PROJECT FINAL REPORT

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1. Executive summary

The World Health Organisation (WHO) has recognized antibiotic resistance as one of the three major threats to global health, and is predicting a forthcoming disaster due to the rapid, unchecked increase in antimicrobial resistance largely as a consequence of the paucity of new classes of antibacterials in development. New drugs or at least new formulations of known drugs that provide better efficacy are urgently needed for a faster, more efficient, and less impairing treatment.

In this context, the NAREB project aimed to develop nanotechnology solutions to the problem of Multi-drug resistant (MDR) *tuberculosis* (TB) as well as methicillin resistant *Staphylococcus aureus* (MRSA) infections, two well-known drug resistant bacterial diseases causing major public health problems in both developed and developing countries.

The project NAREB gathered 16 partners from 8 EU Member and Associated States with outstanding complementary expertise, ranging from material engineering to molecular biology, pharmacology, microbiology and medicine.
From 377 combinations possible according to modellization of interactions between the selected antimicrobials and nanoparticle types, 146 were selected and produced. These combinations were tested using a series of toxicity tests to determine their degree of toxicity and 110 were tested for *in vitro* activity. Only 8 of these combinations showed sufficient *in vitro* activity. These combinations were devoid of toxicity within the range of concentrations used *in vivo* and they were subsequently tested *in vivo* using different animal models.

For all the nanoformulations tested against TB or MRSA there was no evidence of any clear *in vivo* efficacy advantage of encapsulating the antibiotics. The possibility of a lower toxicity during long term treatments, like those required for tuberculosis, remains to be investigated.

Another important result has been the development of two new diagnostic systems that can detect rapidly the different resistant strains of MRSA and MDR-TB infections from clinical specimens, with enough sensitivity and specificity. These products are expected to reach the market in a short time, hence having a strong impact on society and economy.

2. Summary description of project context and objectives

Increases in antibiotic resistances in all genera of bacteria are an increasing concern worldwide. The frequency of antimicrobial resistance in bacteria has increased in line with increasing usage of antimicrobial compounds. The extensive use of antimicrobials in human and veterinary medicine over the past 70 years has now led to a major threat to clinical practice due to a relentless rise in the number and types of microorganisms resistant to these medicines. Infections caused by **Multi-drug resistant (MDR)** ***tuberculosis*** (TB) and **methicillin resistant *Staphylococcus aureus* (MRSA)** infections lead to serious diseases which usually require intensive care treatment with long time of hospitalization, and have high fatality. The portfolio of **available antibiotics for treating these two antibiotic resistant bacterial infections is very limited** and comprises molecules inducing severe side effects and/or are difficult to administrate like aminoglycosides and vancomycin that require parenteral injection. New drugs or at least new formulations of known drugs that provide better efficacy are urgently needed for a faster, more efficient, and less impairing treatment. The possibility of **using novel drug delivery systems for known and new antibiotic drugs** opens the way to an innovative management of infections caused by drug resistant bacteria.

The development of effective and safe nanotherapeutic approaches with targeted delivery of the drugs is particularly relevant in the antibacterial field, where typically high dose levels of drug are administered. Organ and cell targeting and controlled release might allow for administering reduced amounts of active pharmaceutical ingredient, thereby improving safety windows and reducing toxicological side effects. The technology might also allow the use of antibiotic candidates showing a minimal inhibitory concentration (MIC) too high to be used by traditional delivery routes. Indeed, nanotechnology might allow **the selective delivery of antibiotics at the site of infection** by using targeting ligands to target bacterial pathogens or their host cells. More importantly, nanoparticles may also offer the possibility to transport biological macromolecules, like several glycopeptide-based molecules or nucleic acids.

Nanoparticles are structures measuring less than 100 nm, and can be made of very different materials. Certain types of nanoparticles have intrinsic antibacterial properties. Others can be associated with antibacterials and targeting ligands allowing to target their transport to sites of infection with the aim to eliminate the bacteria. A review written by the University of Zaragoza (UNIZAR, Spain) in collaboration the European ITN Network ‘CycloN hit’ described previous experiments of association of nanoparticles with anti-tuberculosis drugs (Costa-Gouveia J et al Drug Discovery Today, 2017). An example of a complex nanoparticle is shown in Figure 1 from the review of Costa-Gouveia et al.

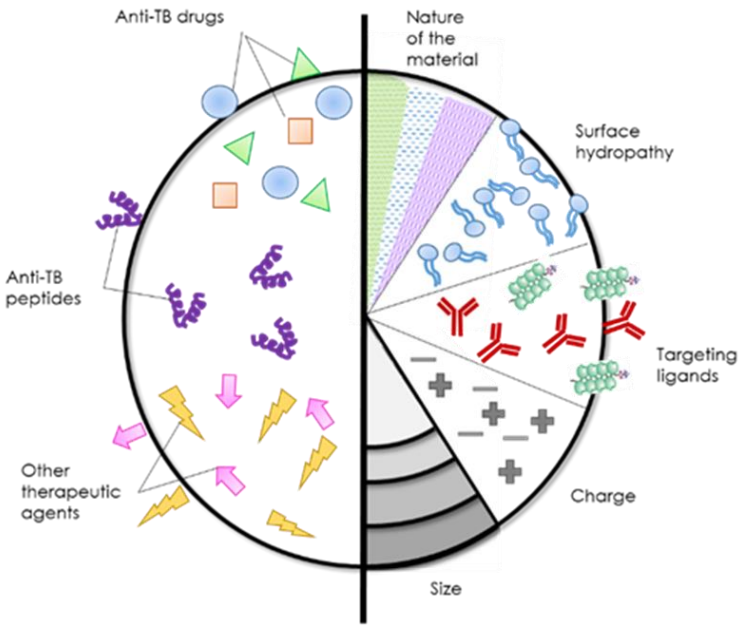


Figure 1. Composition and properties of nanoparticles
a) Composition of the different types of nanoparticles that have been tested for activity against *M. tuberculosis*, such as those encapsulating first or second-line anti TB drugs, alone or in combination, as well as other potentially useful particles such as antimicrobial peptides, other therapeutic agents or small DNA fragments.
b) The main nanoparticle properties that can influence the uptake, distribution and efficacy of nanoparticle-based therapies are: nature of the material, size, surface charge, surface hydropathy, and targeting ligands.
Reprinted from Drug Discovery Today, Vol 22, Issue 3, Joana Costa-Gouveia, José A. Aínsa, Priscille Brodin, and Ainhoa Lucía, How can nanoparticles contribute to antituberculosis therapy?, 600-607, Copyright (2017), with permission from Elsevier

