

Polyhexamethylene Biguanides and Infection Control

How the Unique Method of Action Precludes some Antiseptics from Resistance

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Introduction

Problems associated with the development and spread of antibiotic resistance have been increasing since the early 1960's and, in the clinic, is currently viewed as a major threat to practice [43, 50]. As many as one third of nosocomial infections are believed to be preventable [58]. Common nosocomial pathogens that express resistance towards multiple antibiotics are being increasingly detected both in the clinic and in general practice. Such resistance has been almost universally associated with the overuse and abuse of therapeutic agents, and with the acquisition and fusion of genetic elements encoded within plasmids. It is generally accepted that the main cause of this problem has been, and still is widespread inappropriate use and over-prescribing of antibiotics in clinical medicine, animal husbandry and veterinary practice [1, 4, 26, 28, 49, 52]. Concerns about bacterial resistance have led to calls for increased education, of both public and professionals, on the correct use of antibiotics. Additionally, more stringent infection control measures have been advocated in order to reduce the transmission of infection [2, 3, 5-9, 19, 26, 60]. These measures recognise the tremendous contributions that antisepsis has made, over the last century, towards our current advanced state of public health. Indeed, if reductions in the number of infections requiring antibiotic treatment can be achieved through effective hygiene, including the use of antiseptic products, then this will delay increases in the incidence of antibiotic resistance. Accordingly, it is important to ensure that the

use of antiseptic products is not discouraged in situations where it is part of good hygienic practice and where there may be tangible reductions in the transmission of infection. With respect to the management of post-operative wounds then whilst multiple factors, related to the nature of the surgical procedure, can influence the risk of wound infection, their approximate incidence, for clean procedures is between 2-3% [29]. The normal flora of the skin is an important source of serious post-operational infections with the involvement of skin organisms such as *Staphylococcus aureus* and *Staphylococcus epidermidis* being widely acknowledged. Furthermore, antibiotic resistant coryneform bacteria have been isolated from the skin of both hospitalised patients and control groups [47]. Systemic infections caused by such bacteria are often associated with concurrent use of indwelling medical devices such as central venous lines or catheters [51], but systemic treatment with vancomycin neither eliminates nor prevents colonisation of the device [47]. Topical application of broad-spectrum antimicrobial agents such as quaternary ammonium compounds, biguanides, halogen release agents and triclosan remain safe and effective preventative, and treatment, measures. Whilst such use has generally been confined to medicated soaps, handwashes and bathing formulae, incorporation of these and other antimicrobial agents within the polymer materials and coatings that comprise indwelling medical devices and dressings have demonstrated significant applications in the localised prevention of infection [61].

For over a century cationic antimicrobials have been prominent amongst those used both in infection control and within many consumer products and have often been assumed to possess a single, generic mechanism of action directed towards biological membranes. Cationic antimicrobials that have been in use for

over forty years include a variety of quaternary ammonium based molecules (Cetrimide, Benzalkonium Chloride), bisbiguanides (chlorhexidine) and polymeric biguanides (vantocil). Sadly, in spite of their long and widespread use, assumptions relating to such agents are compounded by a general lack of experimental evidence surrounding their biological mechanisms. Recently the use of such antimicrobial agents has been questioned in many application areas. This questioning is based on an association between trace levels of antimicrobial residue and their implied potential to select of less susceptible bacteria that are co-incidentally resistant to third party antibiotics ^[31, 32]. Polyhexamethylene biguanide (PHMB) is a polymeric cationic antimicrobial agent that has been deployed in consumer applications for over 40 years. Whilst it shares many attributes with the simpler cationic agents it has additional action mechanisms that render it unique amongst this generic class of antimicrobials. The purpose of the current article is to consider the suitability, or otherwise, of PHMB as part of generic wound care systems. In order to do so it will be necessary to consider the whole class of antimicrobial molecules. The molecular basis of antimicrobial action will therefore be considered from the most simple (quaternary ammonium compounds), through bisbiguanides to PHMB. At each stage the potential for resistance development will be considered against a background of published susceptibility surveillance publications ^[32].

