Expert Opinion

informa
healthcare

New drug-targeting strategy from beneath the shell of egg

Hisham R Ibrahim
Kagoshima University, Department of Biochemistry and Biotechnology, Faculty of Agriculture, Kagoshima, Japan

Importance of the field: Antibiotic resistance is a serious problem that continues to challenge the healthcare sectors and has become increasingly alarming in the past few years. To face this emerging global crisis, there is a need to find a new class of antibiotics that act on new microbial targets and/or harness existing antibiotics by developing new drug-targeting strategies.

Areas covered in this review: This review: explores an innovative drug-delivery strategy of using hen egg lysozyme as a carrier to enable water solubilization and to allow specific targeting to the microbial cells of a water-insoluble antimicrobial agent with a powerful killing action; addresses potentials for lysozyme in antibiotics drug targeting; and provides insight for the future direction of this highly prospective technology.

What the reader will gain: The unique features and advantages of lysozyme-based drug delivery system are highlighted. The efficiency of lysozyme in solubilization and delivery of lipophilic antibiotics, to reformulate drugs that may fail clinical trials owing to low solubility, is emphasized.

Take home message: Fewer pharmaceutical companies are inventing new antibiotics because of long development times and high failure rates. Combining lysozyme with a powerful old antibiotic may open doors to revolutionizing medicine, particularly in the treatment of deadly infections.

Keywords: antibiotic delivery, antibiotic resistance, anti-infection, antimicrobial, drug targeting, FabI, lysozyme, phenolic drug, triclosan

Expert Opin. Drug Deliv. (2010) 7(10):1145-1158

1. Introduction

For the last 70 years, the effort to treat infectious diseases has relied heavily on targeting microorganisms themselves with antimicrobial drugs (antibiotics). That approach has been successful, especially against bacterial infections; but the inappropriate use of antibiotics has also resulted in the generation of drug-resistant microorganisms that are threatening to become untreatable. Pathogens have developed strains that are resistant to almost all antibiotics in use today. Particularly worrisome are infections by a large group of bacteria, which cause pneumonia and other often fatal infections. The problem is highlighted by the emergence of multi-drug resistance (MDR) of these organisms, which are resistant to vancomycin, a drug recognized as the last line of defense in many Gram-positive bacterial infections. The manifestation of MDR in bacteria over the past few decades has resulted in one of the most pressing clinical problems in modern medicine. In the face of newly emerging infectious organisms and the global crisis in antibiotic resistance, there is a need to invigorate the basic science and technology of anti-infective therapy. What tips the balance towards this problem? As yet, a renewed commitment to harness the potent hydrophobic antibiotics is the development of new drug-targeting strategies. Even though the therapeutic efficacy of many hydrophobic drugs has been well established, inefficient delivery can result in inadequate therapeutic index and local and systemic side effects. In particular, many intracellular infections

10.1517/17425247.2010.515208 ISSN 1742-5247

Article highlights.

- Several new lipophilic antibiotics with excellent efficacy, targeting the essential type II fatty acid synthesis pathway, have been explored, but their exploitation in therapy is hampered because they are basically insoluble in water, and this paper approaches the issue from the point of their targeted delivery strategy by using lysozyme.
- Using lysozyme, a naturally secreted protein in many of human physiological fluids, as biodegradable drug carrier, a broader range of drugs can be attached and a higher renal selectivity can be attained.
- Lysozyme displays unique features worth using as antimicrobial drug-delivery molecules, including the presence of a hydrophobic pocket with positively charged residues around the active site, and it has a dual behavior: specific recognition and interaction with bacterial surface while its catalytic action enables the release of the loaded drug on binding.
- Accommodation of phenolic antibiotic in the hydrophobic core of lysozyme provides potential protection of both antibiotic and lysozyme against oxidative damage, which might occur during infection-induced inflammation.
- The globular structure of lysozyme, as opposed to the random coil structure of most polymers, makes this cationic enzyme a potential carrier to accommodate several drug molecules for various infectious diseases.

This box summarizes key points contained in the article.

remain difficult to treat simply because of the high hydrophobicity and the poor cellular transport properties of antimicrobial drugs. In addition, toxicity to healthy tissues also poses significant limitations to the use of antimicrobials, thus specific targeting of the drug to the bacterial cells is a top priority.

Food proteins show great promise for developing a range of new GRAS (generally recognized as safe) carriers with the potential to incorporate pharmaceutical compounds and provide controlled release by means of the oral route. Clear advantages of food protein as drug carriers include their high nutritional value, abundant renewable sources, and acceptability as naturally occurring food components degradable by digestive enzymes. As discussed in the paper, lysozyme from egg albumin has unique biophysicochemical properties that can be applied to facilitate the targeting of phenolic antimicrobials, thereby introducing a new drug-delivery strategy. The strategy is to solubilize and specifically deliver the poorly soluble antimicrobial drug by loading the drug into the hydrophobic core of lysozyme, and then deliver the lysozyme to the bacterial cell, because lysozyme can recognize and interact with the cell envelopes of both Gram-positive and Gram-negative bacteria. After interaction and disruption of the cell envelope, lysozyme releases its payload 'antibiotic' into the microbial cell to inhibit multiple cellular processes. The author is developing a lysozyme-based drug-delivery system that can deliver multiple drugs with different hydrophobicity and other characteristics to the bacterial cells in a targeted manner.

2. Phenolic antibiotics and bacterial resistance

Substantial discoveries of antimicrobial agents have been made in the past decades, where most of these antibiotics are a collection of phenolic compounds. Although numerous studies have shown these natural phenolic compounds to be effective against microorganisms, they still have considerable drawbacks. As shown in **Figure 1**, they have limitations that include the potential emergence of resistant strains as they are either kept out of the bacterial cell by the so-called efflux pump present at the surface of most pathogens or by virtue of membrane exclusion. Also, their exploitation in human therapy has not been widespread because most of the powerful agents are basically insoluble in water and have in many cases inherent chemical instability. Furthermore, only a few antimicrobials possess broad-spectrum activity against both Gram-positive and Gram-negative bacteria. With the difficulties of identifying new natural-phenolic antibiotics, largely of bacterial origin, existing natural antibiotics have been used as chemical scaffolds for synthetic antibacterial drugs since the 1970s. Subsequent tweaking of these chemical scaffolds has produced most of today's antibiotics. It is believed that widespread drug resistance among bacterial pathogens is because of the limited choice of antibiotics that exploit a relatively narrow range of mechanisms. Over the years, several new synthetic antibacterials with excellent efficacy as drug candidate have been introduced. Antimicrobial drugs targeting the essential type II fatty acid synthesis (FASII) pathway have recently been explored for their efficacy against infections caused by multi-resistant Gram-positive bacteria [1]. Today, fatty acid synthesis inhibitory antibiotics are revolutionizing the biotechnology platforms, spurring the creation of next-generation therapies with greater effectiveness.

Triclosan (TCS), 5-chloro-2-(2,4-dichlorophenoxy)phenol, is a broad-spectrum antimicrobial agent that possesses potent activity against the most common bacteria responsible for postoperative sternal wound infections [2]. Triclosan is used in topical applications, and it was approved in 1997 for oral care products [3,4]. In the beginning the mode of triclosan action was supposed to be through nonspecific disruption of the bacterial cell membrane [5]. Newer studies, however, revealed that the target of triclosan is the FabI gene product, which blocks bacterial fatty acid synthesis (particularly the enzyme enoyl-acyl carrier protein reductase [ENR]) [6]. The main hurdle in the development of effective therapeutic (topical and postoperative sternal wound infections) formulations is, as in the case of > 50% of the drugs approved for use, the insolubility of triclosan in aqueous media. The problem with preoperative antibiotic prophylaxis is that the need for homeostasis perfusion is markedly reduced

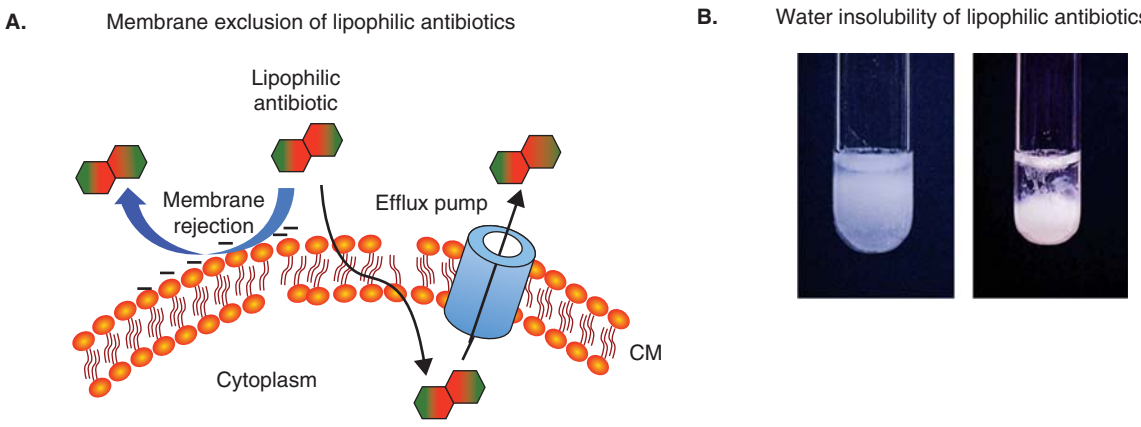

Figure 1. The two common problems encountered with therapeutic application of the lipophilic antimicrobial agents. A. The bacterial resistance through exclusion of the lipophilic antibiotics by the negatively charged surface of CM. Even once inside the cell, efflux pumps can evacuate antibiotics of many classes before they reach their target. **B.** The poor water solubility of the lipophilic antibiotics (such as sulfonamide) will limit their therapeutic manipulation, decrease the oral absorption and thereby decrease the bioavailability and efficacy.
CM: Cytoplasmic membrane.