To tackle the challenge of antibiotic resistance with nanotherapeutics, NAREB has brought together chemists, engineers, microbiologists, toxicologists, and clinicians, experts in the fields of infectious diseases, nanomedicine, and early drug development. Academic partners collaborated with four SMEs and one large pharmaceutical company.

Four different nanoparticles platforms were used to produce the different nanotherapeutics:

- nanostructured lipid carriers made by the Commissariat à l'Énergie Atomique et aux Énergies Alternatives (CEA, France),
- polyester-based particles made by Utrecht University (UU, The Netherlands),
- chitosan-based particles made by the University of Zaragoza/CSIC (UNIZAR, Spain) and

- poly alkyl cyanoacrylate (PACA) particles made by SINTEF (Norway).

However, as each type of NPs can have different charge, size, or polymer composition, we can say that more than 14 subtypes of NPs have been characterized.

The general objectives of NAREB were:

1. To investigate and select drugs for their activity against MDR-TB or MRSA, including those exhibiting side effect and used as second line drugs;
2. To investigate the possibility to associate the selected drugs to different types of nanoparticles;
3. To encapsulate drugs in the nanoparticles;
4. To link ligands to antibiotic containing nanoparticles in order to target receptors on targeted cells and/or bacteria;
5. To test *in vitro* activity and safety of nanoparticles associated drugs with a series of culture tests and inhibitory assays, measuring bacterial growth and toxicity on cultured cells;
6. To assess *in vivo* activity and safety of the most promisings nanoparticle-encapsulated drugs in several animal models including the measurement of bacterial multiplication in animal models and growth in aggregates resulting in biofilms;
7. To develop new prototypes of molecular tests improving MDR-TB and MRSA diagnostics using different PCR assays.

3. Description of main S&T results/foregrounds

NAREB used a structured workflow for organising and accelerating the development of new therapeutic treatments using nanoparticles. This workflow included the following Workpackages (WP) (see Figure 2):

WP1 - Selection of antibiotics and leads
WP2 - Production of drug-encapsulated nanoparticles
WP3 - *In vitro* activity tests of the drug-loaded nanoparticles
WP4 - *In vivo* activity tests of the drug-loaded nanoparticles
WP5 - *In vivo* and *in vitro* toxicity tests of the drug-loaded nanoparticles
WP6 - Regulatory aspects and up-scaling production development
WP7 - Dissemination, exploitation, training
WP8 - Management and coordination

An external advisory board associating expertise in nanotechnology, medical aspects of infectious diseases and ethics provided support for making decisions all along the project.

The workflow including design, synthesis, testing, screening and selection of candidates downstream and upstream the workflow from 1 to 6 is shown in Figure 2.

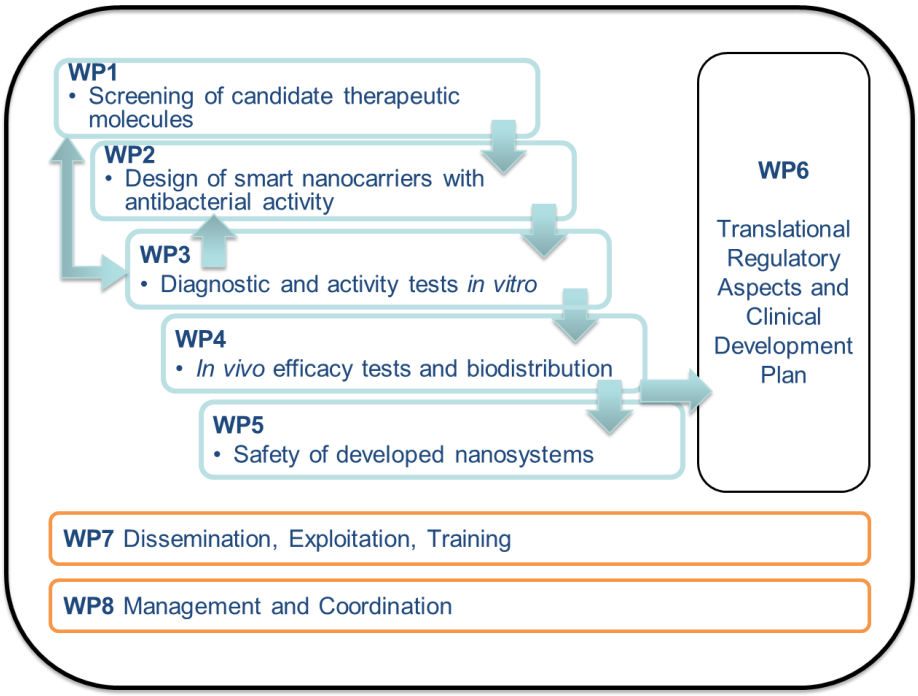


Figure 2: NAREB's workflow

A decision tree has been set-up in order to speed-up and rationalise the selection of relevant candidates to be considered for further *in vivo* testing in animal models. It was decided that drug-loaded particles produced in WP2 had to pass key criteria such as the minimum drug payload, stability and *in vitro* release profile in order to be selected for testing for *in vitro* activity and safety in WP3. Similarly, only combinations exceeding a certain level of *in vitro* activity and safety were tested for *in vivo* efficacy (WP4) and safety (WP5). This process allowed to select the most promising associations between antimicrobials and nanoparticles.

