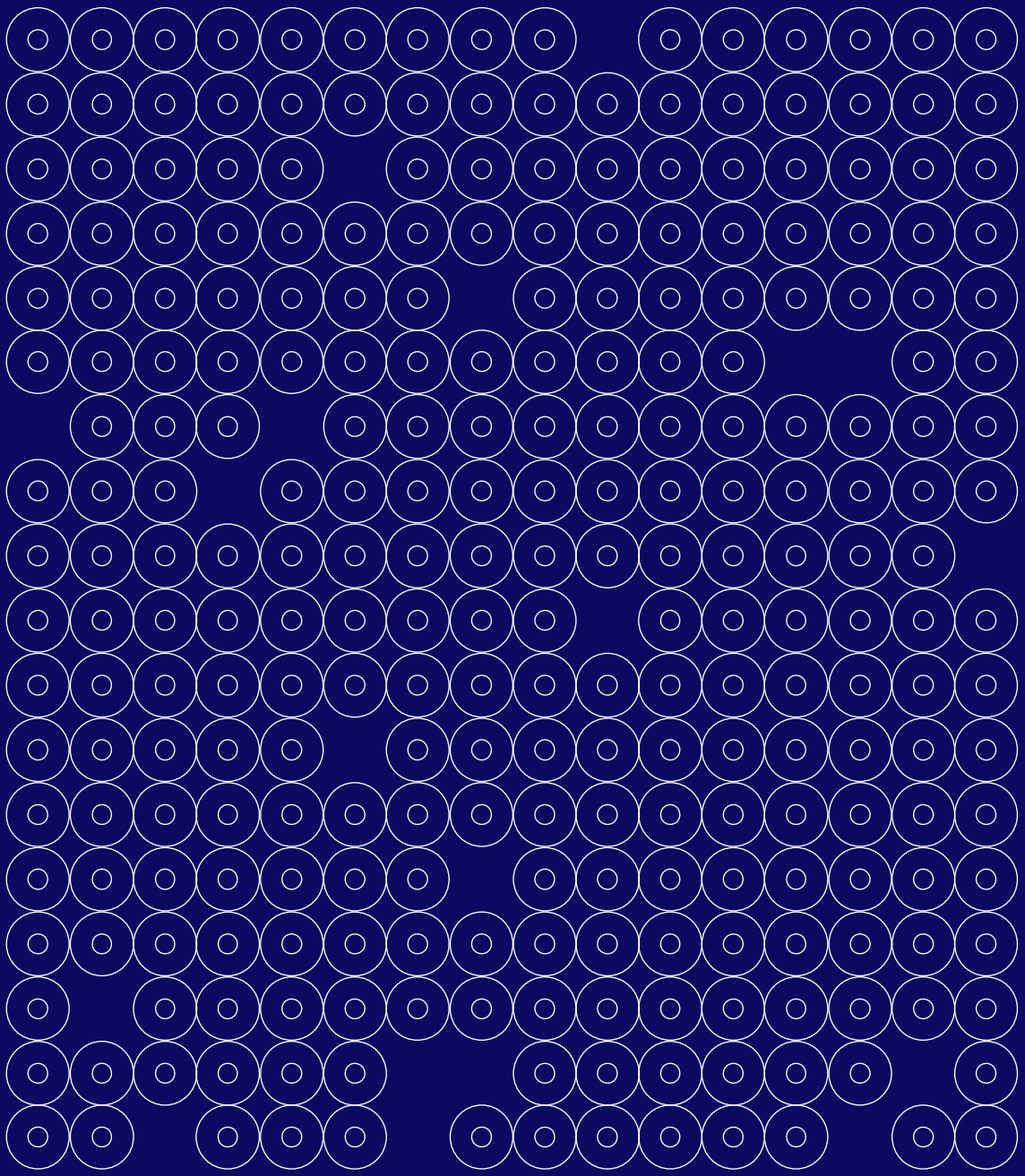
JPIAMR therapeutics discovery pipeline

Outputs, outcomes and impact of the funded projects and networks in the Therapeutics priority topic of the JPIAMR-SRIA



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Joint Programming Initiative on Antimicrobial Resistance

Contents

Summary

The Joint Programming Initiative on Antimicrobial Resistance (JPIAMR) [1] facilitates alignment of research programmes between member countries, as well as initiating and coordinating international funding initiatives on AMR through implementation of priorities identified in its Strategic Research and Innovation Agenda (SRIA)[2]. The SRIA identifies six priority topics that cover the broad scope of the societal challenge posed by antimicrobial resistance (AMR). The JPIAMR SRIA recognises Therapeutics as one of the six priority topics and supports research on the discovery of new antimicrobials and therapeutic alternatives, and the improvement of current antimicrobials and treatment regimens.

To date, JPIAMR has launched 13 joint transnational calls investing approximately 127 million Euros (M€) supporting 137 research projects and networks and connecting 1430 researchers world-wide. Eighteen percent of the total investment has been directed to the priority area of Therapeutics where JPIAMR has invested approximately 24.5 million Euros and has supported 20 research projects through three different transnational project calls and connecting 98 researchers from 17 different countries. These projects include research on discovery of new antibiotics and therapeutic alternatives, repurposing Neglected and Disused Antibiotics (ND-AB), designing combinations of ND-AB, antibiotic and/or non-antibiotic and improvement of current antibiotics and treatment regimens against pathogens identified in the WHO Priority Pathogens List[3]. In addition, JPIAMR also invested approximately 0.35 million Euros to support seven research networks in Therapeutics connecting 156 experts to identify the key bottleneck in anti-bacterial research and antibiotic discovery, the options and effects of minimising barriers for the introduction of novel antimicrobials and anti-infective compounds by simplification of regulatory procedures and by stimulating economic incentives.

Main achievements of the Therapeutics research projects and networks and the impact generated

- The majority of the research projects have pathogen-specific approaches.
- The majority of the research projects are in lead generation phase (hit-to-lead phase).
- The research projects are addressing both traditional (45%) and non-traditional (50%) approaches to develop lead candidate.
- The JPIAMR discovery pipeline resulting from the funded research projects has a high level of diversity including direct acting molecules, potentiators or enablers, anti-virulence agents, repurposed agents, phage and nanobiotics.
- Six new candidates/leads have been identified and five patents have been filed.
- In addition to the peer-reviewed scientific articles in highly-recognised journals, the research projects and networks have also published white papers, roadmaps and position papers that can contribute to evidence-based policymaking.

[1] www.jpiamr.eu
[2] www.jpiamr.eu/app/uploads/2021/06/JPIAMR_SRIA_2021.pdf
[3] www.who.int/medicines/areas/rational_use/PPLreport_2017_09_19.pdf?ua=1

- The research project DesInMBL developed the Beta-Lactamase Database [4] that contains information on all β-lactamases described in the literature, as well as the crystallographic structures and kinetic data of these enzymes that have been deposited in other databases.
- Some research projects have also received additional external funding for complementary activities related to academia-industry research partnership, for e.g, from CARB-X, collaboration with TB-alliance and grants from EU-H2020.

The JPIAMR funded research projects are expanding our knowledge and understanding to improve current antimicrobial treatment by enhancing discovery of novel antimicrobials, novel treatment strategies and alternative therapeutics. An additional outcome from the research networks is the development of policy measures and economic stimuli to minimise barriers for the development, availability and introduction of such new therapies and alternatives for AMR.

[4] http://bldb.eu

Overview of the outputs, outcomes and impact of the funded research projects and networks under the Therapeutics priority topic

JPIAMR has conducted three transnational project calls under the priority topic of Therapeutics and has supported 20 research projects. The research projects have received a total grant amount of approximately 24.5 million Euros and have connected 98 researchers from 17 different countries. In addition, seven research networks have also been funded with a total amount of approximately 0.35 million Euros through various JPIAMR network calls. The research networks connected 156 experts from 25 different countries enabling the JPIAMR to connect researchers and experts in the AMR field across the globe (figure 1).

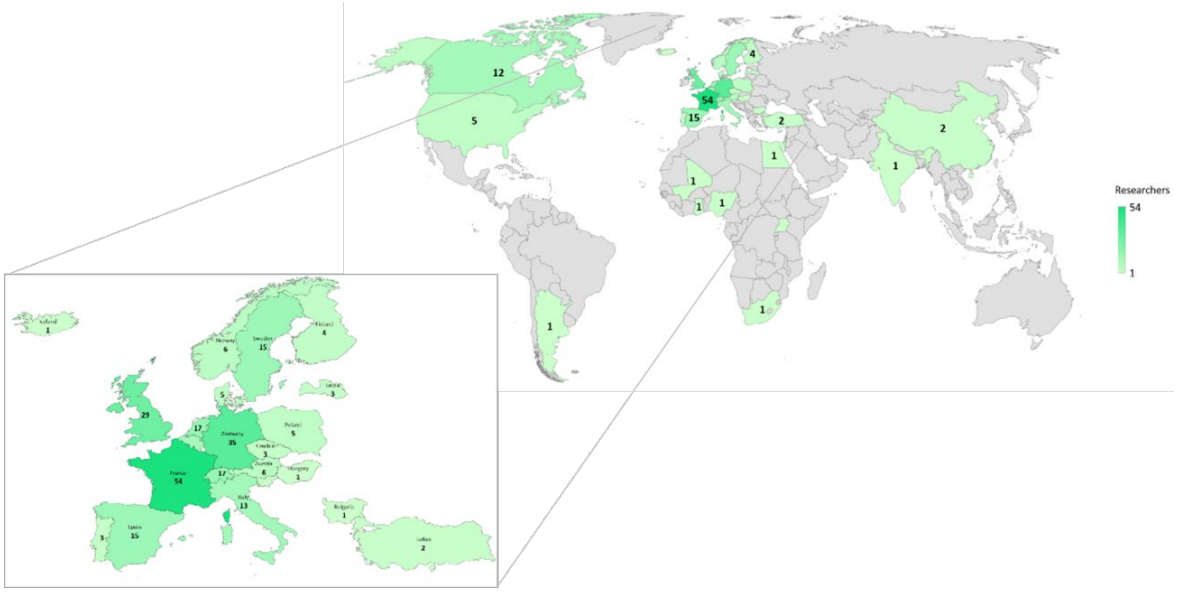

Figure 1. Global distribution of the researchers and experts participating in various research projects and networks supported by JPIAMR in the priority area of Therapeutics.

