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ORIGINAL ARTICLE

Interaction of articaine hydrochloride with prokaryotic membrane lipids

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Abstract

Objective. Local anesthetics are the most commonly used drugs in dentistry, with a wide range of effects, including antimicrobial activity. High antimicrobial effects have recently been reported on oral microbes from articaine hydrochloride, revealed by the minimum inhibitory concentration and minimal bactericidal concentration. Additionally, articaine has recently been used as an alkaline component in endodontic materials with a proposed antibacterial activity. However, the detailed mechanisms of action have not been discussed. ***Material and Methods***. We determined the Langmuir surface pressure/molecular area isotherms of prokaryotic lipid monolayers, as well as the phospholipid phase transitions, by employing differential scanning calorimetry on unilamellar prokaryotic liposomes (bilayers). ***Results***. Articaine hydrochloride was found to interact with the prokaryotic membrane lipids in both monolayers and bilayers. An increase of the phospholipid molecular area of acidic glycerophospholipids as well as a decrease in phase transition temperature and enthalpy were found with increasing articaine hydrochloride concentration. The thermodynamic changes by adding articaine hydrochloride to prokaryotic membrane lipids are potentially related to the effects observed from antimicrobial peptides resulting from membrane insertion, aggregate composition, pore formation, and lysis. ***Conclusion***. Interaction of articaine hydrochloride with prokaryotic membrane lipids is indicated. Hence, further research is necessary to gain insight into where these compounds exert their effects at the molecular level.

Key Words: *Articaine, calorimetry, Langmuir, lipids*

Introduction

Local anesthetics (LAs) are the most commonly used drugs in dentistry. The molecular structure of most dental applied anesthetics is similar, amphiphilic and cationic, having a lipophilic and hydrophilic part, and joined together by an ester or amide linkage component. Accumulating data suggest that LAs have a wide range of anti-inflammatory actions through their effects on the cells of the immune system, as well as on other cells, e.g. thrombocytes, erythrocytes and micro-organisms [1]. The potent anti-inflammatory properties of LAs, superior in several respects to traditional anti-inflammatory agents of the NSAIDs and steroid groups, and with fewer side effects, have prompted clinicians to introduce them in the treatment of various inflammation-related conditions and diseases. The mechanisms of action are not fully understood, but seem to involve a reversible interaction with membrane proteins and lipids.

A widely used LA in dentistry is articaine hydrochloride. Synthesized in 1969, it was offered for clinical use in Germany in 1976, in the USA in 2000 and in Norway in 2001. Articaine hydrochloride (4-methyl-3 [2-(propyl-amino) propion-amido] thiopene-2-carboxylic, acid methyl ester hydrochloride), with molecular wt. 320.84, contains an ester bond and a thiophene ring (Figure 1). Recently, the antibacterial activity of the LAs used in dental analgesia has been analyzed involving articaine hydrochloride [2]. Seven LAs and their active anesthetic components were tested for their antimicrobial activity against 311 bacterial strains from 52 different species and 14 Candida albicans strains. The pathogens tested were members of the oral flora, and some members from the skin and intestinal flora. The trade preparation Ultracaine D-S®,

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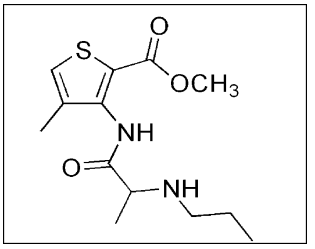

Figure 1. Structure formula of articaine.

with its active substance articaine hydrochloride, showed the most prominent antimicrobial activity with regard to both minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC). An antibacterial activity from articaine by adding the compound to endodontic materials has recently been proposed. Tetrasilicate cements have been developed to obtain materials characterized by a combination of biocompatibility and adequate handling characteristics for use in different clinical conditions, i.e. orthograde endodontic filling materials. Cements were mixed with 4% articaine as an alternative to sterile water in an alkaline pH (11–12.5). The report questions whether the high pH might account for the suggested antibacterial activity of the material [3].