in the sternal wound, and therefore the necessary tissue concentration of antibiotic needed for optimum efficacy is seldom reached. In particular, many intracellular or bloodstream infections are notoriously difficult to treat because the antibiotic concentrations inside the cells are below the minimum inhibitory concentrations. For a dilemma like this, microbe-specific drug-targeting systems could be the solution for many infections. Further, to guarantee a suitable drug solubilization in water and specific delivery to diseased cells, protein-based delivery strategies were suggested, especially to cancer cells [7]. Owing to multiple functional groups in the primary sequences of proteins or peptides and the diversity of chain folding structures, food proteins can be exploited to create different interactions with pharmaceutical hydrophobic compounds, and subsequently form three-dimensional macromolecules to incorporate and deliver them to the target cell in active form [8,9].

3. Squeezing new drug-targeting protein out of egg albumin

Egg albumin, the chemical defense line of avian eggs, represents a complex group of proteins with diverse biological functions that intriguingly interact to protect the chick embryo from microbial infection [10]. The constituent proteins of egg albumin offer tremendous opportunities for drug discovery and hope for the treatment of emerging infectious diseases. In particular, most of the proteins found in egg albumen appear to possess structures homologous to proteins with known functions in the serum of other species, including humans. Lysozyme is a key protein of the hen egg's antimicrobial defense system. It is widely distributed in various biological fluids, including avian egg albumen, human milk, tears, saliva, serum and airway secretions, and is produced by specialized immune cells [11]. The mechanism by which lysozyme kills the sensitive bacteria is known to be the degradation of glycosidic β-linkage between *N*-acetylhexosamines of the peptidoglycan layer in the bacterial cell wall. Its antimicrobial function is limited to certain Gram-positive bacteria [12-15] owing to the differences found in composition as well as the accessibility of the peptidoglycan to the enzyme action (**Figure 2**). Apart from bacteriolytic action, it has been demonstrated that lysozymes have many other functions [16,17], including inactivation of certain viruses by forming insoluble complexes, important roles in surveillance of membranes of mammalian cells, enhancing phagocytic activity of polymorphonuclear leukocytes, and stimulating proliferation and antitumor functions of monocytes.

Studies have shown that lysozyme interacts with and induces fusion of phospholipids vesicles [18]. In parallel, immunochemical *in vivo* studies using confocal microscopy have demonstrated that lysozyme is synthesized and secreted with surfactant apoprotein A by rat alveolar type II epithelial cells and alveolar macrophages, suggesting its role in the extracellular remodeling of surfactant phospholipids in the air-spaces of lung [19]. Lysozyme is known to be specifically bactericidal to certain Gram-positive bacteria, but less effective against Gram-negative ones, despite its strong interaction with and disruptive effect on lipopolysaccharides (LPS) of the latter species [14,20] and phospholipid bilayers [18,21].

4. Lysozyme as a renal drug carrier

The low-molecular-mass proteins with a molecular mass of < 30,000 Da are freely filtered in the kidney and have been considered to be suitable to serve as renal-specific drug

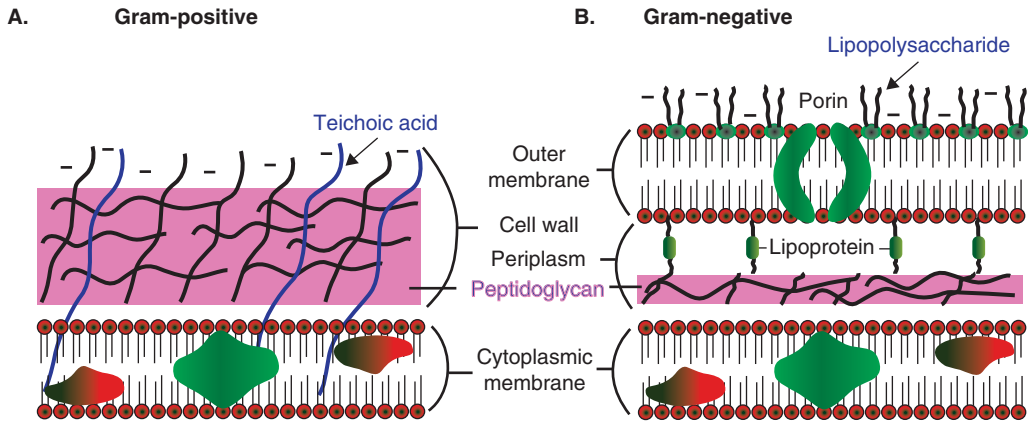

Figure 2. Comparison of cell membranes structures of Gram-positive and Gram-negative bacteria. View of the cross-section in the envelopes of **(A)** Gram-positive and **(B)** Gram-negative bacteria. The negatively charged recognition molecules for lysozyme at the outer surface of both Gram-positive (teichoic acid) and Gram-negative (lipopolysaccharide) bacteria are shown.

carriers that can accumulate in the kidneys by means of endocytosis [22]. The concept is based on four principles. One, the carrier has functional groups allowing drug attachment. Two, the low-molecular-mass protein accumulates specifically in the kidney, in particular in the proximal tubular cells through a luminal reabsorption mechanism. Three, the physicochemical properties of the low-molecular-mass protein overrule those of the linked drug. Four, the drug-low-molecular-mass protein conjugate is stable in the circulation, whereas after arrival in the kidney the active drug is released in the catabolically active lysosomes of the proximal tubular cell [22].

Using lysozyme as the drug carrier, a higher renal selectivity can be attained and a broader range of drugs can be attached, while the rate of drug release can also be manipulated [23]. The studies with captopril-lysozyme [24] and naproxen-lysozyme [25] clearly showed that targeting resulted in a higher renal selectivity and that drugs delivered into and regenerated in the proximal tubular cell exert renal selective pharmacological activity. Further study reported an intracellular delivery approach as a strategy to treat renal fibrosis, by coupling an inhibitor (SB202190) of mitogen-activated protein kinase (MAPK) to lysozyme using either a carbamate bond or a coordinative bond between drug and carrier [26].

Triptolide (TP) is a drug from a Chinese herb with potent immunosuppressive and anti-inflammatory properties, which has been suggested to play important therapeutic roles in treating immunological renal diseases. The clinical use of TP has been limited because of its low water solubility and several toxic effects, which are associated mainly with damaging the digestive, urogenital, blood circulatory and reproductive systems, as well as bone marrow. For clinically safe application of TP, Zhang *et al.* [27] utilized lysozyme as a renal-specific drug carrier for targeted delivery of TP to the renal cortex. The study revealed that the overall targeting efficiency of the succinyl conjugate (TPS-lysozyme) was enhanced significantly compared with TP, from 11.74 to 95.54%. At very low concentration, TPS-lysozyme could significantly reverse the disease progression in renal ischemia–reperfusion injury animal models, whereas the mixture of free TP and lysozyme was ineffective. Further, TPS-lysozyme conjugate presented much lower hepatotoxicity (0.78-fold lower than TP) and no adverse effect on the immune (1.13-fold higher than TP) and genital systems. The authors concluded that TPS-lysozyme represents a very effective drug candidate for specific treatment of immunological renal diseases with low adverse side effects.

Studies on the renal-specific drug targeting by lysozyme indicated that the positive charges on the surface of lysozyme play an important role in renal targeting and excretion of the drug conjugate into the urine [28]. In this way, lysozyme conjugates has been suggested as a carrier for targeting to the urinary tract because positive charges on the protein surface are important for interaction with the tubular uptake-receptor.

5. The new antibiotic-targeting strategy by using lysozyme

Many potentially valuable drugs that look promising are, unfortunately, not soluble in water, their clinical uses are greatly restricted, and their delivery to target cells has always been a challenge. The author and co-workers recently explored a promising strategy by which water solubilization and microbe-specific targeting of phenolic antimicrobial drug can be achieved. This was achieved through incorporation of the hydrophobic drug into lysozyme, a relatively hydrophilic protein able to recognize and disrupt the bacterial envelope [29]. The concept is based on the fact that lysozyme displays unique features worth using as antimicrobial drug-delivery molecules.

