December 2012

AEROPATH

The identification, characterisation and exploitation of novel Gram-negative drug targets

Summary of Final Project Results

1. EXECUTIVE SUMMARY

Despite advances in modern medicine and science, the population of Europe is becoming increasingly susceptible to bacterial infection. Once easily dealt with by antibiotics, severe bacterial infections are increasing sharply in Europe due to growing drug resistance. The problems vary from respiratory to gastrointestinal infections, dermatitis and a range of systemic infections.

New ideas, targets and drugs are urgently sought to fight back against the rise of bacterial infections, in particular to deal with superbug Gram-negative bacteria. Researchers from the Universities of Dundee and St Andrews in Scotland, the Karolinska Institutet in Sweden and two German-based SMEs (LIONEX and mfd Diagnostics GmbH) joined forces under the AEROPATH project to identify and assess new drug targets to address the superbug problem. Funding was provided by the European Union (EU)'s 7th Research Framework Programme (FP7).

The aim of this ambitious research project was to assess targets for the development of antimicrobial drugs and provide new approaches to combating the superbugs and overcoming their resistance to current treatments. At the project onset the coordinator Professor William Hunter explained the overall strategy: "Because these organisms are so tough, we need new ideas for drugs and the way to do that is to find new targets or to apply modern technologies and exploit old targets. We can then search out new compounds that will hit either group of drug targets."

The AEROPATH consortium selected the Gram-negative *Pseudomonas aeruginosa* as both a model system and target since it is a significant medical threat itself, causing around one tenth of infections acquired during a hospital stay. This free-living bacterium is commonly found in soil and water but is an opportunistic pathogen that infects humans with compromised immunity, particularly those in hospital undergoing chemotherapy or suffering from conditions such as cystic fibrosis. These bacteria survive under a wide variety of conditions and are resistant to many antiseptics and all commonly used antibiotics. In addition *P. aeruginosa* is highly adapted to develop resistance to antibiotics making treatment even more difficult.

The AEROPATH consortium advanced understanding, at the molecular level, of the biology of *P. aeruginosa* and identified routes, via inhibition of high value therapeutic targets, that weaken or interfere with the bacterium's ability to survive and cause infection. Some targets that were studied appear not to offer much in regard of new drug development and their identification served to optimise efficiency by guiding us to direct resources to address the high value targets. In so doing the AEROPATH team effectively prioritised and prosecuted research on key proteins.

New approaches to inform about the potential of specific proteins as drug targets and to tie in with the availability of chemical information on compounds that hit the targets, and of how drug-like those compounds are, were developed. Over 35 selected proteins were genetically tested to investigate if they might be drug targets; work that involved modified strains of *P. aeruginosa* and a mouse model of lung infection. Over 100 new protein crystal structures were determined. A new algorithm to predict "druggability", i.e. to estimate the likelihood that a small compound would display the appropriate interactions with the target to effectively block the protein function was developed and applied to over 5000 structures of all *P. aeruginosa* proteins and homologues. These new data were incorporated into the AEROPATH genome annotation. Several key targets involved in fatty acid, folate and sugar biosynthesis progressed through target validation, structure determination, were exposed to compound libraries in screening campaigns after the development of novel assay methods, and in complementary fashion *in silico* compound screening and molecular design methods were applied to exploit selected crystal structures. These approaches served to prioritise these targets and to identify chemical matter, in one case proven on the basis of new chemical synthesis, to support further development. The application of modern approaches to several old targets generated understanding of

structure-activity relationships and, in the case of penicillin-binding protein 3, indicated how novel drugs might now be assembled.

The AEROPATH consortium carried out the programme they promised in full and even extended the studies to prosecute more experiments on high value targets than first envisaged. This success was driven by the efficient manner in which academic and industrial partners combined micro-, molecular and structural biology, with high throughput and biophysical screening approaches to identify starting points for drug discovery programs. Poor targets were identified and dropped quickly. Additional resources were generated by each partner and valued collaborations, e.g. The Oxford Protein Production Facility, established.

AEROPATH has greatly enriched our knowledge of *P. aeruginosa* drug targets and set the stage for new programmes of directed antibacterial drug discovery.

2. PROJECT CONTEXT AND OBJECTIVES

Context

AEROPATH (The identification, characterisation and exploitation of novel Gram-negative drug targets) was a collaborative project funded under the European Union (EU)'s 7th Research Framework Program (FP7). The project was formed to address call topic 2.3.1-1 "Novel targets for drugs against Gram negative bacteria" in the HEALTH-2007 Work Plan. The objective of this topic was to identify and validate novel drug targets in order to select lead compounds for future development of a new class of anti-infective drugs against Gram-negative bacteria.

Work began on the AEROPATH project in November 2008 with a budget of slightly more than €4.5 million funding from the European Union over a 4 year period. Research groups from the Universities of Dundee and St Andrews in Scotland, the Karolinska Institutet in Sweden and two German-based SMEs (LIONEX and mfd Diagnostics GmbH) contributed to the project under the leadership of Professor William Hunter from the University of Dundee. The project greatly benefitted from the robust advice of the AEROPATH Scientific Advisory Group: Dr Bill Primrose (Ithaka Life Sciences), Professor Neil Gow (Professor of Microbiology, Institute of Medical Sciences, University of Aberdeen) and Professor Fritz Winkler (Professor Emeritus of Structural Biology, ETH Zürich) which was established at the start of the project. The three members were selected on the basis of their collective experience in different aspects of drug discovery and microbial pathogens. The group provided advice on scientific direction, decision making and opportunities for exploitation throughout the project.
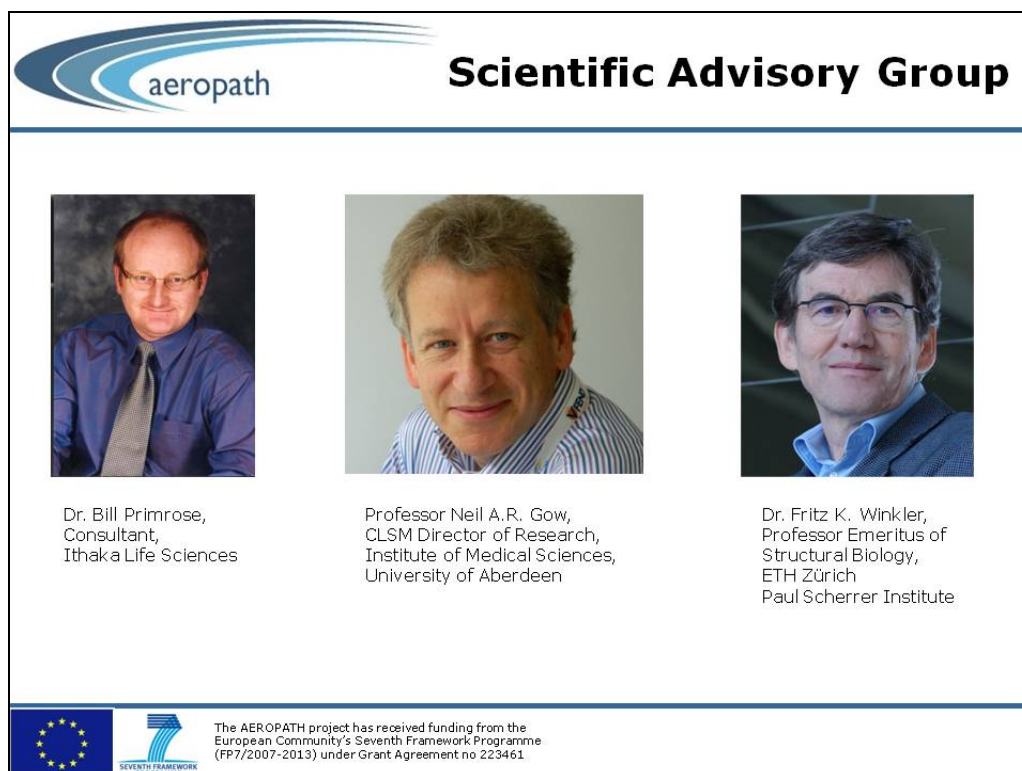

Figure 2.1: AEROPATH Scientific Advisory Group

Scientific background

Since the introduction of penicillin in the 1940s, antibiotics have become essential for the treatment of microbial infections in humans and animals. In addition to the treatment of infectious diseases and hospital-acquired infections, antimicrobials are vital for reducing the risk of complications in relation to complex medical interventions, such as hip replacements, organ transplants, cancer chemotherapy and the care of premature babies. In addition, antimicrobials are used in veterinary medicine and for non-therapeutic purposes.