Many types of drugs share an amphiphilic molecular structure, including antimicrobial peptides. The molecular mechanisms of action of amphiphilic drugs appear to be versatile. Antimicrobial peptides may have a direct effect on the microbe, such as by damaging or destabilizing the bacterial, viral or fungal membrane, or by acting on other targets. Accordingly, they appear to be involved in orchestration of the innate immune and inflammatory responses. Antimicrobial peptides are known for their ability to interact with lipid membranes, resulting in aggregate composition, pore formation, and lysis. The higher proportion of negatively charged lipids on the surface monolayer of the bacterial cytoplasmic membranes is important in the selectivity of antimicrobial cationic peptides for bacterial cells over eukaryotic cells [4]. Accordingly, there are many reasons for elucidating the mode of bacterial model membrane action of the amphiphilic, cationic LA drug articaine hydrochloride.

Several approaches are possible, with the aim of studying interactions between a give effector and cell membranes. One can work with living cells and observe what happens *in situ*, or one can isolate cellular membranes. Natural membranes are complex entities with a great variety of lipids and proteins. If interested in specific aspects of a given biologic phenomenon occurring at membrane level, the best choice is to use membrane models [5]. A large number of approaches have been used in evaluating the great diversity of structural and functional modifications at membrane level [6–9]. The function of the biological membrane cannot be understood without consideration of its thermodynamics [10]. To our knowledge, this is the first report on the molecular antibacterial mode of action from articaine hydrochloride evaluated by thermodynamic parameters.

The aims and hypotheses of the present study were to investigate a prokaryotic membrane lipid mode of action of articaine hydrochloride. Interaction between articaine hydrochloride and negatively charged membrane lipids (i.e. prokaryotic membrane lipids) was investigated. Phosphatidylserines, the predominant anionic species [11], were used as monolayers by the Langmuir technique and as unilamellar liposomes by differential scanning calorimetry [12–14].

Material and methods

Chemicals

1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphoserine (16:0/18:1) and 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (18:0/18:1) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Articaine hydrochloride (powder) was a gift from Denamed AS (Strømmen, Norway).

Monolayer technique

The main parameters, temperature, surface pressure (π), and mean molecular surface area characterize the film state of a given substance spread on an aqueous sub-phase. These parameters are expressed as area per molecule, A. Surface pressure-area ($\pi - A$) isotherm of a monolayer constitutes essential characterization of the film properties. Trough and barriers are made of Teflon, and the entire device rests on an antivibration plate placed inside a thermostatted box. Our experiments were performed using an M 1000 Minitrough ($75 \times 364 \times 5$ mm) made of Teflon (KSV Instruments, Helsinki, Finland). All experiments were done at 20°C or 37°C with two movable barriers and constant speed (5 mm/min). Surface pressure was measured using a Wilhelmy plate detector and an electric balance. Lipids were dissolved in chloroform (1 mg/ml), applied in droplets on either side of the Wilhelmy plate using a Hamilton pipette, and solvents allowed to evaporate before compression of the monolayer. Control Samples of 20 µl were used. Milli-Q water (pH 5.6) or Ringer solution (0.15M NaCl, 5.6mM KCl, 1.7mM $CaCl_2$, pH 7.4) were used as subphases. Samples of 40 µl were used. All experiments were repeated six times and the median value was chosen for statistics.

Liposome preparation

Liposomes were made of glycerophospholipids dissolved in chloroform, lyophilized and allowed to dry overnight. Various concentration of LAs were added in 1.5 ml Milli-Q water to give a 4mM lipid suspension, which contains multilamellar liposomes, and sonicated for 5 min. Unilamellar liposomes were obtained by freeze-thawing six times.

Differential scanning calorimetry

Differential scanning calorimetry measures heat absorbed (or released) by a sample as it undergoes endothermic (or exothermic) phase transition. Parameters of interest are (Table I): 1) area under the transition peak, which is proportional to the enthalpy of the transition; 2) width of transition at half peak height, which gives a measure of the "cooperativity" of transition; and 3) transition temperature itself. The enthalpy of transition describes energy required for melting acyl chains, while cooperativity reflects the number of molecules that undergo a transition simultaneously.