These features include the presence of a hydrophobic pocket and positively charged residues around the active site cleft of the molecule (see **Figure 4** later). This structure confers the molecule a dual behavior: specific interaction with the negatively charged surface of bacteria and accommodation of the hydrophobic, phenolic, drug (see **Figure 3** later). In addition, catalytic action enables the release of the loaded phenolic drug on binding to the bacterial surface.

Before exploring this new antibiotic-targeting strategy by using lysozyme, it would be helpful to discuss what the difference is in the membrane structures of Gram-positive versus Gram-negative bacteria, and what the structural antimicrobial properties of lysozyme are.

Bacteria show a surprising degree of complexity in their envelopes. As shown in **Figure 2**, the basis for this difference relates to the bacterial cell wall. The envelope structures of a typical Gram-positive (**Figure 2**, left) and a typical Gram-negative (**Figure 2**, right) cell are different. The layers of the cell envelope lying external to the cytoplasmic membrane (CM) are referred to collectively as the cell wall. Gram-positive and Gram-negative cells do share one thing in common, that is, the peptidoglycan (PG). The thick PG layer constitutes most of the Gram-positive cell wall, which is sensitive to the action of lysozyme. The cell walls of Gram-negative bacteria have a more complicated structure. There is an extra layer and externally the cell appears convoluted (**Figure 2**, right). Outside the CM is an open area called the periplasmic space containing a thin layer of PG and external to the PG is an extra membrane, the outer membrane (OM), whereas its outer leaflet is composed mainly of lipopolysaccharides (LPS). Both Gram-positive and Gram-negative cells again share one thing in common, that the outer surface of the cell is negatively charged (highly hydrophilic), which serves to exclude lipophilic compounds. The PG in Gram-positive bacteria is much thicker (20 – 80 nm) than in the Gram-negative bacteria (2 nm), and externally it has a smoother appearance (**Figure 2**, left). Peptidoglycan is a rigid layer that is found in both cell types and is composed of an overlapping lattice of 2 sugars, *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM), which are crosslinked by amino acid bridges [30]. Peptidoglycan is not a barrier to solutes, the openings in the mesh are large and all types of molecule can pass through. The cell wall is the site of action of many important antibacterial agents, including lysozyme and penicillin.

The mechanism by which lysozyme acts on Gram-positive bacteria is known to be recognition and hydrolysis of the PG, as it belongs to the class of enzymes that lyse the bond between NAG and NAM of the PG layer [11]. In Gram-negative bacteria, the cell wall includes the OM, lipoprotein, LPS and PG layers. Although lysozyme does not exert antimicrobial activity to many strains of Gram-negative bacteria, it has the capacity to interact with and disrupt the lipid-sugar layer LPS at its cell surfaces [14,20]. Despite its multiple functions, lysozyme does not yet hold a clear place in therapeutics or nutraceuticals, owing to the mystery of its molecular mechanism of action, which remained a dilemma for decades. Surprisingly, studies from the author's laboratory [31,32] and in the literature [33] indicated that antimicrobial activity of lysozyme is independent of its enzymatic function (lysis of PG). The author's study with co-workers revealed that the antimicrobial action of lysozyme rather depends on transition in the molecule on binding to the bacterial surface under certain environmental conditions [17,34].

Advances made in unraveling the mysterious mechanism of lysozyme antimicrobial action lend insight into the exploitation of this fascinating protein in a new drug-targeting strategy [32,35]. Central to this new class of antimicrobial delivery strategy is an engineering complex of the bacteriolytic enzyme, lysozyme, with a phenolic antimicrobial agent that could act in concert to exert optimal efficacy and recruit multiple targets on microbial cells. Basically, this new strategy utilizes the ability of lysozyme to recognize and disrupt the membrane of microbes, regardless of whether they are Gram-positive or Gram-negative bacteria. As recognition sites (receptor-like) of lysozyme, PG and LPS are permanently present at the surface of Gram-positive and Gram-negative bacteria, respectively, specific targeting of a powerful phenolic antimicrobial drug to pathogen cells is thus a new anti-infection strategy (**Figure 3**).

6. The rationale of lysozyme molecule as phenolic drug carrier

Lysozyme is attractive because it is a ubiquitous microbicidal protein, positively charged (with opposite charge to the bacterial surface), specifically interacts with bacterial cell walls (through PG or LPS) and is known to play important roles in immune defense systems [11,36]. Hence, lysozyme may offer exciting new myriad functions beyond its antimicrobial role. Intriguingly, owing to the unique nature of the lysozyme molecule, it can be a potential drug carrier to a specific targeting of the phenolic antimicrobial drugs to pathogen cells, because recognition molecules of lysozyme, PG and LPS, are permanently present at the surface of Gram-positive and Gram-negative bacteria, respectively. Phenolic drug-lysozyme conjugates, through non-covalent affinity, can thus be a new anti-infection strategy and microbe-specific drug-delivery system, to alleviate nonspecific toxicity associated with chemotherapy.

The type of interaction between protein and phenolic compounds is an important factor in producing an effective delivery system. Specifically, it has been demonstrated that weak binding affinity of phenolic drug to the carrier molecule is correlated with the effectiveness of drug delivery [37]. Therefore, the effectiveness of phenolic drug-lysozyme conjugates may be explored by identifying a phenolic antibiotic for lysozyme that shows a lesser degree of association. Triclosan, also known as Irgasan DP 300 or Ster-Zac, a phenolic antimicrobial agent [38], is hydrophobic in nature (**Figure 4**). Triclosan exerts

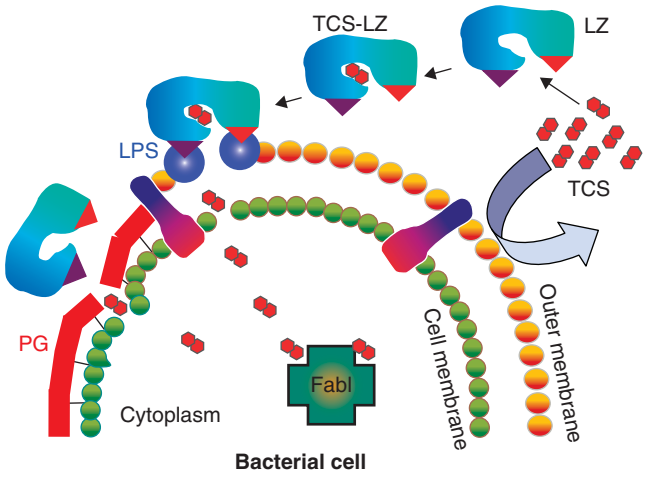

Figure 3. Principle of phenolic antibiotic delivery strategy by using lysozyme. The semipermeable membrane of bacteria excludes phenolic antibiotics but allows the complex (antibiotic engulfed in lysozyme) to enter and be acted on its target inside the cell by the enclosed antibiotic. Lysozyme, loaded with drug, is recognized by either LPS or PG at the surface of bacterial cell. The strategy may be multifaceted, whereas other small molecules (including some anti-inflammatory or anti-oxidants) can be designed to diffuse out from lysozyme into the body during infection for anti-inflammatory therapy.
LPS: Lipopolysaccharide; LZ: Lysozyme; PG: Peptidoglycan; TCS: Triclosan; TCS-LZ: Triclosan-lysozyme complex.

its action by inhibiting an essential enzyme enoyl-ACP reductase (or FabI) [1] that uses NADH to reduce a double bond during each cycle of bacterial fatty acid elongation. At higher concentration, triclosan is likely to damage the bacterial membrane [39]. Triclosan has also been shown to be an effective antifungal agent [40], and to have anti-inflammatory [41] and anticarcinogenic [42] actions. Triclosan has a low toxicity and a very low incidence of contact sensitization [43]. However, several unfavorable biopharmaceutical properties, such as low solubility in aqueous solvents, rapid photodegradation [44] and emergence of bacterial resistance [45], limit triclosan's therapeutic applications [40]. Triclosan was selected for the new strategy of utilizing lysozyme as a specific drug-targeting molecule because it has a powerful antimicrobial action and structurally can be non-covalently accommodated in the hydrophobic pocket of the active site cleft of lysozyme [29]. It has been reported that interaction of triclosan with the ENR is exclusively of hydrophobic nature, with the exception of a strong hydrogen bond between a tyrosine residue and the 2-hydroxyl group of triclosan [38]. The hydroxyl group of Tyr or Ser residues in the hydrophobic pocket of the ENR enzyme functions as an electrophile to stabilize the accumulation of negative charge of triclosan. The hydrophobic residues (Leu, Ile, Ala, Phe and Trp) seem to be important for the formation of the inner surface of the binding pocket and participation in hydrophobic interaction between triclosan and ENR enzyme, whereas NMR study revealed that inclusion of triclosan is preferable when the pH is raised to 9 or 10 [46].