Focal areas of the supported research projects

- The JPIAMR calls on Therapeutics had various areas of interest ranging from:
- Innovative approaches to address antibacterial resistance
- Identification and validation of new targets, the development of new therapies, and new tools for new treatments (including new antibiotics)
- Discovery and implementation of novel therapies to overcome known antimicrobial resistance mechanisms and restore susceptibility to conventional antibiotics
- Reviving ND-AB and research on improving the efficiency of antibiotics by using them in combination with other antibiotics or a non-antibiotic

The focal areas of the supported research projects are represented schematically in the figure 2.

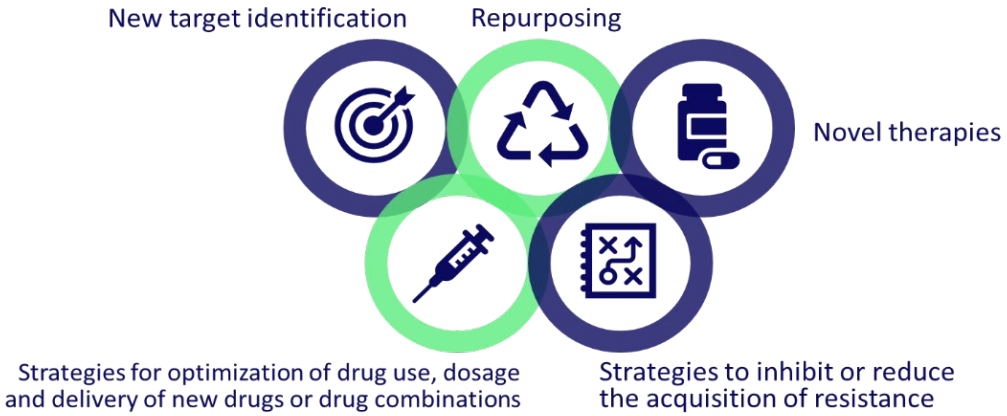

Figure 2. Schematic representation of the focal areas of the therapeutics research projects.

The graphical representation shows that majority of the supported research projects address critical (37%) and high priority (29%) pathogens categorised under the WHO Priority Pathogens List including tuberculosis (TB; 10%) (figure 3).

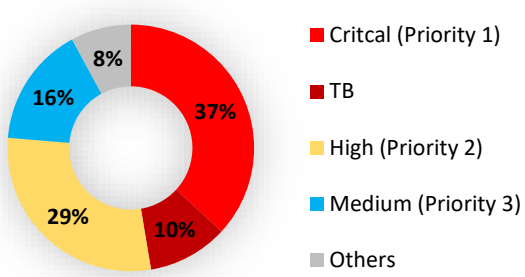

Figure 3. Distribution of JPIAMR supported research projects addressing pathogens categorised under WHO Priority Pathogens List.

JPIAMR discovery pipeline

The funded research projects are diverse and multidisciplinary, covering studies aimed at understanding and overcoming the mechanisms controlling the generation of resistance, studies on new bacterial targets or mechanisms of resistance, re-evaluation of existing antimicrobial compounds either alone or in combination with other drugs, immune-modulators or antibacterial approaches and discovery of new compounds (including new antibiotics and alternatives). The majority of the research projects are in lead generation phase (hit- to- lead phase) working with both traditional (45%) and non-traditional (50%) approaches (figure 4) and are expected to result in preclinical candidates.

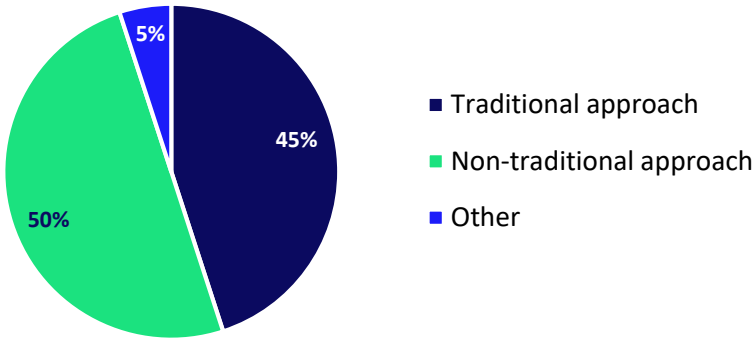

Figure 4. Distribution of JPIAMR supported research projects addressing pathogens categorised under WHO Priority Pathogens List.

The JPIAMR discovery pipeline is diverse. There is also diversity in the non-traditional approaches that the research projects are undertaking for alternative treatments and strategies including potentiators, anti-virulence agents and phage. Potential therapeutic indications of these new leads are for different acute and chronic infections including pneumonia, urinary tract infections and catheter infections, chronic infections in wounds or during cystic fibrosis, medical implant infections, hospital associated infections. The research projects working with new leads/molecules are further grouped into different categories (see Box 1) on the basis of the types of leads generated or to be generated by the projects (table 1).

Box 1. Classification of the projects

The projects are categorised according to their main effect on bacteria into the following groups as per the criteria of the global preclinical antibacterial pipeline[5]:

- Direct acting small molecules: traditional antibiotics that directly inhibit growth or kill the bacteria without requiring any additional therapy
- Repurposed agents/antibiotic: non-antibiotics or antibiotics repurposed in combinations
- Non-traditional approaches:
  - Anti-virulence agent: affects a broad range of virulence factors
  - Immunomodulators
  - Phage or phage derived proteins that directly affect bacteria
  - Potentiators/enablers: that enhance and augment or transform other agents (β-lactamase inhibitors, efflux inhibitors)
- Others: including nanoparticles (nanobiotics) that have antibacterial capabilities.

[5] *Theuretzbacher, U., Outterson, K., Engel, A.* et al. *The global preclinical antibacterial pipeline.* Nat Rev Microbiol *18, 275–285 (2020).*

Table 1. Overview of the JPIAMR discovery pipeline representing the distribution of the JPIAMR supported research projects categorised by types of leads.

Project acronym	Category; the mode of action/target	Lead molecule
DesInMBL	Potentiator/enabler; Metallo-β-lactamase inhibition	Metallo-β-lactamase inhibitor
NAPCLI	Direct acting small molecule; Cell wall synthesis	Bi-substrate inhibitor of L,D-transpeptidases
noTBSec	Direct acting small molecule; Type VII secretion systems	Optimised ESX-5 inhibitor
NPERDMDR	Other ; Nanoparticles	Nanobiotics
REBEL	Potentiator/enabler; β-lactamase inhibition	Not specified
SENBIOTAR	Anti-virulence agent; Biofilm disruption	PqsR inhibitor; PNA (anti-biofilm agent, quorum sensing inhibitor)
ABIMMUNE	Repurposed agents/antibiotic; Immunomodulation	Combination of immunostimulatory molecules and ND-AB/AB
CO-ACTION	Repurposed agents/antibiotic; Not specified	Combination of polymyxin B and spironolactone
Combinatorials	Repurposed agents/antibiotic; Not specified	Three combinations of AB, ND-AB and NA
ANTIBIO-LAB	Phage; Biofilm disruption	Not yet reported; Ongoing project
Anti-Persistence	Direct acting small molecule; RNA synthesis	Not yet reported; Ongoing project
CRISPRattacK	Phage ; Phage as carrier	Not yet reported; Ongoing project
DISRUPT	Anti-virulence agent; Biofilm-disruption	Not yet reported; Ongoing project
EXPLORE	Direct acting small molecule; RNA synthesis	Not yet reported; Ongoing project
FLAV4AMR	Direct acting small molecule; Cell metabolism	Not yet reported; Ongoing project
MTI4MDR-TB	Direct acting small molecule; Other cellular function	Not yet reported; Ongoing project
RESET-ME	Potentiator/enabler; Efflux pump inhibition	Not yet reported; Ongoing project
RIBOTARGET	Direct acting small molecule; Protein synthesis	Not yet reported; Ongoing project
SCAN	Direct acting small molecule; RNA synthesis	Not yet reported; Ongoing project

Outcomes and impact of the concluded therapeutics research projects

Many of the research projects funded from the three different JPIAMR Therapeutics call are still ongoing and are yet to conclude their research activities. However, the outcomes of the recently concluded research projects are highlighted as below and represented schematically in figure 6.

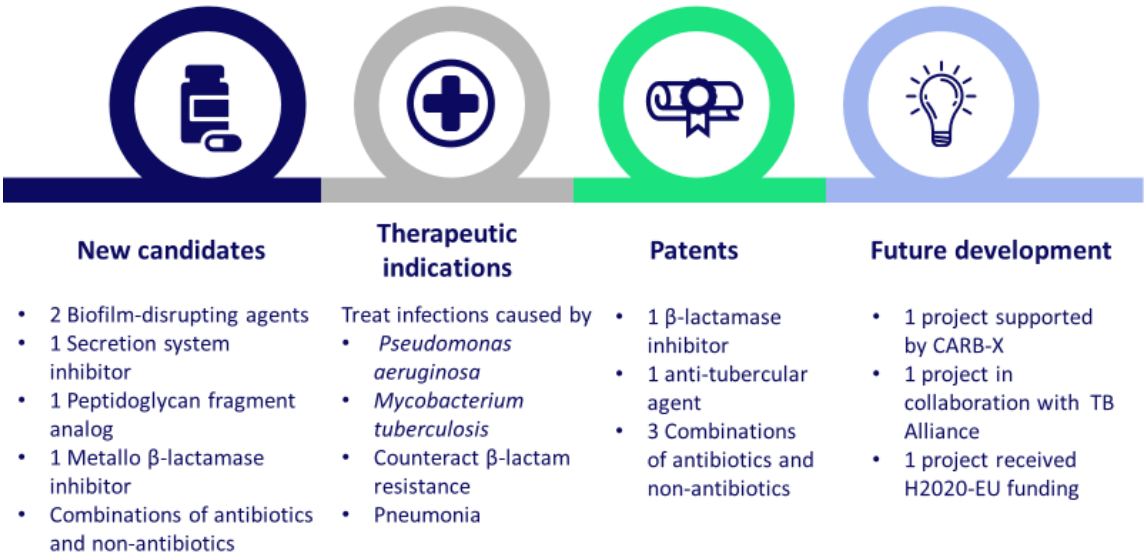

Figure 6. Overview of the outcomes of the concluded research projects supported in the various JPIAMR calls under the Therapeutics priority topic.

- The project **SENBIOTAR** has identified two lead molecules to treat infections caused by *Pseudomonas aeruginosa.* These molecules are:
  - a novel antagonist of the receptor of the *Pseudomonas* quinolone signal pathway (PqsR)
  - a novel antisense molecule (peptide nucleic acid, PNA) conjugate

  These molecules that inhibit quorum sensing, biofilm formation and show synergistic effects with tobramycin in a rat infection model and are under further development as a preclinical candidate.