A Product Development Team (PDT) with three core members (two internal and one external) was established to facilitate the product development process of the NAREB project and advise the project decisional body on the selection process. A landscape questionnaire (requesting information related to laboratory production process and characterization, evaluation in *in vitro* and *in vivo* studies, manufacturing considerations and intellectual property) and a candidate characterisation data sheet were developed and sent to the nanocarrier developers for completion. Based on the information collected, the core PDT members performed a landscape analysis of the various candidates and assessed candidates through a weighted matrix score. The core

members of the PDT followed and monitored the development of various potential candidates; initially through consortium meetings and meetings with developers of nanocarriers. Recommendations and advice were given where appropriate. Product specific PDT meetings were held with PDT members and individual nanoparticle developers of selected candidates to review the progress of product development, to identify any potential regulatory issues including GMP considerations during product development, and plan for further development.

A Product Inventory System was prepared as a tool for product development portfolio management.. An inventory document was sent to nanoparticle developers with selected candidates for completion. This inventory document is a summary of information for each selected candidate, including target product profile, product characterisation, quality & stability data, safety data, biological activities, efficacy data and intellectual property status. This Inventory provides an 'easy to review' document for the decisional body to follow the progress of selected candidates and for use in the portfolio management within the NAREB project. All information in the Product Inventory document was collated and reviewed by the PDT for monitoring purposes and to facilitate discussion in PDT meetings with the nanoparticle developers.
It was planned to prepare a Clinical Product Development Plan as the next step of the development of candidates, but this task was later cancelled due to a lack of candidates with better efficacy compared to the free drugs.

Screening of nanotherapeutics and encapsulation

Extensive efforts have been made to develop nanoparticles adapted to the loading of antibacterial molecules. A number of 17 antibacterial drugs had been chosen among the different drugs used in clinic for the treatment of MDR-TB and MRSA, and leads that are molecules identified with antibacterial activity but not yet fully investigated nor approved by regulatory agencies. Using *in silico* modelisations, we have studied the feasibility of the encapsulation of these 17 antimicrobial agents directed against either MDR-TB or MRSA in 4 different nanocarriers family systems. In addition, transcription factor decoys (TFD) molecules in self-assembled bola amphiphilic-based particles (PROCARTA, UK) were also considered. Modelisations of chemical interactions between nanoparticles and antibacterials were useful for the prediction of potential successful association.

The modelisations of 543 *in silico* associations resulted in the selection of 377 combinations with putative positive outcome. Among them 146 combinations were

effectively produced and only 8 of the 110 tested (5 for MDR TB and 3 for MRSA) showed antibacterial activity (See Figure 3 below).

The 146 candidates (drug loaded nanoparticles) produced for *M. tuberculosis* and *S. aureus* were studied for stability and characteristics before *in vitro* testing. Transcriptional response of human cells to treatment with nanoparticles has also been studied for a few combinations that had shown *in vitro* antibacterial activity against *M. tuberculosis*.

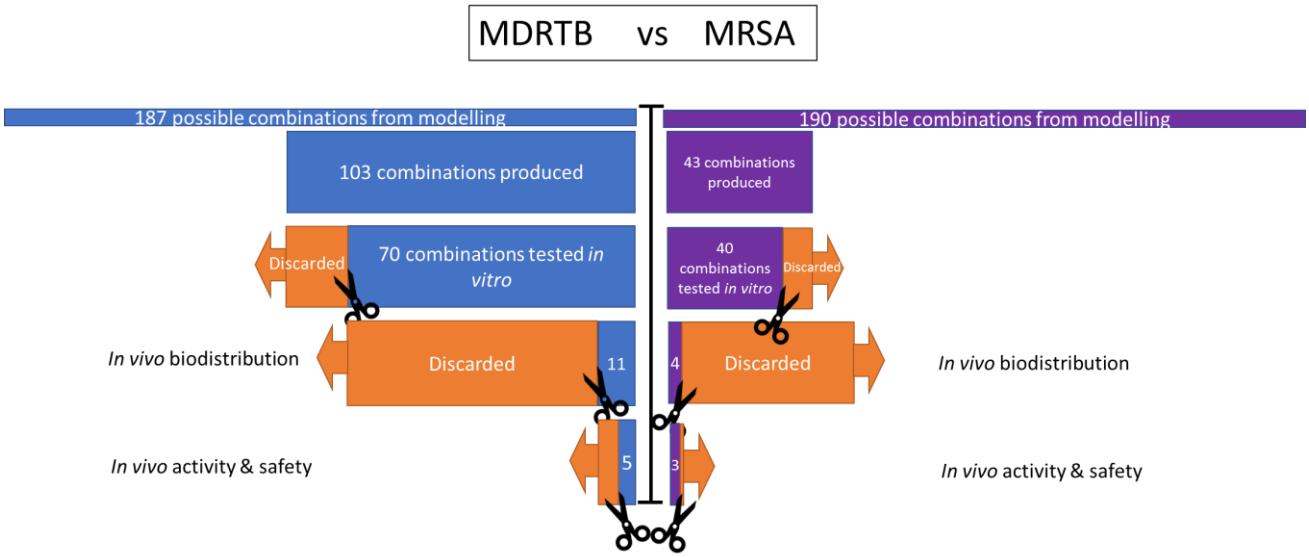


Figure 3: Screening of nanotherapeutics

In addition to drugs and leads selected at a first stage and that were successfully associated with nanoparticles, a number of other leads previously identified by Institut Pasteur (IP) were investigated for possible association to nanoparticles as a back-up in case too few nanotherapeutics would be successful during the first stage. Two families of leads have been investigated by IP:

1) A family of ionophores previously selected as active molecules against fast growing mycobacteria were shown by IP to be active against *M. tuberculosis* in intracellular conditions. Their activity is synergized by beta-lactams. Work was published in Tuberculosis 2017 107:111-118. These molecules were available for encapsulation in case of lack of activity of antimicrobials chosen at the start of the project. This has not been the case.
2) The family of nitazoxanides was also investigated by IP for possible grafting with nanoparticles. They were shown to be active against mycobacterial species like *M. marinum*. Activity against other mycobacterial species was shown to require the

activity of a nitroreductase that is present and can be induced by this chemical family in *M. smegmatis*. However such a nitroreductase was shown to be absent in *M. tuberculosis* thus explaining the limited activity of this class of molecules against this species. Work is published in Journal of Medicinal Chemistry 2017 60:7425-7433. This work shows the usefulness of working with a series of mycobacterial species (including *M. tuberculosis*) for deciphering the activity of antimicrobial candidates. Due to their lack of activity against *M. tuberculosis*, these molecules were not considered for encapsulation with nanoparticles.

