteins and other components, allow researchers to study drug - membrane interactions in a more controlled and manageable way [3].

One of the most used types of membrane models in DSC studies are phospholipid membranes. Phospholipids are the main component of biological membranes, and their amphipathic nature, with a hydrophilic head and hydrophobic tail allows them to form bilayers that closely mimic the structure of cell membranes. Phospholipids can form a variety of structures, including unilamellar vesicles (single-layered vesicles), multilamellar vesicles (multiple-layered vesicles), and supported lipid bilayers, each with their advantages and limitations [4]. Unilamellar

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vesicles provide a simple and homogeneous system for studying drug - membrane interactions. However, their small size and single-layered structure may not accurately represent the complexity of biological membranes [5]. On the other hand, multilamellar vesicles (MLVs) have a more complex structure and, in specific cases, their multiple layers can complicate the interpretation of DSC data [6]. Supported lipid bilayers provide a stable and robust platform for studying drug - membrane interactions, but their preparation can be more complex, and they may not be suitable for all types of studies [7].

These models have been used to study a wide range of drugs, from small molecule therapeutics to larger biologics, and have provided valuable information about the mechanisms of drug - membrane interactions, the factors that influence these interactions, and the effects of these interactions on the chemical and physical properties of the membrane [8]. Despite the wide variety of studies in this area, a clear understanding of these interactions remains challenging due to the complexity of biological membranes and the diverse chemical nature of the drug molecules. A comprehensive review that synthesizes the current knowledge in this field and critically evaluates the methodologies used, particularly DSC, would benefit the scientific community. This review aims to emphasize the relevance of this technique in the study of drug - membrane interactions by providing a comprehensive overview of its use, with a particular emphasis on phospholipid membrane models; these consisted of dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylethanolamine (DMPE), classes of lipids frequently present in the outer and inner layers of most cell membranes. This review aims to offer a comprehensive understanding of the contribution of DSC to the mechanisms underlying drug - membrane interactions and the determinants that influence these interactions. Furthermore, this review will also explore the interactions of specific classes of drugs with phospholipid membranes, including anti-cancer, non-steroidal anti-inflammatory drugs (NSAIDs), and anti-Alzheimer's agents, as well as natural products with antioxidant properties.

2. General theoretical principles

DSC is a thermoanalytical technique that quantifies the difference in heat flow (Δq) between a sample and a reference as a function of temperature (T). The following equation represents this:

$$\Delta q = q_{\text{sample}} - q_{\text{reference}} \quad (1)$$

where q_{sample} is the heat flow into the sample and $q_{\text{reference}}$ is the heat flow into reference [9].

The heat capacity (C_p) of the sample, which is a critical parameter in DSC, is defined as the amount of heat (q) required to raise the temperature (T) of the sample by a certain amount (ΔT). This is represented by the following equation:

$$C_p = \Delta q / \Delta T \quad (2)$$

In DSC analysis, the analyte and reference undergo a controlled thermodynamic transition rate (β), dictated by endothermic or exothermic changes. The transition rate is defined as the change in temperature (ΔT) over the change in time (Δt). This is represented by the following equation:

$$\beta = \Delta T / \Delta t \quad (3)$$

The difference in heat flow (Δq) between the sample and the reference can be attributed to various processes occurring in the sample, such as phase transitions, chemical reactions, and physical changes. These processes can be either endothermic, absorbing heat and resulting in a peak in the DSC curve, or exothermic, releasing heat and resulting in a trough in the DSC curve.

The enthalpy change (ΔH) associated with these processes can be calculated by the integral of the heat capacity over the temperature range, as observed in the DSC thermogram peak (Fig. 1):

$$\Delta H = \int C_p dT \quad (4)$$

where dT is the differential change in temperature [10].

The heating rate (β), sample mass (m), and instrument calibration are all critical factors influencing DSC measurements. The sensitivity (S) of the measurement, defined as the change in response (ΔR) over the change in analyte concentration (ΔC), can be affected by the mass of the sample, as represented by the following equation:

$$S = \Delta R / \Delta C \quad (5)$$

In DSC analysis, a mathematical formulation is introduced, connecting classical differential thermal analysis to both power-compensated DSC and heat-flux DSC methodologies.

DSC has been extensively used in the study of biological membranes, particularly in analyzing lipid bilayers and proteins. The thermotropic behavior of lipid bilayers can be analyzed using DSC, providing valuable information about phase transitions, lipid packing, and the stability of the bilayer.

The temperature-dependent heat capacity curve of a lipid bilayer, obtained using DSC, can provide insights into the phase behavior. Fig. 1 shows a representative scheme of a typical thermogram of a pure phospholipid membrane. The main transition temperature (Fig. 1a; T_m), which corresponds to the temperature at which the bilayer transitions from a gel phase to a liquid-crystalline phase, can be obtained from the peak of the heat capacity profile. The width of the transition, represented by the full width at half maximum of the heat capacity peak (Fig. 1b; $\Delta T_{1/2}$), offers insights into the cooperativity of the transition, which is linked to the number of molecules undergoing a simultaneous transition. A narrow transition indicates a highly cooperative transition, while a broad one suggests a less cooperative one. The enthalpy change (ΔH) associated with the main transition, which can be calculated by integrating the area under the heat capacity peak (Fig. 1b), provides information about the energy required for the transition. This might be

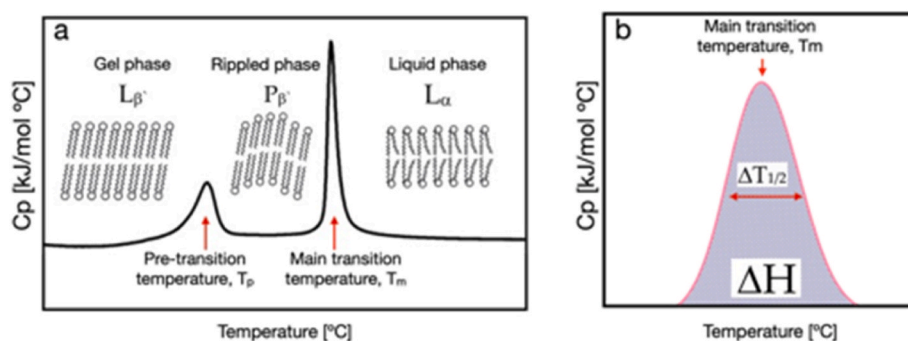


Fig. 1. (a) Representative DSC thermogram of a phosphatidylcholine (PC) bilayer sample, demonstrating its thermotropic phase behavior; (b) Magnified view of the primary phase transition peak in a phospholipid thermogram, highlighting the specific calorimetric changes associated with temperature.

associated with the strength of van der Waals forces acting on the fatty acid chains of lipids.

The effect of various factors, such as pH, hydration, and ionic strength, on the thermotropic behavior of lipid bilayers can be studied using DSC. This can provide valuable information about the stability of the bilayer and its connections with its surroundings.

In the context of protein-lipid interactions, DSC can be used to study the effect of proteins on the phase behavior of lipid bilayers [11]. The presence of proteins can shift the main transition temperature and change the width and enthalpy of the transition, indicating changes in the interactions within the bilayer [12–14]. Moreover, DSC can be used to analyze the thermal stability of proteins in the membrane environment. The unfolding or denaturation of proteins, which is often associated with a significant change in heat capacity, can be detected using DSC [11].

The use of DSC provides a quantitative assessment of drug-induced alterations in membrane stability and dynamics, contributing to a profound understanding of the molecular mechanisms of drug action.

An illustrative example is the interaction of thimerosal (THI) with multilamellar vesicles built by dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylethanolamine (DMPE). Thimerosal is a mercury-containing compound that has been used as a preservative in vaccine formulations. Its interactions with lipid bilayers are crucial to understanding its biological effects and potential toxicity [15,16].

Fig. 2 shows the thermograms of DMPC and DMPE in the presence of increasing concentrations of THI. DSC results underline the pronounced influence of THI on the pre-transition transition of DMPC liposomes, manifesting itself in a concentration-dependent decrease and a shift towards lower temperatures, suggesting an interaction with the DMPC head groups that could increase the spacing between them and disrupt the rippled phase (Fig. 2a). This interaction may alter the arrangement of the acyl chains implying a location in the hydrophobic part of the bilayer, but without affecting the cooperativity region and thus without significantly disturbing the main phase transition [17].

In comparison, the effect of THI on DMPE liposomes is minimal, possibly due to the unique structural properties of phosphatidylethanolamines, such as the small size of the head group and the formation of intermolecular hydrogen bonds, leading to dense packing and structures with higher intrinsic curvature (Fig. 2b). These structural features promote the segregation of THI in the membrane, as reflected in the asymmetry of the thermal transition peaks in the presence of high THI concentrations, suggesting incomplete miscibility and the formation of lipid populations with different melting temperatures [17].

In addition to studying single-drug interactions with lipid membranes, it is crucial to consider the more complex picture that emerges when two or more drugs are administered simultaneously. An exciting work examining the joint action of dimethyl sulfoxide (DMSO) and tilorone on model 1- α -dipalmitoylphosphatidylcholine (DPPC) membranes reveals non-additive effects on membrane melting temperature [18]. While tilorone raises the thermal transition temperature, DMSO lowers it. When introduced together, their interactive effects are evident (Fig. 3). Equilibrium and kinetic DSC studies show that sorption of the drugs onto the membrane and their diffusion through the lipid bilayer stacks occur on different time scales, with sorption occurring within minutes and diffusion within hours [18]. Interestingly, 0.1 mol % DMSO effectively enhanced membrane penetration for tilorone, and the diffusion rate of tilorone depends on the drug delivery scheme. This example emphasizes the importance of considering drug co-administration in studies of lipid membrane interactions. It may serve as a bridge to research that seeks to understand drug synergism or antagonism in complex biological settings.

Modifications induced by drugs in the thermograms of pure phospholipids extend beyond mere shifts in transition temperatures or reductions in cooperativity. Previous works have shown diverse behaviors, including broadening the melting temperature (T_m) peak, the emergence of double thermal transition peaks, or a decrease in enthalpy.