- The project **NAPCLI** designed new inhibitors with antibacterial activity that can act alone or in synergy with β-lactams. A new lead molecule has been identified that is an analogue of a peptidoglycan fragment that acts as a bi-substrate inhibitor of L,D-transpeptidases which are involved in distinct β-lactam resistance mechanisms. The NAPCLI collaboration has received further funding support including funding from CARB-X for an academia-industry research partnership.

- The project **NoTBSec** has identified an inhibitor that targets multiple secretion systems of *Mycobacterium tuberculosis* (ESX-5 inhibitor for Type VII secretion system of *Mtb*). A patent is pending for the identified lead compound. The project has been

successful in receiving follow-up funding and has generated interest for a new collaboration with TB Alliance.

- The project **ABIMMUNE** utilised the strategy to use immunomodulatory drugs in combination with antibiotics to target specifically cells or pathways of innate immunity. The project identified four leads of interest to be used in combination with antibiotics:
  - Monophosphoryl lipid A (MPLA, a Toll-Like Receptor 4 [TLR4] agonist)
  - Pioglitazone, a PPAR-γ agonist
  - Flagellin, a TLR5 agonist
  - Rapamycin, an inhibitor of mTOR pathway
  - The consortium received a H2020 grant for the project "FAIR" that aims to address further the use of flagellin, an immunostimulatory drug, from preclinical to phase 1 clinical trial in the context of antibiotic-resistant pneumonia.

- The project **COMBINATORIALS** identified three types of combinations of antibiotics and non-antibiotics:
  - Combinations that improve the effectiveness of antibiotics against MDR enterobacteria – e.g. vanillin made spectinomycin, a neglected antibiotic effective against MDR UPEC (incl. carbapenem/colistin resistant isolates)
  - Combinations that prevent or decrease antibiotic resistance development by blocking natural competence in *S. pneumoniae* when co-administered with fluoroquinolones, aminoglycosides or β- lactams, competence blockers
  - Combinations that decrease the collateral damage of macrolide and tetracycline antibiotics to the gut microbiome (and hence decrease the population size that can develop resistance that is often horizontally transferred, but allow them to be as effective with pathogens).

All the above combinations were tested and validated *in vivo*. Three patents are filed for each type of combination, including several molecules and combinations.

A brief description of the research aims of the supported research projects, their main findings and the impact generated is provided in Annex I.

Outcomes and impact of the concluded therapeutics research networks

The outcomes of the recently concluded research networks are highlighted as below:

- The network "**BEAM Alliance**" representing 50 European biopharmaceutical companies (small and medium size (SME)) published a position paper[6] regarding establishment of economic incentives to improve sustainability of the innovation efforts of small and medium biopharmaceutical companies involved in developing innovative products to combat AMR.

[6] *https://beam-alliance.eu/web/content/1648?unique=9d2965b066086ab9a451b2705de2a1d5a1ad41f8&download=true*

- The **VeRI BEAM** network gathered 26 SMEs from the BEAM Alliance working in the field of AMR and published a white paper[7] on developing new criteria to improve the comprehensive evaluation of the different features exerted by an antimicrobial drug to enable an informed prescription. Improving the categorisation of the AMR products would increase both their clinical value (to match the patient's needs with the drug actions) and their market value (more benefits for the patient and the health system deserve a better price).

- The network, **IRAADD**, published a roadmap paper that addressed the increasing global spread of AMR and provided a strategic plan for improving the ability to identify and develop novel "resistance breaking" antibiotics.[8]

A brief description of the objectives of the supported research networks, their main findings and the impact generated is provided in Annex II.

[7] *https://beam-alliance.eu/wp-content/uploads/2019/10/beam-alliance-a-new-vision-to-support-amr-innovation.pdf*
[8] *Miethke, M., Pieroni, M., Weber, T. et al. Towards the sustainable discovery and development of new antibiotics. Nat Rev Chem 5, 726–749 (2021).*

Annex. I: Research findings and impact of the therapeutics projects

The following section provides a brief description of the research aims of the supported projects, their main findings and the impact generated in various forms, not only scientific but also policy impact as perceived by the researchers.

DesInMBL: Structure-guided design of pan inhibitors of metallo-β-lactamases

Project webpage: www.jpiamr.eu/project/DesInMBL/

Category; the mode of action/target: Potentiator/enabler; Metallo-β-lactamase inhibition

Activity against WHO priority pathogen(s): *Klebsiella pneumoniae;* carbapenem resistant; Critical priority

Lead molecule: Metallo-β-lactamase inhibitor

Background: The fight against infectious diseases is probably one of the greatest public health challenges, especially with the emergence of pan-drug resistant carbapenemase-producing Gram-negative bacteria. The pandemic NDM-1 and other plasmid-borne metallo-β-lactamases (MBLs) disseminating worldwide in Gram-negative organisms threatens to take medicine back into the pre-antibiotic era since the mortality associated with infections caused by these “superbugs” is very high and the choices of treatment are very limited. DesInMBL combined complementary approaches (microbiology, biochemistry, structural biology, molecular modelling and chemical synthesis) to gain vital insights into the structure-function relationship of MBLs, in order to understand substrate specificities, to determine key residues involved in carbapenem recognition and hydrolysis, to foresee the impact of mutations on the hydrolysis profile, and to develop an MBL pan inhibitor.

Main findings: In-depth biochemical characterization of broad and narrow-spectrum MBLs and their mutants have provided crucial information on the key residues in involved in ß-lactam hydrolysis, and for the development of efficient inhibitors that could serve as leads in drug discovery. Along with the increasing prevalence of MBLs, new variants have been described, with modified hydrolysis properties, which provide further information on the role of different active site residues. The project has developed a novel MBL inhibitor capable of efficiently inhibiting New Delhi metallo-β-lactamase (NDM-1) (100nM IC50) but also Verona integron-encoded metallo-β-lactamase (VIM) and Imipenemase (IMP). Two strategies were used to obtain this MBL pan inhibitor: i) structure- and function-guided optimisation of a previously identified inhibitor, using the data gathered during this project; ii) identification of new pan inhibitors of MBLs using a consensus pharmacophore common to all clinically-relevant MBLs. The results have advanced the development of pan inhibitors of MBLs that, used in combination with β-lactams, will protect the β-lactam antibiotics from degradation by these MBLs.

Impact: The project has resulted in the development of a potent MBL inhibitor with IC50 of 100 nM for which a patent has been filed. The researchers also developed the β - Lactamase DataBase (BLDB, http://bldb.eu), an exhaustive resource containing all β-lactamases described to date in the literature, as well as the crystallographic structures of these enzymes that have been deposited into the Protein Data Bank (PDB). The work of this project has further resulted in the formation of an international workgroup dedicated to the nomenclature of β-lactamases.

NAPCLI: Non-conventional approaches for peptidoglycan cross-linking inhibition

Project webpage: www.jpiamr.eu/project/napcli/

Category; the mode of action/target: Direct acting small molecule; Cell wall synthesis

Activity against WHO priority pathogen(s): *Enterococcus faecum*, *Staphylococcus aureus*; High priority

Lead molecule: Bi-substrate inhibitor of L,D-transpeptidases

Background: Bacterial resistance to antibiotics has been mainly fought by modifications of molecules discovered more than 50 years ago. Consequently, antibiotics available in the clinics belong to a very limited number of chemical classes. The consortium investigated new strategies to inhibit "old" targets, those of β-lactams, that were previously validated by a long-lasting therapeutic use.

Main findings: Peptidoglycan cross-linking by penicillin-binding proteins (PBPs) and L,D-transpeptidases (Ldts) is a two-step reaction involving (i) formation of a covalent adduct with the first substrate (acyl donor) and (ii) nucleophilic attack of the resulting acyl-enzyme by the second substrate (acyl acceptor) leading to cross-link formation. β-lactams are mimics of the first substrate and the corresponding binding site is well characterized, whereas nothing is known on the acceptor site. In this context the project identified the molecular determinants for recognition of the acceptor of the transpeptidation reaction and to design new inhibitors with antibacterial activity that could act alone or in synergy with β-lactams. New binding sites that are distinct from that of penicillin and are essential for the activity of the targets were identified. A new lead molecule that is an analog of peptidoglycan fragment and acts as a bi-substrate inhibitor of Ldts has been identified which are involved in distinct β-lactam resistance mechanisms.

Impact: The project shows how furthering the understanding of peptidoglycan synthesis contributes to the development of new strategies for drug development in academic laboratories with complementary expertise.

noTBSec: New intervention strategy for tuberculosis by blocking multiple essential targets

Project webpage: www.jpiamr.eu/project/noTBsec/

Category; the mode of action/target: Direct acting small molecules; Type VII secretion systems

Activity against WHO priority pathogen(s): *Mycobacterium tuberculosis*; Global priority

Lead molecule: Optimised ESX-5 inhibitor

Background: Mycobacterium tuberculosis is the causative agent of tuberculosis (TB), a disease responsible for almost 1.3 million deaths per year. In recent years, different classes of drug resistant *M. tuberculosis* strains have emerged, making the discovery of novel anti-TB drugs a major global priority. A major disadvantage of most existing and new TB compounds is that they target a single molecule, which significantly increases the emergence of resistant strains. To prevent the rapid generation of antibiotic resistance it would be advantageous to block more than one essential target of the tubercle bacteria with a single drug.

Main findings: The project addressed the problem of emerging resistance in *M. tuberculosis* by identifying compounds that target multiple type VII secretion (T7S) systems that are used to secrete proteins across the cell envelope to the cell surface or into the host environment. *M. tuberculosis* has several different T7S systems, three of which are different but have homologous secretion systems that are essential for growth or virulence (ESX-1, ESX-3 and ESX-5) and blocking multiple T7S systems with a single compound, might considerably reduce the development of drug resistance. A large compound library was screened for activity and toxicity tests and out of the few promising hits a lead compound was extracted as a potent inhibitor of the secretion system that also showed synergistic activity with the antibiotic vancomycin (an antibiotic that targets envelope assembly). After the initial screen on ESX-5 inhibitors the lead compound was also found to block ESX-1. The effect on the ESX-3 system was also determined with increased killing of mutants that are dependent on the ESX-3 system. This confirms proof of principle, but the basis for this (actual target) still needs to be determined. Further analyses are ongoing to elucidate the mechanism of action.

Impact: A patent is pending to be filed for the identified lead compound. This project has resulted in successful follow-up funding for the ESX-5 inhibitor resulting in new collaborations, including collaboration with TB Alliance.