Measurements were performed with liposomes suspended in Milli-Q water with a Microcal VP-DSC differential scanning calorimeter (MicroCal LLC, Northampton, Mass., USA) with cell volumes of 0.5 ml at the indicated scan rates. Milli-Q water was used as the reference. All samples were degassed and the calorimetric cell was kept under an excess pressure of 30 psi to prevent degassing during the scan. A scanning rate of 1.5°C/min was used for all samples. Original scans were processed by subtraction of the Milli-Q water baseline, and further correction by defining a progress baseline from the pre- to the post-transition regions using the Origin TM software (MicroCal LLC) provided with the instrument. Each experiment was repeated three times.

Table I. Effect of articaine hydrochloride on differential scanning calorimetry values (median): transition temperature, half-width, and enthalpy change for 1-stearoyl-2-oleyl-*sn*-glycero-3-phosphoserine and 1-palmitoyl -2-oleyl-*sn*-glycero-3-phosphoserine ($n = 6$)

	T_m(°C)	$T_{C1/2}$(°C)	△H(kJ/mol)
POPS			
Control	11.43	2.80	25.50
10 μg/ml articaine	11.24	2.80	25.32
50 μg/ml articaine	10.27	3.24	20.85
100 μg/ml articaine	9.43	3.99	15.84
250 μg/ml articaine	8.46		
SOPS			
Control	17.76	2.99	33.15
10 μg/ml articaine	17.71	3.37	33.27
50 μg/ml articaine	17.61	3.99	29.28
100 μg/ml articaine	16.93	4.25	16.11
250 μg/ml articaine	13.72		

Results

Monolayer studies

1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphoserine and 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphoserine were used for monolayer studies, representing prokaryotic membrane lipids. The presence of articaine hydrochloride increased molecular areas of 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphoserine at 30 mN/m surface pressure, especially with the Milli-Q water (pH =

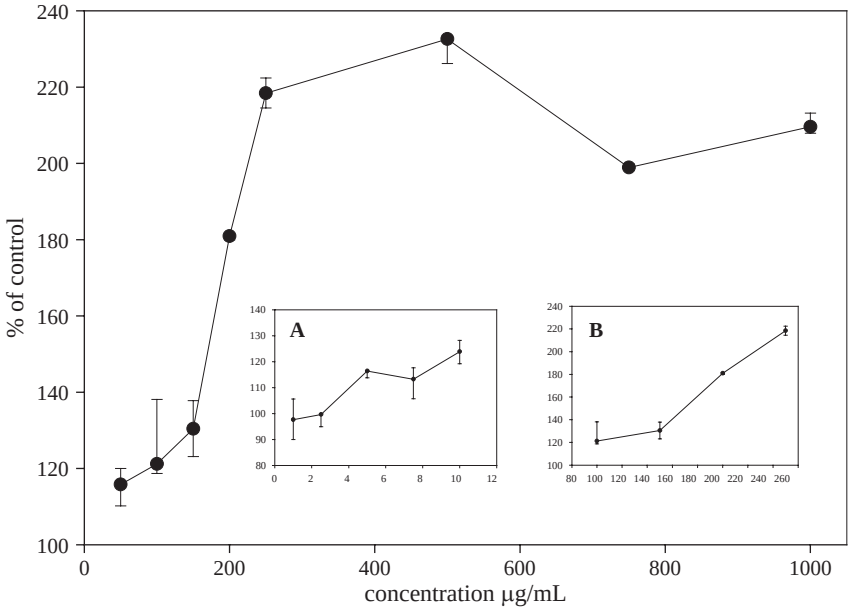

Figure 2. The effects of adding articaine hydrochloride to 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphoserine monolayers. Results are presented as the difference in molecular area at a surface tension of 30 mM/m between control 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphoserine and added samples of different concentrations of articaine hydrochloride at a sub-phase of 37°C Milli-Q water. Insets (% of control and concentration on axis) show plots on articaine hydrochloride at 1–10 μg/ml (A) and articaine hydrochloride at 100–250 μg/ml (B). Minimum and maximum values are indicated ($n = 6$).

