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Location of chlorhexidine in DMPC model membranes: a neutron diffraction study

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ABSTRACT

Chlorhexidine (CHX) is an effective anti-bacterial agent whose mode of action is thought to be the disruption of the cell membrane. It is known to partition into phospholipid bilayers of aqueous model-membrane preparations. Neutron diffraction data taken at 36 °C on the location of CHX in phosphatidylcholine (PC) bilayers is presented. The center of mass of the deuterated hydrocarbon chain of CHX is found to reside 16 Å from the center of the bilayer in 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine (14:0–14:0 PC). This places the drug near the glycerol backbone of the lipid, and suggests a mode of action whereby the molecule is bent in half and inserts wedge-like into the lipid matrix. This mechanism is distinct from detergent-like mechanisms of membrane disruption and more similar to some anti-microbial peptide action, where peptides insert obliquely into the bilayer headgroup region to disrupt its structure.

1. Introduction

An effective biocide, chlorhexidine (CHX, Fig. 1) is a drug used widely in antiseptic products, especially in hand-washing and oral products such as mouthwash, and has potential applications in textile technology (Gilbert and Moore, 2005; Blackburn et al., 2007; Chen et al., 2008). It is a well-known inactivating agent of many nonsporulating bacteria, with some sporostatic and mycobacteriostatic action; it is also effective against yeasts and protozoa (Russell and Day, 1993). With a minimum inhibitory concentration comparable to most antiseptics, CHX is an interesting exception in the field of the physical disruption of lipid membranes by the insertion by amphiphilic molecules, which has been dominated by peptides (e.g. polymyxins and α-helicies) and surfactants (e.g. quaternary ammonium compounds).

Initial investigations of CHX effectiveness as a bactericidal agent began over 40 years ago by Hugo and Longworth (1964), who noted that the uptake of the drug by bacteria was very quick, and its effectiveness was dependent on the concentration of CHX and pH. The uptake by bacteria and yeasts can occur within seconds, and a maximal effect of the drug occurs in as little as 20 s (Fitzgerald et al., 1989).

The path of CHX uptake is of interest, since it can reveal the drug's mode of action. Through hydrophobicity (hydrocarbon response) and antibacterial sensitivity tests, El-Moug et al. discovered that the drug initially causes considerable damage to the outer cell layers of bacteria or yeast (El-Moug et al., 1985). However, this damage was not thought to be a major cause of cell death, since the drug continues on to attack the cytoplasmic membrane of bacteria, or the plasma membrane in yeast. Hiom et al. studied changes in viability, changes in optical density, and looked at the leakage of intracellular components through spectrophotometry, and found that CHX partitions into the plasma membrane and cytoplasm of cells (Hiom et al., 1993). Though the mechanism of this trans-membrane transport is not clearly understood, it is followed by a leaking of intracellular components as a consequence of semipermeable membrane damage. At high concentrations of CHX, there is a coagulation of the intracellular components, followed by a reduction of leakage (Hugo and Longworth, 1964). Although leakage of the intracellular components increases with an increase in CHX concentration initially, beyond a certain critical point, the leakage is reduced as a result of this coagulation. Barrett-Bee et al. called this a "gelling" effect of the drug (Barrett-Bee et al., 1994), not to be confused with the gel phase of the membrane lipids.

Although early research of the mode of action of CHX described an inactivation of ATPase, more recent studies have concluded that it is the membrane disruption by CHX that is associated with its lethal effects. ATP concentrations are reduced by CHX, but it is a very slow process. It is now thought that the lethal effects are a result of the membrane disruption that occurs within seconds (Harold et al., 1969; Barrett-Bee et al., 1994; Kuyyakanond and Quesnel, 1992).

Biological observations show that CHX has little to no-effect on a number of bacterial spores, nor is it effective against many bacterio-

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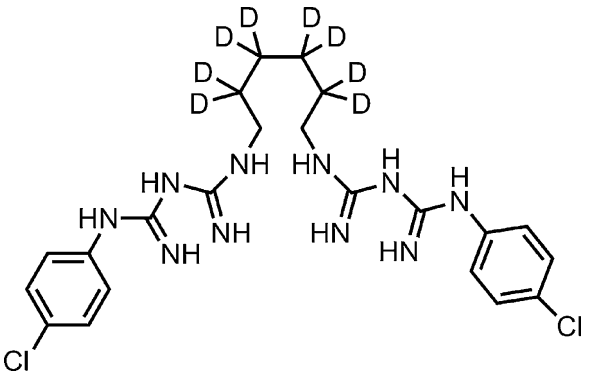

Fig. 1. Chlorhexidine hydrochloride, deuterium-labelled on the hexamethylene.

phages, and its antiviral action is limited to lipid-enveloped viruses (Russell and Day, 1993; Park and Park, 1989). There have been several studies on the effect of sub-lethal doses of CHX on metabolic processes on biofilms encountered in clinical and natural settings (Jones et al., 1997; Lawrence et al., 2008; Dynes et al., 2009). CHX creates morphological changes and tightly associates with the lipid effluent from treated *Pseudomonas flourescens*, and not the protein or lipopolysaccharide components (Dynes et al., 2009). The dose dependence of CHX against natural biofilms is indicated by damage to the plasma membrane (Castillo et al., 2006; Deng et al., 2009). In sub-lethal concentrations, incorporation of CHX drastically alters the lipid composition of *Candida albicans*, shifting the balance of plasma membrane lipids to saturated 16:0 and 18:0 acyl chains and non-polar headgroups, accompanied by a radical adjustment of the sterol content (Abu-Elteen and Whittaker, 1998). This has profound effects on membrane fluidity, indicative of CHX's lipophilic properties. Fluorescence anisotropy was also shown to decrease in the membrane of *Candida albicans* with increasing concentrations of CHX, which indicated that the drug decreased epithelial-cell membrane-lipid packing order (Audus et al., 1992).