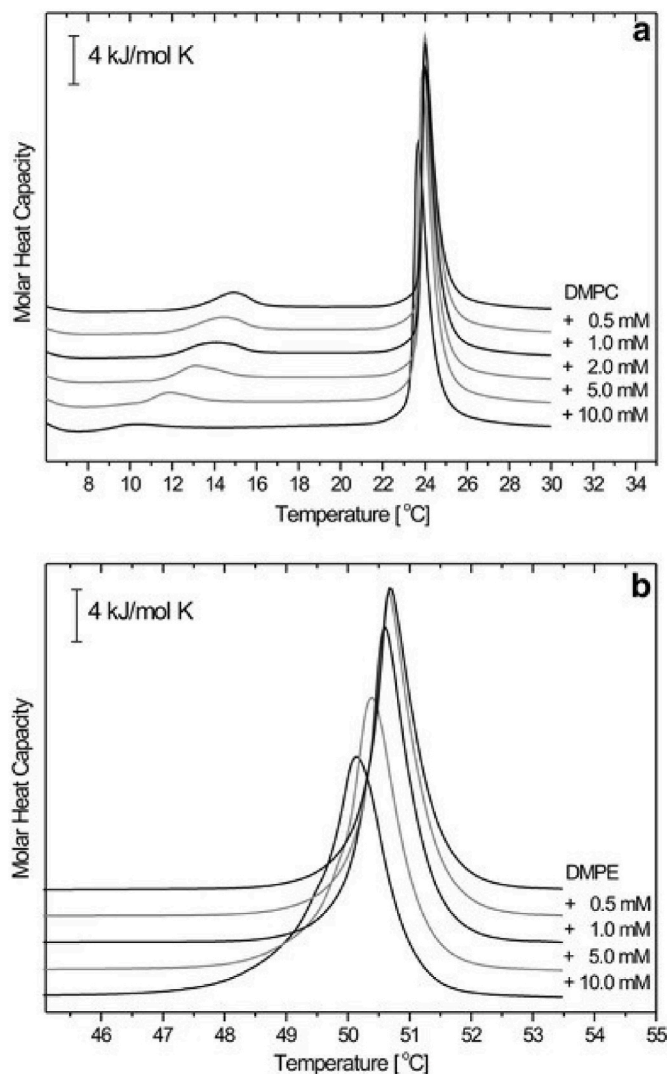


Fig. 2. DSC thermograms of DMPC (a) and DMPE (b) bilayers in the absence and presence of increasing amounts of THI. (a) Pure DMPC bilayers show a pre-transition at 14.91 °C and a main transition at 23.99 °C with transition enthalpies of 2.73 kJ/mol and 18.29 kJ/mol, respectively. (b) Pure DMPE bilayers exhibit a strong and sharp main transition at 50.67 °C with an enthalpy change of 21.17 kJ/mol. THI addition affects the DMPC pre-transition in a concentration-dependent manner, eliminating it at 10 mM THI, while in DMPE, the effect is mild, with a transition temperature shift of no more than 0.72 °C at the highest THI conditions. Reproduced with permission from Ref. [17].

These variations in thermodynamic parameters offer insight into the multifaceted nature of drug-membrane interactions, highlighting the potential for different modes of interaction to occur.

An example of the first case occurs in the interaction of valsartan, a blocker of angiotensin II receptors (ARB). DSC studies found that the inclusion of valsartan in DPPC bilayers induced alterations in molecular packing, thus affecting the conformational state of the lipids. Fig. 4 shows the DSC thermograms of DPPC in the presence of increasing concentrations of valsartan. It is observed that valsartan, even at 1 mol %, can significantly suppress the pre-transition, suggesting its polar interface activity. As the concentration of valsartan increases, a reduction in T_m occurs along with a broadening of the peak. In particular, the total enthalpy of the main transition increases with the valsartan concentration, fluctuating between 7.4 and 9.8 kcal/mol [19].

The division of transition peaks in DSC studies of phospholipids in the presence of drugs is not an uncommon phenomenon and reveals complex interactions at the molecular level. For example, we will

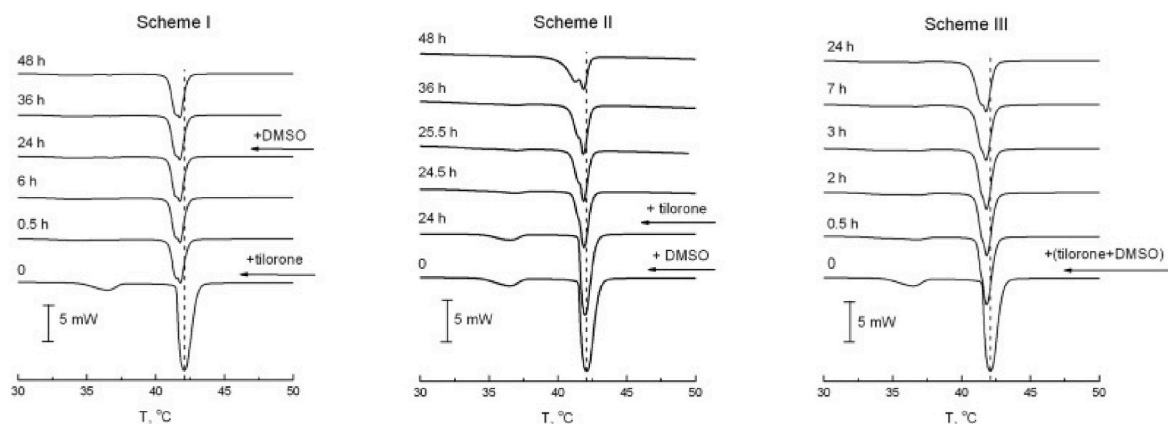


Fig. 3. DSC profiles of DPPC membranes under the introduction of DMSO and tilorone. The graphs show the time elapsed since the beginning of the experiment. Schemes I, II, and III show different times of addition of the components. Reproduced with permission from Ref. [18].

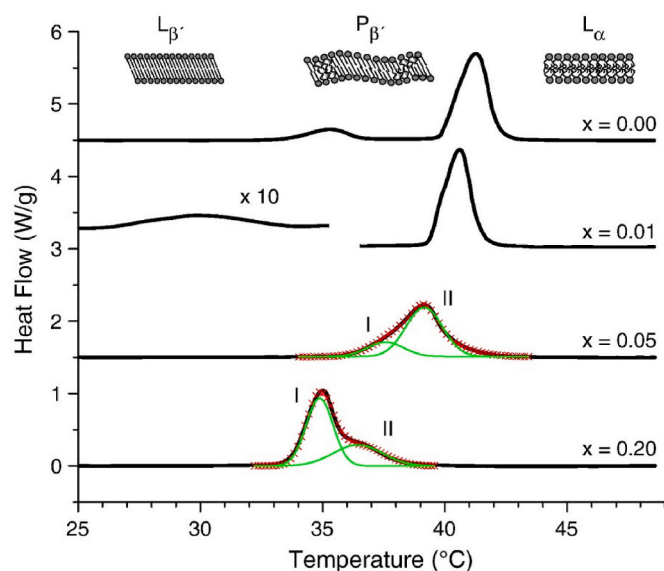


Fig. 4. Example of peak shifting and broadening effects. Thermograms of DPPC bilayers with varying valsartan concentrations at molar ratios of 0.00, 0.01, 0.05, and 0.20. The DSC curve for pure DPPC bilayers exhibits two separate thermal transitions. Adding valsartan disrupts the smaller endothermic peak (the pretransition) at concentrations above 0.01. As the concentration of valsartan increases within the bilayers, the width of the transition becomes broader, and the temperature at which the phase transition occurs decreases. Notably, at valsartan incorporations of 5 and 20 mol%, the primary transition divides into multiple components (green lines). Reproduced with permission from Ref. [19]. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

describe a study on how bromoacylated taxane prodrugs (derived from paclitaxel, an anticancer drug from the cytoskeleton inhibitor family) affects the thermotropic phase behaviour and packing of bilayers in model membranes. DSC data, reflected in both heating and cooling curves, show essentially identical changes, so only endothermic curves were used to characterize the thermotropic phase transitions of binary mixtures of phosphatidylcholines (PCs) and bromoacyl taxane prodrugs (Fig. 5).

This study on the interaction of α -bromoacylated taxane prodrugs with DMPC bilayers revealed significant changes in lipid phase transitions as observed in the endothermic profiles of DSC (Fig. 6). The addition of C-6 taxane prodrug (Fig. 6 A) at concentrations of 1–3% mol caused a noticeable decrease in temperature and peak height of the main phase transition of DMPC and almost elimination of the pretransition

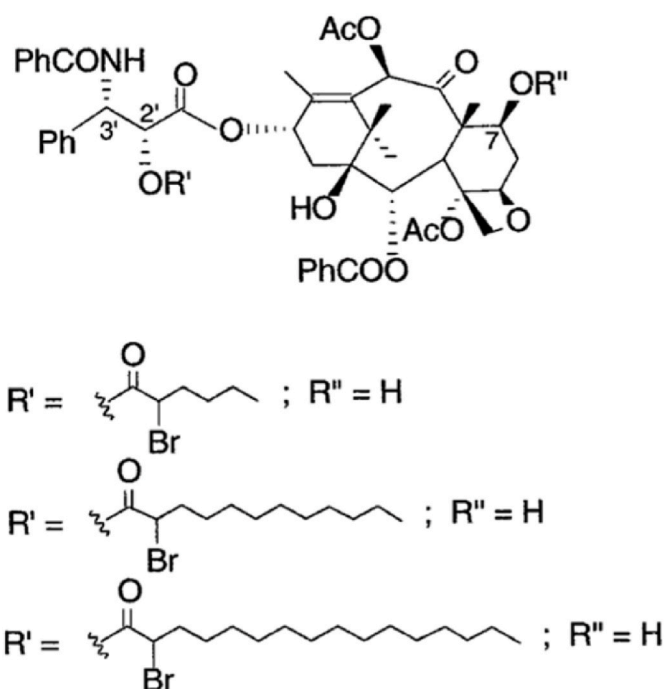


Fig. 5. Representative molecular structures of bromoacyl taxane prodrugs. C-6, C-12, and C-16 are represented in the carbon chain length of R'. Reproduced with permission from Ref. [19].