5.6) sub-phase at 37°C (Figure 2), and in the concentration range 150–250 μg/ml (Figure 2B). 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphoserine added with articaine hydrochloride on the sub-phase milli-Q water at 37°C demonstrated a higher increase of area, i.e. 220% of control at 250 μg/ml (Figure 2), compared to the sub-phase Milli-Q water at 20°C, i.e. 160% of control at 250 μg/ml (data not shown) or a sub-phase of Ringer solution at 37°C, i.e. 120% of control at 250 μg/ml (data not shown). Inclusion of articaine hydrochloride in 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphoserine monolayers on Milli-Q water sub-phases at 37°C resulted in a smaller increase in the surface area compared to 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphoserine (data not shown).

Calorimetry

1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphoserine and 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphoserine were used in this method with articaine hydrochloride present in the range of 10–250 μg/ml. The main transition of these lipids exhibited a concentration-dependent decrease (Figures 3 and 4). The presence of articaine hydrochloride produced a successive reduction of enthalpy with increasing concentrations from 10 μg/ml (Table I) (Figure 3). Enthalpy was about half the value of control at 100 μg/ml for 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (Table I) and about 60% for 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphoserine and, i.e. 16.11 versus 33.15 and 15.84 versus 25.50, respectively. At 250 μg/ml, articaine hydrochloride concentration baseline could not be determined with great enough consistency to calculate the enthalpy (Figures 3 and 4). 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphoserine and 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphoserine added with articaine hydrochloride showed a marked reduction

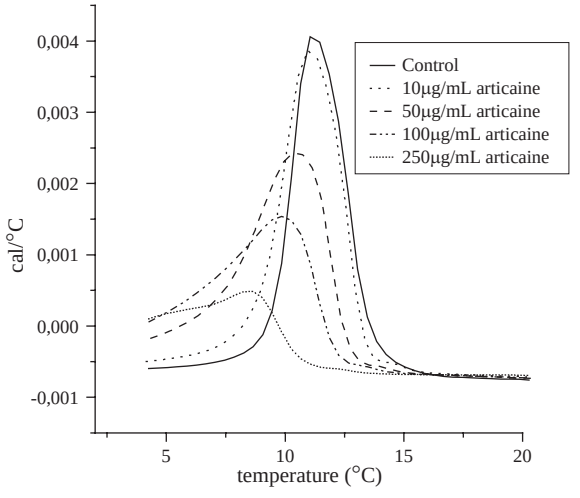

Figure 3. Differential scanning calorimetry thermograms obtained from liposomes. Articaine hydrochloride (10–250 μg/ml) added to 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphoserine (control).

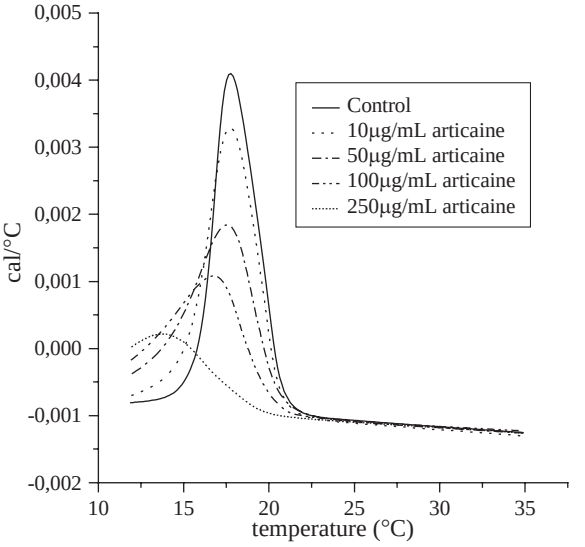

Figure 4. Differential scanning calorimetry thermograms obtained from liposomes. Articaine hydrochloride (10–250 μg/ml) added to 1-stearoyl-2-oleyl-*sn*-glycero-3-phosphoserine (control).

of enthalpy with increasing concentrations from 50 μg/ml (Table I) (Figures 3 and 4). An increase in width of transition of half peak height from 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphoserine and 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphoserine with increasing articaine hydrochloride concentrations was observed (Table I) (Figures 3 and 4). Melting temperature was considerably decreased with articaine hydrochloride concentrations of 250 μg/ml compared to control 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphoserine and 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphoserine, 8.46°C versus 11.43°C and 13.72°C versus 17.76°C, respectively (Table I) (Figures 3 and 4).