A few biophysical experiments have established the lipophilic nature of CHX, but not its mode of action. The pronounced lipophilic properties of CHX are responsible for lowering the inter-facial tension of *n*-hexane/water much more than the air/water interface, and even to a greater degree than the 6-carbon acyl chain of 1-(*p*-chlorophenyl)-5-*n*-propylbiguanide (Heard and Ashworth, 1968). In a follow-up study, CHX exhibited strong association with stearic acid monolayers over stearyl alcohol or N-octadecylacetamide, showing that electrostatic (ionic) interactions also play a significant role in surface interactions (Fisher and Quintana, 1975). The related compound, poly-hexamethylene biguanide (PHMB) shows simply no interaction with neutral PC or PE phospholipids, but a strong preference for the anionic PG headgroup (Ikeda et al., 1983). This indicates a predominately ionic interaction, regardless of PHMB's lack of chlorophenol groups. Finally, compared with arginine based cationic surfactants, CHX's superior antimicrobial effectiveness correlates with its suppression and widening of chain melting transition temperature in DPPC as measured by scanning calorimetry (Castillo et al., 2004).

CHX presents an interesting biophysical modelling challenge. Structurally, CHX is of the family of N^1,N^5-substituted biguanides, in a bis-configuration with a hexamethylene connector, and chlorophenol rings at the ends. The hexamethylene confers a hydrophobic affinity, and the cationic nature of the bigaunide adds a hydrophilic and ionic component. In the literature, both characteristics have been separately advanced as the modes of CHX action against membranes; it is likely that both play important roles. Furthermore, the aromatic rings are lipophilic, since they are now accepted to be lipid/water interface anchoring motifs, especially in proteins (Zhang et al., 2003; Stopar et al., 2006; van der Wel et al., 2007).

The molecular structure in various environments has, to the best of our knowledge, not been highly investigated and there is no *a priori* reason to exclude most conceivable molecular configurations. The hexamethylene linker has the potential to stack the phenol rings through π-interactions, or to extend the molecule's full length to nearly the thickness of a membrane. The later configuration is immediately appealing, since the hexamethylene seems to be safely buried in a hydrophobic environment. However, upon closer inspection, it can be seen that it is not quite long enough to raise all of the amines above the hydrophobic region, which would be energetically unfavourable. Although the pK_a of CHX in various solvents has been measured, the protonation state of the amide backbone in a lipidic environment is not known.

We have begun a series of biophysical experiments to hopefully provide some insight into the molecular basis of CHX's mode of action. In all of our experiments, we use the well-studied, neutral lipid 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine (14:0–14:0 PC, DMPC). The thickness of the DMPC bilayer is comparable to the extended length of CHX, and contrary to some assertions in the literature, we find that CHX has a high affinity for neutral lipids. Neutron diffraction and isotopic/isomorphic substitution by deuterium labelling is used to locate the position of chlorhexidine's central hexamethylene chain in a DMPC bilayer with nanometre resolution, leading to a hypothesis on the mode of interaction of CHX and a bacterial plasma membrane.

2. Methods and materials

1,2-Dimyristoyl-*sn*-glycero-3-phosphatidylcholine was purchased from Avanti Polar Lipids (Alabaster, AL), and used as received. Anhydrous chlorhexidine and chlorhexidine digluconate were purchased from Sigma–Aldrich (St. Louis, MO), and also used as received. Deuterium labelled chlorhexidine hydrochloride was synthesized previously by Moser et al. (2007). The specific label from this synthesis method is 2,2,3,3,4,4,5,5-($^2H_8$)-hexamethylene-CHX, shown in Fig. 1.

2.1. Polarized light microscopy

Stock solutions of lipid and chlorhexidine digluconate in ultrapure water were combined in 10:1 and 3:1 DMPC:CHX ratios, for a total solute concentration of 15% (w/w). The solutions were put through several cycles of vortex/freeze/thaw to ensure thorough mixing and erasing of any lipid thermal phase history. The solution was pulled into a flat-sided capillary and the ends flame sealed. The pH of all solutions prepared this way in pure water were in the range 6.9–7.1.

The samples were examined with polarized transmitted light microscopy (Olympus BX52) with a temperature controlled stage (Linkam THMS600), from room temperature to 80 °C at 5 °C/min, and allowed to cool back to room temperature.

2.2. Langmuir monolayer

A simple test for lipid affinity was designed to use a Langmuir monolayer trough (KSV 5000). 0.1 mg of DMPC was spread from chloroform solution on the surface of ultrapure water, and the solvent allowed to evaporate. From zero surface tension, the trough barriers were compressed to a surface tension of 30 m N/m, and the barriers were continually computer controlled to maintain that pressure. After several minutes, ~0.5 ml CHX digluconate in ultrapure water was injected into the subphase behind the barriers, on the outside of the DMPC monolayer, to bring the 1 L subphase to 10 μM CHX concentration, well below the 6.6 mM critical micelle