(curves b and c). At concentrations of 4.8% mol, the pretransition disappeared entirely and the lamellar transition widened even further (curve d), and at 9.1% mol, the highest concentration used, an additional widening of the endotherm was observed (curve e).

The core observation about C-6 is a weak interaction with PC bilayers, which results in only a reduction in enthalpy (ΔH) without significantly altering the phase transition temperatures. This phenomenon can be attributed to the shorter acyl chain of C-6, which limits its ability to penetrate deeply into the hydrophobic core of the PC bilayers. Unlike the longer-chain prodrugs, C-6 does not span the width of the PC monolayers or disrupt the bilayer structure to the same extent. Instead, C-6 may preferentially associate with the surface of the bilayers or form less disruptive interactions within the bilayer, leading to a decrease in ΔH . In general, the decrease in ΔH observed with the addition of C-6 suggests a diminished capacity for van der Waals interactions between the short acyl chain of the prodrug and the fatty acyl chains of the phospholipids. This reduced interaction likely leads to lesser

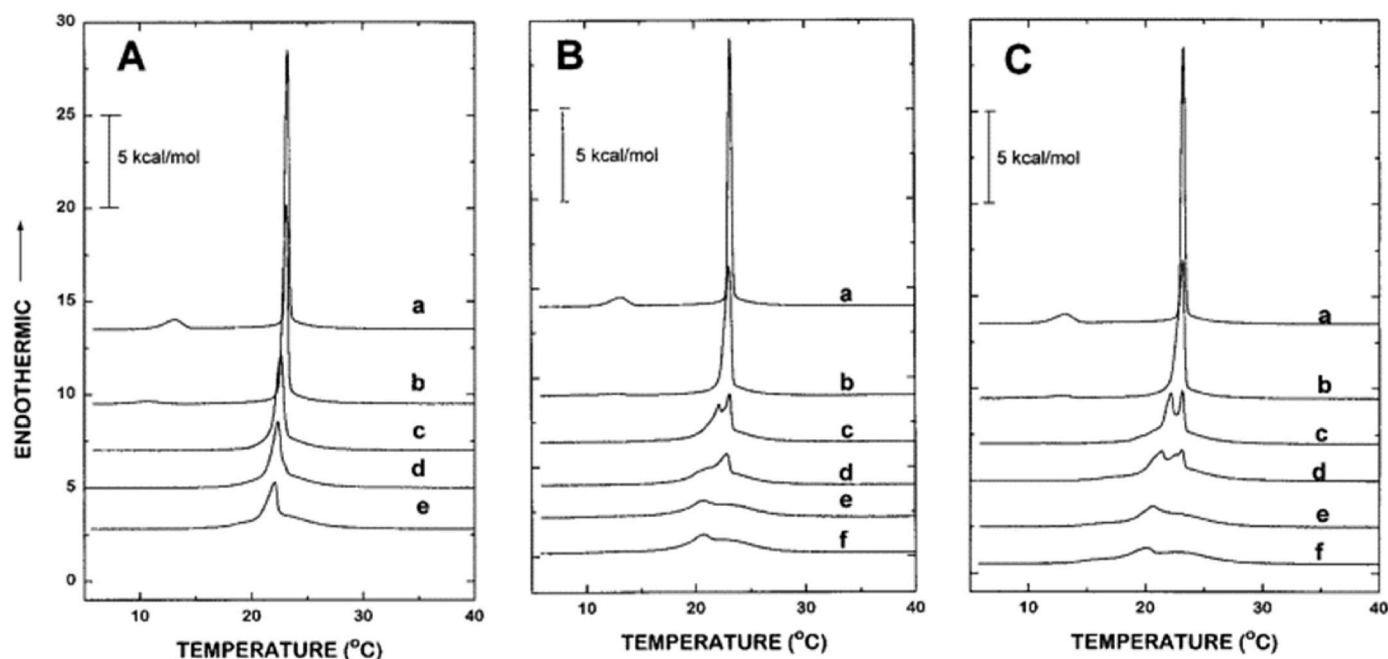


Fig. 6. DSC thermograms for binary mixtures of dimyristoylphosphatidylcholine (DMPC) with varying molar percentages of bromoacyl taxane prodrugs. Displayed across six curves (a–f), the data shows the thermal properties of DMPC with 0, 1.0, 3.0, 4.8, 9.1, and 16.7 mol % of C-6, C-12, and C-16 taxane prodrugs, as illustrated in Panels A, B, and C respectively. These thermograms detail the effects of taxane prodrug chain length on the DMPC lipid bilayer phase transitions. Reproduced with permission from Ref. [19].

perturbation of the lipid bilayer's packing and dynamics, manifesting as a decrease in enthalpy without a corresponding shift in phase transition temperatures. This contrasts with longer-chain prodrugs, which, due to deeper intercalation, cause more significant disruptions in bilayer structure, reflected in shifts of phase transition temperatures and broadening of transition peaks.

Increasing the acyl chain length of the taxane to 12 carbons (C-12) increased its association with DMPC (Fig. 6 B). Adding C-12 taxane to DMPC led to significantly widening the gel to lamellar liquid crystalline phase transition. With only 1 % mol of C-12 taxane (curve b), the pre-transition was almost eliminated, and the main transition significantly widened. At 3 % mol, the pretransition disappeared entirely and the main transition showed two inseparable peaks at about 24 °C, separated by approximately 1 °C, indicating an inhomogeneous mixture and possible phase separation in the gel state. With a taxane content of 4.8 % mol, the two peaks merged into a relatively broad peak with a low-temperature shoulder around 21 °C (curve d). The endotherm widened even further at 9.1 % mol of taxane, maintaining a low-temperature shoulder near 21 °C (curve e). At taxane concentrations of 16.7 % mol, no additional changes in the endotherm were observed (curve f), suggesting saturation in the widening around or above 9 % mol.

The results with C-16 taxane in DMPC (Fig. 6C) were similar to those observed with C-12. Increasing amounts of C-16 taxane (from 1 to 3% mol) showed an endotherm with a $\Delta T_{1/2}$ of approximately 1.75 °C, comparable to a $\Delta T_{1/2}$ of about 1.7 °C for C-12 taxane at equivalent concentrations (curves c, Fig. 5C vs. 5B). Increasing C-16 taxane to 4.8 % mol, the $\Delta T_{1/2}$ increased to 3 °C, similar to the $\Delta T_{1/2}$ of approximately 2.5 °C for C-12 taxane (Fig. 5B). With further increases in C-16 taxane to 9.1% mol and 16.7% mol, there were no apparent changes in the shape of the endotherms with $\Delta T_{1/2}$ of approximately 5–6 °C (curves e and f, Fig. 5C), the same observed for C-12 taxane at equivalent concentrations (Fig. 5B). At higher taxane concentrations (9.1% mol and above), the endotherms remained wide with shoulders at temperatures slightly higher than the main phase transition of DMPC, probably indicating the level of saturation of C-16 taxane in DMPC with inhomogeneous mixing

or domain formation.

In this illustrative example, the length of the chain of the acylated taxane prodrugs plays a crucial role in their mixing behaviour with phosphocholines, where long chains effectively incorporate into short-chain bilayers and vice versa. The composition of acyl chains within the lipid matrix markedly influences the solubility of taxane prodrugs. DSC data indicate that the gel-to-lamellar liquid crystalline phase transition shifts to lower temperatures and widens with the increase of taxane prodrug content, possibly due to the intercalation of the taxane acyl chains into the phospholipid bilayers to maximize van der Waals interactions. Although the depth of penetration of the acyl chain was unknown in that study, the data suggest that the C-16 chain could span the entire width of the PC monolayer in liposomes, disturbing the phospholipid bilayers. The C-6 molecule has limited penetration due to its shorter acyl chain and, therefore, causes less disturbance in the bilayer structure.

In summary, this example illustrates three recurring phenomena observed in studies of drug interactions with cellular membrane models using DSC: a reduction in enthalpy, the creation of double peaks, and the broadening of the peaks. These changes are indicative of the complex dynamics that drugs can induce in membrane structures and highlight the intricate relationship between a drug's chemical makeup, its concentration, and the resulting biophysical properties of membrane models.

In the next sections, we will discuss in detail how these changes in DSC thermography may correlate with the specific localization of drugs in membrane models and how this may affect their biological activity.

3. Drug - membrane interactions: a phospholipid model approach

Phospholipid bilayers as membrane models have emerged as an indispensable tool in studying drug - membrane interactions. These models, designed to emulate the lipid bilayer of cell membranes, provide a simplified but representative environment for investigating the complex dynamics of how drugs interact with and penetrate cellular

barriers. Choosing a phospholipid membrane model is crucial; it can significantly influence the outcome and interpretation of these studies, making it a critical consideration in experimental design [20–22].

Large unilamellar vesicles (LUVs) are among the most widely utilized types of membrane models. These structures consist of a single lipid bilayer that encloses a small aqueous compartment, thereby mimicking the structure of a cell. LUVs are particularly useful for studying the early interaction of drugs with the membrane and their subsequent penetration into the lipid bilayer [23,24]. They are often used in studies involving small, hydrophobic drugs that can readily diffuse across the lipid bilayer. The simplicity of LUVs allows for a precise observation of drug - membrane interactions, making them a popular choice for screening, protein-membrane reconstitution and interaction studies. However, it is essential to note that while LUVs offer a simplified model, they may need to fully capture the complexity of drug - membrane interactions in biological systems. On the other hand, MLVs consist of multiple concentric lipid bilayers [25–27]. These models represent the multi-layered structure of specific biological membranes, such as the stratum corneum in the skin [28].

