Liposomes as novel anti-infectives targeting bacterial virulence factors?

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A recent report commissioned by Prime Minister David Cameron and chaired by former Goldman Sachs chief economist Jim O'Neill warns that the emergence, persistence and spread of antimicrobial resistance could lead to 10 million deaths per year and cause an economic burden as much as US$100 trillion by 2050. In the midst of this global crisis, unprecedented paths are being explored to combat bacterial infection. Virulence factors, and more particularly pore-forming toxins, play a key role in increasing morbidity and mortality caused by drug-resistant bacterial infections. Novel anti-infective liposomes specifically targeting and neutralizing these cytotoxic toxins are potential game-changers in the fight against deadly infections.

Classic antimicrobials target processes that are essential for bacterial growth and survival. Even though some are still effective against multidrug-resistant pathogens, they will eventually stimulate bacterial evolution and elicit resistance. By contrast, therapeutic approaches that do not affect processes required for survival *per se* have the great advantage of not exerting any selective evolutionary pressure which would promote the emergence of drug resistance. Novel classes of antibiotics strive to reach this objective. The recently discovered Gram-positive-specific teixobactin, for instance, targets highly conserved components involved in bacterial cell wall synthesis; this reduces dramatically the likelihood of the rise of drug resistance [1]. Other compounds are also used for their 'anti-resistance' property; β-lactamase inhibitors (e.g., avibactam), for example, act in combination with β-lactam antibiotics to increase treatment efficacy and spectrum [2].

In contrast to classic approaches, 'anti-virulence approaches' are tailored not to kill but rather to disarm bacteria by targeting their virulence factors [3–5]. These approaches are unlikely to disturb the local flora that are free of virulence factors. Monoclonal antibodies targeting specific bacterial toxins and liposomal anti-infective agents are at the forefront of such new strategies to combat infections. Targeting toxins to stand up to bacterial infections has positive precedents: bacterial vaccines made of 'detoxified' toxins raise the level of antibodies neutralizing bacterial toxins. The combined preventive vaccine against diphtheria, tetanus and pertussis, which is composed of inactivated toxins, is a well-known illustration of this approach.

Bacterial toxins are offensive virulence factors which modify and destroy cellular membranes and structures, at epithelial surfaces, in connective tissues, on blood vessels and on host defense cells – possibly even in locations and organs distant from the focus of infection. Pore-forming toxins (PFTs) constitute 25–30% of all cytotoxic bacterial proteins. They make up the single largest category of virulence factors and are produced by both Gram-positive and Gram-negative bacterial species. Among these are clinically highly relevant pathogens such as *Streptococcus pneumoniae* (pneumolysin), group A and B streptococci (CAMP factor or cocytolysin, β-hemolysin/cytolysin, streptolysin O, streptolysin S), *Staphylococcus aureus* (Panton-Valentine leukocidin, α-toxin/α-hemolysin), *Escherichia coli* (hemolysin

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A/α-hemolysin) and *Mycobacterium tuberculosis* (ESAT-6) [6]. PFTs play a fundamental role in the infection process and in the development of severe and fatal complications. On the one hand, they perforate membranes of host cells. They are hypothesized to do so in order to deliver bacterial factors, or to benefit from cell nutrients, or to escape from phagosomes in the case of PFTs acting intracellularly, and to directly kill target cells. On the other hand, they affect host defense processes and trigger undesirable inflammatory responses. Loss of PFTs causes bacteria to be less virulent or completely avirulent, while transgenic expression of a PFT was shown to turn an otherwise harmless bacterium into a harmful pathogen [6]. Because of their almost universal presence in bacterial pathogens, PFTs are important targets for broadly applicable antimicrobial prophylaxis and therapeutics.

Antitoxins strategies indeed are among the most intensively pursued anti-infective strategies. Examples include various monoclonal antibodies, targeting α-hemolysin of *S. aureus* (e.g., AR-301 and MEDI4893), the protective antigen pore-forming component of the lethal toxin of *Bacillus anthracis* (e.g., raxibacumab, Anthim and Valortim), or PFTs A and B of *Clostridium difficile* (actoxumab and bezlotoxumab). These agents, however, have clear limitations. Even if they target toxins associated with virulence, they do not address the broad heterogeneity in bacterial virulence factors. Also, since monoclonal antibodies target a single toxin, sometimes produced by only a specific serotype of the given pathogen, therapeutic interventions are most effective only if the specific pathogen responsible for the infection is readily identifiable. The therapeutic approach is further complicated by the fact that each virulent toxin varies in time and space during the infection process.