Seventy years later, these applications are now seriously jeopardized by the emergence and spread of microbes that are resistant to the affordable and effective first choice medicines, rendering the drugs concerned ineffective for the treatment of the infection. This resistance is a natural biological phenomenon but has been amplified by a variety of factors such as the inappropriate use of therapeutic antimicrobials in human and veterinary medicine, the use of antimicrobials for non-therapeutic purposes, pollution of the environment by antimicrobials and even the increased levels of international trade and travel serve to accelerate the emergence and spread of resistant microorganisms.[1]

The consequences of drug resistance are severe with more than 25,000 patients dying in the EU each year from infections caused by bacteria resistant to multiple antibiotics[2]. Resistance rates to a single antibiotic exceed 40-50% in some European countries[3] and resistance to multiple antibiotics is a common and growing problem. In addition, infectious diseases caused by resistant bacteria lead to additional healthcare costs and indirect costs, such as sick-leave and lost output due to premature death. The overall direct costs to society in terms of extra healthcare costs and productivity losses total €1.5 billion each year in Europe[4]; and the indirect costs to European countries are likely to be several times higher[5]. Antibiotic-resistant germs are now found regularly in many hospitals in the EU, infecting some 4 million patients every year.

The development of resistance, the pressure to reduce the use of antimicrobials as well as the weak market incentives and increasing difficulty and cost to develop new effective antibiotics have discouraged investment in this area with the consequence that only four pharmaceutical companies are researching antibiotics – down from 18 in 1990 – and only two new antibiotics classes have reached the market in the last 30 years.

This situation is particular serious with respect to Gram-negative bacteria such as *Pseudomonas aeruginosa*. Around one tenth of infections acquired during a hospital stay are caused by *Pseudomonas aeruginosa*. This free-living bacterium is most commonly found in soil and water. However, it also occurs regularly on the surfaces of plants and occasionally on animals. For most healthy people, this bacterium is not a problem. Rather, *P. aeruginosa* is an opportunistic pathogen that infects humans with compromised natural defenses. As a result, many *P. aeruginosa* infections occur after patients have been hospitalized. The bacterium causes urinary tract infections, respiratory system infections, dermatitis, soft tissue infections, bone and joint infections, gastrointestinal infections and a variety of systemic infections. *P. aeruginosa* infection is a serious problem for people whose immune system is compromised either due to transplant surgery, chemotherapy, people who have HIV/AIDS, who are recovering from burns and also young people with cystic fibrosis whose lungs struggle to cope with these and other dangerous Gram-negative bacteria. Given medical progress we

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[1] Communication from the Commission to the European Parliament and the Council. Action plan against the rising threats from Antimicrobial Resistance. COM (2011) 748
[2] The bacterial challenge: time to react. ECDC/EMA joint technical report (September 2009).
[3] Goldstein FW. Penicillin-resistant *Streptococcus pneumoniae*: selection by both beta lactam and nonbeta lactam antibiotics. J Antimicrob Chemother 44:141-144 (1999).
[4] The bacterial challenge: time to react. ECDC/EMA joint technical report (September 2009).
[5] Conference report; Innovative Incentives for Effective Antibacterials (2009).

are generating more "hosts" for these bacteria.

Some Gram-negative bacteria, often called 'superbugs', present a serious problem because many, in particular *P. aeruginosa,* are tolerant to a wide variety of physical conditions, including temperature, and are resistant to high concentrations of salts and dyes, antiseptics and most commonly used antibiotics. They are also well adapted to generate or acquire resistance to other drugs, making treatment difficult.

The overall objective of the AEROPATH project was to provide a foundation for the development of antimicrobial drugs by gaining a better understanding of the biology of the 'Gram-negative' type of bacteria at a molecular level using the *P. aeruginosa* bacterium as a model and developing new compounds ('hits') that can weaken or interfere with the bacterium's ability to cause infection. The hits would have the potential to underpin early stage drug discovery to develop antibiotic drugs that kill *P. aeruginosa* itself and other Gram-negative bacteria that are highly resistant to most current drugs.

Since the complete sequence of the *P. aeruginosa* strain PAO1 genome had been determined, this was selected as the reference strain for the AEROPATH project. This species has one of the largest bacterial genomes sequenced with over 5,500 predicted genes on one chromosome. In addition, there are numerous plasmids that have been identified that carry virulence and persistence factors, or that mediate antibiotic resistance.

Analysis of the PAO1 genome data, the scientific literature and consideration of what structural data were available in the Protein Data Bank, guided the selection of an initial list of 50 priority target genes at the onset of the project. These targets were chosen because they were predicted to have an essential function in *P. aeruginosa* but no structural and little biochemical data were available. This initial list was very much a starting point and the project was not constrained to this list, retaining the flexibility to respond as new biological data on potential targets or on active small molecules emerged. In addition, some 15 targets in areas of biology with a history of successful antibiotic discovery were selected for inclusion with a subset taken on by a collaborating group, the Oxford Protein Production Facility.

The AEROPATH partners were uniquely placed to address this challenge, marrying academic excellence in the areas of microbiology, structural biology and bio-chemoinformatics with industry-level technology and experience in high-throughput compound screening and target validation. The industrial partners (LIONEX and mfd Diagnostics GmbH) specialised in target validation and the academic partners contributed understanding of target assessment (including predictions of druggability), microbial metabolism and pathogenesis, identification and characterisation of novel targets and identification of the first inhibitors or hits. In particular, the project was able to take advantage of a new drug-like screening set of compounds assembled at the University of Dundee and some of the largest *in silico* compound databases held in academia.
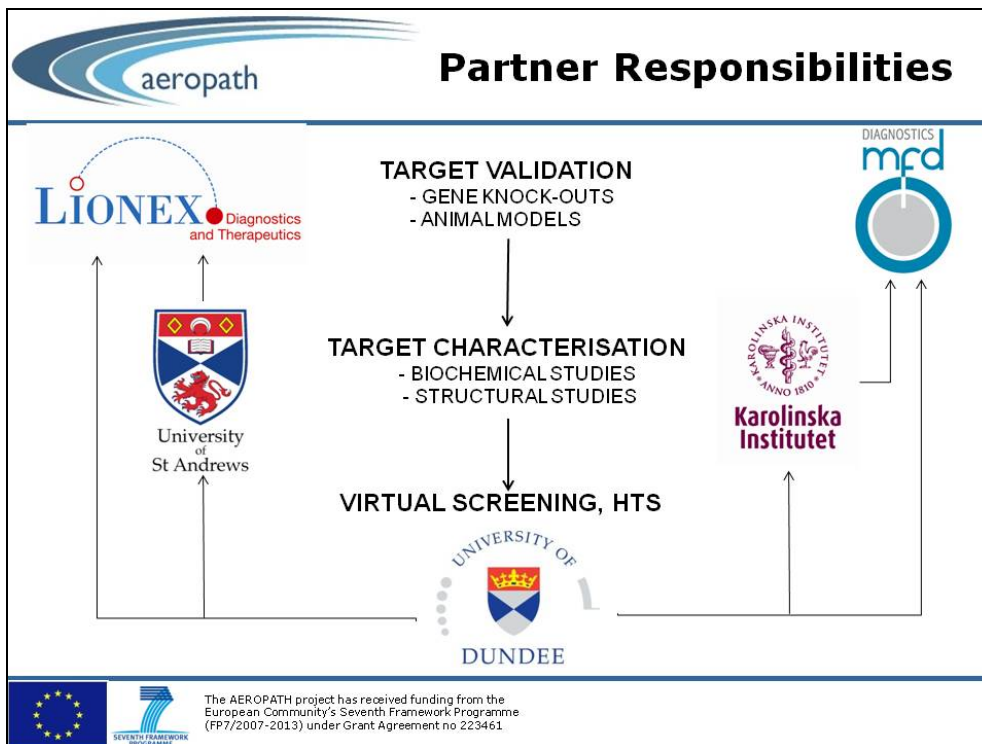
Figure 2.2: AEROPATH Partner Responsibilities

3. THE MAIN SCIENTIFIC AND TECHNOLOGICAL RESULTS

The AEROPATH project was organised into distinct Work Packages, optimized to the expertise of the partners. Progress in each Work Package informed the work in the other Work Packages.

