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Editorial

Potential for inhibition of bacterial efflux pumps in multidrug-resistant *Vibrio cholerae*

The bacterium *Vibrio cholerae* is the causative agent of cholera, a severe gastrointestinal disease characterized primarily by the excretion of large amounts of the so-called rice water stool, which contains critical electrolytes and water. Cholera patients may succumb very quickly to the resulting severe dehydration producing significant morbidity and mortality rates. According to the World Health Organization (WHO), approximately 3-5 million cases of cholera occur each year with 100,000-120,000 yearly estimated deaths. Clearly, *V. cholerae* represents a critical public health concern[1].

Key therapeutic efforts against cholera in humans include electrolyte replenishment, and for severe cholera cases, antimicrobial agents, such as tetracycline, furazolidone, ciprofloxacin, and trimethoprim-sulphamethoxazole[2]. Though antimicrobials shorten the duration of illness and reduce faecal shedding of *V. cholerae*, prolonged antimicrobial use results in the development of antimicrobial resistance. Strains of *V. cholerae* have emerged that are resistant not only to each of these antimicrobial agents but also to multiple drugs, further confounding treatment efforts against cholera[3].

Bacterial antimicrobial resistance mechanisms consist of enzymatic drug inactivation, drug target protection, reduced drug permeability into bacterial cells, biofilm protection, alteration of drug target, alteration of metabolite pathways, and active efflux of single and multiple drugs from cells[3]. Active multi-drug efflux is a major mechanism for bacterial pathogen drug resistance[4]. Efflux pumps are integral-membrane proteins that confer single - and multi-drug resistances by actively extruding drugs from bacterial pathogens[4,5]. We discovered a new multi-drug efflux pump, called EmrD-3, from *V. cholerae* O395[6]. EmrD-3 confers resistance in *V. cholerae* against linezolid, rifampin, ethidium bromide, minocycline, erythromycin, trimethoprim, chloramphenicol, and rhodamine 6G[6]. EmrD-3 and other multi-drug resistance mechanisms allow bacteria to survive in the presence of clinically useful antimicrobials, thus reducing the efficacy of infectious disease chemotherapy[6,7]. Bacterial genome sequencing and comparative genomics have recently become commonplace, and such molecular analyses are important for identifying genetic determinants that confer pathogenesis, including those determinants that confer drug and multidrug resistance[8]. Because of their overwhelming presence in bacterial pathogens, active multi-drug efflux mechanisms remain a major research area, so that measures may ultimately be discovered to inhibit multi-drug efflux[9]. Thus, modulation of multi-drug efflux may restore the clinical efficacy of chemotherapeutics against infectious diseases caused by multi-drug resistant bacterial pathogens.

There are three key energy-dependent solute transport systems. The first is primary active transport, in which ATP hydrolysis is the mode of energy for the entry of molecules into, or efflux from, cells[10]. Another system is the phosphoenolpyruvate-dependent phosphotransferase system (PTS) in which a solute is phosphorylated as it is transported across the membrane[11,12]. Lastly, secondary active transport systems use ion gradients as the energy-mode for transport of nutrients into cells[13] or efflux of molecules from cells[14]. The ion may be a proton (H^+) or a sodium ion (Na^+). Secondary active efflux systems, although poorly understood, are energized by the translocation of the cation across the membrane down its concentration gradient into the cell and the concomitant transport of drug to the outside of the bacterium, a process known as ion/drug antiport[14]. Energy-dependent drug extrusion systems allow cells, including bacteria, to resist potentially lethal molecules like antibacterial agents, heavy metals, toxic metabolites, *etc*[14]. Efflux

pumps may harbour a single drug-substrate conferring resistance to that drug[7,15]. An interesting property of other efflux pumps is that these intrinsically harbour multiple substrates, providing the advantage of resistance to multiple structurally-different drugs[7]. Such multi-drug efflux pumps in bacterial pathogens would make good targets for inhibitors, as reducing multi-drug efflux may restore the clinical efficacy of older drugs and prevent emergence of drug-resistant variants.

Inhibition of drug efflux pumps is a rapidly expanding field of interest, and many strategies have been invoked to do so. Early 'uncouplers' of solute transport, such as cyanide-containing compounds, were non-specific in nature and extremely toxic to hosts[16]. Such compounds were clearly not good for chemotherapy. One strategy sought to bypass the efflux pump directly using synthetic-derivatives of their substrates. A tetracycline-derivative, tigecycline, was not a good efflux substrate[17]. Other strategies include modulation of efflux pump assembly, expression, and energy-coupling[18]. Tremendous efforts are aimed at studying inhibitors that directly bind drug efflux pumps and inhibit transport. Reserpine, for instance, inhibits both primary and secondary active efflux[19]. Such modulators, called resistance modifying agents (RMAs), when used in combination with antimicrobial agents produce a synergistic reduction in drug resistance phenotypes.

Though many newer efflux pumps as wells as the homologues of existing ones are being reported from various bacteria, their clinical relevance remains to be definitively demonstrated. Studies should focus on those efflux pumps which have been shown to confer clinical levels of resistance to understand the molecular basis of antimicrobial efflux and to identify their inhibitors. Though mutagenic studies of some of highly conserved amino acid residues identified by comparisons with their well characterized homologues will provide important information on their functionalities, understanding the interactions of these amino acids with the drugs in three dimensional space is critical to identifying inhibitors with similar binding properties to the preferred drug substrates of the efflux pumps. Though the lack of structural data from protein crystallization or NMR studies is a serious hindrance, this to a great extent can be overcome using modern bioinformatics tools[20]. Takatsuka *et al*[21] showed interactions of the AcrB drug-binding pocket with several substrates using computer docking tools, and reported that the AcrB protein has different binding pockets for different substrates within the main substrate-binding domain, a finding that needs critical consideration when efflux pump inhibitors are selected[21]. This observation of multiple substrate binding sites gains further strength from another study using the resistance-nodulation-division (RND) efflux pump inhibitor Phe-Arg-β-naphthylamide which inhibits levofloxacin efflux by MexAB-OprM, but is not effective against other substrates of the MexAB system such as ethidium bromide and carbenicillin[22]. The unique structural behaviours of efflux pumps such as changes in the transporter conformation following binding with the drug may not be predicted accurately by docking tools, and this discrepancy may result in an inhibitor identified by docking studies not showing any *in vitro* inhibition[23]. It remains to be understood whether putative inhibitors directly bind to and inhibit bacterial drug efflux pumps or if efflux modulation can occur through the regulation of gene expression or of pump assembly. Additionally, such efflux pump inhibitors would need to be demonstrated as non-toxic to humans in order to make this avenue for modulation of multidrug efflux valuable. Because reserpine directly binds and inhibits secondary active efflux pumps, such as Bmr and NorA[24,25], it may be advantageous to explore this area as well, when considering the efficacy of chemotherapeutic restoration. In any case, the vast array of new chemical compounds and naturally occurring agents predict that there are promising avenues for the discovery of novel agents that would inhibit or modulate bacterial drug efflux to help make antimicrobial therapy more effective against infectious disease caused by *V. cholerae*.

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