Discussion

Most of the LAs now in dental use are amphiphilic molecules, which potentially may interact with membranes and causing a variety of effects. The function of the biological membrane cannot be understood without consideration of its thermodynamics. Membrane lipids may undergo order or melting transitions. Application of pressure has pronounced effects on lipid melting. Pressure may be generated by a compound binding to membrane lipids. Compounds binding strongly to the surface generate a large lateral pressure, and can lead to insertion in the membrane monolayer. Such binding insertion phenomena occur mostly with peptides that are partially hydrophobic and partially hydrophilic, i.e. amphiphilic compounds. Prominent examples are mellitin, alamethicin and margainin [10]. In the present study, the amphiphilic LA compound articaine hydrochloride was found to interact with prokaryotic membrane lipids, both in monolayers

and liposomes (bilayers), revealed by the change in thermodynamic parameters.

Considerable advances in our understanding of the effects of drugs on structural and dynamical properties of membrane lipid components have been achieved using spectroscopic and biophysical techniques such as monolayer isotherms and differential scanning calorimetry. Heat capacity measurements and monolayer are complementary useful methods when studying adsorption, binding and insertion of drugs into model membranes. The monolayer technique mimics a single membrane leaflet. It is thus possible to explore potential relationships between the lateral pressure and apparent model membrane lipid area. The region where small pressure changes result in large area changes corresponds to the chain melting regime, where lateral compressibility displays a maximum. Lateral pressure is measured with the Wilhelmy balance, i.e. a small metal rod sensing the surface tension of the film. In our study, prokaryotic membrane lipids made from phosphatidylserine were used with either Milli-Q water or Ringer solution as sub-phases. Since bilayer surface pressure in cell membranes has been reported to be 30–35 mN/m [15], we calculated the difference in corresponding molecular area between added and non-added phosphatidylserine monolayers at 30 mN/m. Articaine hydrochloride induced a considerable increase in molecular area (Figure 2). Previous reports demonstrate that amphiphilic drugs, including LAs, may induce an increased molecular area of glycerophospholipid by the monolayer method [16–18]. Concerning applied drug concentrations in the latter study, it is more like ours, and much lower compared to previous reports on LAs [16,17]. Our phosphatidylserine area increased from 4 nm^2 to 9 nm^2 when 500 μg/ml articaine hydrochloride (i.e. 75 μM) was added, in accordance with the effect from 100 μM chlorpromazine on phosphatidylserine corresponding to an increase from 5 nm^2 to 9 nm^2 [18]. At low concentrations (i.e. 1–10 μg/ml) (Figure 2A), one might propose that an amphiphilic compound such as articaine hydrochloride binds peripherally to the membrane, while they are inserted in the membrane at higher concentrations [10] (Figure 2). Along with the findings of aggregate composition and pore formation in membranes from antimicrobial peptides [19], aggregates of articaine hydrochloride have been suggested to occur by interacting with a phospholipid bilayer [20].

There are two main discrepancies between previous studies on differential scanning calorimetry (DSC)/LAs and the present study. First, the applied lipids are dissimilar. Ueda et al. [21] and Hata et al. [22] employed a classic three-stage model of membrane solubilization, including mixed membranes, membrane-micelle coexistence, and mixed micelles. In our study, unilamellar liposomes mimicking phospholipids from biologic membranes were applied. Second, maximum added amounts of LAs in our report were 1/200 compared to Hata et al. [22]. Nevertheless, fluorescence polarization experiments performed with ropivacaine and bupivacaine [23] have demonstrated, in comparable concentrations to ours, that LAs may fluidize membranes at concentrations lower than those in clinical use. Unilamellar liposomes undergo a phase transition when heated, and the thermodynamic parameters of enthalpy and heat capacity change are measured directly by DSC. Drugs such as general anesthetics, antibiotics, and neurotransmitters have been shown to interact with membranes, and affect the melting points and profiles.