NPERDMDR: Investigating the Mechanism of Eradication of Multi Drug Resistant Bacteria by Inorganic (mixed metal oxides), Organic (antibiotic), and Protein-based Nanoparticles

Project webpage: www.jpiamr.eu/project/NPERDMDR/

Category; the mode of action/target: Other; Nanoparticles

Activity against WHO priority pathogen(s): *Mycobacterium tuberculosis*; Global priority

Lead molecule: Nanobiotics

Background: Nanomaterials have emerged as novel antimicrobial agents because of their high surface area to volume ratio and the unique chemical and physical properties. It appears that their antimicrobial activity is exerted by a combination of different mechanisms, such as reacting with -SH protein groups, uncoupling respiratory electron transport, changing cell morphology, and causing cell membrane disruption and DNA damage. There are four mechanisms by which bacteria exhibit resistance to antimicrobials: antibiotic inactivation/ modification, alteration of target site, alteration of metabolic pathway, and reduced drug accumulation. None of these mechanisms of resistance have been reported in nanoparticles (NPs) with antibacterial activity. Thus, it is crucial to evaluate whether existing or newly developed NPs can inflict genetic changes within the bacteria that will ultimately result in resistance. NPERDMDR developed assays that will allow determining the potential of bacteria to develop resistance to NPs developed and synthesised within the scope of the project.

Main findings: Mixed metal oxide (MMO), antibiotic (ANB), and quorum sensing inhibiting enzyme (QSIE) nanoparticles (NPs) were synthesised, optimized, and chemically characterized. Stable chitosan, aminocellulose and lignin (LN)-capped silver NPs (AgNPs) were produced. The NPs showed strong antibacterial activity against both Gram-positive and Gram-negative strains. However, only LN-AgNPs showed high antibacterial activity against multidrug resistant *P. aeruginosa* and *S. aureus*. The AgNPs were also analyzed for toxicity and found to be non-toxic at concentrations necessary for antimicrobial effects. In addition, it was found that both AgCl and Ag metal must be present for effective antibacterial effects. *In vivo* antimicrobial activity was measured in an abscess murine model. The AgNPs induced a decrease in abscess area as well as a decrease in number of CFUs per abscess; however, quite large amounts of the NPs were required. In these *in vivo* experiments, no inflammation or other signs of local toxicity from the LN-AgNPs were visible. Nanospheres (NSs) of ANBs, penicillin and vancomycin were generated via ultrasound emulsification. The nano-transformation of penicillin G resulted in a disappearance of the β-lactam carbonyl group, but did not compromise the mechanism of the ANB action. In contrast, FTIR spectra of vancomycin NSs didn't reveal any changes in ANB chemical structure upon spherization and showed the formation of hydrogen bond network, contributing to the suspension stabilization. Both types ANB NSs reduced *P. aeruginosa* and *E. coli* planktonic growth, while their bulk counterparts did not affect these Gram-negative bacteria. Hybrid NSs of QSIE and gentamycin were formulated. The NSs interacted better with the bacterial membrane and showed

enhanced membrane disrupting capacity resulting in stronger antibacterial and antibiofilm activity compared to the individual as well as bulk counterparts.

Impact: The project resulted in the development of an assay for the encapsulation of NPs in niosomes to treat intracellular pathogenic mycobacteria. The project is also the first to show the use of ^{111}Ag as an imaging isotope in SPECT for radio imaging of NP distribution in *in vivo* conditions. Antibacterial colloidal solutions developed in this project have potential interest for farming and agriculture and transferring the knowledge to interested companies for commercialisation is envisaged.

REBEL: REpotentiating BEta Lactam antibiotics

Project webpage: www.jpiamr.eu/project/REBEL/

Category; the mode of action/target: Potentiator/enabler; β-lactamase inhibition

Activity against WHO priority pathogen(s): Not specified

Lead molecule: Not specified

Background: The production of β-lactamases by bacteria leads to resistance against β-lactam antibiotics. Resistance can be prevented by combining the antibiotic with a compound able to inhibit the bacterial enzyme. The REBEL project investigated ways to to isolate and identify new β-lactamase inhibitors from a botanical extract library.

Main findings: A large botanical extract library was screened for β-lactamase inhibitory activity, and several hits were identified. Initial hits were evaluated considering expression data yielding a final selection of 75 extracts. The inhibitory activity was verified with additionally obtained extract material and ranked for potency. The isolation and identification of the actual inhibitor molecules in the plant extracts was pursued through bioactivity guided fractionation, combined with several analytical techniques, mass-database searching and NMR. This yielded the identification of active compounds isolated from capsicum and *Tantacetum vulgare*. The compounds were confirmed to inhibit growth of β-lactamase expressing *E. coli* in the presence of ampicillin, and biochemical (nitrocefin) assays showed specific inhibition of in particular SHV-12 β-lactamase. Bioassay guided fractionation of the *Calendula officinalis* extract yielded a highly active fraction for which the active compound(s) are not yet identified. The biochemical and structural characterization of SHV-12 inhibition by the capsicum and *Tantacetum* active compounds, and the identification of *Calendula* inhibitory compounds through co-crystallization experiments is ongoing. Further research on these compounds is needed to determine their suitability for medicinal application, and ultimately to effectively fight β-lactam resistant bacteria.

Impact: The project resulted in methods for isolation /identification of natural compounds and a bioassay for β-lactamase inhibitor screening.

SENBIOTAR: Sensitising *Pseudomonas aeruginosa* biofilms to antibiotics and reducing virulence trough novel target inhibition

Project webpage: www.jpiamr.eu/project/SENBIOTAR/

Category; the mode of action/target: Anti-virulence agent; Biofilm disruption

Activity against WHO priority pathogen(s): *Pseudomonas aeruginosa;* Critical priority

Lead molecule: PqsR inhibitor; PNA (anti-biofilm agent, quorum sensing inhibitor)

Background: The traditional approach to combating bacterial infections has been based on the use of antibiotics which kill bacteria or inhibit their growth. A major problem with therapeutic approaches targeting viability is that they induce strong selective pressures resulting in the rapid emergence of antimicrobial resistance. An alternate approach is to inhibit virulence rather than bacterial viability and this will be explored in the SENBIOTAR project. In the opportunistic human pathogen, *Pseudomonas aeruginosa*, virulence is co-ordinately controlled at the bacterial population level through quorum sensing (QS), a global cell-to-cell communication system employing diffusible signal molecules. SENBIOTAR optimised previously identified hit compounds and peptide nucleic acids (PNAs) which target PQS biosynthesis and/or PQS signal transduction.

Main findings: SENBIOTAR developed novel compounds and antisense molecules that reduce the virulence of *Pseudomonas aeruginosa*, sensitise biofilms to antibiotics and can be formulated to effectively treat and attenuate the impact of infection in a rat disease model. A combination of *in silico* screening of a 90,000 compound library and a medicinal chemistry approaches have led to the discovery two lead compounds. The first is a novel antagonist of the receptor of the *Pseudomonas* quinolone signal pathway (PqsR) and the other is a novel antisense molecule (peptide nucleic acid, PNA) conjugate that has shown inhibition of quorum sensing, biofilm formation and synergistic effects with PqsR inhibitors. Discovery of four novel PqsR antagonists from different structural families, with clear structure activity relationship (SAR) and potency, resulted in a novel set of potent compounds with biological inhibitory activity against both lab strains and clinical isolates of *P. aeruginosa*. The antagonists have an IC_{50} of approximately 50nM and the ability to sensitise biofilms to antibiotics. Combinations of the newly identified compounds with tobramycin showed effectiveness against infection in a rat model and provided mechanistic proof-of-concept. Several novel antisense peptide nucleic acids PNAs with lower toxicity for effective delivery into *P. aeruginosa* have been developed and anti-pqsA PNAs conjugated to these peptides were demonstrated to knock down quorum sensing-mediated activity. In addition, the PNA conjugates did not show any toxicity to human cells and have the potential to be developed into novel treatment for human infections caused by *P. aeruginosa.*

Impact: The SENBIOTAR team has discovered novel approaches to treat *P. aeruginosa* infections that need further development to move towards exploitation in the clinic. The lead compounds developed by SENBIOTAR will also have significant potential for the treatment of wound, bloodstream and medical-device associated infections caused by *P. aeruginosa*.

ABIMMUNE: Repurposing disused antibiotics with immune modulators as antimicrobial strategy for respiratory tract infections

Project webpage: www.jpiamr.eu/project/ABIMMUNE/

Category; the mode of action/target: Repurposed agents/antibiotic; Immunomodulation

Activity against WHO priority pathogen(s): *Pseudomonas aeruginosa, Klebsiella pneumoniae, Staphylococcus aureus, and Streptococcus pneumoniae;* Critical, medium and high priority

Lead molecule(s): Combination of immunostimulatory molecules; MPLA, Pioglitazone, Flagellin, Rapamycin and ND-AB/AB.

Background: Novel antibacterial approaches are needed to treat lower respiratory tract infections. ABIMMUNE project utilised immunomodulatory drugs in combination with antibiotics, to investigate improved use of the current antibiotics that could ultimately minimise both treatment failure and emergence of antibiotic resistance in pathogens.

Main findings: To enhance antibacterial defences, ABIMMUNE targeted innate immune cells and pathways of innate immunity. The combination of therapies with repositioned immune modulators and ND-AB were assessed on:

- antibiotic-susceptible and -resistant bacteria
- mouse infection models of pneumonia for *P. aeruginosa, K. pneumoniae, S. aureus,* and *S. pneumoniae*.

ABIMMUNE identified four leads of interest to be used in combination with antibiotics:

1. Monophosphoryl lipid A (MPLA, a Toll-Like Receptor 4 [TLR4] agonist)
2. Pioglitazone, a PPAR-gamma agonist
3. Flagellin, a TLR5 agonist
4. Rapamycin, an inhibitor of the mTOR pathway

The therapeutic efficacy of systemic administration of MPLA in combination with oral amoxicillin was shown in a mouse model of respiratory infection with an antibiotic-susceptible strain of *S. pneumoniae*. Pharmacokinetics (PK) and pharmacodynamics (PD) were analysed by the dynamic study of bacterial counts and production of immune mediators in tissues, and the use of a highly sensitive bioanalytical assay for amoxicillin. Systemic administration of pioglitazone after the onset of antibiotic-susceptible *K. pneumoniae* infection resulted in decreased bacterial counts in lungs and decreased spread to distant organs compared to the control group. The levels of lung inflammatory mediators were also lower in the pioglitazone-treated group. Respiratory administration of flagellin in combination with oral amoxicillin was effective to treat pneumonia caused by antibiotic-susceptible and antibiotic-resistant *S. pneumoniae*. The immune mechanisms that are responsible are still under investigation. To test the effect of the immunomodulatory drugs, primary cultures of human lung epithelial cells in air-liquid interface were set up. Flagellin was used to stimulate these epithelial cells. ABIMMUNE

identified that the immunostimulation depends on glucose metabolism and activation of the mTOR pathway. Rapamycin was shown to impair the response of epithelial cells by the inhibition of secretion of chemokines and antimicrobial peptides. The relevance of the findings is validated in *in vivo* in *P. aeruginosa* pneumonia models.