Supported lipid bilayers (SLBs) consist of a lipid bilayer maintained on a solid substrate, allowing for direct observation of drug - membrane interactions using surface-sensitive techniques. These models are particularly advantageous for studying the interaction of drugs with membrane proteins and other surface-associated molecules [29,30]. The lipid bilayer remains stable due to the presence of the solid support, allowing for the use of techniques such as atomic force microscopy, which can provide detailed information on the interaction of different molecules with the membrane. However, the preparation of SLBs can be technically challenging, and the presence of solid support may influence the behavior of the lipid bilayer and the drug - membrane interactions.

Each of these membrane models has its advantages and limitations, and the choice of model should be guided by the specific research question at hand. However, despite their numerous benefits, phospholipid membrane models also have certain limitations. The complexity inherent in biological membranes, which contain a variety of lipids, proteins, and other components, may not be entirely replicated by membrane models [31]. They may also not fully represent the dynamic nature of biological membranes, which can change in response to various factors such as temperature, pH, and the presence of other molecules [32,33]. Therefore, while phospholipid membrane models are a valuable tool in the study of drug - membrane interactions, their use should be complemented with other techniques and models to obtain a more comprehensive understanding of these interactions. Although each type of phospholipid membrane model has its unique advantages and applications, it is the MLVs that have shown promise when paired with DSC in the study of drug - membrane interactions [34]. The multilayered structure of MLVs offers a more complex model of biological membranes, making them an ideal choice for these studies. The following discussion will examine the use of MLVs in DSC studies, exploring the unique insights they provide, the challenges they present, and their contribution to the development of more effective and safer drugs.

The use of MLVs in DSC studies of drug-membrane interactions has proven to be particularly useful [34]. This structure (multi-bilayers) provides a robust signal in DSC measurements, allowing for the detection of sensitive variations in the thermotropic behaviour of the lipid bilayer upon drug interaction. Multilamellar vesicles (MLVs), with their multiple concentric lipid bilayers, serve as a more complex model for certain stratified biological membranes, such as the stratum corneum [35]. However, for simulating the plasma membrane, which comprises a single bilayer, unilamellar vesicles provide a more representative model. This complexity is advantageous in DSC studies because it allows for the investigation of how drugs interact and penetrate through multiple lipid bilayers, providing a more comprehensive understanding of these interactions. For example, the use of MLVs in DSC studies has provided information on the mechanisms of action of various antimicrobial and anticancer drugs, which are well known to interact with multiple layers

of the cell membrane. Using MLVs in DSC, the impact of membrane curvature on drug-membrane interactions can be analyzed [36]. MLVs, with their large size and low curvature, can provide different insights compared to smaller, high-curvature models such as LUVs. This is particularly relevant for drugs that target specific membrane structures, such as invaginations or protrusions, where the curvature of the membrane can significantly influence the efficacy of the drug [37]. In some specific cases, the multilayered structure of MLVs can eventually present difficulties in interpreting DSC data, as changes in the heat flow could be due to interactions with one or multiple layers of the lipid bilayer. Despite these challenges, the use of MLVs in the study of molecule-membrane interactions remains a powerful tool, providing unique insight into the complex dynamics of these phenomena.

In conclusion, while the use of MLVs in DSC studies presents certain challenges, the insights they provide make them an invaluable tool in this field. With careful experimental design and data interpretation, MLVs can provide an ample understanding of the interactions between drugs and membrane models, contributing to the improvement of more effective and safer drugs. Through the advancement of technology and methodology, DSC studies using MLVs are expected to continue providing valuable insights into the interactions between drugs and membranes.

4. DSC in drug - membrane interaction studies

Advancing our understanding of drug action and improving drug design has been crucial through the application of DSC in studying drug-membrane interactions in chemistry and pharmaceutical research [38–43]. This section will delve into the exploration of interactions between membrane models and different classes of drugs, each with unique therapeutic implications and modes of action: (i) Non-steroidal anti-inflammatory drugs (NSAIDs), although effective and commonly used, have modes of membrane interaction that can result in adverse effects, emphasizing the necessity to study these interactions; (ii) Natural products, particularly antioxidants, which have been recognized for their potential therapeutic benefits, and their interactions with membranes can provide valuable insights into their mechanisms of action; (iii) Anticancer drugs, a diverse group of compounds that often exert their effects through complex interactions with cellular membranes, making the study of these interactions crucial for understanding and optimizing their efficacy and; (iv) Drugs used in the treatment of Alzheimer's disease, a condition intricately linked with membrane dysfunction, another area where understanding drug - membrane interactions can contribute significantly to the development of more effective treatments. Through the lens of these diverse drug classes, this section aims to demonstrate the broad applicability and value of DSC in studying drug - membrane interactions, emphasizing its integral role in drug development and optimization.

4.1. Nonsteroidal anti-inflammatory drugs

NSAIDs, although chemically diverse, are a heterogeneous group of compounds unified by their shared therapeutic characteristics. The name was given to differentiate these compounds from steroids, the other major anti-inflammatory class of drugs. NSAIDs are effective in reducing inflammation's swelling, redness, and pain, lowering a general fever, and relieving a headache, which renders them highly beneficial for managing inflammatory and degenerative diseases. In the last decades, they have emerged as chemotherapeutics, chemopreventive agents, and a part of complementary therapy for Alzheimer's disease [44]. Due to their comprehensive pharmaceutical benefits, NSAIDs are not only the most frequently prescribed medications worldwide but also commonly accessible over-the-counter for self-treatment. The most famous is aspirin, which has been on the market since 1899. It was created by Felix Hoffman at Germany's Bayer Company in 1897, yet its mode of action was not discovered until 1971. After aspirin, many

NSAIDs were developed [45]. For this reason, NSAIDs are often referred to as aspirin-like drugs. NSAIDs primarily act on the cyclooxygenase (COX) pathway, blocking the production of prostaglandins (PGs) (Fig. 7). Since the substrate of the COX is arachidonic acid, an effective competitive enzyme inhibitor must possess both high lipophilic and acidic properties to mimic the natural substrate. This is clear in the chemical structures of all NSAIDs such as ibuprofen, fenoprofen, naproxen, indomethacin, diclofenac, and piroxicam (Fig. 8). Acidic functionality can be a propionic, acetic carboxylic, or an enolic group [46].

Prostaglandins (PGs) are lipid mediators produced in most cells except red blood cells; they are released by several chemical and mechanical stimuli. PGs generate a notably wide range of effects on the body. They are not accumulated within cells but are promptly synthesized in response to diverse stimuli such as hormones, chemical mediators, inflammation, pain, fever, and cellular injury [47]. With arachidonic acid, a dietary unsaturated fatty acid derived from animal fats, the normal process of prostaglandin synthesis begins [48]. This acid is induced by the COX enzyme to synthesize different PGs. Each type of COX produces different types of PGs. By temporarily blocking the attachment site for arachidonic acid on the COX, most NSAIDs prevent the enzyme from converting arachidonic acid to prostaglandin. The exception is aspirin, which irreversibly inhibits COX-1 by acetylation of serine 530, permanently blocking arachidonic acid access. Some NSAIDs have a stronger and broader range of side effects than others, although their anti-inflammatory action is comparable. This is attributed to the specific affinity each drug has towards different forms of COX.

In clinical practice, this diverse group of drugs is commonly classified based on their inhibition of COX instead of their chemical structure, allowing a classification related to their pharmacological role and side effects, respectively (Table 1). The inhibition of the COX-1 enzyme is the cause of most side effects associated with NSAIDs. On the other hand, the therapeutic benefits of NSAIDs arise from the inhibition of the COX-2 enzyme. Regrettably, most of the commonly used drugs are non-selective inhibitors of COX, affecting both forms of the enzyme. These drugs, despite the variety in their chemical structures, possess the same therapeutic properties, and to varying degrees, they also exhibit several similar side effects. Depending on the dose, they can cause gastric upset, abdominal pain, headaches, dizziness, and nausea. NSAIDs, when administered in high doses, have been reported to postpone the birth process, and in cases of overdose, may inflict damage on the kidney. A

side effect that has been recently identified and is particularly intriguing is the antithrombotic effect. This phenomenon stems from the inhibition of COX in thrombocytes, leading to a reduced production of thromboxane A₂. It results in an extension of the bleeding time and causes a hindrance in platelet aggregation [49]. This property increases the use of NSAIDs as anticoagulant agents to prevent cardiovascular diseases [50,51]. The most used agent is aspirin promoted in the market as Cardioaspirin.

Pignatello et al. published a comprehensive revision of the interaction of NSAIDs and cell membranes in 2013 [52]. However, these drugs are still under study due to the high consumption of NSAIDs worldwide. Past studies have shown that three of the most widely used NSAIDs, namely ibuprofen (Fig. 9 A), naproxen (Fig. 9 B), and diclofenac (Fig. 9C), can modify the thermotropic behaviour and lipid organization of PC and phosphatidylethanolamine (PE) liposomes [53]. The calorimetric results showed a strong fluidization effect of the NSAIDs on the lipid systems, indicating that the highly soluble drugs are mainly located at the water lipid interphase [54–58]. Nonetheless, proposals have been made suggesting that the regular usage of NSAIDs lowers the risk of gastrointestinal cancers, indicating that anti-inflammatory drugs hinder malignant cell formation and, consequently, tumour progression [59–61]. Thus, we also studied the interaction of ibuprofen, naproxen, and diclofenac with phosphatidylserine (PS) lipid bilayers [53]. Since it has been demonstrated that NSAIDs interact with membrane phospholipids in a COX-independent path, the potential binding of NSAIDs to negatively charged phospholipids could be involved in their chemopreventive activity. The findings revealed that the three drugs were capable of inducing alterations in the thermotropic properties of the DMPS liposomes by modifying the phase transition temperature and widening the endothermic peaks as observed through DSC. Even though ibuprofen, naproxen, and diclofenac share the negatively charged solution, the physicochemical properties of these compounds are quite different and induced different effects on DMPS liposomes. According to the results, the smallest size of the ibuprofen favoured the interaction with the liposomes compared to the higher structure of diclofenac [53].

