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Matthew Dyer

Introduction

Antimicrobial resistance (AMR) is a public health threat that was once predicted to cause up to 10 million deaths worldwide annually by 2050 if no new action is taken [1]. That prediction was made in 2016 and since then, there have been meaningful efforts worldwide to change the course of that predicted future. New and novel antibiotics have increased in number, but it's disappointing how many have passed clinical trials and become commercially available [2]. In light of this, I propose the development of a novel antibiotic (NAB) that targets a relatively newly discovered ubiquitous bacterial protein found in both gram-positive and gram-negative species called PopT.

Target Profile

This protein is one of two known undecaprenyl pyrophosphate (UnDP or C55-PP) flippases in bacteria [3, 4]. Proteins with the DUF368 domain have been given the superfamily name PopT (polyprenyl-phosphate transporter) [6]. UnDP is an essential molecule involved in the construction and maintenance of the bacterial peptidoglycan cell wall. The bacterial cell wall is primarily composed of peptidoglycan [5]. Undecaprenyl pyrophosphate plays a pivotal role in the synthesis of this polymer by acting as a lipid carrier. After its role in peptidoglycan synthesis, undecaprenyl pyrophosphate is converted back to undecaprenyl phosphate for reuse in the synthesis cycle. PopT translocates UnDP across the bacterial membrane and allows for this recycling to occur. In in vitro experiments, bacteria that lack PopT have weakened cell walls, viability defects, and deformed shapes [3]. It has been shown that bacteria with inhibited/knocked out PopT are highly susceptible to tunicamycin - an established antibiotic that is not as effective against bacteria with intact PopT [6]. Essentially, PopT helps bacteria maintain their cell wall. Without these proteins, the bacteria are weakened and present easy targets from established antibiotics. I propose targeting PopT in bacteria as part of a multi-pronged approach to combating antibiotic-resistant bacteria. To be explicit, there is no antibiotic on the market that targets PopT and I believe that this represents an exciting opportunity to target a protein that, until recently, was not even known to exist in any significant detail. Any new pathways or modes of attack in antibiotic-resistant bacteria should either be ruled out as ineffective or fully taken advantage of.

As I intimated earlier, combining an antibiotic like tunicamycin with a drug that selectively targets PopT in bacteria shows promise in reducing or eliminating infection from antibiotic-resistant bacteria. This kind of approach has been successfully used by pairing a β-lactam antibiotic (amoxicillin) with a β–lactamase inhibitor (clavulanic acid) [7]. By focusing on weakening the cell's ability to maintain the cell wall with a PopT inhibition drug, an antibiotic like tunicamycin shows improved efficacy in killing the cell [6]. By focusing on the inhibition of PopT, we can theoretically neutralize infectious cells, paving the way for the alleviation of infection-induced symptoms

In the context of singular inhibition of PopT and no combined approach with antibiotics, there is evidence to suggest that targeting PopT alone can reduce infection. In a rabbit model that mimics severe human cholera, the protein with the DUF368 domain (VCA0040) in Vibrio

cholerae was knocked out and compared to infection of V. cholerae with the wildtype protein. The results showed a dramatic competitive defect in the knockout model. It should be noted, however, that this was only observed in alkaline environments and is somewhat limited in effect as a result [3].

Preliminary research, by me, suggests that VCA0040, V. cholerae's protein with the DUF368 domain, has no sequence similarity with humans. Moreover, BLASTp and Interpro domain queries of the fasta sequences of VCA0040 and the proteins with the DUF368 domain of Staphylococcus aureus, Streptococcus pneumoniae, and Enterococcus faecalis also yield no sequence similarity of any known human proteins. There are no experimentally derived structures of PopT, but there are Alphafold-predicted 3D structures for this protein that belong to Vibrio cholerae, Staphylococcus aureus, Streptococcus pneumoniae, and Enterococcus faecalis. After querying Dali server's AF-DB search, the closest human homolog by structure to these proteins is ZINC TRANSPORTER ZIP4, UniProt: Q6P5W5.

Much more work would need to be done to ascertain if any compound that inhibits PopT does so:

1. Selectively (low promiscuity)
2. Effectively
3. With minimal side effects - if it is promiscuous.

There is no antibiotic or ligand to test even in silico at the moment. We have good reason to believe that there is a great enough evolutionary divergence between humans and PopT in bacteria to avoid promiscuous binding, but we can't speak to the toxicity, tolerance, or efficacy of this theoretical compound because it doesn't exist in any published literature.

