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BURNING QUESTIONS

What are the options for treating infections by persister-forming pathogens?

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Persistence is the non-heritable state of dormancy of a small subset of cells that allows these cells to survive a wide range of stresses. These stresses include antibiotics at concentrations well above the minimum inhibitory concentration along with various other stresses including oxidation (which occurs when our immune system encounters pathogens) and starvation (which almost all cells experience, rather than growing exponentially). Hence, persistence may be a nearly universal stress response for microorganisms and appears to be conserved in all kingdoms. Once the stress is removed and nutrients are provided, the majority of persister cells resuscitate, in a matter of minutes, to grow in a pre-stressed manner. As growing cells, the resuscitated persisters may reconstitute an infection or mutate to create resistant cells. With the development of new techniques to make the rare persister phenotype prevalent, antimicrobials can now be screened for activity directly on persister cells. Using this and other techniques, in this opinion piece, we outline several approaches that show promise against persister pathogens as well as review how persister cells form and revive, since a mechanistic understanding of persistence should lead to additional anti-persister compounds and treatment methods.

Persister cells form primarily as a result of stress, but unlike the bulk of the population of cells that actively respond via a stress response (e.g. via sigma factor RpoS), a small population of cells (<1%) enter a dormant state. In the persister state, cells can withstand antibiotics at high concentrations as well as withstand a plethora of other stresses such as acid conditions and hydrogen peroxide treatment (Hong et al., 2012).

To date, there is little to suggest that there are different kinds of persister cells, for example, what have been previously described as 'type I' (pre-existing), 'type II' (spontaneously formed during exponential growth), 'triggered' (formed as a result of stress), or 'spontaneous' (Balaban et al., 2004; Balaban et al., 2019)). Instead, it is far more likely all persisters are formed as a result of elegant regulation in response to stress (Dörr et al., 2009; Kwan et al., 2013; Wood & Song, 2020). Since there is probably only one kind of persister cell and since the cells in a 'viable but non-culturable' (VBNC) population that are capable of life, are equivalent to persister cells (Kim et al., 2018a), a 'dormancy continuum' (Ayrapetyan et al., 2015) is also unlikely. Moreover, it is worth noting that the bulk (over 99%) of the VBNC particles are cell shells that can never grow since they are empty husks, as shown by transmission electron microscopy (Kim et al., 2018a; Song & Wood, 2021b). Furthermore, the degree of protein aggregation is unlikely to create a dormancy continuum (Dewachter et al., 2021) but instead is indicative of the extent of certain stresses and at some threshold, indicates cell death (Baquero & Levin, 2021), because there is no known enzyme which can reconstitute hundreds of different precipitated proteins. Therefore, there is probably only one kind of persister cell (of course, the idea of different kinds of 'dormancy' is

non-sensical), and this dormant state occurs in myriad microorganisms as a result of stress, rather than occurring 'stochastically' as a form of 'bet hedging'. Critically, since persister cells are dormant (Kim et al., 2018b), they cannot mutate (Song & Wood, 2021a); instead, once resuscitated, they grow and mutate.

MECHANISMS OF PERSISTER FORMATION AND RESUSCITATION

The primary mechanism for persister cell formation is reducing protein synthesis, as demonstrated by inhibiting protein production by pre-treating with rifampicin to stop transcription, with tetracycline to stop translation, or with carbonyl cyanide *m*-chlorophenyl hydrazone to inhibit ATP production (Kwan et al., 2013). The link between stress and inhibition of protein production (without invoking toxin/antitoxin systems) is captured by the guanosine pentaphosphate/guanosine tetraphosphate [(p)ppGpp)] ribosome dimerization persister (PRDP) model. The PRDP model proposes that two second messengers, the alarmone (p)ppGpp and cAMP, stimulate hibernation factors to either (i) dimerize 70S ribosomes (creating inactive 100S ribosomes) or (ii) inactivate 70S ribosomes (Song & Wood, 2020c; Wood & Song, 2020). This ribosome dimerization protects ribosomal protein and rRNA from degradation. In *Escherichia coli*, the ribosome dimerization factors are RMF and Hpf, and 70S ribosomes may be inactivated directly through RaiA (Song & Wood, 2020c; Wood & Song, 2020). Note that persister cells are still formed in the absence (p)ppGpp, albeit at 1000-fold lower numbers (Chowdhury et al., 2016a). Critically, these ribosome-inactivating factors are highly conserved in bacteria (Prossliner et al., 2018), and ribosome dimerization occurs in Bacteria (Khaova et al., 2022), Archaea (Yaeshima et al., 2022) and Eukaryotes (Krokowski et al., 2011), so this mechanism of inhibiting protein production is general.