Impact: The project addressed the effect of flagellin, pioglitazone, and rapamycin as immunomodulatory drugs in combination with antibiotics. The partners of ABIMMUNE received a H2020 grant for continuation of the research activities and will feed and cross-fertilize the purposes of studies conducted so far in ABIMMUNE. This new project "FAIR" was granted for five years from 2020 onwards and aims to address the use of flagellin as an immunostimulatory drug, from preclinical to phase 1 clinical trial in the context of antibiotic-resistant pneumonia.

CO-ACTION: Developing combinations of CO-ACTIVE antimicrobials and non-antimicrobials

Project webpage: www.jpiamr.eu/project/co-action/

Category; the mode of action/target: Repurposed agents/antibiotic; Not specified

Activity against WHO priority pathogen(s): *Acinetobacter baumannii, Pseudomonas aeruginosa, Klebsiella pneumoniae;* Critical priority

Lead molecule: Combination of polymyxin B and spironolactone (ND-AB+NA)

Background: An insufficiently explored alternative strategy to overcome resistance is a more efficient use of combinations of existing antibiotics (AB), in particular Neglected and Disused AB (ND-AB). CO-ACTION evaluated the benefit and effectiveness of combining antibiotics and potentially non-antibiotics (NA) in the preclinical setting against multidrug resistant (MDR) Gram-negative bacteria that cause severe infections in patients, with a specific focus on pulmonary infections.

Main findings: The project identified several AB+ND-AB combinations with increased activity against MDR Gram-negative bacteria *Acinetobacter baumannii, Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. Polymyxin B with rifampicin and minocycline was the most promising combinations in *in vitro* extracellular and intracellular studies verified in several animal models (murine thigh and lung infection models, pig model) where pharmacokinetics (PK), pharmacodynamics (PD) and resistance development were studied. Furthermore, in search of effective ND-AB+NA combinations, synergism was found between polymyxin B and some NA with spironolactone identified as a potential candidate for further studies. In addition, a high throughput model for screening multiple ND-AB+AB combinations against large sets of isolates was developed and a method for testing AB+NA combinations was assessed. Finally, PK-PD models were developed by associating drug concentrations with antimicrobial activity and clinical doses for the combined drugs were proposed.

Impact: The team developed novel methods for efficient screening of antibiotic combinations and validated multiple (>14) antibiotic combinations against a large set of clinical isolates. A novel screening method was also developed for the combination of antibiotics and non-antibiotics showing consistent synergism *in vitro* with the most promising combination of polymyxin B and spironolactone against *A. baumannii.*

Combinatorials: Novel drugs and drug combinations against bacterial growth, survival and persistence; from high-throughput screening to mechanism of action

Project webpage: www.jpiamr.eu/project/Combinatorials/

Category; the mode of action/target: Repurposed agents/antibiotic; not specified

Activity against WHO priority pathogen(s): *Acinetobacter baumannii* (carbapenem-resistant)*, Klebsiella pneumoniae* (carbapenem-resistant)*, Escherichia coli* (carbapenem-resistant), *Enterococcus faecium* (Vancomycin resistant), *Salmonella spp.* (Fluoroquinolone-resistant), *Staphylococcus aureus* (Methicillin-resistant, Vancomycin intermediate and resistant), *Streptococcus pneumoniae* (Penicillin-non-susceptible); Critical, high and medium priority

Lead molecule(s): Three combinations of AB, ND-AB and NA

Background: Combination treatments and/or re-purposing of known drugs can provide a cost- and time-efficient solution to antibiotic resistance. Using state-of-the-art high-throughput screening (HTS) and further advanced translational pharmacokinetic (PK)/ pharmacodynamic (PD) modelling, the project has undertaken a comprehensive and powerful strategy to repurpose or improve FDA-approved drugs including neglected/disused (ND) antibiotics, and identify combinations to re-sensitise resistant/tolerant bacteria.

Main findings: The project findings have contributed to new concepts and avenues of using combinations for antibacterial therapies. These include the use of food additives or non-antibiotics to re-potentiate neglected antibiotics against MDR pathogens, the use of approved drugs to stop natural competence of certain pathogens (which leads to spread of AMR), and the use of compounds to decrease the collateral damage of antibiotics to commensal microbes living in/on our body and contributing to our wellbeing. There were three types of combinations of antibiotic and non-antibiotic that were found during this study:

- combinations that made antibiotics more effective against MDR enterobacteria – e.g. vanillin made spectinomycin, a neglected antibiotic effective against MDR UPEC (incl. carbapenem/colistin resistant isolates).
- combinations that prevented development of antibiotic resistance by blocking natural competence in *S. pneumoniae*; when co-administered with fluoroquinolones, aminoglycosides or β -lactams, competence blockers could decrease resistance development.
- combinations that decrease the collateral damage of macrolide and tetracycline antibiotics to gut microbiome (and hence decrease the population size that can develop resistance that is often horizontally transferred, but allow them to be as effective with pathogens.

All combinations were tested and validated in animal models *in vivo*. Three patents were filed for each type of combination, including several molecules and combinations.

Impact: The project findings have led to three patents, which will be the basis of future commercialisation. The team is in discussions with the local technology transfer offices (EMBLEM, Groningen) for options so that some of these findings can move further to application by existing companies or new start-ups. The team also successfully trained highly-qualified personnel that resulted in a recruitment of a faculty position in the University of Wurzburg. The project also opened new research avenues, led to new collaborations within the consortium and new grants/grant applications, as well as new lines of research and collaborations with partners outside of the consortium for each of the partners.

ANTIBIO-LAB: Antibiofilm therapy using Local Application of Bacteriophages

Project webpage: www.jpiamr.eu/project/antibio-lab/

Category; the mode of action/target: Phage; Biofilm disruption

Activity against WHO priority pathogen(s): *Pseudomonas aeruginosa* (carbapenem-resistant)*, Staphylococcus aureus* (Methicillin-resistant, Vancomycin intermediate and resistant)*;* Critical and high priority

Lead molecule: Not yet reported; Ongoing project

Background: The use of medical implants has brought about an enormous progress in the care of patients. However, the development of bacterial biofilms adhered on the implant surface and the resulting resistance to antibiotics can lead to repeated infections. These infections lead to poor treatment success in all medical fields and very often, the implant has to be replaced. ANTIBIO-LAB investigates the use of bacteriophages for the treatment of antibiotic-resistant biofilm infections.

Main objectives: The main goal of the project is to develop a suitable material for local administration of bacteriophages, which can be used for the treatment of antibiotic-resistant biofilm infections. Different methods and experiments for bacteriophage isolation and adaptation, as well as development of local administration materials and clinically relevant infection models are ongoing in this project. The phage collection with isolates targeting hospital-specific strains of MRSA & MDR *P. aeruginosa* (PA) to isolate new lytic bacteriophages have been conducted, followed by antibiogram and genetic sequence analysis of each strain in the collection. Biofilm formation of selected bacterial strains on porous glass beads were optimised. Four phages were selected based on genome analysis and host spectrum for the natural *in vitro* evolution and experimentation is ongoing.

Expected impact: The project partners have already shown that bacteriophage therapy is an effective anti-infective therapy in the laboratory, as well as in patients. Through the collaboration of all project partners, the effectiveness of bacteriophage therapy will be further enhanced by using the latest technologies in local administration materials. It is expected that an effective antibiofilm therapy using bacteriophages would have an enormous impact on the patients.

Anti-Persistence: Fighting antibiotic-resistant superbugs with anti-persister compounds targeting the stringent response

Project webpage: www.jpiamr.eu/project/anti-persistence/

Category; the mode of action/target: Direct acting small molecule; RNA synthesis

Activity against WHO priority pathogen(s): *Acinetobacter baumannii, Klebsiella pneumoniae, Mycobacterium tuberculosis;* Critical and high priority

Lead molecule: Not yet reported; Ongoing project

Background: Pathogenic antibiotic-resistant "superbugs" are a particularly problematic emerging global health threat. These superbugs are typically highly antibiotic tolerant and multidrug resistant. One of the survival strategies of these pathogens is to enter in a seemingly "dormant" state that suspends cell division known as persister state. Disguised as persisters, bacteria become highly tolerant to antibiotics and stress in general, and as such, targeting persisters has become one of the modern challenges of microbiology. Anti-Persistance aims to target the mechanisms of pathogenic bacteria that regulate stress and are involved in persistence to discover novel compounds that could lead to the development of new types of antibiotics.

Main objectives: This project aims to target key steps in the mechanism of ppGpp synthesis and hydrolysis in a variety of pathogenic bacteria. An *in-silico* approach was used to screen an initial set of 500,000 compounds, which successfully unravelled new hits (with a success rate above 15%) that inhibit RSH enzymes *in vitro* and are active *in-vivo* against *E. coli* and *M. tuberculosis*. A framework has been developed to produce high quality samples of SpoT, Rel and RelA from different bacterial species. This has allowed the consortium to already produce diffraction quality crystals of the catalytic domains of *S. aureus* and *M. tuberculosis* Rel besides the enzymes from the model organisms *T. thermophilus* and *C. tepidum*. Using single-cell microscopy, *E. coli*'s SOS system was studied upon treatment with ofloxacin and persister cells were observed at low frequency. The SOS response is induced in persisters at the same level than in non-persisters during ofloxacin treatment. A methodology has been established to work with multidrug resistant clinical isolates of *K. pneumonia* and *A. baumannii* that shows that biofilm development significantly increases persister levels.

Expected impact: In an integrative biochemistry, structural and cellular biology-based approach the team is investigating novel mechanistic aspects of persistence, and aims to deliver novel metabolic biosensors for single-cell analysis and methodologies to study persisters in human pathogens, and discover and validate novel compounds with anti-persister action.

