Writing Assignment: Scientific Research Proposal

Stuck in Transit: a High-Res ssNMR Study on the Antibiotic Murepavadin that Targets the Essential Outer Membrane Protein LptD and Disrupts LPS Transport in Gram-Negative Bacteria

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Part A – Applicant

A.1 Applicant

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Part B – Scientific proposal

B.1 BASIC DETAILS

B.1.1 Title

Stuck in Transit: A High-Res ssNMR Study on the Antibiotic Murepavadin that Targets the Essential Outer Membrane Protein LptD and Disrupts LPS Transport in Gram-Negative Bacteria

B.1.2 Abstract

Antibiotics are extremely important in this day and age for the general health of the populace. There is sadly a major problem with the widespread (over)use of these drugs: cases of multi-drug resistant bacteria, especially Gram-negative bacteria are becoming more prevalent. These Gram-negative superbugs have an additional impregnable membrane that greatly increases their resistance towards their environment and antimicrobial compounds. We will need novel antibiotics to combat these pathogens, however only very few novel antibiotics have been approved for clinical use in the last decades. Murepavadin is a β-hairpin peptidomimetic antibiotic that is active against ESKAPE pathogen *Pseudomonas aeruginosa* (*Pa*). *Pa* is a major cause of death in patients suffering from cystic fibrosis, a genetic disorder that leads to abnormally viscous mucus in the lungs. This altered environment is perfect for opportunistic bacterial infections, which develops into a chronic infection as the *Pa* is impossible to get rid of without the use of antibiotics. Murepavadin targets the lipopolysaccharide (LPS) transport protein LptD that is essential in *Pa* and many more Gram-negative bacteria. Inhibition of the LptD prevents LPS insertion into the outer membrane and will lead to cell death. Thus far, there is no structural information on Murepavadin's mode of action and interaction with *Pa* LptD available. Here, we propose an approach for elucidating Murepavadin's interaction with *Pa* LptD at high-resolution and in physiologically relevant conditions using solid-state NMR. This project will provide not only novel insight about Murepavadin and LptD, but will also provide a basis for improvement of existing and development of novel antibiotics targeting the essential OMP LptD.

B.1.3 Layman's summary

In this day and age, we cannot live without antibiotics anymore. They are extremely important for fighting bacterial infections in hospitals, as the patients there are prone to contract a so called superbug. These opportunistic bacteria are highly resistant towards the most commonly used drugs and can end as a fatal infection. Fifteen out of eighteen most dangerous pathogens as classified by the WHO are Gram-negative bacteria. Gram-negative bacteria contain an additional cell membrane made up of lipopolysaccharide (LPS), which is almost impermeable and helps the bacteria survive the harsh outside world with high and low temperatures and foreign substances, such as antibiotics. We will need new antibiotics to combat antimicrobial resistant bacteria, however the number of drugs that get approved for use that target Gram-negative bacteria is not enough. Murepavadin is a small peptide antibiotic currently being investigated for use against *Pseudomonas aeruginosa*, a Gram-negative bacteria active in most patients suffering from cystic fibrosis and member of the notorious ESKAPE pathogens. Its target is the LPS transport protein LptD, which is a large β-barrel protein in the outer membrane. LPS is made in the cytosol of the Gram-negative bacteria and has to be extracted out of the inner membrane, transported through the periplasmic space and inserted inside the outer leaflet of the outer membrane. LptD performs the last step in this process: it takes LPS from the

bridge spanning the periplasm and inserts LPS in the membrane. Inhibition of this essential protein will lead to cell death, but how this exactly works is unknown. Structural data of the *Pa* LptD and murepavadin interaction are unavailable, which impedes the design of better drugs. Here, we described our methodology for the investigation of the Murepavadin-LptD interaction. We propose to look at this interaction using solid-state nuclear magnetic resonance (ssNMR) spectroscopy. NMR spectroscopy allows us to look at molecules and identify their structure and study their interactions with other molecules at an atomistic level in physiologically relevant model systems. In this case, we can determine where Murepavadin binds LptD, Murepavadin's structure when it is interacting with LptD and which amino acids are important from Murepavadin and from LptD for their interaction. This research will provide us with new insight into Murepavadin, LptD and their interaction, it can also be used to improve Murepavadin's activity against *P. aeruginosa* and in the development of new antibiotics against LptD or similar β-barrel proteins. This can help hundreds of thousands of people suffering from *P. aeruginosa* infections.