Recently, new liposomal nanoparticles, named CAL02, have been discovered as powerful broad-spectrum anti-infective weapons. Liposomes are synthetic nearly spherical vesicular structures made up of one or more lipid bilayers (unilamellar or multilamellar liposomes, respectively). Liposomes can be synthesized from natural lipids exclusively and, therefore, possess limited intrinsic toxicity. They have found commercial applications as drug delivery vehicles in medicine, adjuvants in vaccination, signal enhancers/carriers in medical diagnostics and penetration enhancer in cosmetics. CAL02 consists of a specific mixture of empty, small, uncoated, unilamellar liposomes composed exclusively of cholesterol and sphingomyelin. The surface of CAL02 displays large and stable microdomains similar to cell-surface rafts recognized by bacterial toxins. The cellular raft microdomains are characterized by a tight packing of saturated acyl chains within the liquid-ordered phase, and are unstable structures of <40 nm diameter [7]. In contrast to these rafts, which are formed transiently *in vivo*, the stable liquid-ordered microdomains, artificially displayed in the form of liposomes, bind PFTs with an affinity significantly higher than that between the toxins and the host cells. CAL02 indeed was shown to capture as many toxins as *S. pneumoniae's* pneumolysin, *Streptococcus pyogenes*' streptolysin, *Clostridium tetani's* tetanolysin, *Clostridium perfringens*' phospholipase C-acting α-toxin and *S. aureus's* α-hemolysin [8].

In vivo, CAL02 increases the efficacy of antibiotics when administered as adjunctive therapy, in rescuing infected mice from deadly bacteremia and pneumonia infections induced by *S. pneumoniae* and *S. aureus* [8]. This is all the more relevant since a toxin like *S. pneumoniae's* pneumolysin is released massively following lysis caused by antibiotics, and complications and even death may result days after the onset of antibiotic therapy from the spread of toxins, when tissues are already pathogen-free [9,10]. This agrees with the observation that improved therapeutic efficacy of a β-lactam/macrolide combination in severe pneumococcal disease is associated with a marked reduction in the synthesis and release of pneumolysin [11]. This is also in line with the favorable potential of vaccine formulations containing pneumolysin toxoid, as reported recently [12–15]. Since this PFT is almost uniformly present in all *S. pneumoniae* clinical isolates, CAL02 may be prescribed readily, before serotyping and regardless of any resistance to antibiotics, to avoid tissue damage, promote bacterial clearance and limit the immunomodulatory side effects of antimicrobial therapies. The time frame of intervention appears to be much the same as that necessary for current antimicrobial therapies.

In addition to their direct impact on toxins, liposomes present parallel therapeutic advantages. While they have no direct bactericidal activity, they nevertheless can lead to a significant reduction of bacterial loads in blood and to organ protection when used as monotherapy in severely infected animals [8]. By depriving bacteria of the mechanisms they use to feed and multiply, and by acting as a shield for the immune system, which can then more appropriately clear the infection, CAL02 affects bacterial survival indirectly. Likewise, it attenuates the reduction of blood B-cells and substantially reduces TNF-α blood levels in infected animals. One expects that CAL02 will even be used as prophylaxis, just as the anti-α-hemolysin MEDI4893, currently assessed for the prevention of pneumonia caused by bacterial infection with *S. aureus* in high-risk patients.

Finally, these liposomes have the potential to suppress chronic infections. Indeed, all constituents of the formulation have already been used in other pharmaceutical formulations and multiple administration has proven to be non-toxic in humans. The near-total biocompatibility of CAL02 lipid components, which are ubiquitous, naturally occurring dietary lipids, reduces the potential concern with safety issues related to possible long-term accumulation in the body, a broad issue relevant to most nanoparticles developed currently in nanomedicine [16]. The small and empty liposomes are non-immunogenic and biologically neutral. Liposomes already have multiple medical applications. Moreover, liposomal formulations of antibiotics have been used to target pulmonary alveolar macrophages in the respiratory tract [17], as they are naturally taken up by the mononuclear phagocyte system [16].

Empty liposomes of a particular composition appear to target key virulent factors responsible for the severity of infections caused by many clinically relevant pathogens via PFTs. They

incur milder evolutionary pressure and lead to less resistance to existing antimicrobials when coupled with them. They thus appear to offer a long-term solution to the exacerbating rise of bacterial resistance. They can certainly not resolve, by themselves, all pathogenic processes caused by infection and sequentially triggered by a complex variety of virulence factors. But they can play a beneficial role as add-ons to standard-of-care antimicrobials. They provide the much-needed time that allows antibiotics to realize their full bactericidal potential. They improve the efficacy of antibiotics. They may even lead to a reduction of the duration of antibiotic treatment. Their use as monotherapy or even as prophylaxis is also conceivable; by neutralizing factors that overwhelm the patient's defense system, this non-bactericidal drug may provide sufficient respite for the host immune responses to defeat the infection, in the absence of side effects commonly observed with broad-spectrum agents or with strategies aiming at modulating the natural immune reactions. Therefore, they can also have merit if used in combination with bactericidal chemotherapy in immunodeficient patients, by slowing down the development of a systemic disease. These multifaceted liposomes point to a new era in the fight against severe infections.

Financial & competing interests disclosure

Both authors are employed by LASCCO SA, Geneva, Switzerland, which has rights to license CAL02 worldwide. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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