The Work Packages were grouped around three themes:

- Target assessment and prioritisation
- Hit discovery
- Target-ligand characterisation

The goal of **target assessment and prioritisation** was to determine the subset of the list of target genes with the most potential and support decision-making about introducing additional targets. This involved

- Validating that the target genes are essential to support infection in an animal model (WP1)
- Cloning target genes and structural characterisation of the products by X-ray crystallography (WP2)
- Assessing the druggability of the targets using computational methods (WP3)

The goal of **hit discovery** was to identify compounds (hits) that can bind to the target proteins and decrease or inhibit their activity. Three screening techniques were used:

- *in silico* screening against a virtual compound library (WP4)
- Fragment screening using X-ray crystallography and other biophysical methods (WP4)
- High-throughput screening of compound libraries (WP5)

Finally the goal of **target-ligand characterisation** (WP6) was to determine how ligands (hits or designed/modelled compounds) bind to the target proteins.
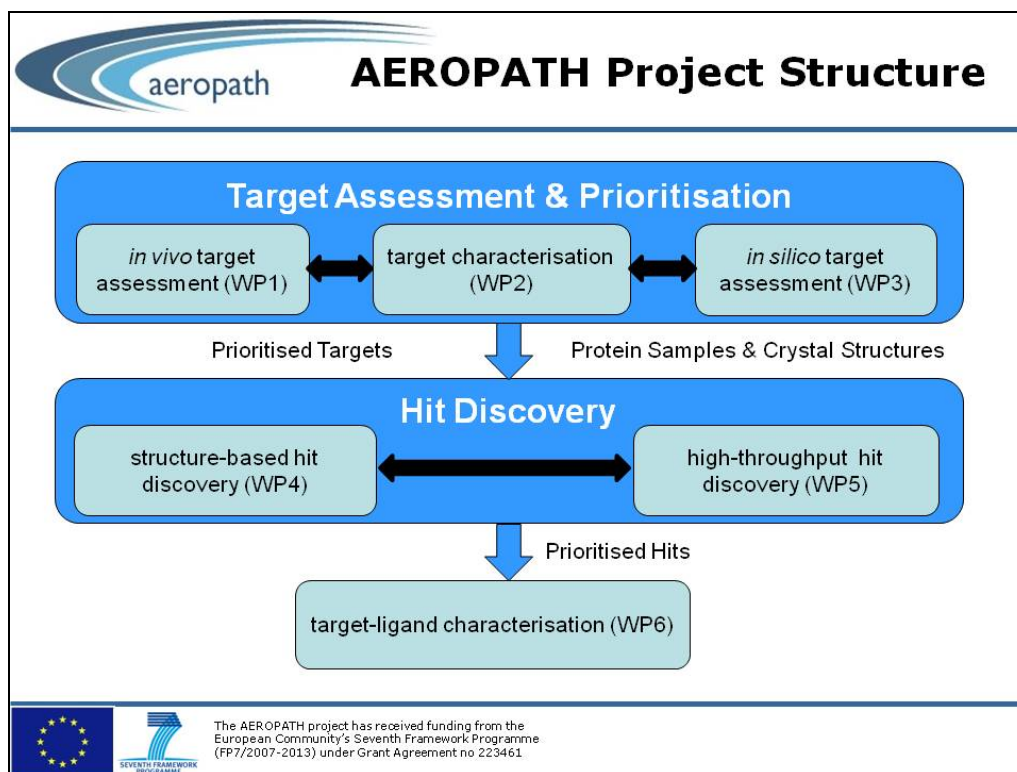

Figure 3.1: AEROPATH Project Structure

3.1 Target Assessment and Prioritisation

Work Package 1: *in vivo* target assessment
The objective of Work Package 1 was to construct knock-out (k.o.) mutants of potential drug targets of *P. aeruginosa* and to validate these by testing if the mutated bacteria can survive and if they can infect mouse models.

LIONEX GmbH developed methods for rapid construction of single gene deletion for each of the targets that had been selected based on bioinformatics and large scale transposon mutagenesis experiments. This involved the construction of genetically modified strains which contained the k.o. gene flanking regions and, in the middle, an antibiotic resistance marker. The marker is needed for genetic selection of the recipient strain of *P. aeruginosa* containing the mutated gene. A significant number of colonies were then tested following which the positive colonies were further modified by removing the carrier DNA molecule. The end result was single colonies containing the k.o. gene with a resistance marker instead of the natural gene (wild type). These strains were analysed and confirmed by DNA sequencing to be correct k.o. mutants, then tested in mice models for survival.

Mfd Diagnostics GmbH tested the infectivity of the k.o. mutants. The aim was to use the k.o. mutants to determine whether the selected targets were suitable for developing inhibitory agents (drugs) against these. If a k.o. mutant grew slowly on synthetic media or it was unable to grow well in the mouse model, it would imply that such a drug target was suitable for developing new drugs against the pathogen.

First, a suitable animal model was developed for infecting the mice with the bacteria. Several mouse strains were tested to find mice that could be best infected with the *P. aeruginosa strain PAO1* used in this study. It was found that optimal infections could be achieved with the NMRI outbred strain. Various trials resulted in an infection method using bacterial loaded agarose beads that were directly installed in the trachea of the animal to guarantee the arrival of the beads in the lung. The number of bacterial colonies in the lungs of mice infected with the mutants compared with mice infected with the *Pseudomonas* wild type provided important information on the targets, indicating which were participating in infectivity and growth and which were not.
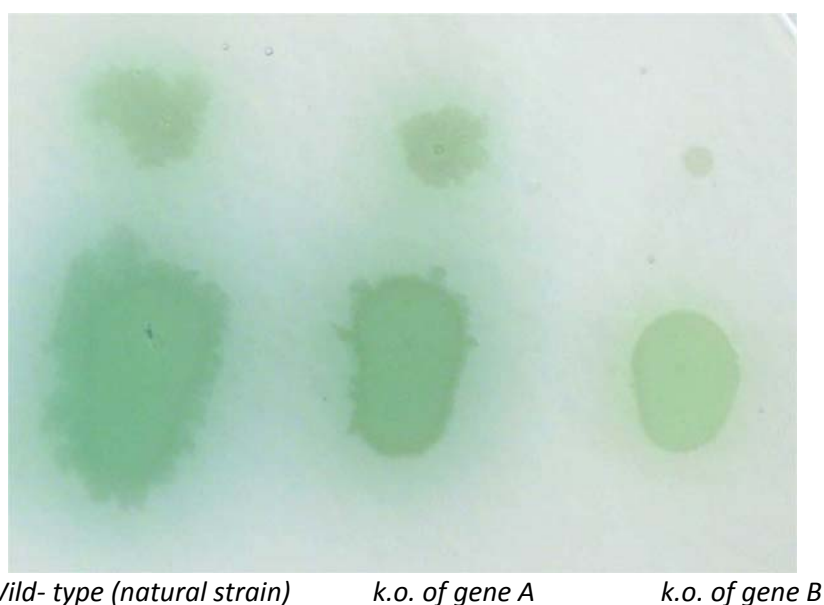

Wild- type (natural strain)   *k.o. of gene A*   *k.o. of gene B*

Figure 3.1.1: Different k.o. mutants show different morphology and growth depending on the function of the gene that has been knocked out

Work Package 1 constructed more than 35 k.o. mutants which are precise deletions of individual genes. This represents one of the largest numbers of such precise gene deletions in *P. aeruginosa*. These mutants provide valuable tools for developing novel drugs against this human pathogen. The growth behaviour of the mutants differ in most cases from that of the wild type PAO1, indicating the importance of the single genes in the infectivity of the bacteria. In addition to achieving its original objectives, Work Package 1 also constructed additional dual copy genes for four targets in the fatty acid biosynthesis pathway. These targets seem to be absolutely essential for growth on plates in a laboratory. As a result, it was not possible to construct k.o. mutants. Instead, a complex but effective strategy of inserting an additional, but functional, copy of the gene concerned was devised and then a k.o. of the natural copy of the target gene was constructed. This showed that the gene in question was really an essential target for the survival of the pathogen. The development of the technology to complement the target is also a significant development that will underpin future efforts in drug discovery against *P. aeruginosa*.