B.1.4 Keywords
Antibiotics, Gram-negative Bacteria, LPS transport, Murepavadin, Solid-state NMR Spectroscopy

B.2 SCIENTIFIC PROPOSAL

B.2.1 Research topic

The Gram-negative problem, outer membrane lipopolysaccharide synthesis and transport

As Antimicrobial Resistance (AMR) is becoming a greater threat to public health[1], the compounds used to combat bacterial infections are in higher demand than ever. However, these compounds are hard and slow to develop which has led to the shortage of novel antibiotics.[2,3] Several strains, including *Pseudomonas aeruginosa* and *Acinetobacter baumannii,* with resistance towards all available antibiotics have been observed.[3] There is a particularly urgent demand for novel compounds against Gram-negative bacteria, which comprise fifteen out of eighteen pathogens on the World Health Organisation's priority list.[4] Infections caused by drug-resistant bacteria is estimated to be over two million[5] in the U.S. alone and these are coupled with a high mortality rate and expensive treatment.[6] Among the pathogens in the critical category[4] is *Pseudomonas aeruginosa* (*Pa*). *Pa* is a Gram-negative bacteria involved in most cases of ventilator associated pneumonia (VAP) in patients suffering from cystic fibrosis (CF).[7,8] CF is a autosomal recessive genetic disorder present in 1 out of every 3,000 newborns[9], that causes mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) protein.[10] This alters the mucus in the lungs to be thicker and is the perfect environment for many bacteria. Thus, CF patients are often subjected to bacterial pneumonia, in the most cases by *Pa*. This infection is difficult to get rid of, as the bacteria can quickly adapt and become resistant to commonly used antibiotics. While the average life expectancy of CF patients slowly increases due to the advances in healthcare, the annual mortality rate is still over 1% in 2019.[11,12]

The Gram-negative bacterial cell envelope is well equipped to deal with environmental stress caused by cell-foreign compounds like antibiotics. It is made up of an inner membrane (IM), a thin layer of peptidoglycan (cell wall), and an outer membrane (OM). The inner membrane is a typical bilayer where both leaflets consist of phospholipids. The cell wall is situated inside the aqueous periplasmic space separating the two membranes and providing rigidity to the cell. The near-impenetrable OM is an asymmetric barrier that contains many efflux pumps that can remove toxic compounds when present in the periplasmic space.[13] The inner leaflet of the OM is made up of phospholipids, while the outer leaflet is mainly comprised of lipopolysaccharides (LPS).[14] LPS has a crucial role in protecting the bacterium from harsh environments and antimicrobial compounds and when LPS production is inhibited, the cells die. LPS has to cross the IM and the periplasmic space before it can be incorporated into the outer membrane. Many different machineries are necessary for the biosynthesis and translocation of the LPS. This essential machinery would be prime targets for antibiotic development.

LPS is a glycolipid made up of three domains: lipid A, the core oligosaccharide, and O-antigen (**Figure 1A**). Lipid A acts as the anchor to the membrane and is typically made up of two glucosamines containing two phosphate groups and a variable amount of acyl groups, ranging from five to seven chains. Attached to lipid A is a species conserved inner-core oligosaccharide structure containing two *D*-manno-oct-2-ulosonic acid (Kdo) and two *L*-glycero-*D*-manno-heptose residues. The heptose residues can be mono-, di-, or triphosphorylated, the last has only been observed in *Pa*. This is all synthesized inside the cytoplasm and cytoplasmic side of the inner membrane. At this point the lipooligosaccharide (LOS) is flipped across the membrane to the periplasmic side by MsbA. Here, the O-antigen is attached to the LOS to form LPS. The O-antigen is a large polysaccharide chain that consists of repeating sugar units and is responsible for the immunological serotyping.[15,16]

Once LPS is located on the outside of the inner membrane, it has to be transported through the periplasmic space to the outer membrane (**Figure 1B**). In the current PEZ model[17,18], LPS is extracted from the inner membrane by the $LptB_2FGC$ ATP-binding cassette transporter. It is thought that for every hydrolysis of ATP by $LptB_2$ allows for a LPS molecule to be extracted by LptFGC to the periplasmic bridge (LptA). Every such event will push the LPS further across the bridge until it reaches the LptDE translocon (**Figure 2A**), where the LPS will move through and exit into the outer leaflet of the outer membrane. This whole process has been likened to a candy (PEZ) dispenser, hence the model's name.