Aloi et al. [62] also studied ibuprofen in combination with biophysical and computational techniques to investigate the association of ibuprofen with DMPC-built MLVs in a physiological temperature range (5–45 °C). The results suggested that ibuprofen attaches at the polar/apolar interfacial region without penetrating the inner hydrocarbon region of the DMPC bilayers. Through DSC, it was observed that the drug stabilizes the lipid fluid phase and promotes the coexistence of gel and fluid phase, implying that the primary action site of ibuprofen is the head group region of DMPC bilayers. Furthermore, Fourier transform infrared spectroscopy (FT-IR) and molecular docking studies detected alterations in hydration through shifts in the PO₂⁻ and C=O group vibrations of DMPC. These results are in accordance with our previous findings published in 2016 [53].

Di Foggia et al. studied the toxic side effects of ibuprofen on cell membranes following the effect of increasing amounts of ibuprofen at neutral (IbuNa) and acidic (IbuH) pH on the behavior of DMPC liposomes using Raman and DSC techniques [63]. DMPC was used because lecithins are the main components of most mammalian membranes. The results showed that the ibuprofen concentration noticeably influenced the liposome structure. At low ibuprofen concentrations, only one peak in the thermograms was observed. The interactions of polar nature between the CO₂⁻ and COOH groups found in IbuNa/IbuH molecules and the choline head of DMPC act as a barrier to their deep insertion, hinting at the concurrent existence of multiple phases within the hydrophobic core of the liposomes. When present in intermediate amounts, the emergence of two DSC peaks alludes to the presence of multiple phases. Both Raman and DSC data implied that at pH 3, IbuH can penetrate within the nonpolar bilayer, whereas at pH 7, the establishment of polar interactions of varying strengths between the carboxylic groups of IbuNa and the choline head of the DMPC obstructs further penetration of the drug [63].

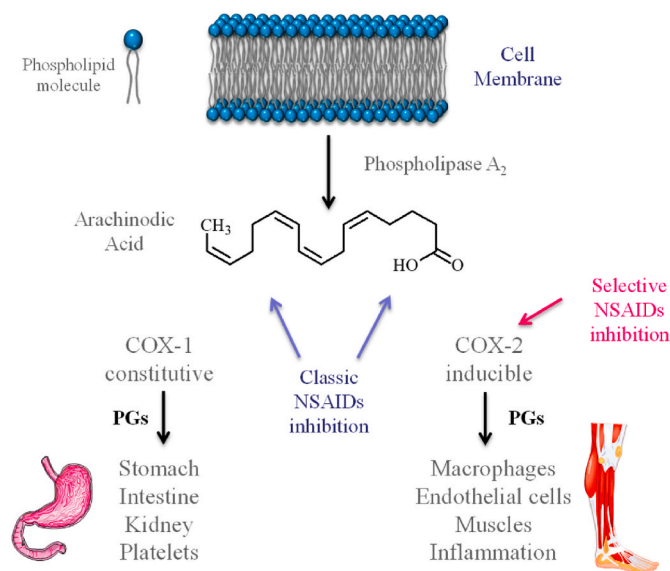


Fig. 7. COX pathway for PG production. The initial step is the liberation of arachidonic acid from phospholipids of cell membrane catalysed by phospholipase A₂. Second step is the conversion of the acid by COX-1 and COX-2 isoenzymes.

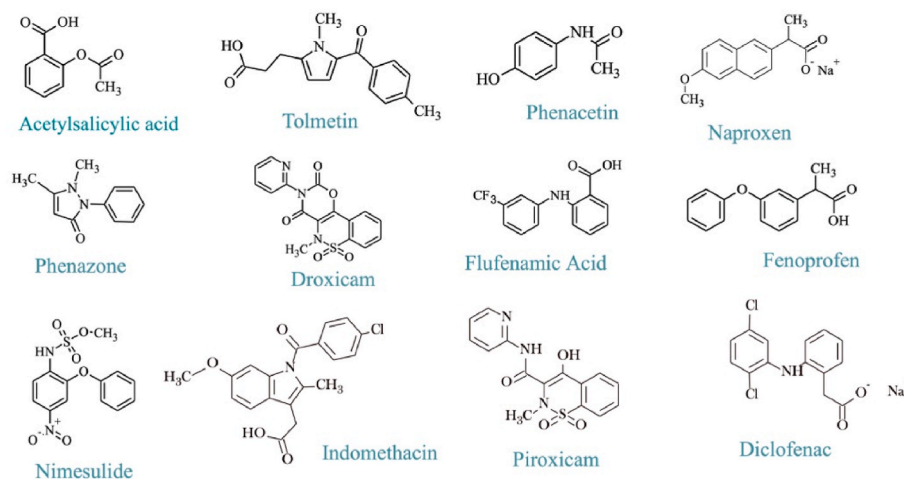


Fig. 8. Chemical structures of the most consumed NSAIDs.

Other biological applications, such as liposome-based transdermal delivery systems, have been studied using DSC. Paliwal et al. studied the incorporation and thermal stability of flurbiprofen and drug-loaded ethanolic liposomes [64]. Ethanolic liposomes have been shown to present fluidity and permeability different from conventional liposomes [65]. Both properties allow them to penetrate the stratum corneum and deeper layers of the skin effectively. In addition, ethanolic liposomes have a higher transdermal flux than conventional liposome formulations, which primarily reach the stratum corneum [66,67]. The results of Paliwal et al. showed the successful incorporation of flurbiprofen into the ethanolic formulation of liposomes. The morphological experiments showed smooth, round, and spherical drug-loaded ethanolic liposomes. Finally, the skin permeation studies using Franz's diffusion cell were performed with formulations of the drug in the ethanolic liposomes. The results demonstrated that the formulations showed a rapid drug release, which could be affected by the lipid: ethanol ratio in the formulations.

The interaction of NSAIDs with lipid molecules does not refer only to the potential side effects of the drug. This interaction could be used as an advantage to change the physicochemical properties of drugs. Amirinejad et al. studied the association between PC and ibuprofen to improve the bioavailability of the drug [68]. Acidic ibuprofen is a poorly water-soluble drug that can be chemically associated with phospholipid molecules. It is defined as pharmacosomes, a vesicular system in which the drug is associated with the carrier, a phospholipid [69]. This complex form possesses both water and hydrophobic characteristics. The drugs present in these systems are conjugated by electron pair sharing and electrostatic forces or by forming a hydrogen bond with lipids [70].

DSC and X-ray diffraction (XDR) analysis confirmed the loss of crystallinity and the amorphous properties of ibuprofen in associations [68]. Associations of ibuprofen can enhance intestinal absorption and diminish gastric irritation in comparison to acidic ibuprofen, attributes that are brought about by an improved surface area, amorphous tendencies, wettability, and particle size. Therefore, the ibuprofen-PC association can potentially increase bioavailability and decrease the gastrointestinal (GI) side [68].

The use of membrane mimetic models and several biophysics techniques like DSC has been determinant in understanding NSAIDs-membrane interactions. These studies have provided information about the molecular basis behind the interaction of NSAIDs with membranes, such as the location of the NSAIDs, alterations of membrane thermotropic behaviour, stability, and affinity. Although this valuable information was obtained using, in most cases, a single lipid component in mimetic models, this simplification has been extensively questioned because it does not represent the membrane properly, especially complexity, fluidity, and charge of the lipid headgroups [32]. The complexity of cell lipids regulates several structural properties of biomembranes, including the lipid environment, the fluidity associated with cellular morphological transformation, and division and differentiation for each kind of membrane.

Primarily, eukaryotic membranes consist of zwitterionic phospholipids like PC and sphingomyelin (SM), which are found in a higher proportion in the exoplasmic leaflet, bestowing an overall neutral charge. Conversely, PE and negatively charged PS are predominantly situated in the cytosolic leaflet [71]. Nonetheless, regarding cancer cells,

Table 1

NSAIDs chemical subgroup classification based on their COX-1 and COX-2 selectivity.

Chemical Classification	COX-1/COX-2 inhibitors	Selective COX-2 inhibitors
Salicylic acid derivatives	Aspirin, Amoxiprin, Benorylate, Choline, Magnesium salicylate, Diflunisal, Ethenzamide, Faislamine, Salicyl salicylate, Sodium Salicylate.	
p-aminophenol derivatives	Acetaminophen and Fenacetine	
Indoles and Indenene acetic acids	Indometacine and Sulindac	Etodolac
Heteroarylacetic acids	Diclofenac, Acemethacin, Alclofenac, Etodolac, Indometacin, Oxametacin, Sulindac, Ketorolac	
Arylpropionic acids	Ibuprofen, Alminoprofen, Benoxaprofen, Carprofen, Fenoprofen, Flurbiprofen, Ibuproxam, Indoprofen, Ketoprofen, Naproxen, Oxaprozin, Tiaprofenic acid.	
Anthranilic acids	Mefenamic acid, Flufenamic acid, Meclofenamic acid, Tolfenamic acid.	
Oxicams or enolic acids	Piroxicam, Droxicam, Lornoxicam, Meloxicam, Tenoxicam.	
Alkanones	Nabumetone	
Pyrazoles	Phenylbutazone, Ampyrone, Azapropazone, Clofezone, Kebuzone, Metamizole, Mofebutazone, Oxyphenbutazone, Phenazone, Sulfinyprazole.	
Sulphonanilides		Nimesulide
Coxibs		Celecoxib, Lumiracoxib, Valdecoxib.