The mechanism for persister cell resuscitation in the PRDP model involves separating inactive, hybridized ribosome dimers via the GTPase HflX upon sensing nutrients and removing the stress; the presence of nutrients is communicated by a reduction in the secondary messengers cAMP and (p)ppGpp (Yamasaki et al., 2020). The persister cells wake in a heterogeneous manner based on the number of active ribosomes; this was first shown with single cells by converting the whole population into persister cells and labelling ribosomes with the green fluorescent protein (Kim et al., 2018b). Once the persisters resuscitate, they grow at the same rate as they did prior to becoming persister cells (Kim et al., 2018b). Remarkably, persister cells do not utilize proteins specific for resuscitation but sense conditions favourable in their environment via membrane proteins utilized for substrate transport, such as those for sugars like glucose and membrane proteins used for chemotaxis, such as those for amino acids (Yamasaki et al., 2020). Also, resuscitating cells commence chemotaxis toward nutrients (who among us has not awaken hungry and propelled ourselves to the kitchen?), and chemotactic proteins facilitate waking (Yamasaki et al., 2020). Furthermore, persister cells do not wake spontaneously as previously proposed from microfluidic results (Balaban et al., 2004) and in the 'Scout Model' (Epstein, 2009), but instead wake based on the detection of a carbon source. This was shown in part by utilizing an *E. coli dadA* mutant which cannot utilize Ala for growth but wakes upon addition of Ala (Yamasaki et al., 2020).

Other mechanisms for persister cell formation and resuscitation have been suggested, primarily as a result of not studying the correct cell population, since many groups fail to wait for persistence to be obtained and instead, study the far more populous dying-cell population (Song & Wood, 2021a). In addition, it is possible that the 'Persistence as Stuff Happens' (PASH) model, in which persistence occurs due to errors in replication (Johnson & Levin, 2013), is correct to the extent that cells that are less capable of responding to stress enter the persister state more readily (Hong et al., 2012). However, although this manuscript (Johnson & Levin, 2013) contains beautiful experimental persister data based on multiple antibiotics, the PASH model does not provide mechanistic details other than suggesting the occurrence of random mutations creates persister cells and also relies on mutations occurring in persister cells which is unlikely due to their dormant state. Critically, the main point with persister cell research is that cells become dormant and thereby tolerant to myriad stresses without undergoing genetic change, so 'persister' mechanisms that invoke mutations are better suited for explaining how resistance arises in growing cells.

Overproduction of active toxins of toxin/antitoxin pairs increases persistence dramatically, and this has been shown by randomly mutating toxins to increase persistence (Hong et al., 2012). However, overproduction of many toxic proteins not related to toxin/antitoxin systems increases persistence (Chowdhury et al., 2016a), and overproduction of toxins is likely not physiologically meaningful (but a fine tool for making persister cells). Furthermore, the *E. coli* toxin variant HipA7 was used by several groups to link persistence to toxin/antitoxin systems (Balaban et al., 2004; Moyed & Bertrand, 1983). Unfortunately, the two amino acid substitutions of HipA7 render it non-toxic (Korch et al., 2003), and several groups have shown deleting multiple toxin/antitoxins has no effect on persistence (Goormaghtigh et al., 2018; Harms et al., 2017; Svenningsen et al., 2019), so toxin/antitoxin systems are probably only weakly associated with persistence.