Cationic Antimicrobial Agents

The outermost surface of bacterial cells universally carries a net negative charge, often stabilised by the presence of divalent cations such as Mg^{2+} and Ca^{2+} . This is associated with the teichoic acid and polysaccharide elements of Gram-positive

bacteria, the lipopolysaccharide of Gram-negative bacteria, and the cytoplasmic membrane itself. It is not therefore surprising that many antimicrobial agents are cationic and have a high binding affinity for bacterial cells. Often, cationic antimicrobials require only a strong positive charge together with a hydrophobic region in order to interact with the cell surface and integrate into the cytoplasmic membrane. Such integration into the membrane is sufficient to perturb growth and at the treatment levels associated with antiseptic formulations is sufficient to cause the membrane to lose fluidity and for the cell to die. Indeed, for many decades such compounds have been loosely designated as 'membrane active agents' or as 'biological detergents' broadly recognising their lack of specificity in mechanism of action. It is however worthwhile considering for a moment the general characteristics of biological membranes in order to fully understand the mode of action of cationic antimicrobials. The membranes are composed primarily of proteins, embedded within a lipid matrix and approximating to a bi-layer ^[59]. The proteins either fully traverse the two sides of the bilayer (integral proteins) or are peripheral and associate with one specific side. Many of these membrane-proteins are required in order to maintain the structural integrity of the membrane, whilst others are functional and associated with catabolism, cellular transport and the biosynthesis of wall and extracellular products (toxins, virulence factors etc.). The functionalities of these proteins is moderated by the hydrophobic environments of the neighbouring phospholipids. Thus each protein is surrounded by particular phospholipids that interact with the protein forcing it into a functional configuration. The lipid bi-layer is further stabilised by di-valent cations such as Ca^{2+} . Cationic antimicrobials are relatively hydrophobic but interact initially with the wall and membrane by displacing these divalent cations. Such action is

shared with simple chelating agents such as EDTA and EGTA that perturb membrane structure solely through the sequestration of stabilising metal-cations. Subsequent interactions of the cationic biocides with membrane proteins and lipid bilayer depend upon the specific nature of the biocide. Many of the cationic antimicrobials have been deployed as surface and topical antimicrobials, in the clinic and in general hygiene delivery, for more than half a century. Notable amongst these agents are the quaternary ammonium compounds (Benzalkonium Chloride, Cetrimide, Barquat), bisbiguanides (Chlorhexidine) and polymeric biguanides (Vantocil, Cosmocil) together with antibiotics such as Polymyxin and Tyrocidin. Of these the quaternary ammonium group of compounds and the polymeric biguanides are mixtures of compounds that share a common generic structure. Such chemical diversity broadens their spectrum of activity but makes standardisation difficult.

In considering the action and utility of the cationic biocides, together with their potential for resistance development, it is worthwhile characterising them according to the number of cationic groupings per molecule. Thus the quaternary ammonium compounds (QAC's) are generally monocationic, whilst bisbiguanides (chlorhexidine) carry two cationic groups separated by a hydrophobic bridging structure (hexamethylene), and polymeric biguanides are polycationic linear polymers comprising a hydrophobic backbone with multiple cationic groupings separated by hexamethylene chains.

Quaternary ammonium compounds (QAC's)

QAC's are amphoteric surfactants, generally containing one quaternary nitrogen associated with at least one major hydrophobic substituent (Figure 1). Cetrimide

USP is tetradecyltrimethylammonium bromide whereas the generic term Cetrimide relates to mixtures of *n*-alkyltrimethyl ammonium bromides where the *n*-alkyl group is between eight and eighteen carbons long. Benzalkonium chlorides are always mixtures of *n*-alkyldimethylbenzyl ammonium chlorides where the *n*-alkyl groups (the hydrophobic moiety) can be of variable length within a specified range. In addition to these various dialkylmethyl ammonium halides and dialkylbenzyl ammonium halides also have antimicrobial activity and are variously deployed as biocides, preservatives and antiseptics [10]. All share a common mechanism of action. The raw materials providing the alkyl group of these synthetic compounds are often natural oils such as coconut or soybean oil. Commercially available QAC's will therefore be more diversified in their fatty-acyl chain length distributions and their degree of C-C saturation, each of which can affect antimicrobial activity.