LptE is essential for the correct folding of LptD as it forms a plug-and-barrel complex and shows in vitro binding to LPS, however its function is not fully understood.[19] LptD is consists of a 26-stranded β-barrel in which LptE is situated, and a β-jellyroll domain, which acts as the last unit of the periplasmic bridge. LPS gets pushed through the hydrophobic internal channel of the β-jellyroll domain into the side of the β-barrel. The acyl chains of LPS can move through the hydrophobic side of the β-barrel, while the sugars are located in the hydrophilic centre of the barrel.

Antimicrobial peptidomimetic murepavadin

The Lpt machinery is an essential protein for cell viability in *Pa*[20] and thus a prime candidate as antibiotic target. Murepavadin (**Figure 2B**) is a narrow-spectrum macrocyclic peptidomimetic antibiotic that targets *Pa* LptDE.[21] It was originally derived in a study to mimic and improve upon the activity hairpin-shaped cationic antimicrobial peptide (CAMP) protegrin I. In contrast to protegrin I, murepavadin (then called POL7080) did not lyse the cells by disruption of the bacterial membranes. Its target was identified as LptD in photoaffinity labelling studies.[21] It has potent anti-*Pa* activity and, due to its specificity, it has been suggested that acquired resistance would be difficult to obtain.[22] Whole genome sequencing of *Pa* where resistance towards murepavadin was specifically induced resulted in many mutations in gene related to the lipid biosynthesis and efflux pumps. Only a few point mutations were observed in LptD itself, which were all located in the periplasmic domains.

Pa LptD contains an additional N-terminal insert domain (about 100 residues) that is only observed in this bacterial species.[23] No structures are present for the *Pa* LptD β-jellyroll domain and insert domain and the function of the insert domain is currently unknown. Murepavadin's specificity to *Pa* LptD must come from its insert domain.

Structural knowledge on the mode of action of murepavadin is critically required to design better antibiotics. Here, we aim to develop a solid-state NMR (ssNMR)-based approach, which allows us to study the binding of murepavadin to the essential LPS transport protein LptD at high resolution and in native-like lipid membranes. This allows for systematic identification of

the binding and mode of action of murepavadin, and it also lays the groundwork for other OMP targeting antibiotics. We first aim to prove the binding of murepavadin to the LptD/E complex in our system using ssNMR, confirm the binding domain and finally obtain high resolution data on the molecular binding site. Furthermore, we will obtain a high resolution structure of target-bound murepavadin, which should help our understanding of the protein-peptide interface. Consequently, we will investigate the inhibitory effect murepavadin has on the LPS transport via LptD by ssNMR. Ultimately, this work will contribute to the understanding of not only murepavadin and its mode of action, but also on the transport of LPS by LptDE.