The 2,4-dichlorophenoxy ring of triclosan (**Figure 4**) is predicted to interact with the faces of several hydrophobic residues (Leu56, 83, 84, Ile55, 58, 98 and Trp62, 63, 108) and hydroxyl-containing residues (Tyr53 and Ser36, 50, 60, 91) lining the inner surface of the active site cleft of lysozyme. In particular, phenol rings (Trp62, 63, 108 and Tyr53 residues) underlying the active site cleft of lysozyme are the preferred conformation for compounds of this class [47]. Tryptophan (Trp) fluorescence is strongly influenced by the indole side chain and has proved to be a useful tool to monitor protein–protein and ligand–protein interactions [48-50]. When buried in a hydrophobic environment, Trp fluorescence generally shows an increase in maximum fluorescence intensity (FI_{max}) and often shifts to a shorter (blue shift) maximal wavelength (λ_{max}). An opposite effect (intensity quenching) is observed when Trp is exposed to the surface (polar environment). **Figure 5A** shows the Trp fluorescence emission spectra of control lysozyme (LZ), TCS-lysozyme complex at 30 mol ratio (TCS-LZ) and triclosan alone (TCS). The fluorescence emission spectrum of lysozyme has a sharp peak with maximum at 345 nm and a steep wavelength tail typical of partially buried Trp within an apolar environment (**Figure 5A**). By contrast, the spectrum of TCS-lysozyme complex showed peaks at 347 nm (red shift) and a progressive increase in emission (more buried Trp). As triclosan alone (TCS) did not show any fluorescence emission spectra at the Trp region (**Figure 5A**), quenching of Trp, that is, increased emission, indicates ground state complex formation, whereas fluorophore (Trp) and the hydrophobic quencher (TCS) come into contact during the lifetime of the excited state. It is known that phenolic compounds, such as triclosan, interact with aromatic residues of protein, such as Trp, through π–π interactions [51,52]. Most probably, the increased emission of Trp residues of lysozyme in the complex is a result of

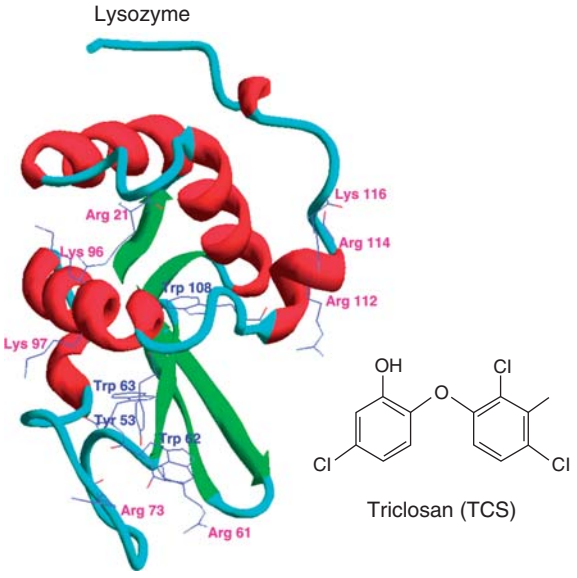

Figure 4. Stereographic representation of the structure of lysozyme illustrating the locations of the aromatic residues in the active site cleft, which could accommodate the phenolic structure of triclosan, and the positively charged residues scattered at the tip of active site cleft, which interact with the negatively charged peptidoglycan or lipopolysaccharide at the surface of bacteria.

interaction with phenolic group of triclosan by means of non-covalent π–π bonding. The red shift (longer wavelength) of the TCS-lysozyme complex emission spectrum and its enhanced fluorescence intensity relative to lysozyme spectra suggest a direct association between lysozyme and triclosan, and is consistent with at least one or two Trp residues being quenched with triclosan in the TCS-lysozyme complex.

7. Physicochemical stability of phenolic drug-lysozyme

The most important goal in the delivery of a drug is to bring the drug solubility to a specific level and maintain its integrity at that level. Stability and solubility are two key physicochemical properties that must be considered when designing a successful drug-delivery formulation. The challenge is to formulate a drug complex that has sufficient chemical and physical stability not to degrade during its shelf-life, yet has sufficient solubility (and dissolution rate) to reach the required therapeutic level. In particular, the ability to formulate a drug with sufficient stability against oxidation is becoming both more important and more challenging. The stability of TCS-lysozyme against photo-oxidative damage has been investigated by using riboflavin as a sensitizer. Electrophoretic and tryptophan fluorescence spectra revealed significant protection of lysozyme conformation in the complex against excessive photo-oxidative treatment using variable mole ratios of riboflavin over lysozyme [53]. Lysozyme alone showed polymerization, fragmentation and quenching of Trp fluorescent on photo-oxidation reaction. Further, the complex retained its strong antimicrobial activity even on photo-oxidative treatment, while causing a dramatic reduction in antimicrobial activity of the free triclosan. The results indicated that the lysozyme interacts with triclosan and that the indole side chains of Trp residues appear to be located in a more hydrophobic environment than in lysozyme, probably the interior of the lysozyme molecule. Taken together, the results confirm that accommodation of the triclosan in the hydrophobic pocket of lysozyme not only protects lysozyme from oxidation-induced loss of structural and biological activities, but also protects phenolic antimicrobial triclosan from oxidative damage until its delivery to the bacterial cell.

An important property of drug carriers that has particular significance in pharmacy is their ability to increase the solubility of poorly soluble drugs in water and have non-covalent interaction with the drug to facilitate delivery [37]. The insoluble triclosan becomes completely soluble in water on complexation with lysozyme (**Figure 5B**), most probably as the phenolic ring is incorporated, non-covalently, into the hydrophobic core underlying the active site cleft of lysozyme, as deduced from fluorescence study. The key residues make up of the lysozyme active site highlights the cleft-like nature of the lysozyme active site, whereas six sugar units (termed A – F subsites) of substrate could accommodate a phenolic agent, such as triclosan, within the cleft. The subsite C, which provides most of the binding energy for the sugar substrate, is formed by the side chains of Trp62 and Trp63, and most importantly a deep hydrophobic hole formed by Ile58, Ile98 and the indole ring of Trp108 [11,54] could accommodate the triclosan molecule (**Figure 4**). The presence of triclosan, therefore, appears to alter the hinge-bending motion of the lysozyme active site cleft as well as the nature of the hydrophobic hole of the subsite C. It is important to note that triclosan possesses three chlorine groups and a hydroxyl group within the two phenol rings, which may provide further hydrogen bonds and van der Waals contacts with the saccharide substrate in the active site of lysozyme, which affects the binding and release of the substrate.

8. Antimicrobial activity of triclosan-lysozyme complex

In a fluorescence study it has been revealed that the combination of lysozyme with triclosan could elicit aromatic stacking interaction (π–π interaction), thus loading the triclosan into the active site cavity of lysozyme molecule. The antimicrobial activity of TCS-LZ when examined against number of Gram-positive and Gram-negative bacterial strains was very promising. **Table 1** shows the antibacterial activity of the complex against four Gram-negative bacteria (*Escherichia coli* K-12, *Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Salmonella typhimurium*) and four Gram-positive bacteria