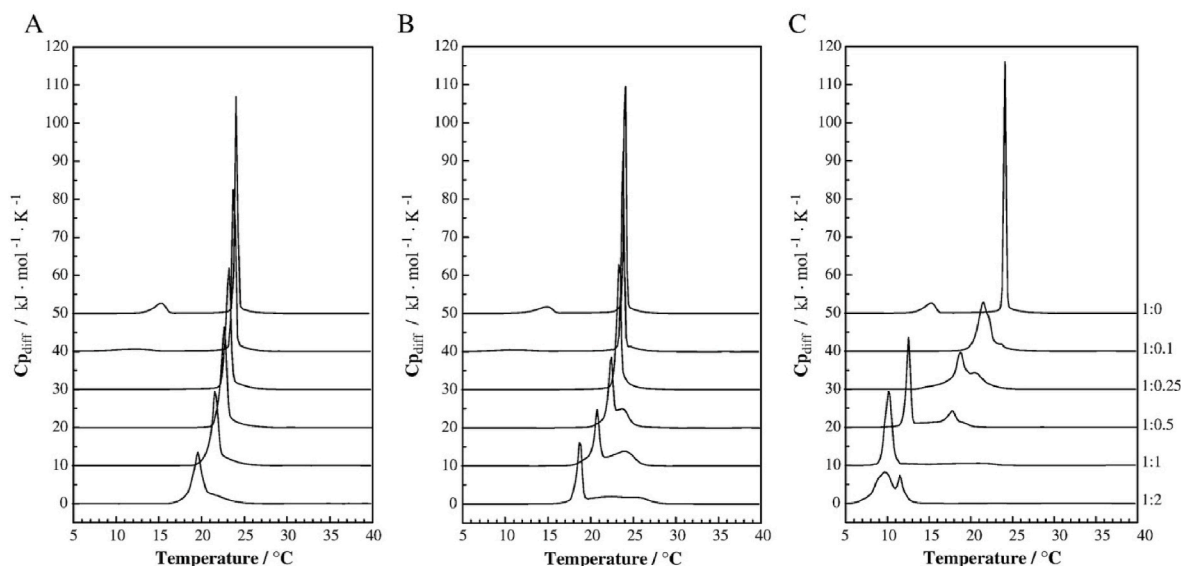


Fig. 9. DSC thermograms for DMPC liposomes in conjunction with (A) Ibuprofen, (B) Naproxen, and (C) Diclofenac. The right side of the figure shows different molar ratios for the mixtures (DMPC:NSAID). The ratios of DMPC to NSAID are as follows: 1:0 (top), 1:0.1, 1:0.25, 1:0.5, 1:1, 1:2 (bottom). Reproduced with permission from Ref. [53].

reports have indicated that the asymmetric distribution of phospholipids between the two leaflets of the cell membrane is disrupted, with alterations in homeostatic mechanisms such as cancer being a contributing factor [72,73]. Therefore, due to the abnormal concentration of PS, the surface of the cancer cell membrane acquires a negative charge [74].

For this reason, several studies have used PS-built liposomes to represent cancer membrane cells. Nevertheless, there is an important difference in the superficial charge of a liposome composed exclusively of PS and a multicomponent liposome with only 10–20% PS [75,76]. Klaiss et al. published a recent study on the interaction of the novel peptide LTX-315, an important oncolytic peptide currently in several clinical trials against different cancer types [77–80], using two artificial systems representative of non-tumour and tumour membranes by DSC and FT-IR [81]. Lipid systems were designed based on the reported composition of the eukaryotic membrane using 16:0 lipids in the proportions PC/SM/PE 4.35:4.35:1 (w/w) and PC/SM/PE/PS 4.35:4.35:1:0.3 (w/w) to represent the non-tumour and tumour cell membranes, respectively [82].

The DSC results showed that the thermograms of the non-tumoral and tumoral systems presented broader and less cooperative in comparison with the pure lipid systems, as it was reported by Lewis et al. [83]. The main transition temperature for multicomponent lipid systems reflects the proportions of the participating lipids and the lipid system. These results are extremely valuable because of the complexity of the membrane under study, and without doubt more closed to the thermotropic behaviour of a real cell membrane.

4.2. Natural antioxidants from plants

Antioxidants are substances that at low concentrations are capable of delaying or preventing oxidation of an oxidizable substrate. Antioxidant compounds act through three main mechanisms: i) hydrogen atom transfer (HAT); ii) single electron transfer (SET) and iii) transition metals chelation [84]. Natural antioxidants from plants are a wide group of chemical compounds that include polyphenols, carotenoids and vitamins [85]. These compounds, particularly polyphenols and carotenoids, show a wide range of biological effects *in vitro* and *in vivo* [86–89]. The therapeutic properties shown by natural antioxidants have been shown to be related to the way they interact with the cell membrane [90–93]. In fact, many researchers have used the DSC technique to study

the changes in the biophysical properties of the lipid membrane due to its interaction with polyphenols and carotenoids and how these changes are related to their antioxidant and therapeutic activity.

4.2.1. Polyphenols

Polyphenols are a large class of natural phytochemicals with enormous structural diversity, including flavonoids, phenolic acids, anthocyanins, lignans, and stilbenes. They are the most common plant secondary metabolites with more than 8,000 known structures [94]. The influence of polyphenols on the thermotropic behaviour of the lipid membrane is related to structural characteristics that reflect the hydrophilicity or hydrophobicity of phytochemicals [95–97]. In general, polyphenols induce greater destabilization in the lipid arrangement during the transition from the gel to liquid crystalline state. However, the magnitude of the thermal changes depends on the structure of the compound and the lipid membrane. Ota et al. [96] investigated how structural differences in p-coumaric, caffeic and ferulic phenolic acids affected the thermotropic properties of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) vesicles. These phenolic acids caused a decrease in the pre-transition and main transition temperature of the lipid. However, p-coumaric acid induced a much greater decrease in the enthalpy value of the main transition (ΔH_m) than ferulic acid, while caffeic acid had no effect on ΔH . The authors attributed these results to differences in polarity between the phenolic acids. (–)-epigallocatechin-3-gallate (EGCG) a flavonoid found mainly in green tea, induced pronounced changes on the T_m and ΔH_m of DPPC. EGCG also caused the separation of the main transition peak into two different peaks of similar enthalpy. Other green tea catechins such as (+)-catechin, (–)-epicatechin and (–)-epigallocatechin had small effects on the lipid melting process, which was correlated with the topological polar surface area (TPSA) and the ability to form H-bonds of these flavonoids [95]. Additionally, it has been reported that octanol-water partition coefficient (logP) also plays an important role in the effect of polyphenols on the thermal properties of lipid membrane [97]. Kaempferol and quercetin produced complete abolition of the pre-transition peak, as well as a slight broadening of the main phase transition peak and its shift at lower temperatures in DPPC, but only quercetin caused a decrease in the ΔH_m value. This suggested that the flavonoids mainly interacted with the polar headgroups of the lipid molecules, although quercetin penetrated deeper into the hydrophobic region of the bilayer. These

phenolic compounds differ only in the number of –OH groups attached to the B-ring of the flavonoid skeleton (kaempferol has one –OH and quercetin has two) [98]. Recently, Altunayar-Unsalan et al. [99] reported the influence of hesperidin and naringin, two flavonoids glycosides that differ structurally only in one additional methoxy group (O–CH₃) in the B ring of naringin, on the thermal properties of DMPC. The DSC data showed that the progressive incorporation of naringin into DMPC liposomes caused the abolition of T_p and reduction of T_m, together with an abrupt increase in ΔH_m and entropy (ΔS_m) as well as a decrease in the cooperative unit. However, only slight changes in these thermodynamic parameters after incorporation of hesperidin were observed. The last two investigations show that even a difference in one chemical group in the polyphenol structure can promote significantly different thermal effects at the level of the lipid bilayer.

Structural differences in the lipid polar headgroups of lipids also determine the interactions of polyphenols with lipid membrane and their corresponding effects observed by DSC. Suwalsky et al. [100] and more recently Colina et al. [101,102] investigated the influence of polyphenols gallic acid, EGCG and caffeic acid, respectively, on the thermotropic behaviour of DMPC and DMPE-built vesicles. According to previous reports, the DSC data showed that the incorporation of the phytochemicals caused the vanishing of T_p, as well as a gradual broadening of the main phase transition peak, together with a continuous decrease in T_m toward lower values in DMPC vesicles. However, these effects were not observed in DMPE. Both DMPC and DMPE have 14 carbon atoms in their two alkyl chains, only differing in their terminal amino group, being + N(CH₃)₃ in DMPC and +NH₃ in DMPE. The smaller size and the greater effective charge of the polar groups allow it to form a strong and compact network of electrostatic interactions and H-bonds that prevents the interactions of this lipid with the polyphenols.

4.2.2. Carotenoids

Carotenoids are the most widespread and important group of pigments, represented by more than 1200 structurally related compounds [103]. Carotenoids are classified into two large subgroups depending on the presence (xanthophylls, polar) or absence (carotenes, non-polar) of oxygenated functional groups [104,105]. Carotenoids are important modulators of the physical properties of lipid membranes [106]. Their influence is determined by the location and orientation of the pigment in the bilayer, which mainly depends on the length of the hydrophobic lipid core, the size of the polar headgroup and the structure of the carotenoid [107,108]. The effect of carotenoids on the phase transition, fluidity, and order of the lipid membrane has been observed to be stronger with dipolar carotenoids, significantly weaker with monopolar ones, and negligible with nonpolar carotenoids [109,110]. The effect of canthaxanthin (CAN), a dipolar carotenoid, on the T_p, T_m and ΔH_m was stronger in DMPC (14:0) compared to DPPC (16:0) and DSPC (18:0) [111]. In these lipids, CAN adopted a transmembrane orientation with its polar groups located in each opposite leaflet of the bilayers. However, because of the thinnest hydrophobic core of the DMPC, CAN adopted a much more tilted orientation with respect to the normal to the bilayer surface, which allowed a greater interaction of the carotenoid with the lipid acyl chains. Although CAN caused a pronounced effect on the thermotropic behaviour of DMPC, it did not produce significant effects on DMPE. This result was also recently reported with crocin (dipolar carotenoid) [112] and was attributed to the small size and compact arrangement of DMPE polar headgroups. Widomska et al. [110] showed that zeaxanthin incorporation decreased the T_p, T_m and cooperativity of DPPC and DMPC vesicles during the change from gel to liquid crystalline phase, in contrast to β -carotene (nonpolar) which had negligible effects. Similar results have been reported elsewhere [113–115].

