IDENTIFICATION AND VALIDATION OF NOVEL DRUG TARGETS IN GRAM-NEGATIVE BACTERIA BY GLOBAL SEARCH: A TRANS-SYSTEM APPROACH

(ANTIPATHOGN)

Executive summary:

The research project "Identification and validation of novel drug targets in Gram-negative bacteria by global search: a trans-system approach" –AntiPathoGN- was launched in February 2009 by a consortium of ten institutions –academic and industrial- from three European countries. With a duration of four and a half years, this Collaborative Project has been supported by funding under the Seventh Framework Programme of the European Union.

The increasing emergence and spread of multidrug-resistant (MDR) pathogens currently constitutes one of the major threats to public health worldwide. The shortage of effective antimicrobials for the treatment of infections caused by MDR Gram-negative bacteria is particularly critical, as strains within this group have already become the main cause of death among patients with hospital-acquired infections. In this context, AntiPathoGN seeks to discover new targets and modes of action, less propitious to the evolution of resistance, for the development of drugs against Gram-negative bacteria. To this end, the consortium has developed a strategy based on a comparative, system-level analysis of proteins and protein-interaction networks of the bacteria of interest with enrichment of factors involved in pathogenesis, virulence, drug resistance and cell division/growth. Main target bacteria of AntiPathoGN include high-priority MDR pathogens such as *Pseudomonas aeruginosa*, *Escherichia coli* and *Acinetobacter baumannii*, and emerging ones such as *Helicobacter pylori* and *Stenotrophomonas maltophilia*. In addition to the identification and validation of new drug targets, the AntiPathoGN project pursues the discovery of novel antibacterial compounds acting against these targets by screening purpose-specific libraries of products derived from natural sources and from synthetic compounds.

At formal closing, the consortium has identified and validated phenotypically eighteen potential antimicrobial targets in Gram-negative bacteria, and has found hit compounds against two of them. Two of the targets are essential for bacterial growth, while the other sixteen are involved in various mechanisms related to virulence or resistance. Cell-based screens have also identified 33 natural products with antimicrobial activity, including three novel compounds. Further studies will be needed to confirm the potential of these targets and compounds for antimicrobial drug development. The experimental interactome data and bioinformatic databases and tools generated by the consortium constitute a fundamental contribution of AntiPathoGN to the research community, within and beyond the field of antimicrobial-drug discovery.

Summary description of the project context and main objectives:

Multidrug-resistant (MDR) bacterial infections are increasing at alarming pace in both developing and developed countries and in both community and nosocomial settings. The spread of multidrug resistance compromises not only the recovery of patients with life-threatening bacterial infections but also the success of seemingly unrelated medical treatments, from surgery to chemotherapy. Indeed, as summarised by the World Health Organization (http://www.who.int), *antimicrobial resistance kills, hampers the control of infectious diseases, threatens a return to the pre-antibiotic era, increases the costs of health care, jeopardizes health-care gains to society, threatens health security and damages trade and economies*. The spread of MDR infections badly combines with the shortage of new antimicrobials arriving to market and the non-encouraging prospects for new developments, as reflected by the antimicrobial pipelines of pharmaceutical companies. The situation is particularly worrying in relation to MDR Gram-negative bacteria. The few antimicrobial agents that have been launched during the last decade have a good activity against Gram-positive

bacteria, but MDR bacteria are often found among the Gram-negative group. A combination of business reasons and scientific challenges (the cell structure of Gram-negative bacteria makes them more difficult to attack with drugs than Gram-positive organisms) has been limiting the interest of the pharmaceutical industry for the development of antimicrobials against Gram-negative bacteria. With the appearance of pan-resistant strains, i.e. strains resistant to every antimicrobial drug, especially in hospital settings, the development of new, effective antimicrobial agents against Gram-negative bacteria has become a pressing need. In this context, the AntiPathoGN project has developed a novel strategy for the discovery of antimicrobial-drug targets starting from the full set of proteins coded in the genome of the organism. While focussing on Gram-negative bacteria, this strategy is broadly applicable to the identification of potential drug targets in other pathogenic microorganisms. It is based on a comparative, system-level analysis of proteins and protein-interaction networks of the bacteria of interest with enrichment of factors involved in pathogenesis, virulence, drug resistance and cell division/growth. Main target bacteria of AntiPathoGN include high-priority multi-drug-resistant pathogens such as *Pseudomonas aeruginosa*, *Escherichia coli* and *Acinetobacter baumannii*, and emerging ones such as *Helicobacter pylori* and *Stenotrophomonas maltophilia*. In addition to the identification and validation of new drug targets, the AntiPathoGN project pursues the discovery of novel antibacterial compounds acting against these targets by screening purpose-specific libraries of products derived from natural sources and from synthetic compounds.