DSC reports on LAs are sparse. In our study, the DSC results demonstrated a concentration-dependent change. A comparable change of the DSC thermogram has been revealed by the addition of compounds such as tocopherol [24], abscisic acid [25], cholesterol [26], and cholesterol/vinblastine [27]. A decreased main transition temperature of di-saturated palmitoyl-phosphatidyl-choline bilayer membranes has previously been found from LAs [22]. In our study, the melting temperature of membrane lipids was significantly lowered (3–4°C) when adding articaine hydrochloride at 250 μg/ml. Our experimental observations may be predicted by thermodynamic theories [10]. Accordingly, a change in the lateral pressure distribution within the membrane is promoted by the insertion of articaine hydrochloride. Therefore, the compound lowers the melting point of membrane lipids. The change in melting temperature is a linear function of the amount of the molecule in the model membrane, which is again a linear function of the partition coefficient. Our report does not directly present data on partitioning. However, the T_m decreasing tendency (Table I) is in accordance with a report from Heimburg & Jackson suggesting that the solubility of small solutes in lipid membranes is directly related to the depression of T_m [28]. Hence, the molecule is readily soluble in the fluid phase of the membrane lipids but insoluble in the gel phase, and a depression of the lipid melting point makes more membrane lipids readily acceptable for the articaine molecules. Additionally, lowering the pH typically increases the melting temperature due to protonation of negatively charged membrane phospholipid phosphate groups [10]. Accordingly, in a clinical situation more antibacterial activity may be introduced either by increasing the concentration of articaine hydrochloride or alkalizing the drug solution (i.e. in endodontic cements). At a pH of 10.0, more than 99% of the articaine molecules are in the uncharged form (pKa of 7.8) [29]; articaine is more hydrophobic [30,31] and more potent [32,33] for membrane lipid interactions than the charged species revealed in our late ^{13}C and ^{31}P solid-state NMR study [20].

Regarding antimicrobial effects, Pelz et al. reported MIC and MBC values from articaine hydrochloride of about 80 μg/ml (i.e. 12 μM). This concentration is in correspondence with reported MIC values from different types of antimicrobial peptides against *E. coli* and *S. aureus* [34], and, compared to our thermodynamic values on DSC (i.e. enthalpy changes) of between 50 and 100 μg/ml of articaine hydrochloride (i.e. 7.5–15 μM), quite in accordance. Thus, indicating an interaction between articaine hydrochloride and prokaryotic membrane lipids, influencing thermodynamic parameters and demonstrating antimicrobial effects at the same level of concentration. Further research is needed to evaluate the antimicrobial effect on a biofilm model from articaine hydrochloride, and additional studies on bioinformatics and atomic force microscopy are in progress to reveal articaine hydrochloride prokaryotic membrane lipid interactions.

The increase in mean molecular area by the inclusion of increasing concentrations of articaine does not mean that the area for each phospholipid molecule increases. It means that the horizontal area of many monolayer phospholipid molecules increases. The most plausible explanation for this increase is by intercalation of articaine between the lipid molecules in the monolayer. This is strongly supported by our calorimetric findings: Both the T_m and the enthalpy decreased by inclusion of increasing amounts of articaine in the liposomes, strongly indicating that articaine and the lipid molecules in the bilayer made an ideal mixture, as if articaine was intercalated between the lipid molecules.

To conclude, articaine hydrochloride demonstrated an increase of molecular area in the monolayer experiments, and a decrease in phase transition temperature and enthalpy. Thus, an interaction of articaine hydrochloride with prokaryotic membrane lipids is indicated. Further research is necessary to gain insight into how these compounds exert their effects at the molecular level.

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