The activity of quaternary ammonium biocides is an approximate parabolic function of the compounds lipophilicity (*n*-alkyl chain length). For gram positive bacteria and yeast, such activity maximises with chain lengths of $n=12-14$, whilst for Gram-negative bacteria, optimal activity is achieved for compounds with a chain length of $n=14-16$. Compounds with *n*-alkyl chain lengths of $<n=4$ or $>n=18$ are virtually inactive. Since the antimicrobial activity of QAC's towards specific species of bacteria is dependent upon the hydrophobicity of the *n*-alkyl chain then, given the raw material source of the *n*-alkyl substituent, the overall activity of individual commercial products can be highly variable [20, 30]. Many quaternary ammonium mixtures are however blended so as to optimise activities against specific groups of bacteria, or to gain as broad a spectrum of activity as is

possible. Pharmacopoeial standards for such molecules define the mixture chain lengths, but such standards differ between regulatory bodies.

The mode of action of quaternary ammonium compounds against bacterial cells is thought to involve a general perturbation of lipid bi-layer membranes as found to constitute the bacterial cytoplasmic membrane and the outer-membrane of Gram-negative bacteria. Such action leads to a generalised and progressive leakage of cytoplasmic materials to the environment. Low concentrations of QAC bind firmly to anionic sites found on the membrane surface, cause cells both to lose osmoregulatory capability and to leak potassium-ions and protons ^[45]. Intermediate levels perturb membrane-located physiologies such as respiration, solute transport, and cell wall biosynthesis ^[55]. The high concentrations used in many biocidal formulations however, kill cells by solubilisation of the membranes to release all of the cells contents, hence their designation as biological detergents ^[56, 57]. Indeed, the surfactant properties of QAC's are often used to good advantage in disinfectant cleansing formulations ^[33]. At a molecular level, action involves an association of the positively charged quaternary nitrogen with the head groups of acidic-phospholipids within the membrane (Fig 2-b). The hydrophobic tail then inter-digitates into the hydrophobic membrane core (Fig 2-b, 2-c). Thus, at low concentration (*circa* minimum growth inhibitory concentrations, MIC) such interaction increases the surface pressure in the exposed leaflet of the membrane to decrease membrane fluidity and phase transition temperature. The membrane undergoes a transition from fluid to liquid crystalline state and loses many of its osmoregulatory and physiological functions (Fig 2-a). The membrane core decreases in hydrophobicity and phospholipids tend towards a stable hexagonal arrangement. At use-concentrations, solutions of QAC's form mixed

micellar aggregates that solubilise hydrophobic membrane components (i.e. Lipid A, Phospholipids etc. see Fig 2-e, 2-f). QAC's have been actively deployed since the 1930's with no apparent reduction in their effectiveness. Nevertheless there are numerous reports of apparent resistance towards quaternary ammonium compounds. Such resistance invariably refers to changes in the MIC and do not affect the activity at use-levels ^[32]. The latter are often 100-1000x higher than the MIC. Where changes in QAC MIC have been demonstrated then these have either been relatively minor (2-3 fold) and associated with changes in the acidic phospholipid content of the membrane ^[63] or they have been associated with the acquisition, or hyperexpression of multi-drug efflux pumps (i.e. qac genes). Such efflux pumps can actively remove QAC from the membrane core and thereby reduce effectiveness at sub-MIC. Acquisition or hyperexpression of multidrug efflux pumps has however been associated with changes in MIC of therapeutically important third party antibiotics which co-incidentally serve as substrates to those pumps. Some species of bacteria, notably *Pseudomonas aeruginosa*, are relatively insensitive to QAC biocides. This is thought to relate to a failure of the compounds to penetrate the outer-membrane and to access the cytoplasmic membrane. Such insensitivity can often be overcome by formulating in solutions of EDTA and EGTA. These sequester divalent cations from the outer and cytoplasmic membrane and thereby aid interaction with QAC.