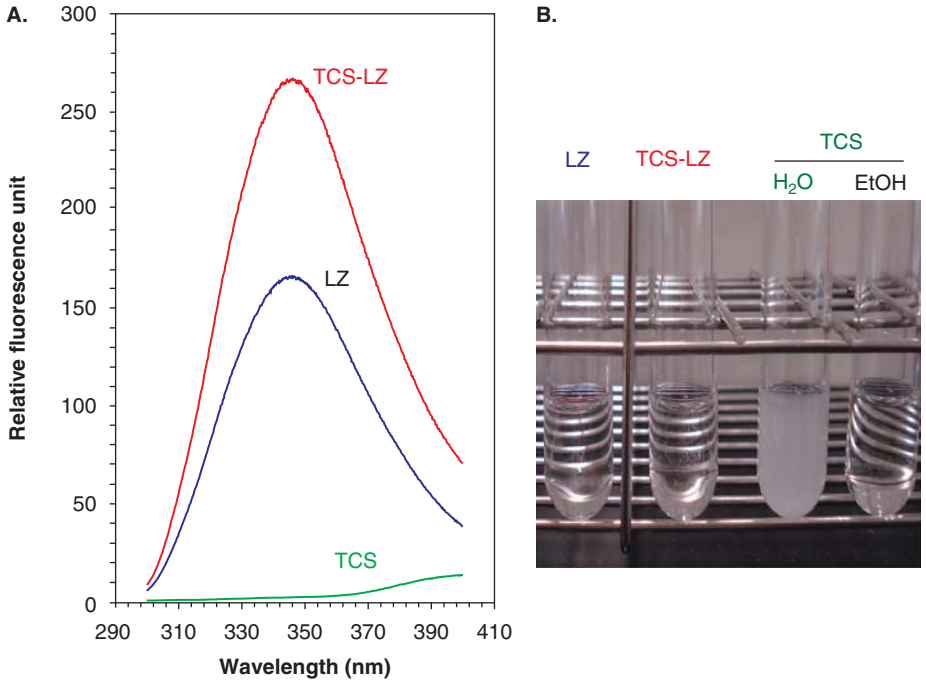

Figure 5. Tryptophan (Trp) fluorescence emission of TCS-LZ and lysozyme alone recorded on excitation at 285 nm. A. Trp fluorescence emission spectra of TCS-LZ and LZ were recorded in solutions containing derivatives (at a LZ-based concentration of 0.1 mg/ml), or free TCS (at concentration equivalent to its content in the respective preparation) in 25 mM Na-phosphate buffer (pH 7.4). **B.** Solution turbidity of LZ, the complex TCS-LZ and free TCS in water (H_2O) or ethanol (EtOH) at concentration equivalent to its content in the complex.
LZ: Lysozyme; TCS: Triclosan; TCS-LZ: Triclosan-lysozyme complex.

(*Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus zooepidemicus* and *Micrococcus luteus*). When incubated with Gram-positive bacteria for 20 h, triclosan alone at concentration equivalent to its content in the respective complex had almost no activity. Although triclosan has been reported to possess antimicrobial activity against *S. aureus*, the resistance of *S. aureus* to triclosan has been reported recently [55,56]. Lysozyme alone was found to exhibit different bactericidal activity ranging from strong to moderate against *M. luteus* and *S. aureus*, respectively, and was markedly weak against *S. epidermidis* and *S. zooepidemicus*. As for Gram-negative bacteria, lysozyme alone was completely inactive against all Gram-negative strains tested. Triclosan alone showed variable bactericidal activity ranging from strong to moderate against *K. pneumoniae* and *E. coli* or *S. typhimurium*, respectively, whereas it was inactive against *P. aeruginosa*. The bactericidal activities of triclosan-lysozyme complex, however, were remarkably stronger than that of triclosan alone against the Gram-negative *P. aeruginosa, E. coli* and *K. pneumoniae*. In particular, the complex showed potent anti-*P. aeruginosa* activity to which triclosan alone was devoid of activity. It is worth noting that the dose dependency of antimicrobial activity of the complex [29] showed the same trend as in **Table 1**. The TCS-lysozyme complex was very effective against all strains tested, resulting in a severe reduction in the CFU of the bacteria (four to eight $\log_{10}$ orders of killing). The results indicate that lysozyme seemed to facilitate the delivery of triclosan to microbial cells and provide a new antibiotic-targeting strategy for the treatment of microbial infections. It is worth noting that TCS-lysozyme complex showed significant bactericidal activity against *S. epidermidis* and *S. zooepidemicus*, to which lysozyme or triclosan alone were almost devoid of activity. Given this degree of TCS-loading dependency, lysozyme seemed to serve as a carrier that allowed specific targeting of triclosan into microbial cells.

9. Lysozyme delivers triclosan into the bacterial cytoplasm

Triclosan blocks the active site of a cytoplasmic enzyme called enoyl-acyl carrier-protein reductase (FabI), preventing the bacteria from manufacturing fatty acids it needs for building cell membranes and other vital functions. The targeted delivery of triclosan into the cell of *E. coli* by lysozyme could be confirmed by assessing the inhibition of FabI in the cytoplasm. To investigate intracellular uptake of triclosan during antimicrobial action of TCS-lysozyme, *E. coli* cells were treated with the TCS-lysozyme complex (20 µg/ml, protein based) and inhibition of FabI enzymatic activity of the cytoplasmic extracts was measured. Cells, suspended in

Table 1. Antimicrobial activity of the lysozyme-triclosan complex against different strains of Gram-negative and Gram-positive bacteria.

	Gram-negative				Gram-positive			
	E. coli	*K. pneumon.*	*P. aerugin.*	*S. typhi.*	*S. aureus*	*S. zooepi.*	*S. epiderm.*	*M. lut.*
Ctrl	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
TCS alone	0.9	2.5	0.0	0.7	0.1	0.6	0.1	6.0
LZ alone	0.0	0.0	0.0	0.0	3.9	0.6	1.1	3.2
TCS-LZ	6.6	5.2	5.3	3.6	5.0	8.0	7.4	6.3

Antimicrobial activity is expressed as bacterial killing power using the formula: ∂log killing = $\log_{10} nc - \log_{10} np$, where *nc* and *np* are CFU per milliliter of mock and treated cells, respectively. The assay was performed in trypticase soy broth at 30°C for 20 h incubation with protein-based 62.5 μg/ml of TCS-LZ or TCS alone or LZ alone at concentration equivalent to their contents in the complex. TCS-LZ was prepared by adding 30-fold molar excess of TCS over LZ [29]. Control, cells treated with solvent without compound. The initial cell number was adjusted to 10^8 CFU/ml.
Ctrl: Control; LZ: Lysozyme; TCS: Triclosan; TCS-LZ: lysozyme-triclosan complex.

trypticase soya broth (TSB), were mixed with an equal volume of TCS-lysozyme complex or its triclosan or lysozyme equivalents. After incubation at 30°C for 2 h, excess protein or triclosan was removed by centrifugation, then cell lysates of the treated cells were prepared by freeze-thawing then sonication of the pellets in 50 mM Tris-HCl, 2 mM EDTA (pH 7.6). Cell debris was removed by centrifugation for 5 min at 12,000 r.p.m., and supernatant was saved as treated cell extracts. Enzymatic activity of FabI (the cytoplasmic target of triclosan) in the treated cell extracts was monitored fluorometrically by measuring NADH consumption for 20 min on addition of crotonoyl CoA as substrate. Fluorometric measurement of NADH oxidation was used in these experiments to obtain a higher sensitivity. The result is interpreted as inhibition of FabI enzymatic activity in the cell extracts as ΔRFU per minute per milligram total protein.

To test the ability of triclosan or the complex to inhibit FabI in the cytoplamic milieu of *E. coli*, first the isolated cytoplasmic fraction, from mock-treated cells, was mixed with triclosan or the complex and inhibition was monitored. Both TCS-LZ and free TCS caused a similar magnitude of severe inhibition of FabI activity when added to the cytoplasmic extract (**Figure 6A**), indicating their comparable potency to inhibit the target enzyme in the complex environment of cytoplasm and validate the assay. **Figure 6B** shows the kinetics of NADH consumption by FabI enzymatic activity of the extracts of pretreated *E. coli* cells with 1.4 μM TCS-lysozyme (carrier based) or equivalent free triclosan. Mock-treated cell extracts (without any test compound) showed profound enzymatic activity, causing linear decrease in NADH fluorescence (**Figure 6B**). Lysozyme alone caused a small decrease in NADH consumption, probably as a result of partial complexation of NADH with the hydrophobic core of lysozyme. The TCS-lysozyme and its free triclosan equivalents showed reduced consumption of NADH (FabI inhibition), but the inhibition by TCS-lysozyme was remarkably higher than that of free triclosan. TCS-lysozyme complex caused > 95% inhibition of FabI activity, whereas 47% inhibition of activity was observed by triclosan alone (**Figure 6C**). The inhibition observed by triclosan is considered to be the entrapment of triclosan in the hydrophilic cell membrane as well as the lethal effect on bacterial cells [39] at this concentration (42 μM corresponding to 12 μg/ml). The results clearly demonstrate that in TCS-lysozyme complex, triclosan is loaded in the hydrophobic cavity of lysozyme where lysozyme facilitates its delivery into bacterial cytoplasm after dissociation at the lipid environment of bacterial cell membrane, thus inhibiting the target enzyme FabI. The potent antimicrobial action of TCS-lysozyme complex together with its specific targeting of triclosan to the fatty acid synthesis pathway (cytoplasmic FabI) of pathogenic bacteria confirms the successful site-specific drug-delivery strategy by using lysozyme as a new approach for therapeutic development.