Another molecule, provided by GSK, was also investigated by IP. Its target was shown to be MmpL3, a transporter that is also involved in the sensitivity/resistance to other chemical families of antibacterials. A collaborative study using a metabolomics-based analysis confirmed MmpL3 as the target for this lead. Works were published in Science Translational Medicine 2018 10 (429).

In vitro evaluation[2]

In vitro antimicrobial activity

70 combinations against MDR TB and 40 against MRSA were tested *in vitro*. These combinations were provided by partners for antimicrobial susceptibility tests against those pathogens. Each candidate was tested for inhibition of bacterial growth and antimicrobial activity *in vitro*, in a series of relevant models, as detailed below. 5 combinations against MDR TB and 3 against MRSA were identified in which antibiotics retained their antimicrobial activity upon encapsulation. Other drug-containing combinations were discarded because of a complete or partial lack of antimicrobial activity. This selection step was decisive for selecting those combinations progressing to further *in vivo* assays.

Candidates for *Mycobacterium tuberculosis*

Minimum inhibitory concentrations (MIC) for activity of NPs on *M. tuberculosis* were tested by using the standard Resazurin Microplate Assays (REMA; Figure 4) for drug susceptible *M. tuberculosis* H37Rv reference strain for safety reasons. Then the Mycobacteria Growth Indicator Tubes (MGIT) system was used for testing samples of a collection of MDR clinical strains of *M. tuberculosis* in order to confirm activity against drug resistant pathogens. The MGIT system is commonly used in clinical

[2] The human biological samples were sourced ethically and their research use was in accord with the terms of the informed consents under an IRB/EC approved protocol

settings for detecting drug resistance in *M. tuberculosis.* First, it was observed that encapsulated drugs were equally active against drug susceptible and drug resistant strains, which validates the selection of the drug susceptible H37Rv strain as a model for studying MDR strains. Second, it is important to note that activity tests done by distinct protocols (REMA and MGIT) gave similar results. Altogether, these two observations have been very relevant results of this project. A selection of candidates was also tested for efficacy in killing intracellular *M. tuberculosis*. TFD containing NPs were tested against *M. smegmatis* (a model organism for *M. tuberculosis*).

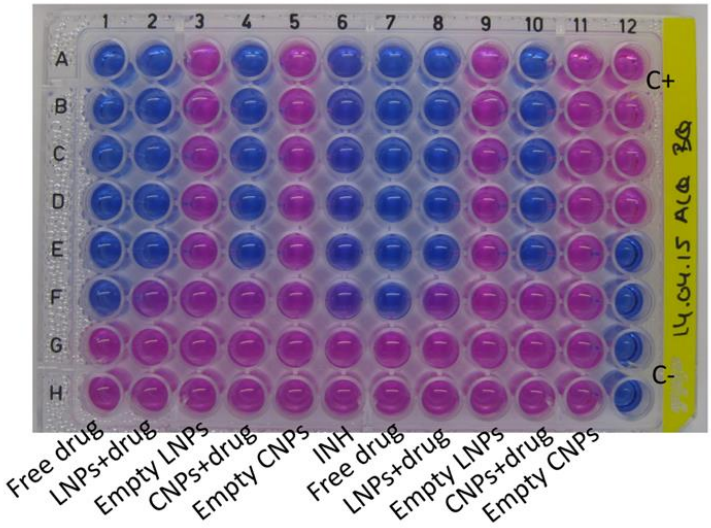


Figure 4. REMA assay

After growth of bacteria with the antibiotics (free or nanoencapsulated), resazurin is added to each well. A change in colour from blue to pink indicates reduction of resazurin and therefore bacterial growth. Free drug: non encapsulated drug; LNPs+drug: drug encapsulated in lipid nanoparticles (LNPs) by CEA; empty LNPs: empty lipid NPs by CEA; CNPs+drug: drug encapsulated in chitosan NPs (CNPs) by UNIZAR; empty CNPs: empty chitosan NPs by UNIZAR; INH: isoniazid, drug control.

<u>Candidates for *Staphylococcus aureus*</u>

Protocols for evaluation of antimicrobial activity consisted in the determination of MIC and minimal bactericidal concentrations (MBC). *S. aureus* ATCC 29213 (methicillin susceptible; MSSA), *S. aureus* ATCC 43300 (methicillin resistant; MRSA) and several MRSA clinical strains in planktonic assays, according to CLSI-guidelines, were elaborated. Assays for evaluation of activity against *S. aureus* biofilms (MBEC-assays) were established and evaluated using reference antibiotics. Most combinations were equally active in both experimental models (planktonic and biofilm), which has been a relevant finding of this project.

In addition to drugs, TFD molecules encapsulated or adsorbed in two different nanocarriers (lipid carrier produced by CEA and chitosan carrier from UNIZAR) were also assayed with *S. aureus*.
Despite promising results obtained for the loading of TFD sequences in nanoparticles, these TFD nanoparticles were not selected for further development due to a lack of evidence of their efficacy.