Work Package 3: *in silico* target analysis

The objective of Work Package 3 was to perform a genome-wide bio-chemoinformatics analysis to assess the druggability of targets to guide target prioritisation.

The availability of complete genome sequences of the causative agents of infectious diseases offers unprecedented opportunities for identifying new drug targets. However, recent experience has been sobering. Previous genomics-led approaches to antibacterial drug discovery, primarily in industry have failed to deliver the anticipated wave of new antibiotics. Conventional biological criteria for target selection include genetic essentiality, similarity of the bacterial target to its human counterparts (an indicator of potential toxicity), likely spectrum of activity and amenability to structural biology. Part of the problem has been the intensive investments in establishing the biological credentials of the potential drug target without regard for their chemical tractability. This is important because not all targets have suitable pockets on their surface for binding small molecule chemical leads with just the right balance of properties to constitute a drug. Those targets that do have appropriate sites are said to be 'druggable'. Making an assessment of likely druggability as early as possible would increase the chance of success of early stage research and could significantly lower the overall product development costs.

Druggability assessment is normally achieved by analyzing a protein's binding sites but this limits assessments to only those proteins for which a three-dimensional structure has been determined (currently less than 5% of the genome). The University of Dundee developed a new approach that "borrowed" the chemical information from closely related proteins. Until recently such an approach would not have been possible as all of the pharmacological information on active molecules and their associated targets that had been published in the medicinal chemistry literature was not available in a form that could be accessed by automated procedures. Now, for the first time, the large scale, publicly available bioactivity data have been extracted from the literature and stored in a database made available by the Wellcome Trust funded ChEMBL group at the European Bioinformatics Institute, based in Hinxton near Cambridge. These data are key to identifying potentially high value targets in bacterial genomes.

In order to assess the quality of a particular target Work Package 3 first assessed the quality of the associated compounds. Compound quality is usually assessed by rules based on their molecular properties (e.g. mass, volume, surface area, hydrophobicity, hydrogen bonding capacity). Molecules either pass or fail these rules. The University of Dundee developed a new numeric measure based on the properties of a set of oral drugs called QED for Quantitative Estimate of Druglikeness[6] which allows a better discrimination across

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[6] Quantifying the chemical beauty of drugs. Bickerton GR, Paolini GV, Besnard J, Muresan S, Hopkins AL. *Nature Chemistry 4 (2), 90-98*

the whole spectrum of compound quality allowing them to be ranked by relative merit. The QED scores of known ligands are aggregated for each target to give an indication of the targets druggability.

All of this information is captured in the AEROPATH Target Database that marries up all of the important chemical and biological decision-making information for every gene in the *P. aeruginosa* genome. The data have been made available to researchers worldwide via the web at http://aeropath.lifesci.dundee.ac.uk .

Figure 3.1.2: Target Listing from AEROPATH Target Database

To help researchers decision making, all of the information can be filtered and sorted by a range of relevant criteria to rapidly identify the most tractable drug targets that meet their desired profile. The database can be explored right down to the level of individual associated chemical compounds. A beneficial outcome of establishing this database is that we now provide a template of how to best organise and mine other pathogen genome datasets to rapidly identify and prioritize the most tractable targets. The approach has already been applied in Dundee to a series of Kinetoplastid genomes – the parasites that cause the important neglected diseases African sleeping sickness, Leishmaniasis and Chagas disease.

Figure 3.1.3: A Venn diagram depiction of the number of *P. aeruginosa* targets selected under different criteria. "Selective" refers to the number of targets that are unique to the bacterium compared to humans. "Perturbative" indicates how many targets are predicted to be essential on the basis of genetic studies. "Chemogenomic Druggability" refers to targets for which there are drug-like ligands in the literature. There are 69 key targets predicted to be bacteria specific, essential and druggable.

Work Package 2: Characterisation of novel targets

The main objective of Work Package 2 was to determine the structures of targeted proteins from *P. aeruginosa*. The structures would be solved to provide three-dimensional templates as part of an effort to design inhibitors against these proteins in other work packages. Work Package 2 was also responsible for generating a supply of protein, suitable assays and materials such as crystals that would allow high throughout screening of the samples to identify promising inhibitor leads.

Work Package 2 aimed to provide novel structural and biochemical data. The initial target was to determine 20 new protein structures and prepare assays for three proteins that could be used in a high throughput screening campaign. The ultimate goal was to bring together data from Work Package 1 and 3 to select targets in Work Package 2 that would then be subject to inhibitor design and identification in Work Packages 4, 5 and 6. The structural data and resources generated in Work Package 2 would be critical to success in Work Package 4.

Bio-informatics and chemo-informatics from Work Package 3 and biological data from Work Package 1 were used to prioritise the proteins for study, in terms of potential drug targets. The gene of interest was then cloned using PCR and *E. coli* was used as a heterologous expression host. Where proteins were soluble, they were purified in large scale for crystallisation and or NMR experiments. The soluble material was critical for high throughput screening and other biophysical work, even where it did not lead to a structure. In several cases the protein from *P. aeruginosa* did not give crystals and a homologous protein from another Gram-negative pathogen provided a surrogate system.

Research in Work Package 2 was carried out primarily at St Andrews and Karolinska, but with some effort in Dundee. Over 100 genes from *P. aeruginosa* and 14 from other bacteria were cloned and expressed. Over 90 of these resulted in soluble proteins suitable for biophysical study. Of the 84 targets in *P. aeruginosa* that were soluble, 39 structures were determined using NMR or protein crystallography. Protein and reagents were supplied for four high throughput screens and numerous fragment screens. A dozen targets were assigned to the Oxford Protein Production Facility and this has produced 2 targets structures. The initial structure determinations then allowed for the characterisation of target-ligand complexes using crystallography so that, in total, in excess of 100 depositions were made in the Protein Data Bank.