Drug Design

The NAB could be either a biologic or a small molecule. However, I believe that due to cost and manufacturing complexities, it would be prudent to first try and target PopT with a small-molecule-based antibiotic. Small molecule drugs are generally less expensive to produce than biologics and are chemically synthesized, which is often a more straightforward and cost-effective process than producing biologics [8]. Moreover, the administration of a small molecule (an orally administered pill, for example) is logistically easier for the application of treatment and transport as it doesn't require strict storage requirements (such as constant refrigeration) [8]. Not all antibiotics are administered in an orally administered pill, but the intent would be to design this NAB to be as straightforward as possible to allow for the NAB to be available worldwide in sundry conditions.

A rational drug design approach makes sense in this case. While we don't fully understand the mechanisms of UndP transport by proteins like PopT [4], we have strong evidence to suggest that targeting this protein can more easily facilitate bacterial cell death [3]. We also don't have an experimentally derived 3D structure of any PopT proteins, but we do have a few species' PopT AlphaFold structures with reasonable confidence in their fidelity [3, 4]. I think it would be

prudent to obtain experimentally derived structures by X-ray crystallography, or similar, before proceeding too far with a rational drug design approach. If we're going to devote millions of dollars and tens of thousands of hours of labor toward a new antibiotic, we should give ourselves the best chance at success. However, that doesn't mean it would be unwise to begin identifying potential drug-binding sites while the true 3D structures are being determined.

We have strong evidence that the PopT protein is evolutionarily conserved across gram-positive and gram-negative bacteria [3]. However, it would be unwise to only consider one species' PopT protein in our drug design. I would recommend using the PopT proteins from Methicillin-resistant Staphylococcus aureus (gram-positive), Vancomycin-resistant Enterococcus (gram-positive),
carbapenem-resistant Klebsiella pneumoniae (gram-negative), and Acinetobacter baumannii (gram-negative) [10, 11]. These are some of the more pervasive and troublesome antibiotic-resistant bacteria currently and if the drug were only effective against these bacteria, it would still be a worthwhile drug to bring to market. Ideally, and obviously, we hope the drug would have broad and general applicable use.

A computational approach is especially wise early n the development process. Whether we have a theoretical or experimental 3D structure, we can begin to identify regions of PopT to target - hopefully, there's a deep pocket or an actionable surface structure for allosteric regulation. Before any physical library development, in silico screening using databases like Mcule.com or Enamine REAL Space [9] will help identify potential ligands with high affinity for the PopT protein - either active site, deep pocket, or allosteric surface regulation surfaces. With the help of a chemistry team, we can synthesize a focused library of compounds based on the in silico hits, prioritizing those with the desired properties for efficacy and safety. We can use docking simulation software like AutoDock and AlloPred to measure efficacy and GROMACS or AMBER to further explore the molecular dynamics of the theoretical drug and PopT bindings.

To be explicit, hopefully the experimentally derived 3D structure is quickly resolved and the work that had been done on the AlphaFold structure transfers with a high degree of relevance. We also will rely quite heavily on the chemist and biochemist team to theoretically determine that our short list of developing leads is non-toxic, what the general pharmacokinetic properties are, and that the lead compounds can permeate the outer membrane of gram-negative bacteria. We also need as much verification as possible that the lead compounds do not promiscuously bind to human proteins such that will cause more harm than good - a minimal amount of promiscuity might be acceptable. We also need (in silico) verification that the lead compounds cause some meaningful conformational change in the PopT protein such that the PopT protein is either functionally knocked out or severely limited - which is related to a bioinformatics software like GROMACS.

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High Level Bioinformatic Workflow for the NAB design

1. **Target Identification & Validation:**
    - Use tools like BLAST, Pfam, and InterPro to identify and validate the presence of PopT/DUF368 domain proteins across diverse bacterial species.
    - Consider phylogenetic analysis of the proteins if appropriate using tools like Clustal and Jalview.
2. **Structural Analysis:**
    - Use PDB or AlphaFold databases to acquire 3D structures.
    - Analyze the 3D structure of PopT proteins and identify potential drug-binding sites using PyMOL and similar.
3. **Ligand Identification:**
    - Use Mcule.com, ChemSpider, or similar to identify possible ligands to bind to the potential binding sites.
4. **Molecular Docking:**
    - Use in silico docking tools such as AutoDock or PyRx to simulate the binding affinity of the ligand and binding site.
5. **Molecular Dynamics Simulations:**
    - Use tools like GROMACS, AMBER, or NAMD to assess the stability of the protein-ligand complex in non-static circumstances.
    - This helps to simulate the variability that comes with in-vivo interactions.
6. **ADME/Tox Prediction:**
    - Use SwissADME or similar to predict the absorption, distribution, metabolism, excretion, and toxicity properties of potential drug candidates.
7. **Literature Mining**:
    - Conduct a thorough literature review to gather existing knowledge and evidence regarding the pathways and known interactions of the protein of interest.
8. **Functional Assays & Validation**
    - Use data obtained so far to carefully plan out and acquire experimental data from in vitro and in vivo studies.
    - Measure for efficiency, toxicity, and off-target effects.