In vitro toxicity evaluation

Empty nanocarriers

The empty NPs provided by the 4 producers were evaluated with respect to toxicity and biocompatibility before drug encapsulation. However, as each type of NPs can have different charge, size, or polymer composition, we can say that more than 14 NP subtypes have been characterized. The first selection was made on those 14 subtypes. Different methods were carried out to study the *in vitro* toxicity i) the classical proliferation assay (MTT) was complemented with ii) Neutral Red Uptake, iii) ROS (reactive oxygen species from cellular stress) and iii) LDH (Lactate Dehydrogenase) release. The selected cell lines were from specific (lung and epithelium) and unspecific target organs (blood and liver). An iterative feedback loop with nanoparticle producers allowed the selection of the safest nanocarriers and the determination of their safety window. Only one nanocarrier was discarded at an early stage due to its toxicity. The maximum tolerated dose was determined *in vivo* to complete the safety assessment of the nanocarriers. No side effect was observed with the maximum feasible dose of the selected empty nanocarriers.

Antibacterial drug loaded nanoparticles

The characterization of the interaction between antibacterial-loaded nanoparticles and cell cultures of THP-1, Jurkat and Raw 264.7 cell lines provided the first set of data for deciding which of the candidate drug-loaded nanoparticles would proceed to further *in vivo* assays. Furthermore, the candidates were analyzed for colloidal stability and aggregation in several buffers and culture media. Most nanoparticles were stable for a minimum of two weeks. Cell viability was not significantly affected for most nanoparticles.

In addition, the toxicity was measured by the ability of candidates to induce necrosis/apoptosis on cell cultures. The extent of cell uptake of nanoparticles was evaluated by using fluorescent NPs and detected either by flow cytometry or fluorescence microscopy. Uptake was mostly dependent on the nature of nanoparticles; whereas for many nanoparticles uptake was stable upon time, uptake of several

nanoparticles greatly increased after prolonged (30-60 minutes) incubation times. Induction of necrosis and apoptosis was estimated by determining the concentration of nanoparticles that produced a 30% reduction in cell viability, which ranged from 10 to over 1000 µg/ml. Several nanoparticles were well tolerated by cells, whereas other NPs triggered apoptosis significantly.

Additional assays were carried out for obtaining a complete cytotoxicity profile of selected candidates. For instance, hemocompatibility tests (haemolysis, coagulation, platelet and complement activation and cytokines profile) were performed with the prototypes that showed efficacy *in vitro*, to ensure animal welfare during *in vivo* experiments.

A second major area of activity has been the *in vitro* testing of cytotoxicity and efficacy of nanoparticles against *M. tuberculosis* infected macrophages. Most candidates maintained their activity against intracellular mycobacteria, to the same levels as it was detected in the *in vitro* activity tests. In this model system, the first biological validation of the targeting of myeloid cells through the grafting of ligands recognized by bacilli receptors was tested. As a result, some ligands-grafted nanoparticles were more efficiently taken-up by macrophages than the corresponding non-grafted ones.

The impact of the grafting of ligands on nanoparticles was explored by using transcriptomic analysis (RNAseq), which revealed major differences in macrophage response to nanoparticles. Basically, for most grafted nanoparticles, it modulated the host transcriptome and most likely the metabolome (Coya JM, et al J Nanobiotechnology. 2019 Jan 25;17(1):15) .These results allow the analysis of host responses when using nanoparticles in order to favour synergy provided by host responses during therapy.

The experiments conducted by the different partners of the consortium have resulted in the selection of:

Five candidates for being tested in *in vivo* preclinical assays of infection by *M. tuberculosis* infected mice:

- Lipid nanoparticles containing a second line antibiotic for MDR-TB
- Chitosan nanoparticles containing the same second line antibiotic for MDR-TB
- PLGA nanoparticles containing an antibacterial lead
- PACA nanoparticles containing the same antibacterial lead
- Lipid nanoparticles containing the same antibacterial lead

Three candidates for being tested in *in vivo* assays with *S. aureus* infected mice

- Lipid nanoparticles containing an anti-MRSA antibiotic
- Chitosan nanoparticles containing the same anti-MRSA antibiotic
- PLGA nanoparticles containing another anti-MRSA antibiotic

In vivo evaluation[3]

In order to reduce to a minimum the number of animals used, only the most promising candidates that passed the *in vitro* screening phase in WP2 and WP3 were tested *in vivo* in WP4 to evaluate their biodistribution, preliminary pharmacokinetics, efficacy and immune system toxicity in relevant animal models as described below. Biodistribution studies on mice using fluorescence optical imaging technologies allowed to further improve the selection of most promising candidates, and to reduce the number of formulations to be tested in the efficacy and toxicity studies.

The mouse was chosen as the animal species for the evaluation of efficacy. The efficacy models developed and utilised with this species allow simulating the MRSA and TB infection conditions in humans and allow to study the various phases of the infection, namely: contamination, virulence, dissemination and treatment efficacy.

Toxicity evaluation focused on the immune system as a target organ, as uptake by phagocytic cells of the reticulo-endothelial system is of major importance in the interaction of nanomedicines with the immune system. The immune function was tested in rats in the T-cell-dependent antibody response (TDAR), which is a gold standard for assessing the impact of a drug on immune-competence. Direct toxicity on immune cells was established by using the BRGS humanised immune system mouse model, which represents a validated tool allowing the monitoring of development of human immune cells *in vivo*.

Preclinical *in vivo* experimental work was conducted in accordance with EU Directive 2010/63 on protection of animals used for research purposes. Experimental protocols were submitted to the local ethical committees for animal experimentation in order to be evaluated and approved before start of any *in vivo* work.

[3] All animal studies were ethically reviewed and carried out in accordance with European Directive 2010/63/EEC and the GSK Policy on the Care, Welfare and Treatment of Animals."