10. Concluding remarks

Despite the supremacy of some phenolic antimicrobials, such as triclosan, acting on weak targets of bacteria, for example fatty acid synthesis pathway, they present formulation difficulties because they are practically insoluble in water. Triclosan is very insoluble in water, but once inside the cell it poisons a specific enzyme that many bacteria need for survival [45]. Triclosan blocks the active site of the FabI, preventing the bacteria from manufacturing fatty acids. Humans do not have this enzyme, so triclosan is harmless to humans. Lysozyme is an antimicrobial enzyme having high water solubility as well as a negligible toxigenic potential beside its known anti-inflammatory activity. Lysozyme, possessing the ability selectively to recognize and disrupt microbial cell walls, could provide almost unlimited opportunities in producing highly efficient and specialized systems for anti-infection drug targeting. The unique properties of lysozyme, such as its high degree of solubility, multivalency of its active site, globular architecture and well-defined molecular mass, make it a promising new scaffold for the delivery of various phenolic drugs with different targets in the microbial cells. The strategy proved to produce high water solubility of the drug-lysozyme complex with significant efficiency against broad spectrum of

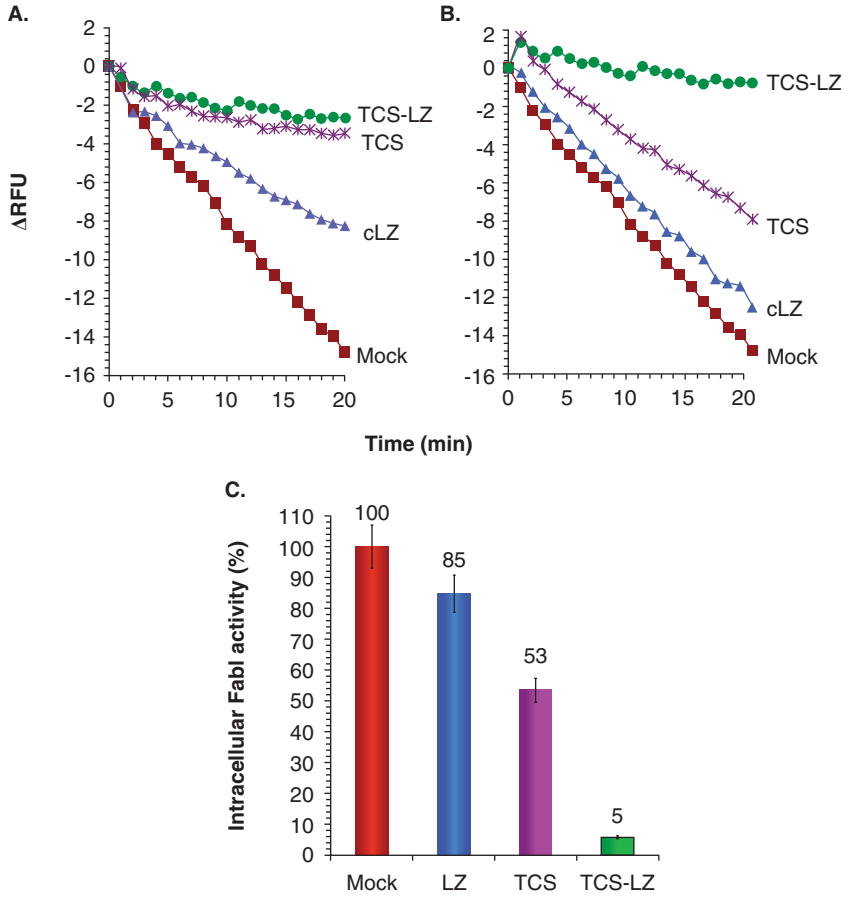

Figure 6. The delivery of triclosan into *E. coli* K-12 cells and subsequent inhibition of the cytoplasmic FabI (intracellular target). Activity of FabI was kinetically monitored by measuring NADH consumption, fluorometrically, on addition of crotonoyl CoA as substrate. **A.** Inhibition of FabI enzymatic activity in the isolated cytoplasmic extract, whereas the complex TCS-LZ or free TCS was added to the extract as inhibitor. **B.** Cells were pretreated with TCS-LZ complex (20 μg/ml, protein-based) or its TCS or LZ equivalents (in TSB). After incubation at 30°C for 2 h, cells were washed, and the lysates of the treated cells were prepared by freeze-thawing then sonication. Total protein in the extract was normalized by using Bio-Rad protein assay. Enzymatic activity of FabI in the cytoplasmic extracts of treated cells was monitored. The result is expressed as inhibition of FabI enzymatic activity in the cell extracts as change in the relative fluorescence units per minute per milligram total protein (ΔRFU). **C.** The residual FabI activity expressed as percentage to the mock-treated cell extract. Each datum point represents the mean plus or minus standard error of triplicates.
cLZ: Control lysozyme; LZ: Lysozyme; TCS: Triclosan; TCS-LZ: Triclosan-lysozyme complex; TSB: Trypticase soya broth.

microorganisms. The strategy provides great promise in alleviating: i) the antibiotic resistance problem; ii) the water solubility problem commonly encountered with pharmaceutical application of phenolic drugs, through loading of a phenolic drug with the highly water-soluble lysozyme; and iii) the non-specific toxicity associated with chemotherapy while improving the therapeutic index of the drug.

Lysozyme as a natural anti-inflammatory protein and regulator of the immune response offers exciting new alternatives to conventional therapeutics and drug-delivery systems. Such a multifaceted delivery system having its individual molecules acting in a coordinated way should allow for delivery of the phenolic antimicrobial drugs with required temporal deposition, particularly in the topical or systemic treatment of surgical wound infections. Regardless of the great contribution of this approach to the basic science and pharmacology, it has a bright future that may yield new avenues in the treatment of different infectious diseases.

11. Expert opinion

Antimicrobial drug delivery sets high demands on the delivery technology to target pathogens specifically and reduce side effects as well as dosing frequency. However, the rise of biological formulations poses fundamental problems with this approach as such molecules are degraded in the stomach,

insoluble, and may target natural microflora such as the intestinal lactic acid bacteria or mammalian cells. Protein-based delivery by stable proteins able to recognize and disrupt membranes of pathogenic cells may circumvent the issue and herald a fascinating opportunity for delivery technology.

The application of protein-based drug delivery is still at a relatively early stage of development, but has experienced rapid growth in the past decade. Recent research has shown that proteins are useful tools for studying fundamental targeting problems and, with their desirable globular structure and polyvalent character, they can also present practical solutions to drug delivery issues such as solubility, biodistribution and targeting [7-9,37]. The lysozyme, from avian egg albumen, structure is likely to find uses in applications involving multifunction particulate systems combining targeting and therapy. This approach utilizes structurally permitted loading of lysozyme with a phenolic antimicrobial drug that has a unique target inside the microbial cells, that is, fatty acid biosynthesis, and thus introduces an innovative strategy that offers a new means in the treatment of infectious diseases. This finding is the first to describe how the membrane disrupting function of lysozyme can be utilized to target antimicrobial drug(s) specifically to pathogen cells and heralds a fascinating opportunity for its potential candidacy as a new antimicrobial or drug-targeting strategy.

Generally, protein drugs are administered as injections and are incompatible with oral administration. The incompatibility of protein drugs with oral administration is a result of poor permeability across the intestinal epithelium [57] and because the intestinal lumen and epithelial cells contain many enzymes that degrade proteins [58]. However, lysozyme as well as cytochrome *c* and bromelain are clinically considered as oral protein drugs. Megalin, a large glycoprotein functioning as an endocytic receptor, is highly expressed in the renal proximal tubule and intestinal epithelial cells [59], whereas its ligands include lysozyme, cytochrome *c* and aminoglycosides [60,61]. Megalin is suggested to be involved in the absorption of intact lysozyme in the intestine [62,63]. Interestingly, it has been found that the intestinal absorption of lysozyme is segment-selective and occurs preferentially from the upper intestine [63].

Notably, the orally administered lysozyme as well as microencapsulated lysozyme can scavenge advanced glycated end products (AGE) *in vivo* and show a protective effect against the development of the initial stages of diabetic nephropathy and renal hypertrophy in an experimental diabetic rat model [64]. In the study, lysozyme was found to be actively taken up by the Peyer's patches, where a remarkable amount of the intact lysozyme was delivered into the systemic circulation [64]. Further, lysozyme was found to be bioabsorbable in human subjects with 48 h circulation time [65].

The gastrointestinal stability of lysozyme has also been evident from several structural studies. In a recent work, it was found that > 60% of hen lysozyme remained intact after reaction with pepsin for 2 or 4 h at either pH 2.0 or pH 4.0 [35]. Fontana *et al.* [66] found that whereas hen lysozyme at pH 2.0 was fully resistant to proteolysis by pepsin, the other members of the lysozyme superfamily were cleaved at different rates at a few sites of the polypeptide chain. They found that horse and pigeon lysozymes were attacked by proteinase K at pH 8.3, whereas dog and hen lysozymes were resistant to proteolysis under identical experimental conditions. In another study, hen lysozyme was found to be fully resistant to proteolysis by thermolysin and was partially nicked in the presence of trifluoroethanol [67].

