Ph.D. Thesis summary

Chemical-genetic profiling to investigate cross-resistance and collateral sensitivity between antimicrobial peptides

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Szeged

2019

Introduction

Development of antibiotic resistance in bacteria has become a serious threat to public health, and a new class of antibiotics is needed to treat resistant bacteria. AMPs are molecules of host-defense system and have been proposed as a novel class of anti-infectives. Considerable efforts have been allocated for developing AMP-based therapeutic strategies against pathogens. Despite the clinical importance of AMPs, the vital concern is that therapeutic use of AMPs holds the risk of cross-resistance to our own immunity peptides. This is an alarming issue as many AMP-based clinical applications are about to reach clinical trials while others are already in clinical use. But are all classes of AMPs equally prone to induce such cross-resistance effects? Our knowledge on the potential for cross-resistance between AMPs remains extremely limited. While most of our knowledge on AMP resistance is coming from case studies focusing on specific AMP, a systematic study on the extent to which the genetic determinants of resistance differ across AMPs is still lacking. Given the therapeutic importance of AMPs, a systematic study on AMP resistance, cross-resistance and collateral sensitivity (evolution of resistance to one drug increases sensitivity to other drugs) between AMPs may help to rationally choose AMPs for clinical development and to minimize the cross-resistance between therapeutic and human host AMPs.

To this end, we applied a genome-wide chemical-genetic approach to map the genetic determinants of AMP resistance in *E. coli*. This approach provides a better understanding of how the differences in mode of action of AMPs shape the resistance mechanisms against them. By analyzing these differences, we could identify a number of genes that induce cross-resistance and collateral sensitivity between AMPs. Moreover, by integrating laboratory evolution approach we demonstrated that chemical-genetic profiles can inform on cross-resistance patterns between AMPs. In future, the chemical-genetic map could inform on how to choose therapeutic AMPs with minimal cross-resistance to human host AMPs.

Aims

In our research, we aimed to systematically investigate the latent and intrinsic AMP resistome of *E. coli* and to understand how the mode of action of AMPs shapes the resistome.

Our particular goals were the following: 1) identifying the resistance determinants of *E. coli* against 15 different AMPs, 2) understanding how the AMPs with different modes of action and physicochemical properties differ in their resistance determinants, 3) investigating whether the differences in AMP's mode of action and resistance determinants advise on cross-resistance and collateral sensitivity interaction between AMPs.

To achieve these goals,

- we established a genome-wide chemical-genetic screening method to measure the effect of overexpression of each of 4,400 *E. coli* genes on bacterial susceptibility against 15 AMPs (latent AMP resistome).

- we explored the intrinsic AMP resistome by performing a chemical-genetic screen with a set of 279 partially-depleted essential genes (hypomorphic alleles) of *E. coli.*

- we examined how the similarity in chemical-genetic profiles inform on mechanism of action of AMPs.

- we interrogated whether the mode of action of AMPs shapes the latent and intrinsic resistome.

- we investigated collateral sensitivity between AMPs and the molecular mechanism underlying collateral sensitivity interactions.

- we investigated whether chemical-genetic profiles can inform on cross-resistance interactions between AMPs**.**

Methods

1. Chemical-genetic screening

We carried out chemical-genetic profiling to determine the impact of the overexpression of each *E. coli* ORF on bacterial susceptibility to each of the 15 different AMPs. To this end, we used the ASKA plasmid library (GFP minus) where each *E. coli* ORF is cloned into a high copy number expression plasmid (pCA24N-ORFGFP(-)). We applied a pooled fitness assay with a deep sequencing readout. The pooled overexpression library and the control strain were grown in the absence and presence of AMP. Following approximately 12-15 generations of logarithmic growth, next-generation sequencing was applied on the resulting plasmid pool to measure the relative abundance of each overexpression strain in the populations.

2. High-throughput sequencing of plasmid pool

To determine the relative abundance of each overexpression strain, the purified plasmid DNA samples were subjected to next-generation sequencing with SOLiD system (Life Technologies). DNA sequencing was carried out by István Nagy and his colleagues from Seqomics Ltd, Szeged.

3. Data analysis and clustering of chemical-genetic profiles

Data analysis and clustering of chemical-genetic profiles were performed by my colleague Gergely Fekete. Following different steps of data normalization and transformation, a differential growth score (i.e. fold-change) was calculated for each gene as the ratio of the normalized relative read counts in treated and non-treated samples at the end of the competition assay. Genes that showed at least 2-fold lower and higher relative abundance with a p-value<0.05 at the end of the competition upon AMP treatment were considered as sensitizing and resistance-enhancing genes, respectively.

We employed an ensemble clustering method to group the AMPs on the basis of their chemical-genetic profiles similarity. This method calculates distance between chemical-genetic profiles of AMPs, and then generates multiple clustering based on perturbing the AMP chemical-genetic interaction data and the clustering parameters. Next, it combines the resulting clusters to obtain a consensus clustering which is robust and the best representative of multiple clustering.