Kostecka-Gugala et al. [116] conducted a systematic study on how different carotenoid structural elements affect the physical properties of membranes. Using MLVs prepared from α -dipalmitoylphosphatidylcholine and performing DSC measurements, it was shown that structurally different carotenoids have different effects on the

thermotropic phase behaviour of the studied liposomes. A broadening of both the pretransition and the main phase transition was observed, as well as their shift toward lower temperatures. The pretransition was specifically strongly affected, indicating a particularly strong effect of carotenoids on the membrane being in the gel phase. The element of the carotenoid structure with the greatest effect on modifying the thermotropic properties of the membrane appeared to be the rigid chain of conjugated double bonds. Carotenoids having ionone rings that contain polar functional groups, e.g. hydroxyl and/or epoxide, were also shown to affect the physical properties of membranes more strongly than carotenoids without polar groups in the rings (carotenes).

The comparison between the geometrical isomers showed that all-*trans* zeaxanthin had a greater effect on the thermal behaviour of DMPC than 9-*cis* and 13-*cis* zeaxanthin, while in DPPC the effect of the isomers was opposite. The *cis* and *trans* isomers of zeaxanthin differ in distance between their polar hydroxyl groups located on their opposite terminal rings, therefore, their effects strongly depend on the thickness of the hydrophobic region of the membrane [110].

In general, dipolar carotenoids adopt a transmembrane orientation and their effects on thermal properties depend mainly on the angle of tilt that the pigments adopt into the lipid bilayer. On the other hand, nonpolar carotenoids are homogeneously distributed in the membrane without a defined orientation, which significantly decreases their influence on lipid molecules during phase changes. The location and orientation of carotenoids in biological membranes allow them to modulate their properties, which have been correlated with their antioxidant and therapeutic effects [88,91,117].

4.3. Anticancer drugs

Anticancer drugs are widely used for the treatment of cancer. These drugs target fast-growing cells, depending on their structure they act through different mechanisms leading to apoptosis [118]. In general, these drugs have intracellular targets and must cross the plasma membrane to exert their pharmacological activity. Therefore, the study of anticancer drugs - membrane interactions allow a better understanding of the partition, orientation, and conformation of these drugs in membranes, which determine important aspects such as transport, distribution, accumulation, and efficacy [119,120]. It has been found that some anticancer drugs, including plant-derived, conventional chemotherapeutic, and membrane-targeted drugs, can alter the thermal properties of the lipid bilayer; the main phase transition enthalpy and main phase transition temperature, as well as the organization of lipid domains, fluidity, and mobility of the cell membrane are some of the effects induced by these drugs. These changes have been extensively investigated using DSC [118,119].

4.3.1. Plant-derived anticancer drugs

Among the main classes of plant-derived anticancer drugs are those that stabilize or destabilize microtubule dynamics known as antimitotic drugs, being the most important Vinca alkaloids and taxanes [121]. Vinca alkaloids were initially isolated from the Periwinkle plant (*Cathartus roseus*) and have been used effectively as monotherapy or in combination with other drugs for the treatment of leukemias, advanced testicular cancer, Hodgkin's disease, lymphomas, breast cancer and lung cancer, as well as Kaposi's sarcoma [122]. The increased concentration of vinca alkaloids has been reported to alter the thermodynamic properties of the membrane, causing destabilization of the lipid bilayer. Maswadeh et al. [123] and Koukoulitsa et al. [124] studied the interaction of vinblastine and vinorelbine with DPPC membranes by DSC. They found that the addition of 17 mol % of vinblastine or vinorelbine caused the elimination of DPPC pretransition, which was related to interactions between drug molecules and lipid head groups. Furthermore, they observed a marked increase in the ΔH_m (approx. 20 %), which was attributed to partial interdigitation of the acyl chains caused by an increase in the lateral area between the lipid head groups

accompanied by the formation of voids in the hydrophobic core of the bilayer.

Taxanes are anticancer drugs initially isolated from the bark of the Pacific yew tree (*Taxus brevifolia*) [125], which stabilize microtubules and inhibit their depolymerization and disassembly, leading to cell death by apoptosis [126]. Taxanes are used in the treatment of a broad spectrum of disease, especially ovarian [127,128], breast [129], non-small cell lung [130], gastric cancer [131] and Kaposi sarcoma [132]. Incorporation of taxanes into membrane model systems has been reported to induce changes in the physical and thermotropic properties of the lipid bilayer, including changes in phase transition temperatures and lipid order parameter [133,134]. Different researchers have used DSC to study the incorporation of paclitaxel, the main taxane anticancer drug, into membrane model systems. Zhao et al. [135] found that 0.5 mol % paclitaxel suppressed DPPC membrane pretransition and induced broadening of the main phase transition peak, slightly shifting T_m toward lower values. The authors concluded that paclitaxel was preferentially inserted into the hydrophobic zone of the bilayer, particularly in the C1–C8 region of the acyl chains, through van der Waals interactions; however, paclitaxel also established interactions with polar head groups of the lipids [135–137]. On the other hand, the influence of the length of the lipid chain on the interactions of paclitaxel with the bilayers of DMPC, DPPC, and 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) was studied by DSC and it was shown that paclitaxel had a higher ability to partition into bilayers of phospholipids of shorter chain length [136]. Similarly, Zhao et al. [137] studied the effect of lipid chain unsaturation and head group on paclitaxel incorporation into model membrane systems; for this purpose, they used bilayers of DPPC, 1,2-dierucoyl-sn-glycero-3-phosphocholine (DEPC), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE) and DSPC and observed that the thermotropic properties of DPPC were altered to a greater extent than DPPE bilayers. This is since DPPE has a protonated amino group in its head group that allows it to establish a network of H bonds with neighbouring molecules, making it difficult for paclitaxel to incorporate into the bilayer. The authors concluded that paclitaxel showed a higher affinity for DPPC than DPPE. On the other hand, when DSPC and DEPC were compared, they showed that the incorporation of small amounts of paclitaxel in DEPC bilayers caused large perturbations in their thermotropic properties, including abolition of the pretransition, broadening of the main transition peak, and shift of the T_m toward lower values with respect to saturated lipids.

Similar results were reported for docetaxel, a semisynthetic analogue of paclitaxel. Incorporation of docetaxel into DMPC and DPPC bilayers perturbed the thermotropic and structural parameters of the bilayers, leading to abolition of pretransition, shift of T_m towards lower temperatures and resulting in phase separations in the presence of higher drug concentrations [138,139].

4.3.2. Conventional anticancer drugs

Anthracyclines antibiotics, like doxorubicin, epirubicin and daunorubicin are cytotoxic that have been widely used in the treatment of different types of cancer such as breast, ovarian, bladder, gastric lymphomas, acute leukemias and multiple myelomas [119,140]. Recently Petit et al. [141], studied the effect of epirubicin on DMPC and DMPE bilayers, and found that with increasing drug concentration the thermotropic behavior of the lipids was disturbed (Fig. 10). Epirubicin partitioning in the bilayers induced the decrease of DMPC pretransition (Fig. 10 A), and the broadening of the main transition peak and the shift of the T_m to a lower temperature value for both phospholipids. Mady et al. [142] reported similar results for anthracycline doxorubicin, which caused a significant effect on the hydrophobic core of DPPC bilayers, inducing a decrease in the transition cooperativity of the lipid acyl chains and leading to the formation of lateral domains at higher concentrations [115]. On the basis of these results, the authors concluded that anthracyclines could be inserted into lipid bilayers and establish two types of interactions because of their amphiphilic

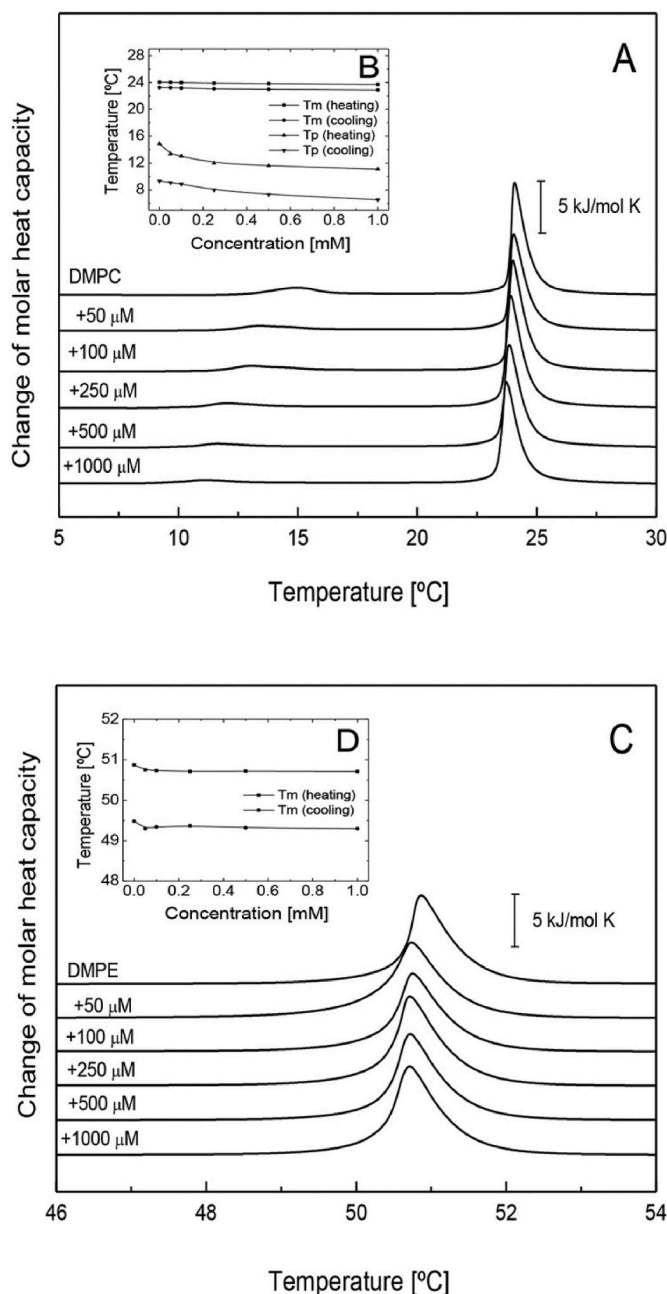


Fig. 10. DSC thermograms during heating for multilamellar liposomes composed of (A) dimyristoylphosphatidylcholine (DMPC) and (B) dimyristoylphosphatidylethanolamine (DMPE), which incorporate varying concentrations of epirubicin. The figure also includes graphs for the phase transition temperatures of (C) DMPC and (D) DMPE multilamellar liposomes, calculated from both heating and cooling cycles and plotted against the concentration of epirubicin. These measurements were taken at a constant heating rate of 1 °C/min, where T_m represents the main transition temperature, and T_p indicates the pretransition temperature. Reproduced with permission from Ref. [141].