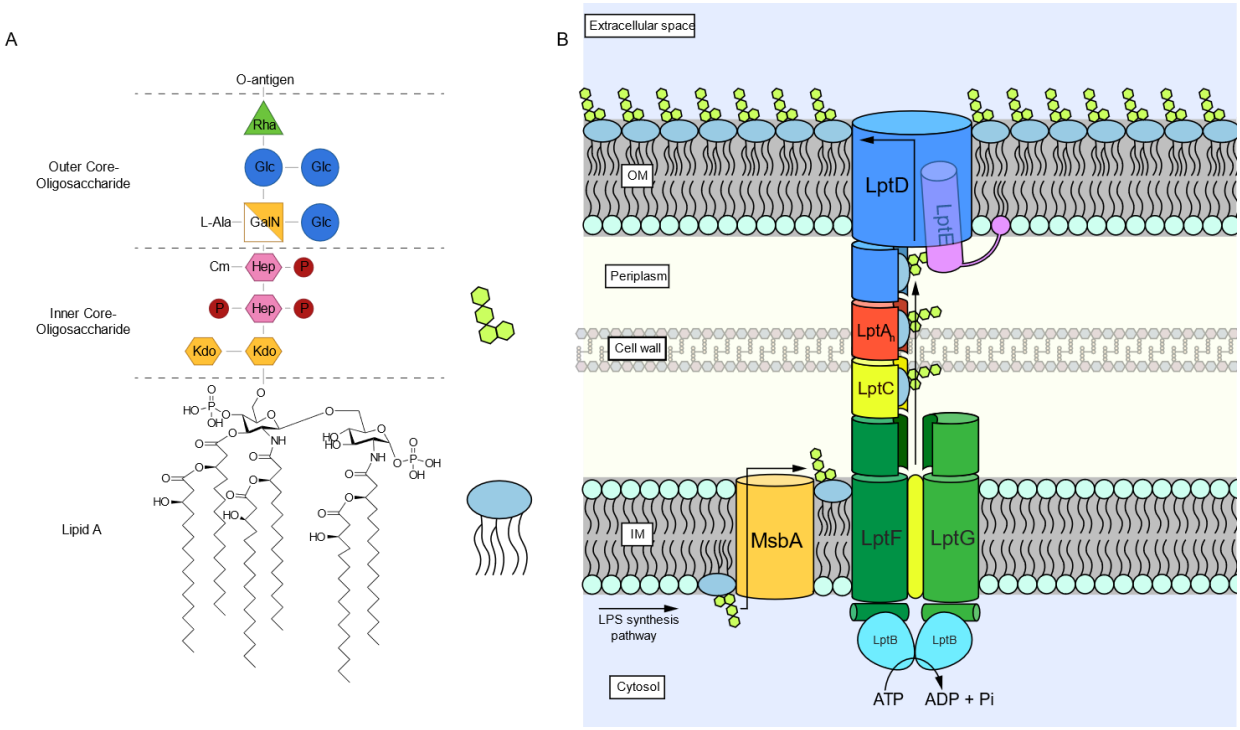

Figure 1. Schematic of an LPS molecule and the LPS transport pathway. A LPS consists of three major parts: Lipid A, the core oligosaccharide and the O-antigen. Lipid A is an acylated diglucosamine diphosphate structure. The conserved inner core oligosaccharide is made up of two 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) and two heptose sugars, which contain phosphate (P) and a 7-*O*-carbamyl (Cm) group(s). The outer core contains different glycoforms (only one depicted here) made up of N-acetyl galactosamine (GalN), glucose (Glc) and rhamnose (Rha). The O-antigen is a polysaccharide chain containing a repeating subset of sugars, typically totalling over 100 sugars long. On the right is a simplified depiction of LPS as used in **B**, where only lipid A and the inner core sugars are visualized. **B** Once LPS is synthesized and flipped to the inner membrane, it can be inserted into the cavity between LptFG. Then, LptC will dissociate between LptFG and using ATP-hydrolysis by $LptB_2$, the LPS is pushed up the periplasmic bridge consisting of the jellyroll domains of LptC, LptA and LptD. Once LPS has reached LptD, its hydrophobic part will insert in between the beta barrel stands of LptD and its sugars will insert into the hydrophilic centre of the beta barrel. LPS will eventually be pushed out through the opening in the side of the beta barrel and insert into the outer leaflet of the outer membrane.

B.2.2 Approach and Methodology

Expression, purification, and membrane-reconstitution of the LptD/E complex *(6 months)*

The expression and purification of *Pa* LptD/E has already been established in literature[19,23] and we have obtained the plasmid containing the *Pa* LptD/E recombinant protein for expression in *E.coli* from prof. dr. John A. Robinson (University of Zurich). The established method of the Robinson group will be utilised to obtain Pa LptD/E.[23] Briefly, B21 DE3 *E. coli* will be transformed with a pET3a (Novagen) vector containing the *lptD* gene and a pCDFduet-1 vector (Novagen) containing *lptE*, both from *P. aeruginosa* PAO1 (PA0595 and PA3988). Expression will be induced by addition of ITPG and will result in LptD containing its native N-terminus (res. 34-924) and LptE with a N-terminal lipid anchor and C-terminal His_6 tag. The LptD/E complex will be isolated using Ni-affinity chromatography and further purified by gel filtration. Additionally, a LptD mutant lacking the *Pa* specific N-terminal insert will also be expressed, purified and used in the study of the murepavadin binding.