AntiPathoGN follows two objectives: the generation of new knowledge and, building on this, the advancement toward novel solutions. Thus, at the first level, AntiPathoGN aims at increasing current knowledge on biological processes and mechanisms related to pathogenicity of Gram-negative bacteria. This is achieved using a combination of computational and experimental proteomic and systems-biology approaches to unveil proteins relevant to pathogenesis and virulence, resistance mechanisms and bacterial growth. At the second level, AntiPathoGN aims at finding new drug targets or combinations of targets in Gram-negative bacteria and active compounds against them. To this end, a multi-disciplinary approach is followed, bringing together expertises in the fields of microbiology, systems biology, computational biology, structural biology, and drug discovery. The process is guided by expert clinical knowledge as well as by studies on socio-economic factors.

Description of the main S & T results/foregrounds:

Existing antimicrobials target mechanisms or structures that are essential for bacterial survival or growth. These may be proteins involved in essential processes such as genome replication or cell division, their inhibition disabling bacterial growth, or components of the cell wall, their attack leading to the destabilisation or permeation of the bacterial cover and eventually to cell lysis. Thus, the strategy dominating antimicrobial-drug development is based on straight elimination of the microorganism. While in principle this offers a rapid and definitive solution to the problem, it also has a number of undesired side effects. The first one is drug resistance. Indeed, there is no better way to select for drug-resistant mutants than to eliminate completely the non-resistant ones. The number and growth rate of bacterial cells in a site of infection can be such that the probability that drug-resistant mutants arise by chance is far from negligible. These mutants will clearly have a growth advantage over the drug-susceptible, dying populations (which may include other coexisting bacterial species, as discussed below). When considering an entire human community, the probability that some of these individual mutations will be fixed is also notable, as current indicators on antimicrobial-drug resistance demonstrate. A second side effect originates from the fact that essential proteins tend to be more conserved among bacteria than non-essential ones. This results in wide-spectrum antimicrobials, i.e. antimicrobial drugs that kill indistinguishably a series of bacterial species, whether pathogenic or not. While this simplifies treatment options and supports the economic viability of antimicrobial-drug development, it can disrupt the equilibrium of the natural microbiota of the patient and, under certain circumstances, promote disease.

An alternative strategy to antimicrobial-drug development consists in targeting mechanisms related to pathogenicity or virulence (although such drugs would not be strictly antimicrobials, we shall keep this terminology for simplicity). For example, mechanisms related to the interaction of the

pathogen with host cells, to the cross-talk between bacteria (quorum sensing), the production of toxic agents, etc. The rational behind this approach is that by reducing the capacity of the microorganism to effectively infect or compromise the health of the host, the main threat is eliminated and bacterial clearance can be left to the immune system. As mutants are not actively selected (the survival advantage over the parent strain is much lower than in the previous scenario), the probability of fixing resistant phenotypes may be reduced. In turn, the immune system may be given additional time to develop protective immunity against the pathogen. The level of conservation among bacterial species, and therefore the spectrum of the antimicrobial drug, will depend on the targeted mechanism, but will be generally lower than for essential targets. This approach is however not without risks. For example, it is not clear how immunodepressed patients, the most sensitive group today in relation to infection by MDR Gram-negative pathogens, would respond to such a treatment.

The use of drug combinations offers additional opportunities for combating resistance. Under this premise, the effect of an antimicrobial drug may be enhanced in several ways. For example, by combination with 1) a second antimicrobial drug targeting a different mechanism, such that the probability that resistance will emerge at the same time against the two compounds is minimal; 2) a drug targeting a specific mechanism of resistance against the antimicrobial; 3) a drug activating or inhibiting a mechanism in the host that may contribute to combating the pathogen or its effects. Examples of each of these already exist in the medical practice. Future combinations may also include drugs limiting bacterial pathogenicity or virulence or drugs enhancing the effect of the antimicrobial by targeting a mechanism without which the pathogen becomes more vulnerable to it.

By applying an integrative approach with strong roots in network biology, AntiPathoGN has been able to extend its focus to the various alternatives explained above. Knowledge generation has followed the path summarised in the following paragraphs:

Comparative genomic analysis: the translated genomes (full set of proteins coded for in the genome) of the Gram-negative species of interest constitute the first level of information exploited by the project for the identification of potential antimicrobial-drug targets. The analysis is based on multiple strains to enable the assessment of conservation of candidate proteins at various levels, i.e. species, Gram-negative bacteria and humans (as a relevant indicator of potential toxicity). It also considers the presence of isoforms and paralogs (which could facilitate functional replacement and development of resistance), existing annotations on essentiality, virulence and previous use of the protein as an antimicrobial target, and annotations and predictions on the presence of active and/or allosteric sites in the protein (relevant to the assessment of target druggability). Additional analyses such as presence of the protein in only pathogenic species of a genus (for the enrichment of pathogenicity and virulence factors) and codon-level evidences of positive selection of mutations (indicative of selective pressure, potentially by contact with the host immune system) are also performed. The consortium has developed bioinformatic tools to mine information and perform the analyses, including the antibacTR database and ranking tool, accessible at www.antipathogn.eu.