This workflow provides a (high-level) comprehensive approach to drug development for PopT/DUF368 domain proteins using bioinformatics tools and resources. It's essential to iterate and refine the workflow based on new data and insights, ensuring the development of effective and safe drug candidates.

Screening Campaign

My intuition is that we would use biochemical screens. We already have a (hopefully) strong library of leads to test. Phenotypic screening may or not be a useful secondary screening. Antibiotic research has been so thoroughly combed over that drug repurposing is becoming one of the remaining possibilities for finding a new antibiotic [5]. I would not place much stock in the hope of a serendipitous, or even well-planned phenotypic screening in identifying an antibiotic that works in disabling PopT.

The biochemical screens would be performed in vitro very similarly to what's been described in prior research [3, 4, 5]. However, the bacteria would be wild-type and not have knocked out PopT genes. To ensure that any effective compounds are not false positives or those that appear to be active due to nonspecific effects rather than true interaction with the target, we would follow up with counter screens. I can think of two approaches. The first would be to label the compound with a fluorescent tail and observe where the compound congregates in the cell. This would be difficult for its own reasons as the addition of the tail might very well affect the binding of the compound. The second would be to use PopT knockout bacteria. We would add the compound and compare an experimental batch of knockout bacteria growth in the presence of the compound with a control batch of knockout bacteria growth without the presence of the compound. One control and one experimental. It would also be prudent to have additional controls of the wild-type bacteria growing with or without the presence of the same compound. This will provide more evidence and statistical power as to whether the compound affects more than the PopT proteins.

Next, we would want to perform physical in vitro screenings of the compound on a variety of human cell lines. We might be especially interested in observing how the compound might bind to any human proteins identified as possible suspect and promiscuous off-site binding proteins in prior research. This could be done by using E. coli to produce the protein in vitro and adding the compound in various concentrations.

Lastly, throughout these physical experiments, we want to provide our chemists with an optimal amount of data. What are the structure-activity relationships (SARs) of the compounds with the target (and non-target) proteins? Can we modify the compounds to be more specific, potent, and less toxic (if applicable)? If we can refine the compounds to be more ideal and repeat our experiments with ever-improved compounds, we increase our chances of finding a drug that will pass clinical trials. However, time and resources are finite, so we might only be able to repeat our biochemical and counter screens so many times.

In Silico

Physical data is the most reliable information we can get from our lead optimization, but we will constantly rely upon in silico approaches and applications to minimize unnecessary physical experiments. As I've already written, we can use tools Like GROMACS, AMBER, AutoDock, and AlloPred to try and obtain the best physical library of compounds to use in physical testing. Concurrently, tools like SwissADME would be leveraged to predict the absorption, distribution, metabolism, excretion, and toxicity properties of potential drug candidates. We would hope that we can use in silico approaches to find the "perfect" compound and only use one or two physical interactions of experiments to verify that our "perfect" compound is ideal.

The integration of in silico approaches began at the beginning of this project and will continuously be used in combination with physical in vitro/in vivo testing. With a well-defined target and a robust set of computational and experimental tools at our disposal, this strategy outlines a comprehensive approach to identifying and developing a novel antibacterial agent targeting the PopT protein.

Biomarker Strategy

The primary biomarker is the presence of antibiotic-resistant bacteria with a PopT protein sensitive to the NAB. More specifically, the biomarker is the presence of those same bacteria infecting a host and causing symptoms related to infection.

The purpose of the primary biomarker is to give diagnostic, pharmacodynamic, and efficacy-related information about the NAB and its utility concerning the patient. The diagnostic data identifies patients who are likely to benefit from the NAB - are they experiencing symptoms related to infection and first-round antibiotics haven't worked? The primary biomarker is more relevant in the presence of secondary biomarkers such as fever or an elevated white blood cell count. If the presence of antibiotic-resistant bacteria with a PopT protein sensitive to the NAB is discovered in a patient in this context (or similar), there is likely strong enough evidence to prescribe the patient the NAB. Other biomarkers have been shown to demonstrate clinical utility in the need to prescribe first-round antibiotics, but our NAB is not intended to be a first-round antibiotic [12].