In the Utrecht University NMR section, several studies were successful in the reconstitution of β-barrel membrane proteins for ssNMR, *e.g.* PagL[24] or BamA.[25,26] We will apply the same strategies to reconstitute the LptD/E into liposomes. Here, we can vary the lipid composition to increase in the complexity and biological relevance, from simple liposomes containing only phosphatidylcholine to liposomes containing mostly LPS. An even more compelling lipid system to study LptD/E in is their native system. While in-cell experiments would be very difficult, there is an alternative which is bacterial outer membrane vesicles (OMVs).[27] This will be the ideal environment to study any OMP in. Almost if not all Gram-negative bacteria naturally produce OMVs containing OMPs without the need for induction, but the yield can be boosted as OMVs are part of the bacterial stress response.[28] The OMVs contain proteins in their native lipid environment, meaning preferential lipids are in close proximity and membrane properties are still intact, such as membrane asymmetry. By studying LptD/E in both a phospholipid

and a more native environment, we will also be able to compare and gauge the influence of a more native lipid environment on the protein structure and dynamics, and its interaction with antibiotics such as murepavadin.
An efficient murepavadin chemical synthesis has been established by our collaborator prof. dr. Nathaniel I. Martin (Leiden University) and we already have been provided several mg of murepavadin for use in our lab. Using commercially available isotope-labelled precursors, Prof. Martin's group can selectively introduce ^{13}C, ^{15}N and/or ^{19}F isotope labels in murepavadin. This allows us to explore different labelling approaches to investigate the binding of murepavadin on LptD/E at high-resolution.

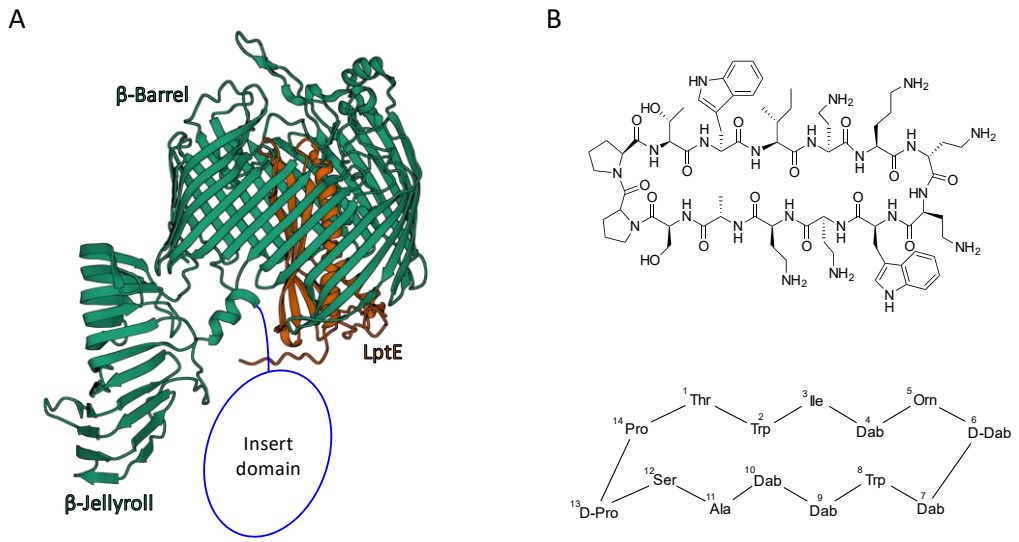

Figure 2. The domain organization and structure of *Pa* LptD/E and murepavadin. A *P. aeruginosa* LptD (green) contains 3 domains: a 26-stranded β-barrel (pdb 5IVA), a β-jellyroll (pdb 4Q35) and *Pa*-specific insert domain. As the structures of the β-jellyroll and insert domain are not known for *Pa*, the β-jellyroll shown here is based on *Shigella flexneri*. LptE (orange) is a lipoprotein that forms a plug-and-barrel conformation with LptD. **B** The structure and amino acid sequence of murepavadin. It is a 14 residue long β-hairpin peptidomimetic antibiotic.