CRISPRattacK: Advancing CRISPR antimicrobials to combat the bacterial pathogen Klebsiella pneumoniae

Project webpage: www.jpiamr.eu/project/crisprattack/

Category; the mode of action/target: Phage; Phage as carrier

Activity against WHO priority pathogen(s): *Klebsiella pneumoniae (multiple MDR strains);* Critical priority

Lead molecule: Not yet reported; Ongoing project

Background: The project is developing CRISPR antimicrobials against *Klebsiella pneumoniae*, a major cause of multidrug resistant infections worldwide. The goals are to identify the most active CRISPR nucleases and DNA target sites for programmed killing, to engineer bacteriophage delivery vehicles that can efficiently deliver CRISPR to a large fraction of the clinical isolates and to ensure CRISPR can eliminate cells carrying antibiotic resistance.

Main objectives: Nineteen strains were selected from an extensive list of sequenced clinical isolates of *Klebsiella pneumoniae* representing different capsular groups, multidrug resistance spectra and geographical origins, to identify the most potent CRISPR nucleases for DNA targeting. Three different types of CRISPR nucleases were screened for their antimicrobial activity showing that Cas12a nucleases exhibited the most consistent killing across target sites. The GO-TRAP approach was developed and applied to boost delivery of a T7 phagemid. One variant was identified that could deliver the phagemid to multiple *K. pneumoniae* isolates and further work is ongoing to boost delivery efficiency. Gut colonisation models that can be used for assessing the capacity of the CRISPR antimicrobials to clear colonised multidrug-resistance *K. pneumoniae* are also under development.

Expected impact: The project resulting in optimised CRISPR antimicrobials will represent a leap forward toward the commercial development of novel antimicrobials against *Klebsiella*, and development of similar CRISPR antimicrobials against other multidrug resistant pathogens.

DISRUPT: Fighting antimicrobial resistant infections by high-throughput discovery of biofilm-disrupting agents and mechanisms

Project webpage: www.jpiamr.eu/projects/disrupt/

Category; the mode of action/target: Anti-virulence agent; Biofilm-disruption

Activity against WHO priority pathogen(s): *Pseudomonas aeruginosa* (carbapenem-resistant), *Enterobacteriaceaea* (UPEC), *Staphylococcus aureus* (MRSA, VRSA), *Streptococcus pneumoniae* (penicillin non-susceptible); Critical priority

Lead molecule: Not yet reported; Ongoing project

Background: The DISRUPT project aims to identify new strategies to treat biofilm-associated infections. The project focuses on four bacteria which has been listed as high-priority pathogens by the WHO. These are (1) uropathogenic *E. coli* (the major cause of urinary tract infections and catheter infections), (2) *Pseudomonas aeruginosa* (whose biofilms are associated with chronic infections in wounds or during cystic fibrosis), (3) *Staphylococcus aureus* (causing biofilm-associated chronic wound and medical implant infections), and (4) *Streptococcus pneumoniae* (whose biofilms have been associated with middle-ear infections and pneumoniae). By inhibiting bacterial biofilms, the likelihood of infection will be reduced, and which will resensitise the bacteria to existing antibiotics.

Main objectives: State-of-the-art genetic technologies (transposon sequencing, CRISPR interference) combined with high-throughput screens (screen for chemicals and microfluidic antibody screens) are being developed and used to identify anti-biofilm agents and mechanisms. The final optimisation of the bioinformatics analysis and robotic protocols are ongoing. The CRISPRi-Seq protocol has also been optimised and will be important to standardise the CRISPRi method within the project. The CRISPRi-Seq method has been used to determine the essentialome under different conditions for *S. pneumoniae* and *S. aureus*. The high throughput assays that will be used to monitor biofilm formation (structured macrocolonies and/or crystal violet assays) have been developed. For *S. aureus*, the pooled CRISPRi library has been used in a random screen, and novel biofilm candidate genes have been identified.

Expected impact: The project aims to identify novel strategies to treat biofilm-associated infections, including the development of antimicrobials based on CRISPR-Cas immune systems that can be used to treat multidrug resistant bacterial infections.

EXPLORE: Exploration of the TPP riboswitch as a new target for antibiotics

Project webpage: www.jpiamr.eu/projects/explore/

Category; the mode of action/target: Direct acting small molecules; RNA synthesis

Activity against WHO priority pathogen(s): *Enterobacteriaceaea, Escherichia coli, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus pneumoniae, Enterococcus faecium;* Critical, high and medium priority

Lead molecule: Not yet reported; Ongoing project

Background: The TPP riboswitch, that controls the expression of essential bacterial genes, has already been validated as a drug target. However, potent and drug-like ligands with antibiotic activity are needed as starting points to develop novel strategies for anti-infective treatments. The project aims to develop these ligands that would serve as starting points for drug discovery for future antibiotics.

Main objectives: The proof-of principle of the TPP riboswitch *in vitro* transcription assay has been established. Currently, work is going on to transfer the assays to the CZ-Openscreen screening facility in preparation for the High Throughput Screening (HTS). An *in vivo* reporter gene assay was developed for testing of TPP riboswitches. With this assay, the influence of potential ligands on regulation of TPP riboswitch-controlled genes can now be tested. The assay also will allow the determination of the mode of action (transcriptional control or translational control) of the compound. So far, reporter strains for nine different TPP-riboswitches from ESKAPE-pathogens were constructed. For these riboswitches the mode of action (transcriptional control or translational control) was determined. Twenty-two potential ligands were already tested with regard to their effect on gene expression employing a TPP-riboswitch from *Klebsiella pneumoniae*. One compound was found to be active in comparably low concentrations. Further work on exploring previously discovered TPP riboswitch fragment hits is ongoing. Several different routes to obtain the target compounds have been explored with varying success. One target compound was prepared and several more are on the way.

Expected impact: The project explores the TPP riboswitch as a new drug target for antibiotics for key ESKAPE pathogens (*E. coli, K. pneumoniae, A. baumannii, P. aeruginosa, S. aureus*) and *Streptococcus pneumoniae* and will pave the way for urgently needed new antibiotics.

FLAV4AMR: Flavodoxin inhibitors to kill resistant bacteria

Project webpage: www.jpiamr.eu/projects/flav4amr/

Category; the mode of action/target: Direct acting small molecules; Cell metabolism

Activity against WHO priority pathogen(s): *Pseudomonas aeruginosa* (Carbapenem, metronidazole resistant), *Helicobacter pylori* (resistant to clarithromycin, amoxicillin, tetracycline, metronidazole, macrolides, quinolones), *Streptococcus pneumoniae* (Metronidazole resistant, penicillin-non-susceptible), *Klebsiella pneumoniae* (carbapenem-resistant, 3rd generation cephalosporin-resistant); Critical, high and medium priority

Lead molecule: Not yet reported; Ongoing project

Background: Helicobacter pylori (Hp) has been identified by the WHO as one of the pathogens for which it is urgent to find new antibacterial compounds, due to the high incidence of antibiotic-resistant strains, as well as the fact that half of the world's population suffers from gastric infections caused by Hp. In addition, Hp infection constitutes a risk factor for developing gastric cancer. The project aims to determine the relevance of flavodoxin as a novel drug target to treat bacteria that pose problems associated to antimicrobial resistance.

Main objectives: The essentiality of flavodoxin from *H. pylori*, *E. coli* and related enterobacteria such as *K. pneumoniae* and *C. jejuni*, other Gram-negative bacteria such as *P. aeruginosa* and *S. maltophilia*, and Gram-positive *Streptococcus pneumoniae*, were determined and conditional knock-down mutants constructed for screening and characterization of novel flavodoxin inhibitors. Lead compounds were identified and pilot studies in biological model systems are ongoing. The susceptibility of Hp strains (two reference strains, and one pathogenic for mice) to currently used antibiotics and to compound IV was determined by E-test and in broth, to identify the best culture conditions in liquid medium and to identify the most reproducible method to assess antibiotic activity. Tests related to combinations of antibiotics and lead compounds are ongoing. Experiments have been performed to test the efficacy of the lead compounds to inhibit the colonisation of the mice gastric mucosa by Hp. The analysis of gastrointestinal microbiota of treated mice is under investigation.

Expected impact: This transnational collaboration aims to develop new antimicrobial compounds that could enter clinical testing and to determine the overall importance of flavodoxin as a novel drug target.

MTI4MDR-TB: Development of novel Mycobacterial Tolerance Inhibitors (MTIs) against MDR/XDR tuberculosis

Project webpage: www.jpiamr.eu/projects/mti4mdr-tb/

Category; the mode of action/target: Direct acting small molecules; Other cellular function

Activity against WHO priority pathogen(s): *Mycobacterium tuberculosis* (MDR, Isoniazid resistance, Rifampicin resistance)*;* Global priority

Lead molecule: Not yet reported; Ongoing project

Background: The inadequacies of present TB therapies demand discovery of new agents with unique mechanisms of action to treat *Mycobacterium tuberculosis* (*Mtb* infection). Towards this end, we have discovered and developed a new family of ring-fused 2-pyridones (termed Mycobacterial Tolerance Inhibitors, MTIs) that potently sensitise *Mtb* to stresses encountered during infection and restores activity to the frontline antibiotic isoniazid (INH) in otherwise INH-resistant *Mtb* isolates. The primary objectives are to demonstrate preclinical proof-of-concept for MTIs to combat *Mtb* infection, optimise the current lead MTIs for translation to a therapeutic and reveal new insights into pathways of drug tolerance and resistance.

Main objectives: Mycobacterial Tolerance Inhibitors (MTIs) have been optimised through structure-activity relationships and structure-property relationships studies. New MTI analogs have been designed and synthesised to systematically explore chemical modifications that improve activity in *Mtb*, solubility in water and pharmacokinetic (PK) properties. Based on the peptidomimetic thiazolino 2-pyridone central fragment two different compound collections have been developed and solubility studies were performed to identify suitable vehicles. The activity has also been confirmed with a collection of clinical isolates. PK studies in mice are under investigation. The activity of MTIs on wildtype and drug-resistant strains *in vitro* were determined. The mode of action and the detailed mechanisms by which MTIs impact antibiotic and stress sensitivity is under study. By using forward genetics approach a *Mtb* mutant was identified that can grow in the presence of both MH44 and INH. Whole genome sequencing revealed that this strain harboured a single point mutation in katG. This data suggests that the ability of MH44 to reverse INH-resistance is dependent on KatG activity.

Expected impact: This transnational collaboration aims to develop a new orally available antibiotic that improves the current regimens for patients with drug-resistant TB.