As hen lysozyme has been found to be bioabsorbable and relatively stable against proteolysis in the gastrointestinal tract, drug-lysozyme complex is expected to circulate in the blood long enough to accumulate at the sites of infection, but can also be eliminated from the body at a reasonable rate. A comparison of the features of lysozyme with other polymers shows that the lysozyme architecture can provide several advantages for drug delivery applications. For example, the controlled multivalency of the active site cleft of lysozyme can be used to accommodate several drug molecules in a well-defined manner. In addition, the low polydispersity of lysozyme should provide reproducible pharmacokinetic behaviour, in contrast to that of some polymers containing fractions with vastly different molecular mass within a given preparation. As cationic polymers have shown great promise in the development of stomach-targeted antimicrobial drug delivery systems [68], the application of the cationic lysozyme in this area is particularly interesting. Furthermore, the more globular shape of lysozyme, as opposed to the random coil structure of most polymers, makes this enzyme a potential drug carrier for antibacterial treatment.

Food proteins show great promise for developing a range of new carriers with the potential to incorporate pharmaceutical compounds and provide controlled release via the oral route [8,69,70]. Notably, lysozyme has been affirmed as GRAS by the FDA, with its tentative final rule dated 13 March 1998 (21 CFR Part 184, docket number 89G-0393). The USDA 2009 (Doc. No. AMS-TM-09-0060; TM-09-07) has authorized the use of lysozyme (CAS# 9001-63-2) as an ingredient in or on processed products labeled as 'organic', whereas the FDA established GRAS status when it is identified as 'egg-white lysozyme'. In addition, a recent study concluded that the use of lysozyme as an additive in Grana Padano cheese does not appear to be harmful in egg allergic subjects [71]. Clear advantages of lysozyme as a drug carrier include its abundant renewable sources, and acceptability as naturally occurring food components.

Although the extra effort required for the synthesis of lysozyme complexes with phenolic drugs means that these molecules must possess distinct advantages over the polymer analogues to be useful in practical terms, research has shown that lysozyme does indeed have many unique pharmaceutical features that warrant its further exploration in drug delivery. Inflammation is a biological response of immune cells (macrophages, dendritic cells and neutrophils) already present

in vascular tissues to microbial infections. At the onset of an infection, these cells undergo activation and release inflammatory mediators responsible for the clinical signs of inflammation. Reducing the inflammatory barrier is essential to allow penetration of the antibiotic. Lysozyme is known for its anti-inflammatory function through inhibition of chemotaxis of activated neutrophils, inhibition of mitogen-induced lymphoblastogenesis and autologus mixed lymphocyte reaction [72,73]. Besides, studies have shown that lysozyme is also capable of directly modulating the entire activation of complement system reaction cascade, inhibiting the classical pathway, and inhibiting polymorphonuclear leukocyte response towards complement-derived chemotaxins [13]. One of the areas that remains to be addressed is the biodistribution behavior and allergenicity of drug-lysozyme complexes. For example, the tissue localization of drug-lysozyme complex is easy to predict in advance, but more studies are required to determine its allergenic reaction on long-term build-up. A further area that has to be investigated is the release of drugs from lysozyme. The steric hindrance associated with the dense globular lysozyme architecture is a useful scaffold for the exploitation of various alternative release mechanisms, including enzymatic cleavage. Some work in this area is required.

Declaration of interest

The work is supported in part by a Scientific Aid Fund from Mandom Co. Ltd, Japan.

Bibliography

1. Heath RJ, White SW, Rock CO. Inhibitors of fatty acid synthesis as antimicrobial chemotherapeutics. Appl Microbiol Biotechnol 2002;58:695-703
2. Nudera WJ, Fayad MI, Johnson BR, et al. Antimicrobial effect of triclosan and triclosan with gantrez on five common endodontic pathogens. J Endod 2007;33:1239-42
3. Bhargava HN, Leonard PA. Triclosan: applications and safety. Am J Infect Control 1996;24:209-18
4. Jones RD, Jampani HB, Lee AS. Triclosan: a review of effectiveness and safety in health care settings. Am J Infect Control 2000;28:184-96
5. Paul KS, Bacchi CJ, Englund PT. Multiple triclosan targets in Trypanosoma brucei. Eukaryot Cell 2004;3:855-61
6. Heath RJ, Li J, Roland GE, Rock CO. Inhibition of the Staphylococcus aureus NADPH-dependent enoyl-acyl carrier protein reductase by triclosan and hexachlorophene. J Biol Chem 2000;275:4654-9
7. Bhutia SK, Maiti TK. Targeting tumors with peptides from natural sources. Trends Biotechnol 2008;26:210-17
8. Liu X, Sun Q, Wang H, et al. Microspheres of corn protein, zein, for an ivermectin drug delivery system. Biomaterials 2005;26:109-15
9. Torchilin VP, Lukyanov AN. Peptide and protein drug delivery to and into tumors: challenges and solutions. Drug Discov Today 2003;8:259-66
10. Ng H, Garibaldi JA. Death of Staphylococcus aureus in liquid whole egg near pH 8.0. Appl Microbiol 1975;29:782-6
11. Jolles P, Jolles J. What's new in lysozyme research? Always a model system, today as yesterday. Mol Cell Biochem 1984;63:165-89
12. Lee-Huang S, Huang PL, Sun Y, et al. Lysozyme and RNases as anti-HIV components in beta-core preparations of human chorionic gonadotropin. Proc Natl Acad Sci USA 1999;96:2678-81
13. Ogundele MO. A novel anti-inflammatory activity of lysozyme: modulation of serum complement activation. Mediators Inflamm 1998;7:363-5
14. Ohno N, Morrison DC. Lipopolysaccharide interaction with lysozyme. Binding of lipopolysaccharide to lysozyme and inhibition of lysozyme enzymatic activity. J Biol Chem 1989;264:4434-41
15. Sava G, Ceschia V, Pacor S, Zabucchi G. Observations on the antimetastatic action of lysozyme in mice bearing Lewis lung carcinoma. Anticancer Res 1991;11:1109-13
16. Ibrahim HR, Aoki T, Pellegrini A. Strategies for new antimicrobial proteins and peptides: lysozyme and aprotinin as model molecules. Curr Pharm Des 2002;8:671-93
17. Ibrahim HR, Thomas U, Pellegrini A. A helix-loop-helix peptide at the upper lip of the active site cleft of lysozyme confers potent antimicrobial activity with membrane permeabilization action. J Biol Chem 2001;276:43767-74
18. Posse E, De Arcuri BF, Morero RD. Lysozyme interactions with phospholipid vesicles: relationships with fusion and release of aqueous content. Biochim Biophys Acta 1994;1193:101-6
19. Gibson KF, Phadke S. Intracellular distribution of lysozyme in rat alveolar type II epithelial cells. Exp Lung Res 1994;20:595-611
20. Ohno N, Morrison D. Lipopolysaccharide interactions with lysozyme differentially affect lipopolysaccharide immunostimulatory activity. Eur J Biochem 1989;186:629-36
21. Gorbenko GP, Ioffe VM, Kinnunen PK. Binding of lysozyme to phospholipid bilayers: evidence for protein aggregation upon membrane association. Biophys J 2007;93:140-53
22. Haas M, Moolenaar F, Meijer DKF, De Zeeuw D. Specific drug delivery to the kidney. Cardiovasc Drugs Ther 2002;16:489-96
23. Haas M, Kluppel AC, Wartna ES, et al. Drug-targeting to the kidney: renal delivery and degradation of a naproxen-lysozyme conjugate in vivo. Kidney Int 1997;52:1693-9
24. Kok RJ, Grijpstra F, Walthuis RB, et al. Specific delivery of captopril to the kidney with the prodrug captopril-lysozyme. J Pharmacol Exp Ther 1999;288:281-5
25. Franssen EJF, Moolenaar F, De Zeeuw D, Meijer DKF. Low molecular weight proteins as carriers for

renal drug targeting: naproxen coupling to lysozyme via Spacer L-lactic acid. Pharm Res 1993;10:963-9

26. Prakash J, Sandovici M, Saluja V, et al. Intracellular delivery of the p38 mitogen-activated protein kinase inhibitor SB202190 4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole] in renal tubular cells: a novel strategy to treat renal fibrosis. J Pharmacol Exp Ther 2006;319:8-19

27. Zhang Z, Zheng Q, Han J, et al. The targeting of 14-succinate triptolide-lysozyme conjugate to proximal renal tubular epithelial cells. Biomaterials 2009;30:1372-81

28. Kok RJ, Haas M, Moolenaar F, et al. Drug delivery to the kidneys and the bladder with the low molecular weight protein lysozyme. Ren Fail 1998;20:211-17

29. Hoq MI, Mitsuno K, Tsujino Y, et al. Triclosan-lysozyme complex as novel antimicrobial macromolecule: a new potential of lysozyme as phenolic drug-targeting molecule. Int J Biol Macromol 2008;42:468-77

30. Salton MRJ, Pavlik JG. Studies of the bacterial cell wall. VI. Wall composition and sensitivity to lysozyme. Biochem Biophys Acta 1960;39:398-407