Biguanides

Order of magnitude increases are noted in the antimicrobial activities of n-alkyl-QAC's when the n-alkyl chain length is increased beyond 10. This is related to a concentration independent dimerisation of the molecules in solution. Above these

critical chain lengths attraction between the adjacent hydrophobic chains exceeds the electrostatic repulsion of their charged nitrogen head-groups. QAC dimers are formed that bear bi-polar positive charges in conjunction with interstitial hydrophobic regions. Such dimers both interact more strongly with the cytoplasmic membrane, than the monomeric form of QAC, and are able to more easily solubilise within it [20, 30]. Bisbiguanides, such as chlorhexidine, provide similar bi-polar configurations of cationic and hydrophobic domains within a single molecule and are potent antibacterial agents. Biguanides were first synthesised in the early part of the 20th century and have since provided a variety of drugs with a broad range of pharmacological activities (anti-malarial, blood-sugar lowering, antiseptic, anti-protozoal). Marked antibacterial activity was noted for mixtures of polyhexamethylene biguanide (PHMB) salts produced by the reaction of hexamethylene bis-dicyanodiamide and hexamethylene diamine [53]. PHMB (Fig 3) produced by this route contains polydisperse oligomers with molecular weights of between 500 and 6000, in which *n* varies. The mixtures have a mean *n* of 5.5 and the tetramer is the dominant species. Due to the method of synthesis each member of the series might have either amine or cyanoguanidine groups at either end position. Attempts to rationalize the PHMB mixtures were unsuccessful at that time, and precluded their use in pharmaceutical products. Further synthesis led to the development of the closely related bisbiguanides. In this series of molecules, optimal antibacterial activity was exhibited by the bisbiguanide, 1,6-bis(4'-chlorophenylbiguanide) hexane [25] (Fig 4a). This molecule became marketed as chlorhexidine. Alexidine (Fig 4b), a related molecule with ethylhexyl end-groups replacing the 4-chlorophenol endgroups was developed for its activity against plaque-forming organisms.

The bisbiguanides chlorhexidine and alexidine

Chlorhexidine, is active towards a wide range of Gram-positive and Gram-negative bacteria and is compatible with a variety of commonly used antibiotics. Whilst the molecule had little systemic activity in mice, it was found to be highly effective against wounds infected with haemolytic streptococci.

Chlorhexidine has since been widely deployed in surgical handwashes, as an antiseptic and in various topical treatments for wound sepsis. Chlorhexidine has also been marketed extensively within various oral hygiene products as an anti-plaque agent, and within topical slow release vehicles for the treatment of periodontal disease. There have been few if any reports of chlorhexidine resistance at use concentrations, in spite of its widespread use for almost fifty years in clinical and domestic settings, but small changes (c. 5-fold) in MIC have been noted [44, 62]. The latter is thought to relate to changes in envelope composition relating to anionic targets and cation binding rather than to target modification and or efflux [64, 44]. Notable, in the spectrum of activity of bisbiguanides is however their ineffectiveness against some Gram-negative bacteria particularly *Pseudomonadaceae* and *Providentia* spp [62]. As with the QAC biocides such insensitivity can often be overcome by formulating together with a chelating agent such as EDTA.