structure: the positively charged amino sugar fraction interacts electrostatically with the polar head groups, while the dihydroxyanthraquinone ring would insert into the hydrophobic zone of the membrane, disturbing the acyl chains. On the other hand, it has been reported that tamoxifen, a non-steroidal antiestrogenic agent widely used in the treatment of breast cancer [143], affected the main phase transition and fluidity of membranes [144,145]. Studies also revealed that increasing tamoxifen concentration resulted in the disappearance of pretransition, broadening of the main phase transition peak and shift of

DMPC, DPPC and DSPC T_m to lower values, indicative of destabilization of the hydrophobic bilayer core and intercalation of the drug in this region of the membrane [146,147].

4.3.3. Membrane targeted anticancer drugs

Although most anticancer drugs exert their cytotoxic action directly on DNA or proteins, in recent years evidence reveals the important role of membrane lipids in the regulation of numerous fundamental cellular functions such as signal transduction and in the activity of membrane proteins; thus, membrane lipids emerge as a molecular target for the development of anticancer drugs associated with modulation of the biophysical properties of the cell membrane [148,149]. In fact, a new class of anticancer drugs analogues of membrane phospholipids called alkylphospholipids has been developed. Through the modulation of membrane permeability and fluidity, phospholipid metabolism, and proliferation signal transduction, these compounds act on cell membranes. It has been demonstrated that these drugs induce selective apoptosis in tumour cells and animal models [150,151]. Biophysics techniques such as fluorescence, X-ray diffraction, and DSC have been used to study the interaction of alkylphospholipids in cell membrane models. Recently, the effect of miltefosine, an alkylphospholipid used in the local treatment of cutaneous metastases of breast cancer [152], was studied by DSC in DMPC and DMPE. The results showed that the incorporation of the drug into DMPC caused the abolition of pretransition, decrease of ΔH_m , accompanied by phase separation and the formation of lateral domains above 0.25 mM miltefosine. Furthermore, the presence of miltefosine destabilized the cooperativity of the DMPE phase transition which was evidenced by a decrease and broadening the main transition peak and by the shift of T_m toward lower temperatures [153]. Similar outcomes were reported after the addition of miltefosine in DPPC [154]. From these results, it was concluded that due to its amphiphilic structure, miltefosine inserts into the lipid bilayer with its acyl chains parallel to the hydrophobic core of the membrane while its hydrophilic moiety establishes electrostatic interactions with the polar head groups of the phospholipids.

On the other hand, DSC was used to study the effect of incorporation of 10-(Octyloxy) decyl-2-(trimethylammonium) ethyl phosphate (ODPC), an alkylphospholipid highly effective against various tumour cell lines including human brain tumour and human leukemic cell lines, into DMPC and DMPC: cholesterol [155]. The results indicated that ODPC perturbed DMPC inducing a reduction in the variation of ΔH_m and T_m in a concentration-dependent manner. Furthermore, these perturbations were observed to be greater in the DPPC: cholesterol, which is consistent with the hypothesis that alkylphospholipids preferentially interact with highly organized cholesterol-rich lipid domains present in cell membranes.

4.4. Anti-Alzheimer drugs and drug-candidates

Characterized by the deterioration of memory and a decline in cognitive functions, Alzheimer's disease (AD) is a progressively worsening neurodegenerative condition [156,157]. Despite significant advances in understanding the cause of disease, treatment remains a considerable challenge. Currently, drugs such as memantine, rivastigmine, and donepezil are used to treat the cognitive and behavioural symptoms of the disease [157]. Memantine, an NMDA receptor antagonist, is used to treat moderate to severe symptoms of AD [158]. On the other hand, rivastigmine and donepezil, acetylcholinesterase inhibitors, are used to treat mild to moderate symptoms [159]. Despite their therapeutic effects, the exact mechanism by which they act remains unknown. There has been a limited focus on the interactions between these drugs and cellular membrane components and how these interactions may impact their pharmacological activity. Within this context, the present review aims to provide an analysis of recent studies that shed light on the specific interactions between memantine, rivastigmine, and donepezil and cellular membrane phospholipids.

Zambrano et al. conducted studies on the thermotropic behavior of memantine [160], rivastigmine [161], and donepezil [162] on multilamellar vesicles (MLVs) of DMPC and DMPE. In the first study, memantine showed a notable interaction with DMPC vesicles, altering their thermotropic behavior. This was manifested in a shift of the main phase transition and pretransition as a function of memantine concentration. These results were experimentally supported by additional experiments by X-ray diffraction of DMPC multibilayers, in which a substantial interaction was also observed [160]. However, in DMPE vesicles, memantine had a minor effect, with a transition temperature shift not exceeding 0.1 °C. This suggests that memantine may interact differently with different types of phospholipids. In a second study, rivastigmine in the concentration range of 0–500 μ M did not induce significant changes in the main phase transition of DMPC vesicles [161]. At a concentration of 1 mM, a significant decrease in the area under the curve of the main phase transition was observed, indicating a change in the cooperativity of the phospholipid during this phase transition [115]. The term cooperativity is used to denote the number of acyl chains that can transition from the gel phase to the liquid crystalline phase [163]. The DMPC pretransition was strongly affected by the presence of rivastigmine, causing a shift in the pretransition temperature. In DMPE vesicles, rivastigmine had a mild effect on thermotropic behavior [161]. On the other hand, a third study was focused on donepezil [162]. The addition of this molecule to DMPC altered its thermotropic behavior leading to a substantial decrease in the main phase transition temperature towards lower values and complete abolition of the pretransition peak. In DMPE MLVs, donepezil had a moderate effect, although an increase in the width of the base of the peak was observed at a concentration of 1 mM [162].

In recent studies, Zambrano et al. have extended the use of DSC to molecules with a high potential to be considered drug candidates for the treatment of AD. Such is the case with a donepezil-huprine hybrid (AVCRI104P4 [164,165]) and a rhein-huprine hybrid (RHE-HUP [166]), both of which induced a similar thermotropic behavior in DMPC and DMPE multilamellar vesicles.

In summary, these studies provide invaluable initial insight into how drugs used in Alzheimer's disease treatment interact with the phospholipids of cellular membranes. However, there are numerous promising directions for future research that could provide a more comprehensive and nuanced understanding of these interactions and their implications for Alzheimer's disease treatment.

5. Perspectives

Drug - membrane interactions are crucial in the search for deciphering the mechanisms of action of already approved drugs, and in improving the properties of new materials. DSC has played a fundamental role in understanding the properties of these molecules and in elucidating the mechanisms of action of a large number of them, whose main function lies in interaction with cellular membranes. From a technological perspective, the DSC technique has faced no major obstacles for its automated development or for the creation of new protocols. Current technological tools have partially overcome the technical challenges that existed in the field of biologically oriented calorimetric sciences a decade ago. Currently, calorimeters and equipment have high sensitivity, enough for most biochemical or biological experiments. In addition, advances in computing and artificial intelligence have allowed the rapid development of software that makes scientific activity in the field of calorimetry more comprehensible and manageable.

Calorimetric experiments with biological membranes or multilamellar vesicles of different phospholipids can be carried out in an automated manner, saving significant time. The implementation of automation in almost all calorimetric devices has made a significant contribution to science, allowing researchers to carry out a variety of experimental projects in a relatively short time. However, the main challenges of applying DSC to the study of molecule-membrane

interaction lie in the appropriate choice of membrane models to use and the type of phospholipid (and membrane model) with which the experiments will be carried out. Phospholipid systems composed of a single species of molecules have great experimental advantages, but they do not exactly reflect the biophysical and chemical properties of natural membranes. Therefore, two major experimental challenges arise: (i) to find a suitable membrane model (SUVs, MLVs, etc.) that is most suitable for DSC experiments and (ii) to find the most suitable and representative lipid composition of the natural system to explore. The latter could be achieved by mixing different types of phospholipids, with different chain lengths, unsaturation grade, etc. However, not all phospholipid combinations are suitable for calorimetry assays, so many screening experiments are necessary to find favourable conditions.

In the case of the molecules discussed in this review, it is necessary to implement membrane models more similar to the membranes of neurons in the case of anti-Alzheimer drug interactions. Similarly, the composition and physical properties of the cellular membranes of cancer cells differ significantly from those of a normal cell, so it is essential to explore biophysical methods of vesicle preparation (unilamellar and multilamellar) that reflect that scenario as faithfully as possible.

In general, the study of drug interactions with cellular membranes through DSC has been a fundamental pillar in the field of membrane biophysics, generating a vast amount of knowledge regarding the thermotropic behavior of a wide variety of phospholipids and materials. To enhance these investigations, the exploration of phospholipids beyond DMPC and DMPE could be considered, given that cellular membranes encompass a variety of distinct phospholipids. In addition, supplementary studies could be conducted to discern how these interactions with phospholipids translate into effects at the cellular and tissue levels. It would also be beneficial to examine how these interactions may be influenced by other factors such as pH or the presence of other compounds in the cellular environment, yielding more realistic results at the molecular level.

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CRediT authorship contribution statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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