31. Ibrahim HR. On the catalytically-independent novel antimicrobial function of hen egg-white lysozyme: a conformation dependent activity. Nahrung Food 1998;42:187-93

32. Ibrahim HR, Matsuzaki T, Aoki T. Genetic evidence that antibacterial activity of lysozyme is independent of its catalytic function. FEBS Lett 2001;506:27-32

33. Laible NJ, Germaine GR. Bactericidal activity of human lysozyme, muramidase-inactive lysozyme, and cationic polypeptides against Streptococcus sanguis and Streptococcus faecalis: inhibition by chitin oligosaccharides. Infect Immun 1985;48:720-8

34. Ibrahim HR, Higashiguchi S, Juneja LR, et al. A structural phase of heat-denatured lysozyme with novel antimicrobial action. J Agric Food Chem 1996;44:1416-23

35. Ibrahim HR, Inazaki D, Abdou A, et al. Processing of lysozyme at distinct loops by pepsin: a novel action for generating multiple antimicrobial peptide motifs in the newborn stomach. Biochim Biophys Acta 2005;1726:102-14

36. Imoto T, Johnson LN, North ACT, et al. Vertebrate lysozymes. In: Boyer PD, editor. The enzymes. Volume 7. Academic Press, New York; 1972. p. 665-868

37. Lao BJ, Tsai WLP, Mashayekhi F, et al. Inhibition of transferrin iron release increases in vitro drug carrier efficacy. J Control Release 2007;117:403-12

38. Stewart MJ, Parikh S, Xiao G, et al. Structural basis and mechanism of enoyl reductase inhibition by triclosan. J Mol Biol 1999;290:859-65

39. Villalain J, Mateo CR, Aranda FJ, et al. Membranotropic effects of the antibacterial agent Triclosan. Arch Biochem Biophys 2001;390:128-36

40. Schweizer HP. Triclosan: a widely used biocide and its link to antibiotics. FEMS Microbiol Lett 2001;202:1-7

41. Panagakos FS, Volpe AR, Petrone ME, et al. Advanced oral antibacterial anti-inflammatory technology: a comprehensive review of the clinical benefits of a triclosan copolymer fluoride dentifrice. J Clin Dent 2005;16(Suppl):S1-19

42. Liu B, Wang Y, Fillgrove KL, Anderson VE. Triclosan inhibits enoyl-reductase of type I fatty acid synthase in vitro and is cytotoxic to MCF-7 and SKBr-3 breast cancer cells. Cancer Chemother Pharmacol 2002;49:187-93

43. Dastychova E, Necas M, Pencikova K, Cerny P. Contact sensitization to pharmaceutic aids in dermatologic cosmetic and external use preparations. Ceska Slov Farm 2004;53:151-6

44. Lores M, Llompart M, Sanchez-Prado L, et al. Confirmation of the formation of dichlorodibenzo-p-dioxin in the photodegradation of triclosan by photo-SPME. Anal Bioanal Chem 2005;381:1294-8

45. Yazdankhah SP, Scheie AA, Hoiby EA, et al. Triclosan and antimicrobial resistance in bacteria: an overview. Microb Drug Resist 2006;12:83-90

46. Paulidou A, Maffeo D, Yannakopoulou K, Mavridis IM. Crystal structure of the inclusion complex of the antibacterial agent triclosan with cyclomaltoheptaose and NMR study of its molecular encapsulation in positively and negatively charged cyclomaltoheptaose derivatives. Carbohydr Res 2008;343:2634-40

47. Tsai PJ, She CH. Significance of phenol-protein interactions in modifying the antioxidant capacity of peas. J Agric Food Chem 2006;54:8491-4

48. Bhattacharyya R, Samanta U, Chakrabarti P. Aromatic-aromatic interactions in and around alpha-helices. Protein Eng 2002;15:91-100

49. Chapman-Smith A, Cronan JE Jr. Molecular biology of biotin attachment to proteins. J Nutr 1999;129:477-84

50. Samanta U, Chakrabarti P. Assessing the role of tryptophan residues in the binding site. Protein Eng 2001;14:7-15

51. Haslam E. Polyphenol-protein interactions. Biochem J 1974;139:285-8

52. Kapoor M, Reddy CC, Krishnasastry MV, et al. Slow-tight-binding inhibition of enoyl-acyl carrier protein reductase from Plasmodium falciparum by triclosan. Biochem J 2004;381:719-24

53. Hoq MI, Aoki T, Ibrahim HR. Triclosan-lysozyme complex: a promising anti-microbial macromolecule stable against photooxidative damage. Food Res Int 2009;42:298-306

54. Cheetham JC, Artymuik PJ, Phillips DC. Refinement of an enzyme complex with inhibitor bound at partial occupancy. Hen egg-white lysozyme and tri-N-acetylchitotriose at 1.75 A resolution. J Mol Biol 1992;224:613-28

55. Seaman PF, Ochs D, Day MJ. Comment on: triclosan resistance in methicillin-resistant Staphylococcus aureus expressed as small colony variants: a novel mode of evasion of susceptibility to antiseptics. J Antimicrob Chemother 2007;60:175-6

56. Tkachenko O, Shepard J, Aris VM, et al. A triclosan-ciprofloxacin cross-resistant mutant strain of Staphylococcus aureus displays ann alteration in the expression of several cell membrane structural and functional genes. Res Microbiol 2007;158:651-8

57. Aungst BJ. Novel formulation strategies for improving oral bioavailability of drugs with poor membrane permeation

or presystemic metabolism. J Pharm Sci 1993;82:979-87

58. Woodley JF. Enzymatic barriers for GI peptide and protein delivery. Crit Rev Ther Drug Carr Syst 1994;11:61-95

59. Christensen EI, Birn H. Megalin and cubilin: multifunctional endocytic receptors. Nat Rev Mol Cell Biol 2002;3:256-66

60. Leheste JR, Rolinski B, Vorum H, et al. Megalin knockout mice as an animal model of low molecular weight proteinuria. Am J Pathol 1999;155:1361-70

61. Yammani RR, Seetharam S, Seetharam B. Cubilin and megalin expression and their interaction in the rat intestine: effect of thyroidectomy. Am J Physiol Endocrinol Metab 2001;281:E900-7

62. Nagai J, Katsube T, Murakami T, Takano M. Effect of gentamicin on pharmacokinetics of lysozyme in rat: interaction between megalin substrates in the kidney. J Pharm Pharmacol 2002;54:1491-6

63. Takano M, Koyama Y, Nishikawa H, et al. Segment-selective absorption of lysozyme in the intestine. Eur J Pharmacol 2004;502:149-55

64. Cocchietto M, Zorzin L, Toffoli B, et al. Orally administered microencapsulated lysozyme downregulates serum AGE and reduces the severity of early-stage diabetic nephropathy. Diabet Metabol 2008;34:587-94

65. Hashida S, Ishikawa E, Nakamichi N, Sekino H. Concentration of egg white lysozyme in the serum of healthy subjects after oral administration. Clin Exp Pharmacol Physiol 2002;29:79-83

66. Fontana A, De Laureto PP, Spolaore B, et al. Probing protein structure by limited proteolysis. Acta Biochim Polonica 2004;51:299-321

67. De Laureto PP, De Philippis V, Scaramella E, et al. Limited proteolysis of lysozyme in trifluorethanol: isolation and characterization of a partially active enzyme derivative. Eur J Biochem 1995;230:779-87

68. Inouye K, Machida Y, Sannan T, Nagi T. Buoyant sustained release tablets based on chitosan. Drug Des Deliv 1988;2:165-75

69. Latha MS, Rathinam K, Mohanan PV, Jayakrishnan A. Bioavailability of theophylline from glutaraldehyde cross-linked casein microspheres in rabbits following oral administration. J Control Release 1995;34:1-7

70. Leclerc PL, Remondetto GE, Ramassamy C, Subirade M. In: Conference on Bioencapsulation. Kingston; 2005. p. 24-6

71. Iaconelli A, Fiorentini L, Bruschi S, et al. Absence of allergenic reactions to egg white lysozyme additive in Grana Padano cheese. J Am Coll Nutr 2008;27:326-31

72. Goldman AS, Goldblum RM, Hanson LA. Anti-inflammatory systems in human milk. Adv Exp Med Biol 1990;262:69-76

73. Gordon LI, Douglas SD, Kay NE, et al. Modulation of neutrophil function by lysozyme. Potential negative feedback system of inflammation. J Clin Invest 1979;64:226-32

Affiliation

Hisham R Ibrahim
Associate Professor,
Kagoshima University,
Department of Biochemistry and Biotechnology,
Faculty of Agriculture,
Kagoshima 890-0065, Japan
Tel: +81 99 285 8656; Fax: +81 99 285 8525;
E-mail: hishamri@chem.agri.kagoshima-u.ac.jp