4. Chemical-genetic profiling of hypomorphic alleles

Construction and chemical-genetic profiling of the hypomorphic alleles was carried out by our collaborator Mohan Babu and his colleagues at University of Regina, Regina, Canada

5. Calculating physicochemical properties of AMPs

For each AMP, different physicochemical properties (like amino acid frequency, isoelectric point, hydrophobicity, net charge) were calculated. These calculations were made by my colleague Ádám Györkei. An in-house perl script was used to count protein amino acid frequencies. Isoelectric point, hydrophobicity, hydrophobic moment and net charge was calculated with the peptides R package, version 2.462. AMP length and molecular weight were calculated using the ExPasy Prot Param tool. The alpha-helical content and the disordered structure of AMPs were calculated using PASTA 2.0 server.

6. Calculating the overlap in resistance-enhancing and sensitivity-enhancing genes between AMP pairs

To calculate the overlap in resistance and sensitivity genes between AMP pairs, we used a modified version of the Jaccard index that takes into account measurement noise. For each AMP pair, we calculated the Jaccard index of overlap between the resistance gene sets and performed a correction by dividing this value by the average Jaccard index of overlap between replicate screens of the same AMPs. Corrected Jaccard indices were calculated separately for resistance and sensitivity genes.

7. Enrichment analysis of collateral sensitivity-inducing genes

For each AMP pair, overrepresentation of collateral sensitivity-inducing genes was calculated over random expectation.

8. Gene ontology (GO) enrichment analysis of resistance-enhancing and sensitivity-enhancing genes

Biological Networks Gene Ontology tool (BiNGO) was used to determine which Gene-ontology (GO) terms are significantly enriched in the resistance-enhancing and sensitivity-enhancing genes.

9. Minimum inhibitory concentration (MIC) measurement

Minimum inhibitory concentrations (MIC) were determined with a standard serial broth dilution technique with a minor modification. Specifically, instead of a two-fold dilution series, smaller steps of AMP concentration were used (typically 1.2-fold) to capture small changes in the growth inhibition.

10. Measurement of membrane surface charge

To measure bacterial membrane surface charge, we performed a fluorescein isothiocyanate-labelled poly-L-lysine (FITC-PLL) (Sigma) binding assay. FITC-PLL is a polycationic molecule that is used to study the interaction between cationic peptides and anionic lipid membranes. The lower the amount of bound FITC-PLL, the less negatively charged the cell surface.

11. Membrane potential measurement

BacLight™ Bacterial Membrane Potential Kit (Invitrogen) was used to measure transmembrane potential (Δψ). In this assay, a fluorescent membrane potential indicator dye (DiOC2) exhibits green fluorescence in all bacterial cells and the fluorescence shifts to red in the cells that maintain a high membrane potential. The ratio of red/green fluorescence provides a measure of membrane potential.

12. Growth curve measurement

To determine the difference in bacterial growth in the presence of an AMP, we performed growth curve analysis. Growth curve measurement for the overexpression strains was done by measuring OD_{600} of the liquid bacterial cultures at different time points in a standard 96-well plate containing a 12-step dilution series of the tested AMP in MS medium.

13. Construction of *mlaD* mutant

We constructed *mlaD* knockout mutant of *E. coli* BW25113 by removing the kanamycin resistance cassette present in the position of the gene in *mlaD* knockout strain from KEIO collection. This mutation was performed by my colleague Csörgő Bálint.

Results

1. Chemical-genetic profiling and its validation

We performed a genome-wide overexpression screen to examine the effect of overexpression of each of 4,400 *E. coli* genes on bacterial susceptibility against a set of 15 AMPs. The AMPs used in this study are diverse in their physicochemical properties, mechanism of action and importantly some of them have clinical relevance. Next, we tested the accuracy of our chemical-genetic workflow by applying three different approaches. First, we tested correlation of chemical-genetic interaction scores between replicates. We found that the overall correlation was good ($r = 0.63$ from Pearson's correlation) and was in agreement with previous studies, showing reproducibility of chemical-genetic interactions. Second, we randomly selected 19 overexpression strains and determined minimum inhibitory concentrations (MIC) of the interacting AMPs. In total, we performed 92 measurements representing 4-5 AMPs per strain on average. In total, 83% cases showed a MIC difference in the expected direction, demonstrating that the majority of the chemical-genetic interactions can be confirmed by an independent experiment. Third, we compared the chemical-genetic interactions with literature curated list of resistance determinants in *E. coli*. To this end, we collected those genes that provide resistance or sensitivity to AMPs when overexpressed in *E. coli*. We found that 70 % of the 13 cases were in agreement with our chemical-genetic screen. Altogether, these results show the high sensitivity of our workflow in determining chemical-genetic interactions between AMPs and gene overexpression.