Biodistribution of antibacterial loaded nanoparticles by Fluorescence Optical Imaging

Fluorescence optical imaging *in vivo* biodistribution data have been generated by Forschungszentrum Borstel (FZB, Germany) and BIOASTER (France) for nanoformulations against TB and MRSA, respectively. As biodistribution was a selection parameter for the selection of candidates, the number of nanoformulations tested was higher than the number tested for *in vivo* activity and safety.
A total of 11 different nanoformulations against TB (including 3 with ligands grafted) and a total of 4 different nanoformulations against MRSA were tested for biodistribution.
The biodistribution of empty nanocarriers was also evaluated (6 for each type of infection). Comparative study between the biodistribution of different empty nanoparticles contributed to improving the selection of nanocarriers for further *in vivo* studies.
Main biodistribution for formulations against **TB** studies at FZB were performed on C3HeB/FeJ mice chronically infected with MTB. Nanoformulations were administered every 2nd day for a period of two weeks. Signals of nanoformulation accumulation over time in infected lungs or liver revealed differences regarding signal intensity and preferential targeting of lung or liver according to the type of nanoparticle utilised.
Biodistribution studies on anti-**MRSA** nanoformulations were performed at BIOASTER with empty and loaded nanoparticles in infected and non-infected animals using Dynamic Contrast-Enhanced optical imaging (DyCE) to visualize and measure the organ-specific uptake during the first 30 min post injection. Subsequently, biodistribution was monitored at specific time points up to a maximum of 24 hours. No differences in biodistribution were observed in all nanoformulations between MRSA infected and non-infected animals.

Bioavailability and tissue concentration

Nanoimmunotech (NIT, Spain), SINTEF, BIOASTER and GSK produced data on the pharmacokinetics of NP encapsulated and free drugs. SINTEF conducted the low-level quantification of the drugs in tissues and blood by LC-MS/MS quantification. For example, lipidots loaded with drugs for MDR-TB or MRSA gave a biodistribution that was quite similar to the free drugs with comparable drug levels in liver, kidney, spleen and lungs.
GSK produced PK data during the TB infection models for encapsulated and free drugs. In general drug exposure after administration of nanoformulations was similar to the free drug with the exception of one formulation for which the exposure (for equivalent

amount of drug administered) of the nanoformulation was higher. However, the efficacy at similar exposures was lower for the nanoformulation.

In vivo efficacy of antibacterial loaded nanoparticles

Efficacy of selected nanoformulations was evaluated by GSK for TB using the H37Rv reference strain and by BIOASTER for MRSA in murine infection models.
Acute and chronic models of infection, and a model developing necrotic lung lesions (Kramnik model), were utilised to compare the efficacy of drug loaded nanoparticles compared to the free drugs. Septicaemia and thigh models, as well as a biofilm model were used to test the efficacy of anti-MRSA drug formulations.
For all the nanoformulations tested against TB or MRSA there was no evidence of any clear *in vivo* efficacy advantage of encapsulating the antibiotics. The possibility of a lower toxicity during long term treatments, like those required for tuberculosis, remains to be investigated.

In vivo safety evaluation of the nanoformulations

During the last period of the project, the *in vivo* toxicity of the loaded NPs was evaluated in parallel with the *in vivo* efficacy assays. Immunotoxicity *in vivo* was carried out in rats to evaluate after 28 days repeated dose the effect on the immune system. Two of the five selected candidates for TB showed inhibition of the secondary immune response. *In vivo* toxicity was also carried out in humanized mice, i.e. mice with a human immune system in order to ensure the safety for human use. We showed no toxicity on human leukocytes of the selected prototypes at the maximum administrable doses.

Development of diagnostic tests

CORIS Bioconcept (Belgium) successfully developed and tested a series of molecular diagnostic tools targeting MRSA and MDR-TB including quantitative PCRs to follow the *in vivo* efficacy tests of the free or encapsulated drugs.

Development of novel diagnostic systems for *S. aureus* and *M. tuberculosis* advanced progressively since the launch of the project. The technology selected for developing the diagnostic tests for MRSA was the PCR-OC (Polymerase Chain Reaction followed by OligoChromatographic detection), which consists in a multiplex PCR amplification of the DNA followed by a detection using specific probes spotted on a membrane. These steps are performed on the TRAPIST microfluidic platform. TRAPIST V6 is a fully

automated instrument that performs multiplex diagnostic testing intended to be used in clinical laboratories.

Initially, six targeted genes were selected to detect *Staphylococcus* spp., *S. aureus*, *S. epidermidis*, methicillin resistance (*mecA* and its variant *mecC*) and PVL (Panton-Valentine Leukocidin). Specific primers and probes were validated on clinical bacterial strains and on blood cultures and optimized for sensitivity and specificity. This MRSA test was included in a large panel of tests for Gram positive bacteria in a sepsis application.

In parallel, a MRSA qPCR test was developed including DNA extraction control and mouse endogenous control (for normalization), and validated on different mouse organs (spleen, muscle, kidney, lung and liver) to follow *in vivo* efficacy of the free or encapsulated drugs against MRSA.

For *M. tuberculosis*, mutations that confer resistance to rifampicin were identified and specific primers and probes were designed. The test is able to identify *M. tuberculosis* complex strains as well as nine different mutational positions (both WT and mutated forms) of the *rpoB* gene, conferring rifampicin resistance. At a later stage, an amplification control and an additional PCR allowing the detection of a new mutation were also incorporated in this TB/RIF chip. The TRAPIST instrument and the dedicated software were adapted to allow the single point mutation identification. Finally, the test was implemented in order to target the detection of the main single mutations involved in isoniazid (INH) resistance. The *katG* and *inhA* genes were selected, the 4 most prevalent mutations of each gene were selected, and probes were optimised for increasing sensitivity and specificity.

Following the example of the qPCR developed for the MRSA *in vivo* infections follow-up, a TB qPCR test was validated on DNA extracted from bacterial cultures and optimised for analysis of murine specimens for the follow-up of experimentally infected mice treated with nanotherapeutics to fight MDR-TB.

4. Potential impact and the main dissemination activities and exploitation of results

Potential impact

A Committee composed of Intellectual Property specialists and/or Business developers from each partner institution was created to review NAREB results and discuss their

exploitation. The committee met first during a seminar in the middle of the project to identify the products/systems with good chances of exploitation and prepare the first draft of the exploitation plan, which was afterwards reviewed and updated regularly. The Committee gathered at the end of the project for a second seminar, whose purpose was to discuss NAREB's latest results and fine-tune the exploitation plan.