A major outcome of Work Package 2 was the determination of many novel structures that could serve as the basis for rational drug design. Two examples are presented in Figures 3.1.4 and 3.1.5. Since the results are all deposited in the Protein Data bank we have greatly enriched the number of *Pseudomonas* structures available to the scientific community. The mechanism and assay for the protein FabA (part of the fatty acid cycle in Gram-negative bacteria) was a particular highlight. This has laid the groundwork for tackling the cycle as individual proteins and thus opening up the possibility of entirely novel classes of inhibitors. Previous attempts had only been able to look at the entire cycle. Complementary enzymatic assays for FabG and FabV, two other enzymes from fatty acid biosynthesis, have also been developed.
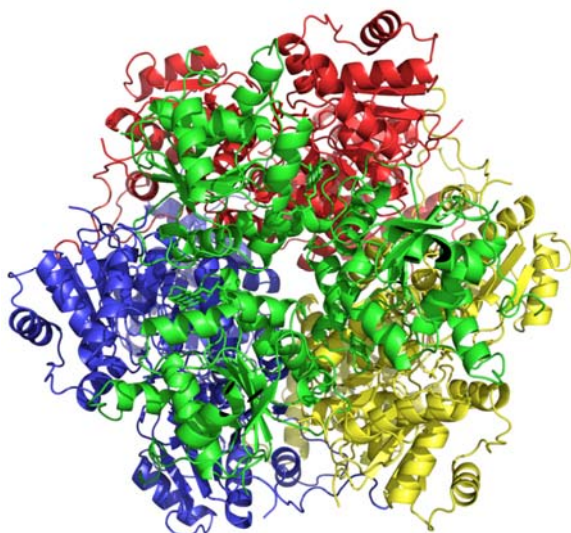
Figure 3.1.4: The structure of the putative aromatic decarboxylase, UbiX, from ubiquinone biosynthesis in *P. aeruginosa.*
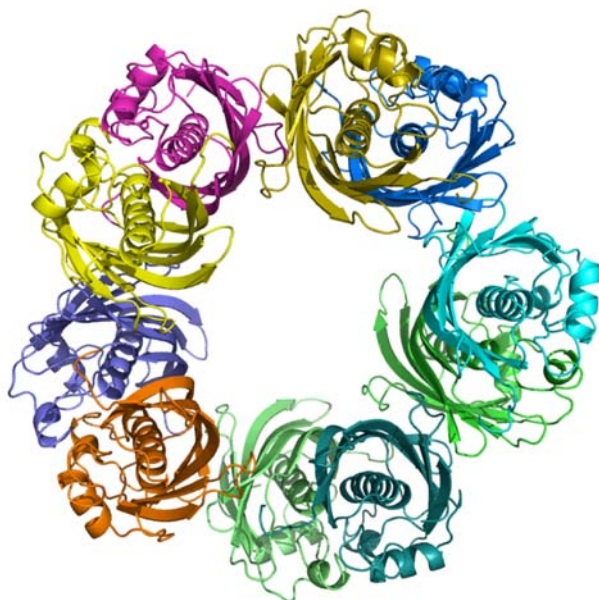

Figure 3.1.5: The structure of the dual function (dehydratase, isomerase) enzyme FabA, from the fatty acid pathway in *P. aeruginosa.*

Work Package 2 proved that structural studies can be accelerated such that they can usefully contribute to early stage inhibitor design in an academic setting. The success of Work Package 2 shows that early detailed biochemical assessment of proteins is essential if they are to be screened for inhibitors. It also shows that such ambitious undertakings require academic-industrial collaboration.

The results of Work Package 2 are available for other researchers to use with over 40 new structures and data sets being deposited in the public domain to which can be added over 60 structures of protein-ligand complexes. These structures help researchers to understand the basic biochemistry of the important pathogen *P. aeruginosa*. An assay for the fatty acid biosynthetic pathway that can be widely applied to other pathogens was also devised including the application of organic synthetic chemistry to prepare reagents. This data supported high throughput screening campaigns, which have identified lead compounds that can be tested against pathogens and these methods and approaches are available to other researchers.

3.2 Hit Discovery

Work Package 4: Hit discovery by structure-based methods
The objective of Work Package 4 was the identification of potential ligands for a subset of the targets using computational and experimental approaches. Putative ligands were characterised experimentally with respect to binding and/or inhibition. Crystal structures of the targets with these ligands were determined in order to provide templates for further improvement of the inhibitory potency of these compounds by modelling.

Virtual screening is a computational approach that identifies putative ligands for any given target protein by screening a large virtual library of small, drug-like molecules. Experimental approaches were based on the screening of fragment libraries that contain smaller chemical entities that often are found as building blocks in larger drug-like compounds. Identification of fragments that bind to the target proteins relied initially on biophysical methods such as differential scanning fluorimetry (DSF) and NMR. At the onset of the project DSF had garnered support from industry as a useful fragment screening technology. This was not our experience and, although DSF worked well for a couple of targets, it was highly prone to false positives and we quickly dropped the method in favour of biolayer interferometry (BLI). Of note is that the establishment of a NMR-based screening pipeline at St Andrews proved extremely useful for work carried out in this work package. Putative ligands identified by any of these methods were then further examined experimentally by structural investigations and/or by enzyme assays. The latter required the adaptation and *de novo* development of several enzyme assays in the course of the project. Finally crystal structures of the ligands bound to their target proteins were sought. How a target responded to the screening was taken to inform on the druggability of the protein, e.g. too high a hit rate could suggest difficulties in regard of being able to attain specificity.

Our initial work in the area first identified issues with DSF, and some of the first targets, which rendered it difficult to obtain useful structural data on protein-ligand complexes. Once the problems were recognized we were able to overcome them, use the experience and ultimately Work Package 4 has been more productive than originally anticipated with several milestones being achieved ahead of schedule. Fragment-based screens at St Andrews, Karolinska and Dundee have been carried out for 23 targets, which significantly exceeds the original objective of 4 to 8 screens and directly led to 26 crystal structures of enzyme-ligand complexes.

Two particular highlights from Work Package 4 involved realising the crystal structures of RmlA (glucose-1-phosphate thymidylyltransferase) with hits from virtual screens and the identification of a novel allosteric inhibitor-binding site in FabG following NMR fragment screening.

RmlA is an enzyme of the rhamnose biosynthesis pathway and this metabolic route has been shown to be essential for many bacteria. Virtual screening and fragment-based screening identified a series of potential ligands and several of these displayed inhibition of the enzyme to various extents. Crystal structures of the enzyme with seven hit compounds from Virtual Screening were then obtained validating the screening data and providing molecular details of how new chemical matter inhibits the enzyme.
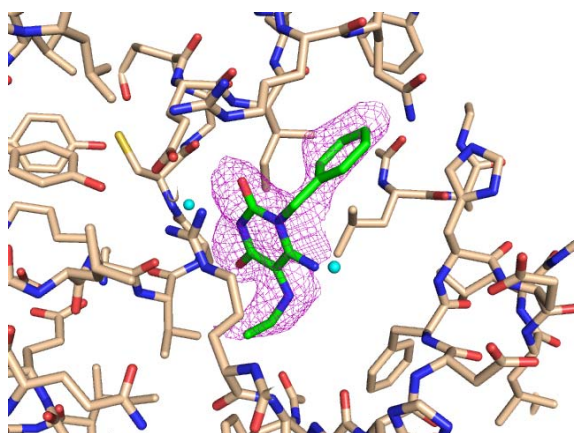
Figure 3.2.1: Crystal structure of a complex of RmlA with a hit compound, shown in green, obtained by Virtual Screening. The chicken wire represents the omit electron density map.

Fatty acid biosynthesis has been validated as a promising target for the development of antibacterial compounds. Fragment-based screening of FabG, 3-oxo-acyl-ACP reductase, one of the enzymes from this pathway, identified several hit compounds, four of which showed weak inhibition. The crystal structure of one of these fragments bound to the enzyme unexpectedly revealed a previously unknown allosteric inhibitor-binding site which is located at the interface between two subunits. This binding site was further explored using High-Throughput-Screening in Work Package 5 and has yielded a series of potent inhibitors that all bind at this position.
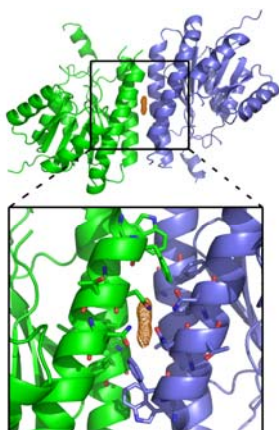

Figure 3.2.2: Structure of a FabG-inhibitor complex highlighting the allosteric binding site between two of the subunits, shown in green and blue. The electron density for the bound inhibitor is shown as a brown mesh.

The structural information generated in Work Package 4, together with the functional characterisation of the compounds derived from virtual and fragment-based screening approaches, form a sound basis for the assessment of these proteins as targets for antibacterial compounds. The achievements and results of this work package also provide frameworks for the design and development of improved inhibitors for several of the target enzymes.