Using ssNMR to show murepavadin binding to the LptD/E complex *(9 months)*
Solid-state NMR spectroscopy can provide detailed insight into the atomic-scale structural and dynamical organization of biomolecules, even in complex environments such as lipid bilayers or cell membranes.[29] We will first prove the binding of murepavadin to the LptD/E complex in our system using ssNMR. Therefore, we will acquire 2D ^{15}N-^{1}H and 2D ^{13}C-^{13}C ssNMR spectra of (^{13}C,^{15}N)-labelled LptD/E in the presence and in the absence of murepavadin and analyse drug-induced chemical shift changes. This analysis will substantially benefit from access to ultra-high magnetic fields (1200 and 950 MHz). These results will be supported by isothermal titration calorimetry (ITC) data, where we will titrate large unilamellar vesicles (LUVs) containing LptD/E to murepavadin. This will show binding and give us information about the binding affinity and stoichiometry in our system.

The structure of target-bound murepavadin *(9 months)*
Structural details about the murepavadin - LptD interaction are completely unknown. To elucidate the binding site, we will first need to determine the structure of LptD-bound murepavadin. Therefore, we will use unlabelled (^{12}C,^{14}N)-LptD and fully (^{13}C,^{15}N)-labelled murepavadin. Assignments of murepavadin in it's bound state will be performed with 1H-detected 3D experiments.[30] Distance information for structure determination will be obtained from 2D ^{13}C-^{13}C experiments with different magnetization transfer times[31], complemented by chemical shift dihedral angle restraints.[32] These experiments and analyses are straightforward and will provide crucial data. Additionally, we will use free murepavadin in solution and determine its structure. From this data, we will be able to model the structure of target-bound murepavadin and gives insight on which residues are involved in the binding interface. Furthermore, we will measure ^{15}N-(T^{1rho}) dynamics[30] of bound murepavadin to obtain additional information on its conformational space.

Studying the interaction of murepavadin with the LptD/E complex *(12 months)*
The *Pa* LptD/E complex is around ~ 130 kDa [23,33,34] and it will be very difficult to assign every ^{13}C signal. However, this is not the goal of our research and not needed. We will first identify the chemical shifts corresponding to the *Pa* specific insert domain

that is attached to the N-terminus [23]. Since murepavadin is narrow-spectrum antibiotic active against *P. aeruginosa*, it is highly likely that the insert domain will be target and our main interest in the NMR experiments. To do this, we will prepare two ssNMR samples: full length *Pa* LptD and the *Pa* insert domain deletion mutant. Additional signals observed in a 2D NH and 2D CC experiment when comparing the spectra from the full length *Pa* LptD and the deletion mutant will correspond to the insert domain (**Figure 2A**). Previous research performed by Robinson's group to express a recombinant protein of the insert domain did not yield a stably folded protein, thus full length LptD is required for its study.

Once we have identified the *Pa* specific insert domain in our NMR spectra, we can use this together with the confirmation that murepavadin binds to LptD in our system to determine if the binding site is located on the *Pa* specific insert domain. Furthermore, we will use different labelling strategies (described below) to find and verify residues important for the binding. Variations in the degree of isotope labelling and differently labelled molecules are essential with the increasing molecular size and complexity of the system when studying protein-protein interactions.

First, we will orthogonally ^{13}C-label both murepavadin and LptD/E and we use ^{13}C-^{13}C 2D data to determine the protein-protein interface. We can use isotopically labelled amino acids for incorporation in murepavadin and with forward labelling we can introduce ^{13}C-labeled amino acids into the growth media that will be incorporated into LptD. By creating labelling schemes that have 1 or 2 orthogonally labelled amino acids in both LptD and murepavadin, we can reduce spectral crowding and obtain contact information on the interface between the proteins.