The Committee identified two types of Key Exploitable Results:

- Tools and technologies that will be either exploited commercially or within the framework of other projects.
- Scientific knowledge or practices that can be applied to new projects and also be used to inform the general public on topics of social interest such as the safety of the nanotherapeutics.

Tools and techniques

Diagnostic tests

Two diagnostics tests developed within NAREB will be further refined to become available for a product offer by CORIS. One is dedicated to the diagnosis of different strains of MRSA infections and the second one to MDR-TB. These products, based on PCR, offer a faster solution for the diagnosis of antibiotic resistant infectious diseases. In microbiology laboratories, the main conventional methods for the diagnostic of bacterial infections essentially still rely on bacterial cultures. The newly developed diagnostic products offer a faster answer in less than 2 days from sampling to result, especially for TB infections where bacterial cultures are close to several weeks. These molecular tests developed on the TRAPIST automate give a complete answer on the identification and antibiotic resistance profile with a turn-around-time of less than 90 min. Moreover, PCR-based methods are often more sensitive than the conventional culture methods, but a complete clinical comparison will be conducted in a near future for more precise data.

Antimicrobial nanotherapeutics

In NAREB a very large number of antibacterial drug-loaded nanoparticles have been produced and the most promising have been tested *in vivo*. Most of the selected drug-loaded nanoparticles were equally active and efficacious as their corresponding free drugs; hence, the association of antimicrobial drugs and nanoparticles performed in the NAREB project, apparently, does not present any significant advantage over the use of conventional antimicrobial drugs. However, the possibility of a lower toxicity during long term treatments, like those required for tuberculosis, remains to be investigated. In addition, we can expect that further improvements in carrier composition and properties,

or the selection of other antimicrobial drugs, could lead to the development of new nanopharmaceuticals to improve on current antimicrobial chemotherapy.
It is important to note that most combinations were equally active in experimental models to evaluate *in vitro* activity either for TB (REMA and MGIT) or MRSA (planktonic and biofilm). For the former, this result the selection of the drug susceptible H37Rv strain as a model for studying MDR strains. NAREB also showed the importance of targeting ligands in nanopharmaceuticals for infection such as TB as some ligands-grafted nanoparticles were more efficiently taken-up by macrophages than the corresponding non-grafted ones. Of importance is also the absence of side effect of the selected empty nanocarriers with the maximum feasible dose and several combinations were well tolerated by cells.

Techniques and methods

New analytical methods have been developed during the lifetime of the project. These methods will be exploited by their owners in the framework of other projects. They are now enriching their portfolio of analytical assays.

This set of methods and techniques has allowed to evaluate the *in vivo* biodistribution, pharmacokinetics and efficacy of the encapsulated compounds that were selected in previous *in vitro* studies. We have set up and deployed technologies and animal models that allowed us to screen and rank a number of nanoformulations to feed the clinical development plan.

These assays include:

- Fluorescence Optical Imaging procedures for analysing the biodistribution of nanoparticles in the whole body of animals tested, thus allowing in some cases the detection of nanoparticles at the site of infection and encouraging their use for localised infections (FZB and Bioaster).
- Analytical methods for quantification of drug loading and drug release (SINTEF, CEA, UU, UNIZAR)
- *In vivo* efficacy models of infection for testing compounds to treat MRSA and MDR-TB (Bioaster and GSK)

Scientific Knowledge and practice

Delivery platform for antimicrobial drugs for infectious diseases

A large number of combinations associating different antibacterial drugs and different kinds of nanoparticles was produced, thanks to the four platforms of nanoparticles of

CEA, UNIZAR, UU and SINTEF. These partners had experience in developing nanoparticles for other diseases such as cancer or inflammatory disorders. The NAREB project was for them a good learning platform for dealing with an infectious disease from a nanopharmaceutical point of view. It appeared that the existing platforms that were developed for non-communicable diseases needed to be adapted. The transfer was successful given that a high degree of control and flexibility over particle size, drug loading and surface charge, and other surface properties could be modulated by changing the fabrication process. Also, the antibacterial nanoparticles demonstrated high stability in biological media.

Safety of nanotherapeutics

All drug-loaded candidates have been tested for toxicity by the different laboratories producing them but also more extensively by NIT, an independent platform offering a large number of tests for the detection of toxicity and allowing a ranking of all products. Indeed, safety profile is one of the most important criteria after the *in vitro* activity to select new potential nanotherapeutics to be tested in *in vivo* models.

Most importantly, all this information contributes to the better understanding and assessment of the safety of the nanoparticles. Reliable safety assessment methods and their stringent application may improve the acceptance by the general public of the use of nanocarriers for drug delivery or other applications in human health, as toxicity of the nanomaterials is a social concern.