Work Package 5: Hit discovery by high-throughput screening (HTS)

The objective of this Work Package was to identify four protein targets that had been shown to be essential for bacterial growth, develop the appropriate HTS assay conditions and then screen these against a diverse collection of small molecules. The output would be a number of small molecules that would be validated as binding to the proteins by crystallography or by detailed binding studies. These small molecules could represent a starting point for a drug discovery campaign, informed by the crystal structures. How the targets

behaved in the screen, in similar fashion to the fragment screens, would be taken to infer on druggability aspects of the target, ie. is the protein likely to represent a tractable target.

Work package 5 was successfully completed with seven protein targets assessed in total. Work on an early target, DapD was stopped as it had been assigned as non-druggable. MurA and FabV proved to be problematic in designing an appropriate assay technology. Although assays were developed for both enzymes, the protein construct used for MurA gave inappropriate control data when tested with a compound known to be active in the scientific literature. Despite extensive effort, a new construct could not be obtained. FabV is highly active and we have struggled to determine a suitable time-course for screening although effort continues for this target.

FolD was successfully screened against 65,000 small molecules. Active compounds were discovered and further profiled. The molecules proved to bind in the cofactor site and may be tools for scientific research. However, their binding mode and weak efficacy suggests that they are not suitable starting points for drug discovery and that FolD represents a difficult protein target for drug discovery. The data and this conclusion have been published.

RmlA was successfully screened against 16,000 small molecules after a change in strategy to look at fewer molecules in order to allow more targets to be screened was agreed. Active small molecules were discovered which, after a small amount of crystal structure driven optimisation, led to high affinity molecules. These tool molecules were used to characterise the mechanism of action of the enzyme and demonstrated an elegant but complex means of controlling the enzyme through an alternative or allosteric binding site. The molecules were characterised in a number of bacterial growth experiments but showed little activity against Gram-negative bacteria. At this stage we do not know if this is due to a failure in compound uptake by the cells, being actively pumped out or whether the enzyme is actually not essential, despite the previous genetic work. However, given the affinity of these inhibitors for the target and the recognition that RmlA might also be a potential anti-tuberculosis target, researchers at St Andrews, in collaboration with a group in the USA tested the molecules directly against *Mycobacterium tuberculosis*. The compounds display activity against this pathogen. The University of Dundee have taken the compounds into an alternative collaboration specifically focussing on drug discovery for TB and are actively cloning and expressing the relevant form of the enzyme. The data has been published in the American Chemical Society's Chemical Biology journal.

FabG was successfully screened against 16,000 small molecules using a novel and potentially patentable assay system. Active small molecules were discovered and 16 have so far provided crystal structures in combination with the enzyme. These molecules, like RmlA, appear to bind in an allosteric site. Further characterisation work is ongoing to determine if these are good starting points for drug discovery.

FabA was the last target and has only been run recently. Significant interference from fluorescent compounds has slowed progress and it took some effort to develop the appropriate assay. The screen identified two compounds with possibilities for generating crystal structures. These molecules have been ordered and we are awaiting delivery.

As well as the protein based screens we undertook a phenotypic screen against *P. aeruginosa* (PA01). Using a simple absorbance measure of growth, we screened 65,000 small molecules. Active molecules were discovered however, on detailed analysis, these proved to be parts of known antibacterial drugs. Although the screen was successfully prosecuted, we did not discover any new starting points for drug discovery.

target	Assessed	Assay Development	Screen	Output	Publication	Follow up
FolD			65,000	NADP site only – crystals obtained	Published 2012	None planned
MurA		Stopped – literature compound did not give correct response – protein construct suspected				Potential fragment screen
DapD	Non-druggable					
Phenotypic			65,000	Known pharmacophores only		None planned
RmlA			16,000	14 confirmed hits pXC50 >5 – crystals obtained	Published 2012	Compounds developed to 70nM and taken into TB-accelerator project
FabA			16,000	44 confirmed hits		ongoing
FabG			16,000	35 confirmed hits pXC$_{50}$ 16 – crystals obtained		ongoing
FabV		Ongoing – issues around reaction speed				

In summary, Work Package 5 has been successful, running five screening campaigns instead of the planned four. Crystal structures have been described for several complexes so far with one awaiting compound resupply. A single target has been shown to be non-druggable due to the nature of the binding interaction, while two others interact with an allosteric site. Excitingly, as a fortuitous bonus, one series of compounds showed good activity at killing *M. tuberculosis* and has been taken into another collaboration specifically working on drug discovery for tuberculosis. Two papers have resulted to date, with two more papers and a technology assay patent planned.

3.3 Target-Ligand Characterisation

Work Package 6 (Characterisation of target-ligand complexes) continued with the hit molecules identified in Work Packages 4 and 5 and using materials generated in Work Package 2. Once molecules are identified that function as inhibitors of relevant bacterial metabolic pathways or targets, they need to be further optimised in terms of their potency to block their target enzyme. By crystallising the hit molecules in complex with their target enzyme, a three-dimensional picture of the interacting molecules can be derived. These pictures give information about the forces that make the molecules block the enzyme and reveal which parts of the molecules are important for binding. As an example,
Figure 3.3.1 shows the known antibiotic ceftazidime binding to the enzyme "penicillin-binding protein 3". A computational approach was subsequently used to identify molecules with similar properties.

The objective of this work package was to understand which parts of the hit molecules are important for being recognized by their target enzymes. By systematically modifying the chemical structure of the molecules, an increase or a decrease in their activity can be observed. Thus, knowledge about the features important for chemical affinity and biological activity can be derived and used for further optimisation towards new anti-bacterials. The AEROPATH project was not designed to progress into medicinal chemistry, but with an understanding of the target-ligand interactions, it was considered possible to exploit computational methods that would allow for the development of sound structure-activity relationship data.
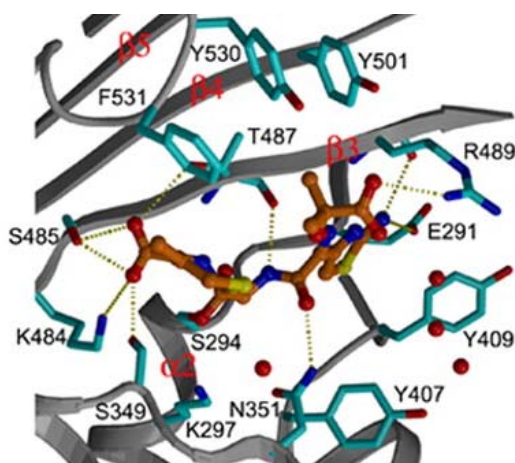

Figure 3.3.1: Ceftazidime (orange) blocking the target enzyme active site (cyan/grey)[7]

Work Package 6 successfully achieved the goal of providing more than 80 new crystal structures of relevant target enzymes with bound molecules. 52 of these molecules were identified previously in Work Packages 4 and 5.

The highlight of Work Package 6 was the provision of high quality three-dimensional descriptions of newly discovered inhibitors bound to their target enzyme. For three such enzymes the data for a series of molecules has been used to derive detailed knowledge about the most important features for affinity against the target and the mode of action of the compound. A fourth protein FabA will very shortly join this list as ongoing work is completed. In one case the feedback between the structural biology, biochemistry and synthetic chemistry has led to optimisation of inhibition to low nM. These "structure-activity-relationships"

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[7] Picture from *Crystal structures of penicillin-binding protein 3 from Pseudomonas aeruginosa: comparison of native and antibiotic-bound forms*. Sainsbury S, Bird L, Rao V, Shepherd SM, Stuart DI, Hunter WN, Owens RJ, Ren J. J Mol Biol. 2011

provide valuable information for promoting the molecules towards drug candidates and have transformed the potential to develop new antimicrobial drugs.

The results from Work Package 6 give valuable starting points for the development of new molecules with antibacterial activity. The knowledge about their mechanism of action on a molecular level will help to direct the development of initial hit molecules from screening towards highly potent "lead compounds" and further on to drug candidates.