Bisbiguanides antiseptics have a very similar mechanism of action to the QAC biocides in that the biguanide groupings associate strongly with exposed anionic sites on the cell membrane and cell wall, particularly acidic phospholipids and proteins [17, 18]. Binding to such sites is stronger than that of the QAC's and can cause displacement of wall and membrane associated divalent cations

(Mg²⁺, Ca²⁺) [22, 42]. A major difference between the bisbiguanides and QAC biocides is that the hydrophobic regions of the QAC biocides become solubilised within the hydrophobic core of the cell membrane whilst those of chlorhexidine do not. Being six carbons long, rather than 12-16 carbons, the hydrophobic region of chlorhexidine is somewhat inflexible and incapable of folding sufficiently to interdigitate into the bi-layer. Chlorhexidine therefore bridges between pairs of adjacent phospholipid headgroups each being bound to a biguanide moiety and displaces the associated divalent cations [22] (Figure 5).

Interestingly the distance between phospholipid headgroups in a closely packed monolayer is roughly equivalent to the length of a hexamethylene grouping. Such binding to the cell membrane is critical for the bisbiguanides since activity is reduced significantly if the polymethylene bridge is made longer or shorter than six-carbons [25]. An interaction with the cell membrane, such as this, will reduce membrane fluidity at low concentrations and affect the osmoregulatory and metabolic capability of the cell membrane and its contained enzymes [36]. These effects have been variously reported as cellular leakage of potassium ions and protons [27, 34-36, 54], and inhibition of respiration and solute transport. At higher, in-use, concentrations the interactions are more severe and cause the membrane to adopt a liquid crystalline state, lose its structural integrity and allow catastrophic leakage of cellular materials [16-18, 48]. Whilst the action of multi-drug efflux pumps is able to moderate the action of QAC's at low concentrations they have no effect upon the action of bisbiguanides. This is presumably because the bisbiguanides do not become solubilised within the membrane core.

Polyhexamethylene biguanides

As antibacterials, PHMB (Fig 3) was recognised as possessing superior antimicrobial effect to other cationic biocides, but it could only be poorly defined chemically. Early attempts to rationalize the PHMB mixtures were unsuccessful and precluded their use in pharmaceutical products. Nevertheless, PHMB was marketed as a broad-spectrum antimicrobial agent in a number of diverse applications. These included their use as swimming pool sanitizers (Baquacil), as disinfectant cleansers of Beer-glasses (Vanquish), as well as well as general purpose environmental biocides and antiseptics. The antimicrobial activity of PHMB is superior to that of the bisbiguanide molecules subsequently derived from it. Analysis of the antimicrobial activity of PHMB reveals an enigma. When mixtures were purified with respect to polymer chain length then it was observed that antimicrobial activity increased dramatically, on a mass basis (i.e. $\mu\text{g/mL}$), with increasing polymer chain length ^[11]. Thus, the amine-ended dimers, corresponded crudely to the bisbiguanides, but were only poorly active, whilst high molecular weight materials with $n > 10$ were highly effective. Clearly whilst there were broad similarities between the actions of chlorhexidine and PHMB ^{[23,} ^{24]} the latter had additional properties that rendered it a superior biocide.

As with the bisbiguanides, PHMB was shown to bind rapidly to the envelope of both Gram-positive and Gram-negative bacteria and in doing so displaces the otherwise stabilising presence of Ca^{2+} ^[12]. This binding is to the cytoplasmic membrane itself, and also to lipopolysaccharide and peptidoglycan components of the cells wall. The interstitial hexamethylene groups of the polymer, as with the bisbiguanides, are hydrophobic yet sufficiently inflexible that