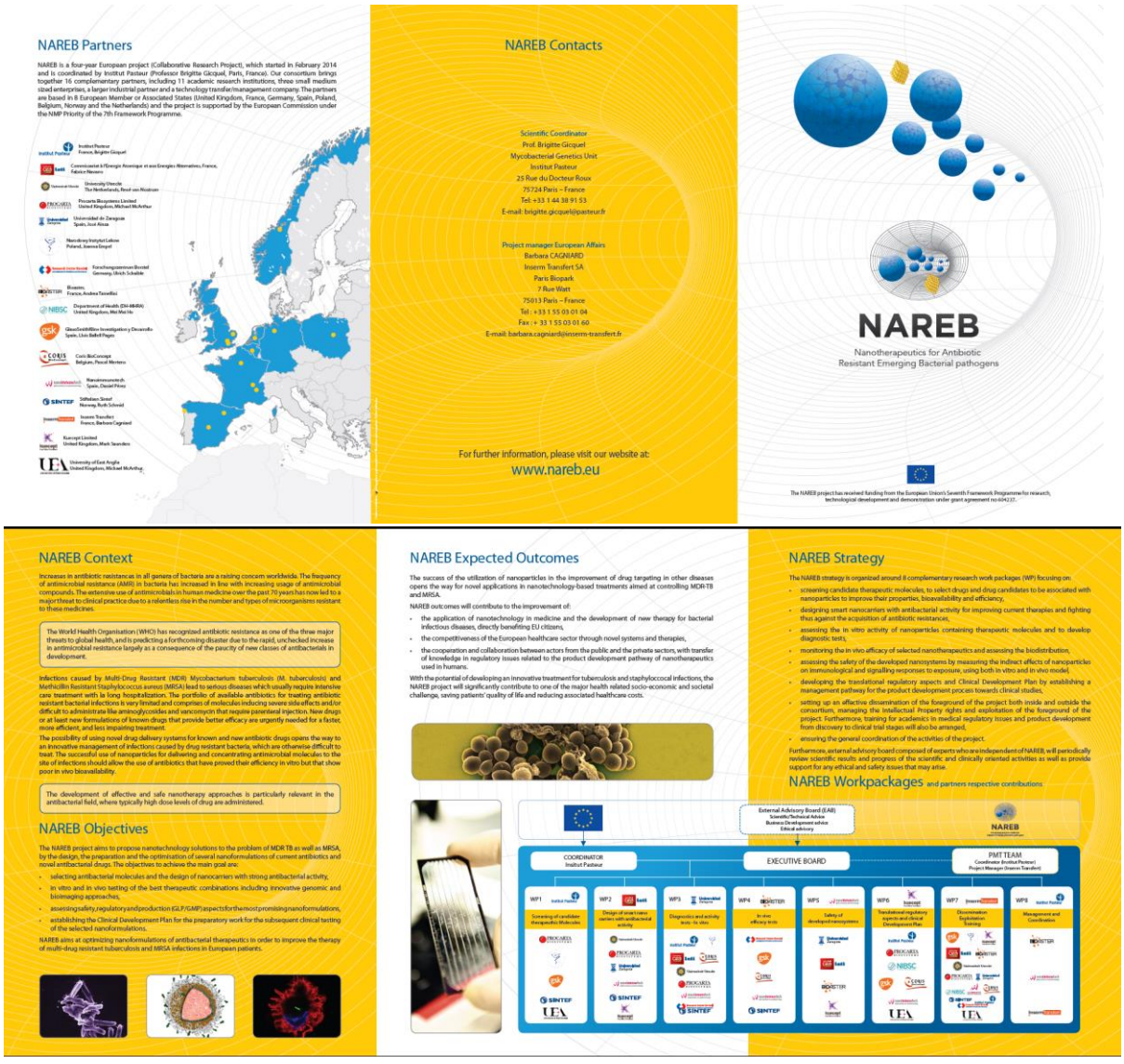
New approaches for nanomedicine scientists

The synergetic capacity of the participating laboratories for efficiently developing and testing a wide range of novel nanoparticle systems has been instrumental for testing a large number of candidates and setting up a decision tree to select a limited number of promising candidates. The most promising candidates have been tested *in vivo* and investigated for their bio-availability and biodistribution. Overall, this procedure has reduced considerably the required number of tests in animal models.

The early stages of product development are very critical. The key role of the PDT was to establish a product development structure with a team of internal and external experts in this area to assess the product development process, to provide relevant regulatory advice where appropriate and to train the scientists on the product development process. The nanoparticle developers within the consortium are indeed from either academia or research organizations. A concept of product development comprising key manufacturing & regulatory considerations was introduced at an early stage to facilitate and streamline the subsequent product development pathway, a knowledge they will also apply in future projects.

Dissemination

All the partners participated in the dissemination of NAREB information and results. The NAREB website provides project information and news from the field of infectious diseases and nanoparticles to the general public. A downloadable brochure explaining the project is also available on the website.



A total of 10 articles in peer-reviewed journals (4 with open access) were published by the end of the project. In addition, consortium members shared their knowledge with the scientific community through 41 oral presentations and 17 poster presentation at a variety of conferences in Europe and US, as well as 6 press releases. Two dissemination workshops were also organised. The first one focused on “Novel approaches to fight bacteria” and gathered researchers involved in four EU projects (IMI-NBB-Translocation, PneumoNP, FORMAMP and NAREB) aiming to increase the efficiency of antibiotics for fighting antibiotic resistant bacteria. The topic of the second workshop

was “Nanomedicine & Antibiotic resistance” where several topics were covered such as the ‘One-health approach’, targeted nanomedicines, nanotechnologies for infectious diseases vs other disease, etc. All these activities allowed to diffuse information to specialists and experts in the fields of infectious diseases and nanomedicines. The list of dissemination activities is available on the NAREB website.

Conclusion

In conclusion, after 4.5 years of operation, the NAREB project has delivered a huge amount of scientific results and knowledge, which will serve for further exploitation. Furthermore, all partners have increased their expertise in Nanomedicine and in infectious diseases. Certain Key Exploitable Results like analytical methods and analytical tests will be directly and immediately valorised by their owner either as a service or as an additional tool in their technology portfolio.

The exploitation of the most promising nanotherapeutics will take more time to further refine their composition, the understanding of their mode of action and later the preparation for regulatory approval. The preclinical results acquired during NAREB are positive enough for some nanotherapeutics that their owners are already planning a follow up project, e.g. within the IMI programme. This would reinforce the industrial drive of the development in close connection with leading clinical teams.

As a reminder, the World Health Organisation (WHO) has recognized antibiotic resistance as one of the three major threats to global health, and is predicting a

forthcoming disaster due to the rapid, unchecked increase in antimicrobial resistance, largely as a consequence of the paucity of new classes of antibacterials in development. Nanotherapeutics may considerably contribute to addressing one of the major health related socio-economic and societal challenges, improving the patients' quality of life and reducing the associated healthcare costs. Moreover, the applications of such nanoformulations of antimicrobial agents may also be extended to treat other infectious diseases.

5. Address of the project public website and relevant contact details

Website: http://nareb.eu/