4.   POTENTIAL IMPACT, THE MAIN DISSEMINATION ACTIVITIES AND EXPLOITATION OF RESULTS

Potential Impact

The prime objective of the AEROPATH project was to target the serious pathogen *P. aeruginosa* by blending expertise in structural biology, biology and biochemistry with the explicit goal of developing potential drug starting points. We achieved this important goal and have shown drug starting points are transferable to other serious Gram-negative pathogens. The second aim of the project was to demonstrate that such trans-EU Industrial Academic partnerships could deliver concrete results and thus re-invigorate antibiotic development.

In addition to the starting points, during the four years of this multidisciplinary project, the AEROPATH Consortium generated a wide variety of resources and a significant body of data. The lessons and developments have been placed in the public domain either via web sites, databases, presentations or publications. The AEROPATH output will support current antibacterial drug research as well as having enormous potential to underpin future, much longer term, efforts toward this goal by consortium members, by other academic researchers and also biotech-pharma interests.

The project successfully achieved the original goals of identifying and assessing novel drug targets, seeking out and finding novel hit compounds and hit series that have the potential to pave the way towards new and urgently needed antibiotics. We also learned important lessons that inform on future development of antibiotics.

- More than 35 k.o. mutants have been constructed representing one of the largest number of such precise gene deletions in *P. aeruginosa* including complementation of genes encoding components of the fatty acid biosynthetic pathway. These mutants provide valuable tools for developing novel drugs against this human pathogen. The growth behaviour of several mutants differ significantly from that of the wildtype PAO1, indicating the importance of key genes in the infectivity of the bacteria.
- A mouse lung model of *P. aeruginosa* infection was developed and mutants tested for virulence and capacity to survive in the animal model. A key lesson from the genetic and mouse studies is that large scale datasets of gene essentiality generated by transposon insertions are severely compromised by the presence of false positive data. Operon disruption rather than single gene k.o. may explain this finding.
- The AEROPATH project has shown that early detailed biochemical assessment of proteins is essential if they are to be screened for inhibitors. The results are available for other researchers to use with over 39 new structures and 100 diffraction data sets being deposited in the public domain. These structures will help researchers to understand the basic biochemistry of *P. aeruginosa* and related bacteria.
- An assay that supports compound screening for the fatty acid biosynthetic pathway and that can be widely applied to other pathogens has been devised. This supported high throughput screening campaigns, which identified lead compounds that can be tested against pathogens. These methods and approaches are available to other researchers.
- As a result of the high-throughput screening, a single target has been shown to be non-druggable due to the nature of the binding site, while two others interact with an allosteric site. Excitingly, one series of compounds showed activity at killing *M. tuberculosis* and has been taken into another collaboration specifically working on drug discovery for TB.
- The structural information generated in the AEROPATH project, together with the functional characterisation of the compounds derived from Virtual and fragment-based screening approaches formed a sound basis for the assessment of a number of proteins as targets for antibacterial compounds. The work helped us to understand how and why hit molecules, identified by compound screening, interact with their enzymatic targets on a molecular level. Providing such structural information is highly

desirable for optimising the initial hits in terms of their potency and will enable us to identify compounds with more drug-like properties.

- The incorporation of chemogenomics information (known ligands) to assist annotation of the PAO1 genome provided an assessment of druggability that has been combined with an assessment of target structures themselves. The combination offers an unprecedented look at a genome in terms of where potential drug targets might exist. The data provides confidence in some identified targets and clearly marks some targets out as difficult if not un-druggable. Critically, the assessment suggests that there exist several new areas of research that might be exploited in antibiotic drug discovery.

AEROPATH has provided access to this genome-wide assessment of druggability for Gram-negative pathogens via the AEROPATH Target Database. The data have been available to researchers worldwide via the web for more than two years and has been regularly updated. The approach has already been applied to a series of Kinetoplastid genomes – the parasites that cause African sleeping sickness, Leishmaniasis and Chagas disease, important neglected diseases. Our database has provided a template of how to organise and best mine pathogen genomes to rapidly identify and prioritize the most tractable drug targets.

In addition to the impact on antibacterial drug research, the AEROPATH project will have a beneficial economic impact on the SME partners who have been involved in the project. LIONEX will use the technology and the know-how they developed to introduce new services that can be offered to the pharmaceutical industry involved in drug development for infectious diseases. Mfd Diagnostics will use their experiences in further studies where infections in animals are necessary. This will benefit not only in the internal projects of mfd Diagnostics, but also in offering such studies as contract research to industry.

Finally, individual researchers benefited hugely from the experiences shared and lessons learned in this project. A specific output is the creation of academic groups primed to directly contribute to early stage drug discovery. Many of the academic researchers working on the AEROPATH project had the opportunity to visit the labs of the other academic partners, participate in training, join in with project meetings and to generally increase their knowledge, which will be of benefit in their future careers. The project brought in several students who were able to participate in the research as part of undergraduate and postgraduate programmes.

Main Dissemination Activities

The project has been widely disseminated and promoted both to the scientific research community and the general public. The main outreach to the research community has been publications in scientific journals, presentations at scientific meetings and conferences, depositions in public databases and the use of our project WEB site. Although accessible to the general public the web site was not designed to give anything other than a general introduction. Rather, for outreach to the general public we concentrated on press releases to announce major achievements.

Over the course of the project, AEROPATH has been promoted at more than 20 scientific conferences and meetings. This has involved a combination of invited talks, presentations and posters communicating the objectives of the project and results achieved. Examples are shown in Figure 4.2.1 below.
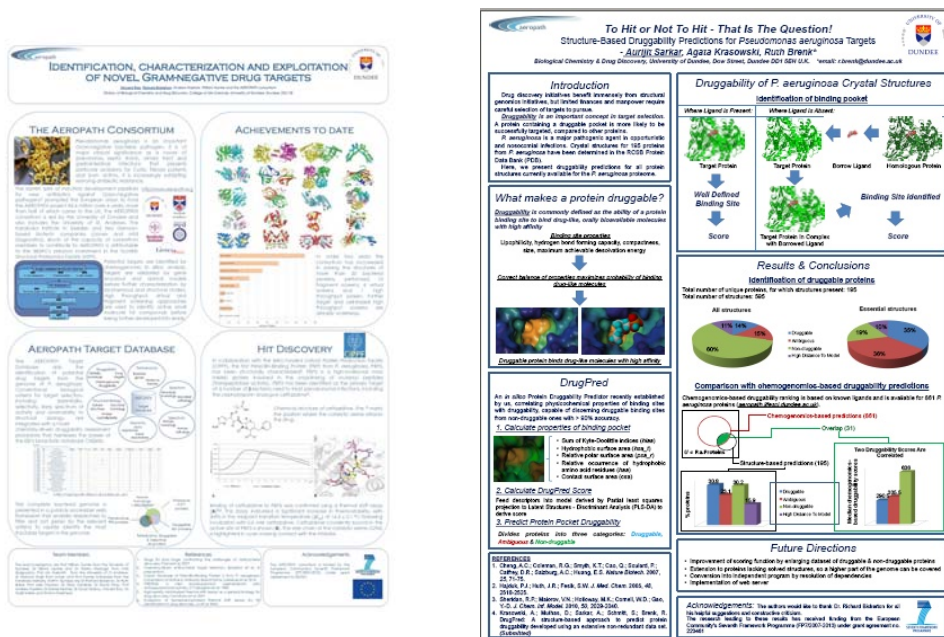
Figure 4.2.1: Examples of posters produced by the AEROPATH Project

In addition, the AEROPATH partners have disseminated the project results via more than 15 papers published in scientific journals, the majority of which have been made open access due to additional means of support, and with over 100 structures and data sets being deposited in the most widely used structural biology public database, the Protein Data Bank. See Figure 4.2.2 for examples of journal papers.