The pharmacodynamic purpose of the biomarker is to provide evidence of the direct effect of the NAB. This can be measured by either a discrete or continuous measurement of target bacteria in the patient. We can also use secondary biomarkers such as fever, white blood cell count, C-reactive protein (CRP), or procalcitonin (PCT) to measure this[12]. We can also collect samples of the bacteria directly from the patient and analyze them directly using microscopy, which has been successfully done in animal models [3]. Related to the pharmacodynamics is the efficacy of the NAB. We can use qPCR or microarray assays at various time intervals during treatment to understand the efficacy of the NAB both from an overall and time efficiency perspective - are the levels of the offending bacteria dropping and at what rate? These data can inform if continued treatment with the NAB is justified or not.

Safety biomarkers are essential to assess any potential off-target effects of the drug on the patient. This might involve monitoring for biomarkers indicative of liver function (ex: alanine aminotransferase), kidney function (ex: serum creatinine), cardiac (ex: troponins), immune system (ex: eosinophil counts), or gastrointestinal (ex: lactate and fecal calprotectin). These are relatively standard safety biomarkers for assessing the effects of drugs like antibiotics in a patient [13]. These safety biomarkers can be used in preclinical, clinical, and commercial use - for various purposes. Some emerging safety biomarkers deserve consideration [14] - but these are beyond the scope of this paper.

Pre-clinical validation of the primary biomarker for diagnostic, pharmacodynamic, and efficacy can be established using in vitro studies with bacteria in various circumstances. In vivo studies

using animals such as mice or rabbits can be used to further establish the legitimacy of these biomarkers' utility in humans. The safety biomarkers should be as well understood as possible in an in-silico environment before proceeding with animal models. Animal models can be used to establish or confirm the maximum tolerated dose, minimum effective dose, minimum toxic dose, no observed adverse effect level (NOAEL), etc. The validation of safety biomarkers in animals such as mice does not perfectly laterally transfer to humans, nor do the doses, but this data allows us to gain confidence in the pharmacodynamic effects of the NAB as well as general principles of the efficacy and safety in the administration of the NAB. This data will be instrumental in predicting optimal dosages of the NAB in an orally administered pill for phase 1 trials.

The presence of antibiotic-resistant bacteria with a PopT protein sensitive to the NAB is generally a predictive biomarker. The treatment of this biomarker with the NAB should give evidence that patients show an improvement. To be explicit, if the patients were untreated, the biomarker could be seen as a prognostic biomarker - the presence of the bacteria would lead to a decrease in the health of the patient. However, we are talking about the biomarker in the context of treating with the NAB, so the biomarker is predictive. Patients with this biomarker should indicate that treatment with the NAB would improve their health. Likewise, patients without this biomarker would likely see no improvement in their health in response to treatment.

Randomized discontinuation design (RDD) trials offer a relatively safe and effective way to test the NAB on those who will benefit most from it and protect those who will not [15]. RDD trials can be used to collect observational data on the effectiveness and safety of the NAB in a way that allows for robust statistical analysis. I would recommend an RDD trial or a similar trial during NAB phase II of clinical trials. This strategy ethically allows for all patients to receive an optimal standard of care most safely. Will the NAB be more effective than standard care? Does the NAB work in certain patients but not others? This approach allows for the meaningful generation of data regarding many issues. By starting with all patients receiving the NAB in an RDD, we cast the widest net but allow for patient safety above all else.

I believe the general regulatory process and development of the drug would look like the following:

1. **Discovery and Preclinical Testing** - Safety, efficacy, PK/PD, Diagnostic testing, and data generation. Revise as needed.
2. I**nvestigational New Drug (IND) Application** - After successful preclinical testing, we submit the IND application to the FDA with all relevant data. The IND is necessary to ship across state lines and allows for human administration [16].
3. **Clinical Trials (Phases I, II, III)** - Test for optimal dosage levels/safety in phase I. Begin Randomized discontinuation design after phase I
4. **New Drug Application (NDA)** - I believe that an NDA is more relevant than a PMA or 510(k) as our NAB is a novel pharmaceutical that uses a novel mechanism [17].
5. **FDA Review and approval**
6. **Post-Marketing Surveillance**

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