DISCOVERING ANTI-PERSISTER COMPOUNDS FOR PATHOGENS

There are three main approaches (Wood, 2016) to combatting persister cells: (i) kill persisters as they sleep using compounds that rely on passive diffusion to work intracellularly or utilize membrane-corrupting compounds that do not have to be transported, (ii) wake persisters to convert them into an antibiotic-sensitive state since most antibiotics utilized to date kill growing cells, or (iii) prevent persister cell formation. The ability to convert exponentially growing cells into a homogeneous persister cell population by inhibiting translation (Kwan et al., 2013) allows antimicrobial discovery to focus directly on killing persister cells, rather than focusing on growth inhibition initially. Unfortunately, current antimicrobials are not usually tested on persister cells.

Using this approach for the first time of targeting sleeping *E. coli* persisters directly by converting the whole population into persister cells prior to screening, the indigoid 5-nitro-3-phenyl-1*H*-indol-2-yl-methylamine hydrochloride (NPIMA) was discovered by screening a 10,000 compound library of pharmaceutically relevant chemicals (Song et al., 2019). Notably, NPIMA eradicated the persister cells for the pathogens *Pseudomonas aeruginosa* and *Staphylococcus aureus* by damaging their cell membrane, and NPIMA was effective in an in vitro wound model (Song et al., 2019). Furthermore, as we reviewed previously (Song & Wood, 2020a), indigoids like NPIMA remain a rich source of molecules that eradicate pathogenic persister cells.

Using the same approach of screening initially with a homogenous population of persister cells to identify compounds that wake persisters, 2-[[2-(4-bromophenyl)-2-oxoethyl]thio]-3-ethyl-5,6,7,8-tetrahydro[1]benzothieno[2,3-d]pyrimidin-4(3H)-one (BPOET) was identified (Song & Wood, 2020b). To determine how BPOET resuscitates persister cells, each *E. coli* protein was overproduced prior to converting all the cells into the persister state, and it was determined that BPOET wakes persister cells by modifying hibernating ribosomes via 23S rRNA pseudouridine synthase RluD, which converts uridine to pseudouridine at three positions in 23S rRNA to stabilize its structure (Song & Wood, 2020b). These results also lend credence to the ribosome hibernation mechanism for persister cell formation and resuscitation.

Another robust approach for identifying novel compounds that are active on persister cells involves machine learning: a deep neural network was trained on 2560 FDA-approved antibiotics and natural products, followed by analysing experimentally 99 putative antibiotics from a 6111 molecule library of human drugs to identify halicin (a repurposed nitrogen-sulfur kinase inhibitor) that kills *E. coli* persisters at 40 μg/ml by dissipating the proton motive force (Stokes et al., 2020). Additionally, halicin was effective against the pathogens *Mycobacterium tuberculosis*, *Clostridioides difficile* and *Acinetobacter baumannii* in a murine model (Stokes et al., 2020). This approach was then used to screen *in silico* over 100 million compounds of the ZINC15 database, which led to the identification of ZINC000225434673 that kills *E. coli* persisters (Stokes et al., 2020).

Since the primary mechanism of persister cell formation is inhibition of protein production (Kwan et al., 2013), it is reasonable to focus on increasing ATP to prevent persister cell formation, since reducing protein production by reducing ATP increases persistence (Cheng et al., 2014; Dörr et al., 2010). For example, in *Mycobacterium marinum*, a close relative of *M. tuberculosis*, inactivation of the high-affinity potassium transport system that depends on ATPase activity increases ATP levels and thereby reduces persistence (Liu et al., 2020). Hence, ATP analogues should be screened for preventing persister cell formation without stimulating the growth of the pathogen. Of course, resuscitating persister cells and converting them into growing cells makes them susceptible to antibiotics (Allison et al., 2011), but this approach stimulates the growth of the pathogen and may lead to its proliferation.