they cannot interdigitate into the hydrophobic core of the cell membrane. Once again, therefore, a bridging of adjacent acidic-phospholipids is brought about by interaction of the antimicrobial with the cell membrane ^[15, 39, 40]. One additional feature of this interaction is that it will tend to become concentrated around any points of maximum charge density within the membrane ^[37, 38]. It has been shown that integral proteins constitute such sites. Thus, the initial interactions of PHMB and the membrane will be concentrated around such proteins leading to a loss of their function by inflicting changes in their boundary phospholipid environment ^[12]. This manifests itself, as with the bisbiguanides as a loss of transport, biosynthetic and catabolic capability. The unique polymeric nature of PHMB means that, unlike for the bisbiguanide, such bridging is not restricted to pairs of adjacent phospholipids. Rather adsorption to the cell membrane will lead to a sequestration of common acidic phospholipids into separate membrane domain ^[13-15, 39, 40]. Thus, in the presence of PHMB the homogeneous distribution of phospholipids normally associated with biological membranes is transformed into a mosaic of individual phospholipid domains. Each of these will have different phase transition properties causing the membrane to fragment into fluid and liquid crystalline regions. As with other cationic biocides this is manifested as a generalised cellular leakage, first of small cationic materials such as potassium ions and later by losses of intra-cellular pool materials ^[11]. A secondary consequence of domain formation is that the separated phospholipid types will assume their energetically favoured position of a hexagonal phase leading to a total loss of the membrane permeability barrier ^[40]. The ability to form lipid domains is clearly a function of polymer chain length with longer polymers being able to form the larger domains and hence the greater perturbation of membrane function ^[41].

PHMB therefore not only embodies the attributes of the bisbiguanides in terms of antimicrobial action but possesses additional molecular actions. As with chlorhexidine there is no evidence that PHMB susceptibility is affected by the induction or hyperexpression of multi-drug efflux pumps, neither have there been any reports of acquired resistance towards these agents. Rather, as with all membrane active antimicrobials, small changes in MIC have been reported that correlate with alterations in envelope lipid composition and cation binding ^[15, 21].

Conclusions

PHMB was the forerunner of the highly successful antiseptic agent chlorhexidine, and falls into a general category of antibacterial agents that are cationic, displace divalent cations from the wall and membrane of bacteria and bring about a disruption of the lipid bilayer. The biguanides and polymeric biguanides differ from other cationic biocides in that they interact only superficially with the lipid bilayer altering fluidity through cation displacement and head-group bridging. QAC's on the other hand interact fully with the membrane and are therefore susceptible to resistance mechanisms mediated through multidrug efflux pumps. The activity of biguanides and bisbiguanides is unaffected by such hyper-expression of efflux. Their deployment in the clinic cannot therefore have implications towards the selection of multi-resistant organisms through this mechanism ^[32]. Both the bisbiguanides and the polymeric biguanides are recalcitrant in the soil. This implies that there are currently no microbial degradative pathways that might be high-jacked into a resistance pathway. Indeed the multiplicity of critical lethal targets affected by the interaction of biguanides with the membrane dictate

against singular mutations leading to changes in susceptibility ^[31, 32]. This is borne out by a lack of evidence to suggest that the use of either compound over forty years has affected their activity in the field.

The toxicity profile of both the biguanides and the polymeric biguanides is excellent. Neither molecule is a primary skin irritant nor a hypersensitising agent and oral tolerances are good. Whilst the use of PHMB has until recently been restricted to environmental applications (Swimming pool sanitiser, beer glass sanitiser, topical antiseptic), such restriction relates to difficulties in standardization of PHMB formulations rather than to toxicity issues. Second generation PHMB formulations such as Cosmocil CQ give a much better definition of polymer dispersity that was previously possible with Vantocil IB. Such PHMB formulations are now widely deployed in clinical applications such as the treatment of *Acanthamoeba* keratitis ^[46] and have been included within certain contact lens cleansing formulations.

With respect to the deployment of PHMB as part of a wound care system there is little or no evidence to suggest that this would lead to the emergence of PHMB resistant nor antibiotic resistant strains of nosocomial pathogen. Use of the agent within a barrier wound dressing such as Kerlix AMD would impair the growth and penetration through the dressing of adventitious pathogens both from the environment to the dressed wound and from the wound to health care workers and other human contacts. Such action can only contribute to breaking the cycle of nosocomial infection and will inevitably reduce the usage of antibiotics currently used in the care of such infections.

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