# A new strategy to combat biofilm antibiotic tolerance: Eradication of bacterial persister cells by targeting membrane potential

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**Statement of Purpose:** The extensive use of biomaterials to replace medical or implantable devices has increased the risk of biofilm associated infection. According to the National Institute of Health, biofilms account for up to 80% of microbial infections in human (Davies D. Nat Rev Drug Discov. 2003;2(2):114–122); and biofilms are up to 1,000 times more tolerant to antibiotics than planktonic cells of the same strain (Sharma, D., Misba, L. & Khan, A.U. Antimicrob Resist Infect Control 8, 76 (2019)). One major mechanism that contributes to the failure of current antibiotic treatment is the formation of persister cells, a dormant subpopulation exhibiting extremely high-level tolerance to antibiotics. During antibiotic treatment, persisters remain arrest and transiently repopulate once the antibiotic is withdrawn, causing the failure of treatment and relapse of the infection. It is believed that all bacterial species can form persisters including those causing medical-device associated infections such as *Pseudomonas aeruginosa*, *Staphylococcus aureu*s, and *Escherichia coli*. In addition, it has been shown that persistence promotes the development of resistance through genetic mutations overtime. Despite extensive research, controlling persister cells still remains a challenge. Here we report a new strategy of persister control by targeting the very mechanism that causes tolerance. We hypothesize that dormancy associated decrease in membrane potential reduces drug efflux and subsequently causes more accumulation of antibiotics that do not require active transport to penetrate bacterial membranes.

**Methods:** To test this hypothesis, we first tested tetracycline and minocycline on normal and persister cells of *Escherichia coli* (*E. coli*) HM22. Both antibiotics are from the tetracycline family of antibiotics and target protein translation by binding to the ribosome complex. These two antibiotics are also the substrates of the resistance-nodulation-cell division (RND) efflux pumps. We focused on *E. coli* HM22 as the model strain because it contains the *hipA7* allele that leads to high-level persistence.

**Results:** The results show that at a concentration of 100 μg/mL of minocycline killed *E. coli* persister cells by 70.8 ± 5.9% while it did not have significant killing of normal cells. In addition, the results show that persister cells accumulated ~2.6 times more minocycline per cell compared to normal cell. Consistently, the results were corroborated with tests using efflux pump mutants and efflux pump inhibitors. Specifically, treatment with carbonyl cyanide m-chlorophenylhydrazone (CCCP), an efflux pump inhibitor, at 10μM led to 94.7 ± 2.5% killing of *E. coli* normal cells by minocycline. This is a 4-time increase compared to treatment without membrane depotentiation, which only led to 22.0 ± 3.3% killing. We demonstrate here that *E. coli persister* cells have reduced efflux and thus more accumulation of minocycline than normal cells, leading to effective killing of this dormant population. Encouraged by these findings, we then sought out to determine if persister killing can be further enhanced by increasing the target binding affinity of antibiotics. Through literature research, we found eravacycline, which also targets the ribosome but has stronger binding than minocycline. Our results demonstrated eravacycline can kill *E. coli* persisters by 3 logs when treated at 100 μg/mL. In addition, it has a strong synergy with ampicillin, eradicating both normal and persister cells of *E. coli.*

To validate this new strategy, it is necessary to test if these antibiotics are also effective against persister cells of other pathogenic bacteria. This motivated us to test eravacycline on *P. aeruginosa* and uropathogenic *E. coli* (UPEC). Our results demonstrated that eravacycline is effective in killing both UPEC normal and persister cells; by 97.8 ± 0.7% and 99.9 ± 0.1%, respectively. In addition, we tested the effects of eravacycline on *P. aeruginosa* PAO1 (wild-type) and PDO300 (mucoid mutant) persisters cells. Our results show CCCP-isolated PAO1(wildtype) and PDO300 (mucoid) persisters can be effectively killed by eravacycline (by 99.7 ± 0.0% and 99.0 ± 0.1%, respectively). Lastly, we tested the effects of eravacycline on biofilm associated persister cells given that biofilms play a major role in chronic infections. Our results revealed that at the concentration of 100 μg/mL eravacycline reduced the number of viable biofilm cells of UPEC, *P. aeruginosa* PAO1, and *P. aeruginosa* PDO300 by 99.2 ± 0.5%, 99.9 ± 0.8% , and 99.6 ± 0.4%, respectively.

While it is commonly stated that persister cells are tolerant to conventional antibiotics, our study reveals that these dormant cells can be killed by selecting the right antibiotics with appropriate treatment conditions. Specifically, we demonstrate that antibiotics capable of penetrating bacterial cells by energy-independent diffusion and binding to their target strongly can kill persister cells during wake-up. These results can help developing better strategies to combat medical device-associated infections.