Differential proteomic analysis: a second level of information for the identification of potential antimicrobial-drug targets derives from the comparative proteomic analysis of full protein extracts (or specific fractions, such as outer-membrane proteins and outer-membrane vesicles) from clinical and attenuated strains, aiming at the identification of differentially expressed proteins that may be involved in pathogenesis, virulence or resistance mechanisms.

Interactome analysis: a major novelty and achievement of the AntiPathoGN strategy. The functional role of a protein in a system cannot be fully explained without proper description of the system. For example, a seemingly uninteresting protein may reveal relevant to bacterial growth, pathogenicity or antimicrobial resistance only when placed in its functional context, i.e. the network of protein-protein interactions governing the system. Building on this concept, AntiPathoGN has generated a database with the most complete and accurate interactome networks available today for *E. coli* (K12 and EHEC), *P. aeruginosa*, *A. baumannii* and *H. pylori*, combining bioinformatics and experimental approaches. At the experimental level, yeast-2-hybrid screens have enabled the determination of high-quality and comprehensive intra-species interactomes of *E. coli* K12 and *H. pylori*. The *E. coli* interactome is a milestone in microbial protein interactome research, being the

first binary interactome of a major model prokaryote. In addition, the consortium has determined pathogen-host interactomes using EHEC-human and phage lambda-*E. coli* as model species. The results of this analysis may open up new ways to use phages to kill *E. coli* and other gram-negative bacteria. Importantly, the binary interaction data has been integrated with structural information, protein complex data, and genetic interactions. The structural characterisation of protein complexes has included the analysis and prediction of sites for the design of protein-protein-interaction inhibitors. To organise and facilitate access and further analysis of the data, the consortium has developed tools to host and query all the interactome data generated (In-silico networks), annotate protein networks with 3D structures of complexes (Interactome3D) and align protein networks for comparative interactome analysis (NetAlign). These tools are accessible at www.antipathogn.eu.

Intracellular expression of antibodies targeting the full proteome: a final target-discovery approach implemented in the project is based on *in vivo* intracellular conditional expression of anti-proteome single-domain camel antibodies (VHH), for which a VHH library against the proteome of *E. coli* K12 and EHEC has been developed. Selective, intracellular expression of the antibodies *in vivo* enables the identification of VHH clones whose expression turns out to be inhibitory for growth of the bacteria that harbours them.

The information obtained in the previous four types of studies is used to prioritise potential antimicrobial-drug targets. Once a potential target has been identified, it is transferred to a pipeline that validates the phenotypic effect of the suppression of the target in the bacterial genome, including assays of virulence in a *Caenorhabditis elegans* model of infection when available (*P. aeruginosa and S. maltophilia*), to continue with recombinant production of the validated target, determination of its three-dimensional structure (when a homolog with high sequence identity is not available), biochemical characterisation (binding-assay development) and, finally, the screening of synthetic fragment compounds and natural products for ligand discovery. Bound to this latter step, the consortium has devoted a full line of work to the discovery of new natural products with antimicrobial activity. The screens of natural compounds are performed both *in vitro* (on target proteins) as well as in cell-based assays.

Description of the potential impact, main dissemination activities and exploitation of results:

The identification of novel antimicrobial-drug targets and modes of action to combat Gram-negative bacteria is a grand challenge as much as a pressing need. The efforts in this direction are countless, as a quick look at the scientific literature demonstrates, but the path from the promising lab candidate to the approved drug is long, extremely expensive and full of yet uncontrolled variables, both of scientific and business-related nature. AntiPathoGN has made a contribution to this collective effort at two levels:

1) Providing the research community with new valuable data for the interpretation of the biology of these microorganisms as well as with tools to efficiently use this data. Indeed, the experimental interactome data and bioinformatic databases and tools generated by the consortium constitute a fundamental contribution of AntiPathoGN to the research community, within and beyond the field of antimicrobial-drug discovery.

2) Arriving to a short list of candidate targets and compounds that, after the initial identification process, have passed a first round of phenotypic and activity assays, respectively. At formal closing, the consortium has identified and validated phenotypically eighteen potential antimicrobial targets in Gram-negative bacteria, and has found hit compounds against two of them. Two of the targets are essential for bacterial growth, while the other sixteen are involved in various mechanisms related to virulence or resistance. Cell-based screens have also identified 33 natural products with antimicrobial activity, including three novel compounds. Further studies will be needed to confirm the potential of these targets and compounds for antimicrobial drug development.

AntiPathoGN has appeared in the media in 17 occasions, the project or its results have been presented in 19 scientific conferences and, at formal closing, has generated 54 publications in

scientific journals, including top-ranking journals such as Nature Methods, Proceedings of the National Academy of Sciences of the USA or PLoS Pathogens. The dissemination activities of AntiPathoGN will continue after the formal closing of the project and are accessible at www.antipathogn.eu.

Project Information

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Project web-site: www.antipathogn.eu

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