Second, ^{19}F-labelling of murepavadin and/or LptD/E. ^{19}F labelling will be great for determining the structure and binding site of bound murepavadin. Like protons, ^{19}F is a spin ½ nucleus with a very high gyromagnetic ratio and near 100% natural abundance. This together with the fact that ^{19}F does virtually never occur naturally in proteins, will ensure high sensitivity without background noise.[35] The ssNMR probe for ^{19}F measurements on the 1200 MHz magnet (1.3 mm 1H/^{19}F/X/Y 4-channel) will be available in the summer of 2022 in the NMR facility. Murepavadin contains 2 tryptophan (Trp), 2 prolines (Pro), and 1 isoleucine (Ile) which can easily be fluorinated[36–39] and incorporated in murepavadin. ^{19}F analogues of these residues are commercially available and, with the help of our collaborators, ^{19}F-murepavadin can be readily synthesized. Fluorinated LptD variants can be obtained by addition of ^{19}F-analogues of aromatic residues to the growth media. We can fluorinate the tyrosine (Tyr), Trp, and phenylalanine (Phe) residues for incorporation in LptD. We can then probe the binding interface using ^{19}F-Trp-murepavadin and ^{13}C-LptD/E by measuring the ^{19}F-^{13}C long-range contacts (2D ^{13}C-^{19}F REDOR[40]), use orthogonal labelling with ^{19}F-Tyr LptD/E and ^{19}F-Trp murepavadin (2D ^{19}F-^{19}F spin diffusion[41]), and use ^{19}F-Tyr/Trp LptD/E and ^{13}C murepavadin (2D ^{13}C-^{19}F REDOR).

Mutagenesis and structural modelling *(12 months)*

The above-mentioned experiments can be combined with mutagenesis to confirm the residues of the binding site. As there is not structural data on the insert and β-jellyroll domains, it is difficult to predict which residues are of importance. Single mutations of these residues are likely to reduce the binding affinity of murepavadin to *Pa* LptD. This can be easily observed with ITC and more intimately investigated using ssNMR with the same experimental set-up.

We can use the obtained structure of target-bound murepavadin together with structures available of LptD. There are homology models available for the N-terminal domain and insert domain from *P. aeruginosa*. Robinson *et al.* show a high homology model [23] based on Ig-like V_H domain (pdb 1T2J) that was found with a sequence motif search. We can use HADDOCK to model the acquired murepavadin structure on the speculative homology model to gain insight on the binding of murepavadin on *Pa* LptD.

With the knowledge of the binding site, we can further investigate the mode of action of murepavadin and improve upon its binding. By substituting the residues of murepavadin that are important for binding, we can obtain a higher affinity for the target. There are UV-photo-crosslinking studies showing where LPS can bind on LptD [18,42]. By comparing this literature to our acquired binding site, we can determine if there is direct competition, where murepavadin directly blocks the binding of LPS or if there is another non-competitive allosteric effect, where a conformational change induces inhibition of LPS transport.

B.2.3 Feasibility & Risk assessment

The methods described in the approach section are almost all established and robust ways to obtain the data on the molecular interaction with LptDE and murepavadin. The purification of LptDE has been extensively performed and described[23]. The production and incorporation of isotopically labelled amino acids in murepavadin has already been performed and is ready for use. The ssNMR experiments are all established[30,32,40], we have strong in-house expertise on β-barrel membranes proteins[24–26], and we have access to state-of-the-art NMR equipment. In addition, the Utrecht NMR section has had extensive experience with mutagenesis studies and computational modelling of protein-peptide interactions.[31,43–45] Still, we are aware of the troubles that may arise during the project.

The use of OMVs from *P. aeruginosa* is relatively new. This would be a very interesting way to resolve structural data and compare the effect of the lipid environment on form and function of LptDE, however it is not necessary for the big picture. The LptD/E complex can be reconstituted into any membrane composition that we chose and therefore we can be as close as