RESET-ME: Restoring *E. coli* Sensitivity for Antibiotics by blocking TolC-Mediated Efflux

Project webpage: www.jpiamr.eu/projects/reset-me/

Category; the mode of action/target: Potentiator/enabler; Efflux pump inhibition

Activity against WHO priority pathogen(s): *Escherichia coli* (Carbapenem-resistant, 3rd generation cephalosporin resistant)*;* Critical priority

Lead molecule: Not yet reported; Ongoing project

Background: Overexpression of efflux pumps is a major factor for drug resistance in Gram-negative bacteria. In *E. coli*, the AcrAB-TolC efflux pump complex transports antibiotics from the periplasm or cytoplasm into the external medium. TolC deletion has been shown to result in increased susceptibility of *E. coli* to several antibiotics and will be further explored in this project as an attractive drug target.

Main objectives: Identification and optimisation of previously identified and novel TolC blockers using *in silico* techniques such as molecular docking, 3D QSAR and scaffold hopping techniques were performed. Several compounds were screened and based on docking scores as well as visual analysis, more than 100 compounds were identified as potential TolC blockers and their optimisation are ongoing. Compounds were investigated for restoring sensitivity of antibiotic piperacillin against *E. coli* BW25113 strain. One compound (MZ-1054) showed promising results with the ability to reduce efflux of H33342 and synergise with piperacillin and also it did not present any antibacterial activity when tested on its own. Thus, the compound represents a promising lead for the development of novel adjuvants. Other set of compounds identified from the virtual screen, that was not commercially available, were synthesised experimental validation is ongoing.

Expected impact: The identification and optimisation of efflux pump blockers which sensitise *E. coli* bacteria to antibiotics is investigated in the project. If successful, the approach will be applied also to other human pathogenic bacteria and as a consequence, the efficacy of antibiotics can be improved significantly.

RIBOTARGET: Development of novel ribosome-targeting antibiotics

Project webpage: www.jpiamr.eu/projects/ribotarget/

Category; the mode of action/target: Direct acting small molecules; Protein synthesis

Activity against WHO priority pathogen(s): *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Mycobacterium tuberculosis;* Critical, high and global priority

Lead molecule: Not yet reported; Ongoing project

Background: The RIBOTARGET consortium aims to discover novel ribosome-targeting antibiotics with improved activity and selectivity against WHO Priority 1 pathogens and *Mycobacterium tuberculosis*, with chemical scaffolds that target novel sites on the ribosome and different steps of the translation cycle.

Main objectives: Compounds were synthesised with modifications in ring 1 of 2-deoxystreptamine aminoglycoside antibiotics (AGA) and assessed in various assays for antimicrobial activity (MIC) and target inhibition in cell-free translation. A series of 2' NH2 modifications in the 4,5 AGAs neomycin, paromomycin, and ribostamycin were also targeted with the view to develop compounds which can resist the aminoglycoside-modifying enzyme, AAC2'. Proline-rich antimicrobial peptides such as abaecin and drosocin, as well as mammalian peptides riptocin, Bac5 and arasin 1 were also developed as novel antimicrobial agents. Experiments revealed that drosocin targets translation termination analogous to the PrAMP apidaecin, whereas Bac5 targets translation elongation analogous to the PrAMP Bac7 resulting synthetic Bac5 and Bac7 PrAMPs as lead compounds. High Throughput Screening Assays on large chemical libraries have identified more than 10 potential molecules interfering with trans-translation that will be studied further. A synthetic platform for organic synthesis pipelines for derivatisation of ppGpp molecular scaffold has been set up that yielded a targeted library of compounds which was then tested *in vitro* and *in vivo*. This resulted in the development of non-hydrolysable ppGpp / ppApp analogues and identification of the allosteric site targeting by ppGpp in RSH enzymes. The two advances would be crucial for further development of specific ppGpp-based RSH inhibitors.

Expected impact: Multidrug resistant pathogens are making our current arsenal of ribosome-targeting antibiotics obsolete, highlighting the need for development of new antimicrobial compounds that this project aims to achieve.

SCAN: Design, Synthesis and Lead Generation of Novel Siderophore Conjugates for the Detection and Treatment of Infections by Gram-Negative Pathogens

Project webpage: www.jpiamr.eu/projects/scan/

Category; the mode of action/target: Direct acting small molecules; RNA synthesis

Activity against WHO priority pathogen(s): *Escherichia coli* (Penicillin G, Oxacillin, Vancomycin, Lincomycin, Bacitracin, Clindamycin, Linezolid, Nystatin, Dalfopristin, Teicoplanin, Piperacillin resistant)*, Staphylococcus aureus* (Oxacillin, Aztreonam, Gentamycin, Erythromycin, Lincomycin, Norfloxacin, Colistin, Pipemidic acid, Kanamycin, Clindamycin, Nystatin resistant)*, Klebsiella pneumoniae, Acinetobacter baumannii (Penicillin G, Oxacillin, Ampicillin, Cefalotin, Cefazolin, Vancomycin, Lincomycin, Pipemidic acid, Bacitracin, Clindamycin, Linezolid, Nystatin, Dalfopristin,Teicoplanin resistant), Pseudomonas aeruginosa* (Penicillin G, Oxacillin, Ampicillin, Mezlocillin, Cefalotin, Cefazolin, Cefotaxime, Chloramphenicol, Gentamycin, Vancomycin, Erythriomycin, Lincomycin, Ofloxacin, Norfloxacin, Pipedemic acid, Nitrofurantoin, Bacitracin, Kanamycin, Neomycin, Ceftriaxone, Clindamycin resistant)*;* Critical, and high priority

Lead molecule: Not applicable; Ongoing project

Background: There is a strong need for novel, innovative therapeutic solutions for infections caused by Gram-negative pathogens. A major hurdle is the limited understanding how to get drugs into Gram-negative bacteria. In addition, there is a lack of tools to diagnose bacterial infections at deep body sites, e.g. on implant surfaces. The project is applying a rational design approach to establish a targeting conjugate platform that can be used to both diagnose and treat bacterial infections ('theranostics' principle). The conjugates are actively transported into bacteria through their iron transport machinery that accepts siderophores as substrates. These will be coupled with hitherto unexplored effectors: RNA polymerase inhibitors are employed as potent antibiotics, and dioxetane-based chemiluminescent probes will be used for imaging.

Main objectives: Desferrioxamine (DFO)-dioxetane conjugates with enzyme-responsive linkers (NAD(P)H-dependent quinone reductase & β-galactosidase) were synthesised and tested in the presence of (i) chemical or enzymatic inducers, (ii) the six pathogens of the ESKAPE panel in iron-depleted cation adjusted medium (ID-CAM), as well as in a competition experiment with *P. aeruginosa*- or MRSA-infected A549 lung epithelial cells. Bacteria-specific PET tracers were synthetised and evaluated in siderophore-deficient bacteria. The 68Ga-(III)-complexation efficacy, plasma complex stability, logD value and cytotoxicity of the PET tracers were evaluated and indicated that the compounds qualify for a progression with *in vivo* studies. Antibiotic conjugates were also synthesised for the DOTAM and MECAM siderophores with ampicillin, amoxicillin, daptomycin, vancomycin and rifamycin SV as payloads. DOTAM and mono-catechol derivatives with daptomycin and rifamycin payloads showed potent antibiotic activity in Gram-negative, MDR *A. baumannii* and *E. coli* strains in ID-CAM compared to the inactive, free drugs. MECAM-ampicillin and amoxicillin conjugates showed a significant (>20 fold) increase in antibiotic activity in MDR *E. coli*, *A. baumannii*, as well as in MRSA and *E. faecium* strains.

An uptake assay based on cellular fractionation followed by LC/MS revealed an internalisation for both synthetic siderophores, MECAM and DOTAM, into the periplasm and cytosol of Gram-negative bacteria, respectively. Uptake studies using single, double, and triple knockout mutant strains revealed that MECAM conjugates are transported into *E. coli* via outer membrane receptors fepA, cirA and fiu, and into *P. aeruginosa* by PfeA and PirA, and DOTAM conjugates are transported into *P. aeruginosa* by PirA. The uptake is strictly TonB-dependent.

Expected impact: The project would yield novel antibiotic lead structures as well as activatable bacterial probes with proven efficacy *in vivo* to detect and treat infections. Taken together, the afforded antimicrobials and moreover the novel theranostics could be tools that allow for strain-specific, potent treatment and monitoring of bacterial infections, addressing a major medical need expressed by the WHO.

Annex. II: Research findings and impact of the therapeutics networks

The following section provides a brief description of the aims of the supported networks, their main findings and the impact generated in various forms, not only scientific but also policy impact as perceived by the researchers.

BEAM Alliance

Network webpage: www.jpiamr.eu/project/beam-alliance/

Background: The network gathered the leading SME C-level motivated executives (CEOs, CMOs, CBOs and CFOs with exceptional experiences both in antibiotic R&D, biotech entrepreneurship and pharmaceutical development and market access) from at least 20 EU companies to mobilise, study and express the aggregated SME position on the current issues, recommendations and actions for AMR. The long-term objective was to integrate into the global AMR agenda the insight accrued from the key opinion leaders innovating and struggling day after day to bring to market the much needed new drugs and devices. This sapience shall be decisive in pushing forward conceptualization of ideas towards market access of novel products that tackle the AMR crisis.

Main findings: SME-driven innovation in the AMR R&D field is key for future success. The network provided key recommendations to support SME needs including:

- Adequately-shaped incentive mechanisms that ultimately rewards R&D evidence
- Health Technology Assessment recognising the true value of SME innovation
- Dedicated regulatory pathways to support the specific needs of AMR projects and act as pre-qualification criteria to some PUSH/PULL incentive mechanisms
- PUSH incentives and funding mechanisms that are directed to SMEs, calibrated and accessible for SMEs in practice
- Calibrated Market Entry Rewards (MER) to ensure continuous and sustainable innovation from academics to biotech companies and to large pharma players
- R&D prizes and phase entry rewards as effective PULL mechanisms for SMEs to incentivise the most underserved indications in AMR
- Targeted tax incentives specifically addressing SMEs to incentivise private investments into AMR-focused companies and/or avoid de-prioritisation
- Going beyond to exploit all possibilities for AMR from SMEs
- Support education to strengthen attractiveness of the field for R&D professionals/scientists
- Long term thinking and wisely usage of AMR innovations combined with appropriate diagnostics development

Network outcomes: The BEAM Alliance updated the pipeline [9] of products in development by SMEs in Europe to quantify the innovation supported by SMEs and communicate on the tremendous potential of the European SMEs as a whole to tackle AMR. An early audit suggested that the BEAM Alliance members are collectively

[9] *https://beam-alliance.eu/pipeline/*

developing more than 100 new products focused upon tackling AMR. BEAM Alliance has compiled and published a position paper[10] that summarises the practical problems and needs of SMEs which relates to both conducting R&D evaluation criteria at early and clinical stages, regulatory pathways, etc. and funding of business models that are from R&D and market perspectives both unpredictable. The aim of the position paper is to provide guidelines to support policy makers in validating that the actions they prepare ultimately reach the target to drive incentivisation for AMR therapies. The network BEAM-ALLIANCE was further supported in the JPIAMR-VRI Network call 2018 as VeRI-BEAM.