Similarly, reducing (p)ppGpp levels should prevent formation of persister cells, and the first success using this approach was Relacin, a (p)ppGpp analogue that inhibits RelA (synthesizes (p)ppGpp) and reduces *Bacillus subtilis* survival in the stationary phase (Wexselblatt et al., 2012). Relacin has been used to increase antibiotic killing in *C. difficile* (Pokhrel et al., 2020), and a more potent Relacin variant was synthesized for preventing *Mycobacterium smegmatis* persister formation (Syal et al., 2017). In addition, a screen of two million pharmaceuticals for inhibition of the (p)ppGpp synthase Rel of *M. tuberculosis* yielded compound X9 (4-(3-methyl-4-(2-(4-methylthiazol-5-yl) ethoxy)styryl)benzoic acid) that reduces persistence by enhancing isoniazid killing (Dutta et al., 2019). Hence, reducing (p)ppGpp levels remains a robust method to reduce persistence.

Since exposure to oxidative stress increases persister cell formation dramatically (e.g. hydrogen peroxide increases *E. coli* persistence by 12,000-fold) (Hong et al., 2012), another approach to prevent pathogen persister cell formation is to reduce reactive oxygen species seen by intracellular pathogens by reducing the immune response in macrophages (Beam et al., 2022). Hence, the immunosuppressive chemotherapeutics dexamethasone and rosiglitazone and the anti-inflammatory agent sulforaphane have been used to pretreat macrophages before *S. aureus* infection to reduce reactive oxygen species in the macrophages; after treating with rifampicin, less *S. aureus* intracellular persisters remained (Beam et al., 2022).

For killing persister pathogens as they sleep, the best compound identified to date is mitomycin C, a

repurposed, FDA-approved, anti-cancer that crosslinks DNA; it was first shown to kill commensal *E. coli* persisters along with the persisters of pathogens *E. coli* O157:H7, *S. aureus*, *P. aeruginosa* and *A. baumannii* (Cruz-Muñiz et al., 2017; Kwan et al., 2015) and subsequently has been utilized successfully by various other groups. Although mitomycin C is somewhat toxic when used internally, the concentrations that are effective for killing microbial persister cells in wounds are at least an order of magnitude lower than the toxic concentration (Kwan et al., 2015). Similarly, cisplatin, another repurposed, FDA-approved, anti-cancer compound, kills persister cells of the pathogens *E. coli* O157:H7, *S. aureus*, *P. aeruginosa* (Chowdhury et al., 2016b) and is also probably best-suited for wound treatments.

A novel mechanical-like device for eradicating pathogenic persisters as they sleep is the molecular machine consisting of naphthalene moieties that upon visible light activation, physically disrupts *S. aureus* membranes by 'drilling into' them (Santos et al., 2022). This permeabilization of the membrane also facilitates the use of conventional antibiotics, and resistance was not detected (Santos et al., 2022). Also, tools of the cell's worst enemy, phage, are being utilized to kill persisters, such as endolysins (Pacios et al., 2020).

PERSPECTIVES

It is imperative that there should be a paradigm shift in that new antimicrobials should be tested for their activity on persister cells, along with growing cells. This entails converting a large population of pathogens into *bone fide* persister cells to facilitate this assay or using time-kill assays that investigate long exposures to antimicrobials, to probe the extent of killing. Alternatively, the visual interaction tolerance detection test may be used to rapidly determine whether persister cells are killed by antimicrobial treatments (Liu et al., 2022).

Moreover, as in many fields, there is a trend to combine treatments for killing persister cells. For example, combining traditional antibiotics like ampicillin (to kill metabolically active cells) with the DNA-crosslinker mitomycin C (to reduce the concentration required) (Zheng et al., 2020), and combining a lytic phage with mitomycin C to kill *Klebsiella pneumoniae* persisters (Pacios et al., 2021). So the answer to the 'burning question' of the title is that there are many options for killing persister cells formed by pathogens. Also, based on recent insights into the mechanism of persister cell formation and resuscitation, we can be optimistic about the future prospects of treating successfully infections.

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CONFLICT OF INTEREST

Authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

All data are available in this manuscript.

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