possible to the OM composition of *Pa* [46] as possible. We can reduce the complexity of the liposomes or eliminate the problem by using a liposome made up of phosphatidylcholine lipids.
The largest setback that we could encounter is not observing the binding of murepavadin to LptDE using ssNMR. This could be by a too crowded spectrum or by murepavadin not being able to bind LptD inside a liposome. We can reduce the spectral crowding with more specific labelling schemes of the protein. Murepavadin will likely still be able to bind to the periplasmic domains of LptD without membrane disruption of the liposome due to LptD barrel protein. We can make sure murepavadin can bind the periplasmic domains of LptD by including it in the proteoliposomes preparation buffer. This way, the murepavadin will also be present inside the liposomes. Even if exact binding site cannot be found on LptD, by determining the structure of murepavadin in the target-bound state and comparing it to its free-state, we already gain critical information on the binding mode of murepavadin
The *Pa* insert domain contains only five aromatic residues that can be used for ^{19}F-labeling: 2 tyrosine and 3 tryptophan residues. Without the structure of this domain, we cannot predict if these residues are exposed and in close proximity to the binding site. We will need to pay mind to the few residues that we can use if we want to use orthogonal ^{19}F labelling with ^{19}F-Tyr-murepavadin. However, using ^{19}F, we can probe distance up to 10-15 Å, which is sufficient to observe binding between murepavadin and the few ^{19}F-labelled residues of LptD. By selectively ^{19}F-labelling tryptophan residue and substituting two out of the three tryptophan residues we can obtain valuable about the residues involved.

B.2.4 Scientific and societal impact

Gram-negative bacteria are becoming a bigger and bigger threat to general healthcare. These pathogens are difficult to get rid of due to their growing catalogue of antimicrobial resistances. The development pipeline of antibiotics is almost dried up, thus there is a need for novel antimicrobial compounds and novel targets. Here, we advocate more research into murepavadin, a peptidomimetic antibiotic that specifically targets *Pseudomonas aeruginosa* (Pa). As one of the eighteen priority pathogens as classified by the WHO[47], *Pa* is an opportunistic Gram-negative bacteria that infects airways, urinary tracts, and wounds. It is one of the main perpetrators of pneumonia in cystic fibrosis (CF) patients. Cystic fibrosis is a monogenic recessive disorder that is most commonly found in Caucasian populations[48]. It stems from mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. Beside the myriad of health related issues caused by this defect, the most obtrusive one is chronic lung infections. Around 80 to 95% of people with cystic fibrosis (CF) die by respiratory failure caused by bacterial infection of *Pseudomonas aeruginosa*[49]. Murepavadin is a drug that specifically targets *P. aeruginosa* and such a narrow-spectrum antibiotic is very appealing, maybe not as prophylactic therapy or initial treatment of an infection, but when the causative pathogen is known, it is a very effective treatment method. Narrow-spectrum antibiotics are actually unlikely to negatively impact a patient's native bacterial flora, which are unfortunate consequences of treatment with broad-spectrum antibiotics. Furthermore, the use of broad-spectrum antibiotics causes a selection for antimicrobial resistance, while this is not the case for narrow-spectrum antibiotics. E.g. Murepavadin acts on the OMP LptD, which is responsible for the LPS transport to the outer membrane and causes cell death in *Pa*. Given its specific mode of action on *Pa*, it is unlikely to naturally develop antimicrobial resistance. However, not much is further known about the specific binding and mode of action. That is why Polyphor has received approval to start phase 1 clinical trial for inhaled murepavadin.[51]
The here proposed research aims to better understand the target binding and mode of action of murepavadin as model peptidomimetic. Our results could have a huge impact on the structural knowledge of murepavadin and its interaction partners on LptD, and will help the development of these drugs to better combat *Pa* infections. With more knowledge about murepavadin, its relative toxicity or affinity towards host and foreign cells can be improved and a higher chance of a successful drug against *Pa* infections for CF patients. Different substitutions of residues in murepavadin can then be done on a knowledge basis instead of with random mutations and serendipitously finding an improvement.
Lastly, this research will show that with the right labelling schemes and mutagenesis experiments, we can study interactions of very large OMPs with antibiotics in a native-like environment. LptD contains a 26-stranded β-barrel and is one of the largest characterized at this time.[34,52,53] We can identify important residues in both the protein and antibiotic for its binding and activity.

B.2.5 Ethical considerations

Any and all research and communication about this research will be done openly, honestly and without exaggeration about the aims and objections, the funding, and the results and discussions. Furthermore, all researchers included will be treated fair and evenly, and any possible conflicts of interests will be declared.

B.2.6 Literature/references

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