Histidine Kinase Inhibitors as Novel Anti-infectives

Network webpage: www.jpiamr.eu/projects/histidine-kinase-inhibitors-as-novel-anti-infectives/

Background: This transnational network intended to align research activities and devise a strategy to develop new anti-infective drugs targeted to histidine kinases (HK) in multidrug resistant pathogens. Selective inhibition of target bacterial histidine kinases involved in the regulation of virulence or antimicrobial resistance is a promising strategy with low potential for resistance development and interference with the host microbiome.

Main findings: The network looked into various approaches on anti-virulence strategies based on selective histidine kinase inhibition. In the further development of HK inhibitors, it was deemed important to know whether the selected anti-virulence targets are inhibited *in vitro* as well as *in vivo*. HK inhibitors were also considered likely to inhibit multiple HK, which might cause lethality even *in vitro* under normal laboratory conditions. The catalytic domain of the HK was considered the most druggable and best target for inhibitors although if identified the extracellular recognition domains of HK could be targets for agonists or antagonists to disrupt proper regulation of the system. A promising new approach to identify new hit molecules is the identification of inhibitors via synthetic chemical strategies and X-ray structure-guided, synthesis-aligned fragment medicinal chemistry.

Network outcomes: The network focused on molecular basis of selective inhibition and prioritising targets for selected multidrug resistant pathogens by taking account of international state-of-the-art research. The purpose was to foster cooperation and collaboration to accelerate progress on developing inhibitors of histidine kinases.

[10] *https://beam-alliance.eu/wp-content/uploads/2019/03/2017-11-16-beam-alliance-position-paper.pdf*

Phage Forward

Network webpage: www.jpiamr.eu/project/phage-forward/

Background: The aim of PhageForward was to overcome the hurdles that slow down the (re)introduction of phage therapy in Western medicine and raise awareness of the regulatory issues with regard to sustainable phage therapy approaches.

Main findings: Phage therapy, the use of bacterial viruses (phages) to combat bacterial infections, is increasingly put forward as an alternative/addition to antibiotic therapy. However, the conventional medicinal product (drug) pathways are developed to cater for static drugs such as aspirin or antibiotics, but are less suitable for sustainable (evolving) phage therapy products. As such, there are no phage medicinal products on the Western markets, and very few phages are available to conduct the necessary safety and efficacy studies.

Network outcomes: PhageForward facilitated a series of meetings, workshops and a scientific publication, which contributed to the awareness that there is a need for an adapted phage therapy regulatory framework and to the elaboration and implementation of such a regulation. This led to the implementation of a prototype phage therapy framework in Belgium, that also contributed to the elaboration of a local (Belgian) solution and its spread to other EU Member States such as France, Germany and The Netherlands.

EXPLOIT: Inhibition of antimicrobial drug resistance: Exploiting an old drug as a basis for inhibitory discovery

Network webpage: www.jpiamr.eu/project/exploit/

Background: The alarming increase in the number of infections by multidrug-resistant Gram-negative pathogens in the EU calls for new strategies and solutions to address bacterial resistance mechanisms. The EXPLOIT network highlighted the efforts made in the AMR field by academics and industry alike and raise awareness on the urgency of action and proposes the use of an old nitrofuran drug for future development of potent antimicrobials by intense study of its use, potency, and the fundamental science behind its mode of action and resistance mechanisms.

Main findings: The network identified that there is an unmet need for a new oral agent active against multidrug resistant *Enterobacteriaceae* causing urinary tract infections (UTIs) including in the elderly. The network focussed on several aspects:

- basic mechanism of uptake and efflux of Nitrofurantoin *in vitro*;
- mode of Nitrofurantoin action; resistance mechanism, resistance evolution *in vitro*;
- the molecular design and synthesis of new compounds based on the nitrofuran scaffold;
- design and synthesis of inhibitors and delivery systems, thereby increasing/modulating and/or potentiating the permeability/activity of nitrofurans against *E. coli* and in particular multidrug resistant isolates;

- determination of efficacy, PK/PD, resistance evolution *in vivo*; transfer to clinical studies (SME-supported);
- dissemination, prediction of economic burden, demographics, cost-saving measures.

Network outcomes: The network included researchers with expertise in medical microbiology, pharmacokinetics, *in vivo* models, and drug discovery/development in industry to carry out underpinning science required for the development of a new antibacterial based on an old drug such as nitrofurantoin and to formulate a research programme that would be the basis of an application in future research call.

IRAADD: International Research Alliance for Antibiotic Discovery and Development

Network webpage: www.jpiamr.eu/project/iraadd/

Background: The IRAADD network aimed to promote and accelerate translational science in the early stages of novel antibiotic discovery and lead candidate development.

Main findings: The areas of research leveraged by the network include hit identification and hit-to-lead programs, aiming at novel preclinical candidate nominations. These initial stages of drug development are essential to find and validate novel drug candidates, which are effective to fight AMR. However, such early-stage projects are mainly embedded within the academic sector and are greatly underfunded. Partnering with external funders, e.g. from pharmaceutical industry, is in most scenarios only realistic after nomination of preclinical lead candidates, which most often cannot be achieved by academic funding and infrastructures alone.

IRAADD also worked on developing strategies for an increased awareness of the need for novel antimicrobial therapeutics mainly within the public sector in order to enhance chances of sustainable funding for the initial phases of anti-infective drug development. Several global health organisations and public-private partnerships do currently address this gap, but still fail to help academic researchers to efficiently translate their findings into novel and useful therapeutic products. Thus, IRAADD devised blueprints together with stakeholders in industry and politics that shall serve as a guidance how to overcome this severe funding problem, which would be a big step forward to boost the production of new antibiotics and to improve the global situation of spreading AMR. These aims are also in line with the current "One Health Action Plan against Antimicrobial Resistance", introduced by the European Commission, which explicitly demands for the implementation and support of "research into the development of new antimicrobials" and the establishment of sustainable research networks in this area.

Network outcomes: The network published a roadmap paper[11] that addresses the increasing global spread of antimicrobial resistance and provides a strategic plan for improving the ability to identify and develop novel resistance breaking antibiotics.

[11] *Miethke, M., Pieroni, M., Weber, T. et al. Towards the sustainable discovery and development of new antibiotics. Nat Rev Chem 5, 726–749 (2021).*

TT: Translocation Transfer

Network webpage: www.jpiamr.eu/project/tt/

Background: The network aimed to improve academically driven antibiotic drug discovery on a key bottleneck in anti-bacterial research, namely how compound penetration determine efficacy and resistance properties.

Main findings: Translocation-transfer (TT) brought together experts from two major publicly funded programs, with the goal to improve the process of academically driven antibiotic drug discovery by capitalising on recently gained insights into a key bottleneck in anti-bacterial research, namely how compound penetration properties determine efficacy and resistance properties. Three main communities form the TT network: i) the partners associated with the multinational program Translocation (www.translocation.eu), part of IMI ND4BB; ii) partner sites from EU-OPENSCREEN, the European Research Infrastructure for chemical biology and screening (www.eu-openscreen.eu) and iii) partners from the wider global community working on AMR issues and research. This resulted in transfer knowledge between Translocation and EU-OPENSCREEN to fully incorporate compound permeation and efflux considerations into academic antibiotic drug discovery.

Network outcomes: The network provided a platform for exchange of expertise on the uptake of antibiotics into Gram-negative bacteria and offered regular seminars on related questions. The network also resulted in the production of an overview of the current research focusing on the transport of antibiotics as well as on the current approaches to measure antibiotic uptake.

VeRI BEAM

Network webpage: www.jpiamr.eu/project/veri-beam/

Background: The goal of the VeRI BEAM network was to highlight the need to develop new criteria to evaluate more comprehensively the different features exerted by an antimicrobial drug to enable an informed prescription, with the drug that is the most suited for a particular condition. Improving the differentiation of the AMR products would increase both their clinical value (to match the patient's needs with the drug actions) and their market value (more benefits for the patient and the health system deserve a better price).

Main findings: The network defined a categorisation framework based on the infectious pathogenesis cycle allowing the identification of one or multiple medicinal activity for each drug. To exemplify the concept, the 140+ products of the BEAM pipeline were categorised accordingly (https://beam-alliance.eu/pipeline). Since many products may simultaneously act through various strategies, thus belonging to multiple categories at the same time, correlations analyses were run to check whether the level of overlap between categories was acceptable. The categorization underlined the lack of criteria to evaluate the performance of AMR products in most strategies. Although assays to do so

are available, there is a pressing need to validate their usefulness/robustness and translate them into regulatory guidelines for AMR drug development.
Contacts with the regulatory agencies such as the EUCAST Steering Committee and EMA were established to acknowledge the relevance of the approach and to agree on the way to establish new criteria. Focus was laid on a particular category, regrouping microbes able to modulate their metabolism to become tolerant to the antimicrobials. Finally, relevant assays to support the definition of appropriate evaluation criteria were identified and a decision tree to help patient management by clinicians was designed to support clinicians' practice facing a putative case of infection with a pathogen prone to metabolic evasion.

Network outcomes: A white paper summarising the main concept was written[12] and widely distributed to more than 400 AMR stakeholders. A peer-reviewed scientific article on the need for better criteria for products acting against tolerant pathogens was also published[13]. A reminder was organised during WAAW2020 through a layman article published in Global Cause and The Guardian. The business consequences were discussed at an EU-JAMRAI round table. The need for dedicated research funding was highlighted both within the IRAADD VRI network workshop and at the EU R&I Days organized by the European Commission. The regulatory path to get new criteria accepted is a long way to go, however, the network managed to get traction from the regulatory bodies and have them acknowledging the highlighted needs. Nevertheless, it is of utmost importance that the work is being pursued as the future of the current product pipeline depends on it. More relevant criteria aim to support product differentiation and valuation and would lead to improved prescription.

[12] *Concept of categorizing the AMR products according to their action on the pathogenesis cycle*
[13] *Tasse J, Dieppois G, Peyrane F, Tesse N. Improving the ability of antimicrobial susceptibility tests to predict clinical outcome accurately: Adding metabolic evasion to the equation. Drug Discov Today. 2021 Sep; 26(9):2182-2189.*