Figure 4.2.2: Examples of scientific publications from the AEROPATH Project

Press Releases have been used from the very start to promote the project in general as well as highlighting key achievements. For example, the University of Dundee issued a press release at the start of the project,

which was featured on a variety of UK and European news, medical and business WEB sites as well as the local Dundee press and on radio. See Figure 4.2.3 for examples of media coverage resulting from press releases issued by the AEROPATH partners.
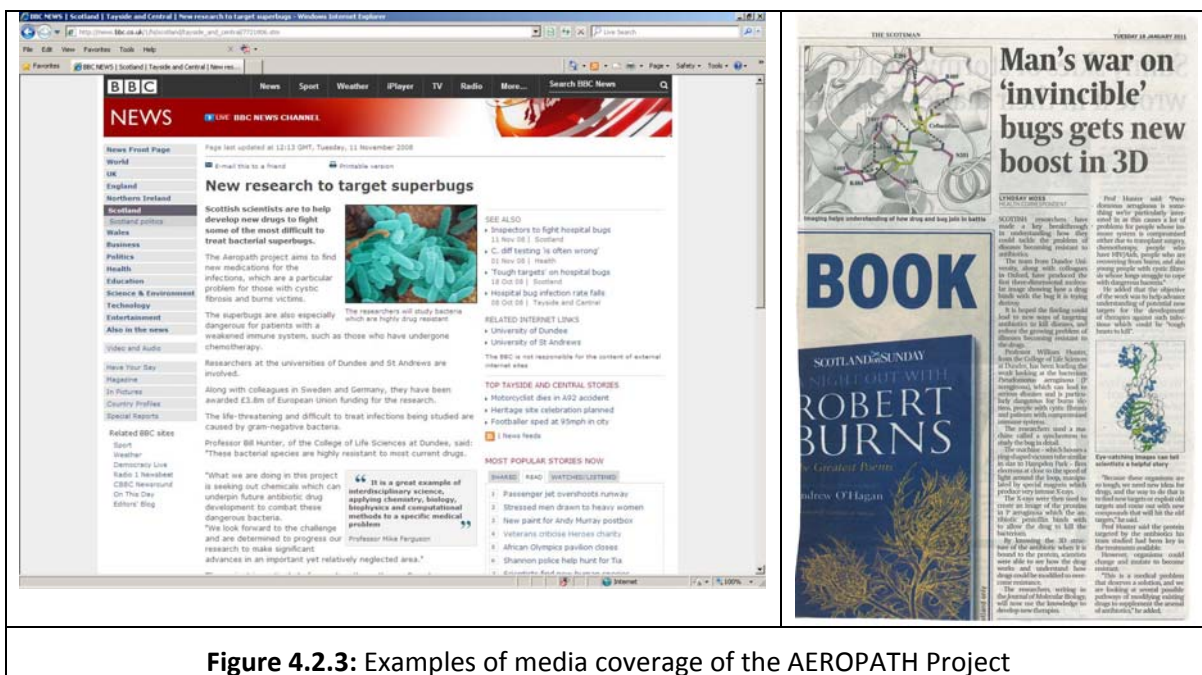

Figure 4.2.3: Examples of media coverage of the AEROPATH Project

The AEROPATH Project WEB site – www.aeropath.eu - has acted as the main communication tool of the project since its launch in January 2009. It has been updated on a regular basis and contains areas for both the general public and the research community. For example, the **News** page is aimed at a wide audience and contains links to all project-related publicity, press releases and news articles, whereas the **Publications and Presentations** page is aimed more towards the research community. This page contains downloadable copies of many of the project presentations and posters as well as links to the relevant journal WEB sites where copies of the scientific papers can be downloaded. See Figure 4.2.4 for the AEROPATH Home Page.
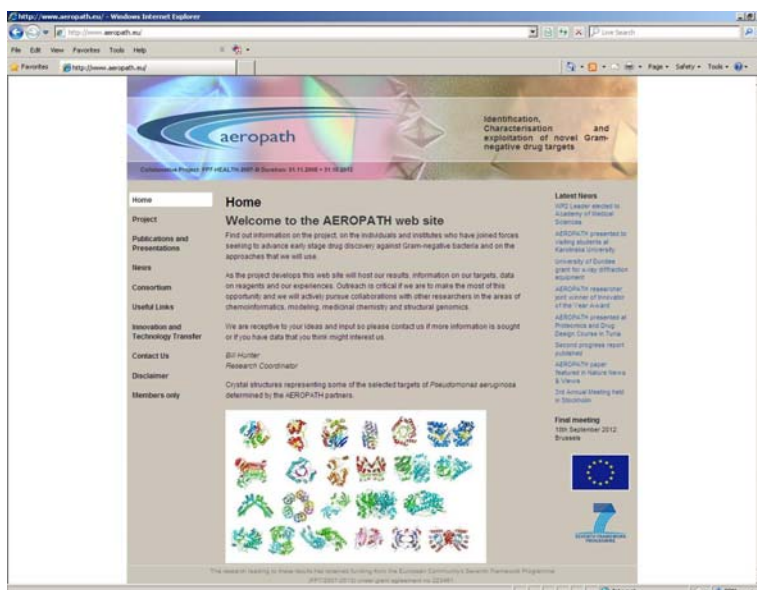

Figure 4.2.4: AEROPATH Home Page

The AEROPATH partners have also engaged with various other projects funded under the HEALTH-F3-2009 call to share ideas and identify opportunities for future collaborative studies. This has included the coordinators of the AntiPathoGN and Divinocell projects attending the AEROPATH project Kick Off meeting in February 2009 and a joint meeting with the partners of the NABATIVI project in March 2011.

Figure 4.2.5: Joint meeting with the NABATIVI project

As well as disseminating the specific objectives and scientific results of the AEROPATH project, many of the partners have also been involved in educational activities and events aimed at school children and undergraduate students. For example, AEROPATH researchers at St Andrews University were involved in a number of lectures and presentations to school children and students about how science is being used to tackle bacterial infections.

Exploitation of results

The results of the AEROPATH project will be exploited in a number of ways:

- **Capability to create mutant *P. aeruginosa* strains and a mouse lung infection model**
  The developments by the SME partners provide commercial opportunities to assess new chemical hits and leads *in vivo*. A range of mutant strains of *P. aeruginosa* and a mouse lung infection model are now available to assist future drug discovery.

- **Informatics analyses and database**
  There is potential to extend the approach behind the AEROPATH Target Database beyond *P. aeruginosa* to inform on other infectious diseases and our database provides a template of how to best mine other pathogen genomes to rapidly identify and prioritize the most tractable targets.

- **Capability for phenotypic screen of antibacterial agents**
  An efficient, cost effective, high throughput method for screening compound libraries against Gram-negative bacteria has been established. This technology will come to the fore when focused compound library sets become available in the future.

- **Chemical entities that bind and inhibit potential drug targets**
  Numerous hit compounds have been identified against a range of targets. This chemical matter provides a basis to inform on future medicinal chemistry approaches in particular in the area of fatty acid biosynthesis.   Some uses of the chemical matter extend beyond the sphere of *P. aeruginosa* and are already being used in follow on projects.

- **New crystal structures that enrich the collection of models in the Protein Data bank**
  More than 100 crystal structures have been determined that enrich the collection of models in the Protein Data bank. This represents a major contribution to the structural biology of *P. aeruginosa* proteins and of protein-ligand complexes for the scientific community to exploit.

- **Protocols for high-throughput *in silico* chemical screening**
  Efficient methods for high-throughput *in silico* (computer-based) chemical screening have been develop making automated large scale protein-based druggablity predictions possible for the *P. aeruginosa* proteome for which structures are determined. These methods are now so robust that they can be applied to any organism of interest and used to embellish genome annotation as outlined above.

- **Provision of services to biotech or pharmaceutical companies**
  The academic partners now have established capabilities to supply services covering all aspects of molecular biology, recombinant protein production, crystallographic services, screening technologies on a commercial basis. The customers of such services could be biotech or pharmaceutical companies.

5. PROJECT DETAILS

Title

AEROPATH: The identification, characterisation and exploitation of novel Gram-negative drug targets (GA no. 223461)

Coordinator

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Consortium

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Duration

1 November 2008 – 31 October 2012 (48 months)

Funding Scheme

FP7 HEALTH, topic HEALTH-2007-2.3.1-1 "Novel targets for drugs against Gram negative bacteria"

Budget

EU contribution: 4,591,463 €

Website

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