2. Chemical-genetic profiles group AMPs with similar mechanisms of action and physicochemical properties

We next tested whether AMPs with similar mechanistic and physicochemical properties cluster together based on their chemical-genetic profiles. We performed consensus clustering analysis using the chemical-genetic interaction profiles of AMPs. This analysis grouped the 15 AMPs into four different clusters (referred to as C1-C4). We found that membrane-targeting and intracellular-targeting AMPs grouped into separate chemical-genetic clusters. Notably, membrane-targeting (C1and C2) and intracellular-targeting AMPs (C3 and C4) can also be differentiated based on their physicochemical properties. Our results suggest that similarity in chemical-genetic profiles reflects similarity in broad mechanisms of action and physicochemical properties of AMPs. Therefore, chemical-genetic profiles can capture subtle differences in how these AMPs interact with the bacterial cell.

3. A large and functionally diverse set of genes modulate bacterial susceptibility to AMPs

Our chemical-genetic screen revealed that a large number of functionally diverse genes modulate AMP resistance in bacteria. These results substantially broaden the scope AMP resistance modulating genes, as a small fraction of resistance genes has been reported previously. Furthermore, we found that resistance-enhancing genes substantially differ across the AMPs with different modes of action. The differences in the chemical-genetic profiles were prominent between AMPs with different modes of action. In particular, resistance-enhancing genes showed significantly low overlap between membrane (C1 and C2)- and intracellular-targeting AMPs (C3 and C4). These results reveal that AMPs differ considerably in their resistance determinants and these differences are shaped by mechanistic action of AMPs.

4. Partial depletion of essential genes reveals the intrinsic AMP resistome

Resistance upon gene overexpression informs on latent resistome while sensitivity upon gene depletion reveals intrinsic resistome. To investigate the intrinsic AMP resistome, we carried out a chemical-genetic screen with a set of 279 partially-depleted essential genes (hypomorphic alleles) of *E. coli*. We found that 75% of tested hypomorphic alleles show chemical-genetic interaction with at least one AMPs. AMPs with similar modes of action showed substantial overlap in their intrinsic resistomes whereas this overlap was significantly lower between functionally dissimilar AMPs. These findings reflected differences in the resistance determinants between AMPs, and showed that mode of action of AMPs shapes both the intrinsic and the latent AMP resistomes.

5. Collateral sensitivity interactions are frequent between functionally dissimilar AMPs

The limited overlap in resistance determinants across AMPs prompted us to hypothesize that some of the gene overexpressions might even show collateral sensitivity between AMPs. Interestingly we found that collateral sensitivity-inducing genes are widespread. Notably, collateral sensitivity interactions were significantly overrepresented between C1, C2 (membrane targeting) and C3, C4 (intracellular targeting) AMP clusters.

6. Perturbed phospholipid trafficking as a mechanism underlying the collateral sensitivity interactions between AMPs

Next, we investigated the underlying molecular mechanism of the observed collateral sensitivity interactions between membrane-targeting and intracellular-targeting AMPs. By studying four different mutants including *mlaD*, a member of clinically relevant phospholipid transport pathway, we demonstrated that decreased negative surface charge and increased membrane potential result in collateral sensitivity between membrane- and intracellular-targeting AMPs. Decreased negative surface charge results in weaker electrostatic interactions between bacterial membrane and membrane-targeting AMPs and therefore confers resistance against membrane-targeting AMPs. At the same time, the increased membrane potential helps in translocation of intracellular-targeting AMPs inside the bacterial cell and in exerting their antimicrobial activity.

7. Chemical-genetic profiles inform on cross-resistance spectra of AMPs

Since bacterial susceptibility can be defined by different gene sets for AMPs with distinct modes of action, we hypothesized that chemical-genetic profiles that differentiate these AMPs can advise on the cross-resistance spectrum. To test our hypothesis, we used our recently published dataset of antimicrobial susceptibility profiles of AMP-evolved lines. By integrating this comprehensive dataset with chemical-genetic profiles of AMPs, we identified that cross-resistance interactions (at 2-fold MIC increase) were significantly overrepresented between exclusively membrane-targeting AMPs or between exclusively intracellular-targeting AMPs. Interestingly, the chemical-genetic clustering provided additional insights into the cross-resistance patterns that could not have been predicted based on the broad mode of action of AMPs. Altogether, these results demonstrate that chemical-genetic profiles capture subtle differences in resistance determinants between AMPs with similar broad modes of action, and therefore inform on cross-resistance patterns between AMPs.

List of publications

MTMT identification number: 10060960

*Kintses B[#], **Jangir PK[#]**, Fekete G[#], Számel M, Méhi O, Spohn R, Daruka L, Martins A, Hosseinnia A, Gagarinova A, Kim S, Phanse S, Csörgő B, Györkei Á, Ari E, Lázár V, Faragó A, Bodai L, Nagy I, Babu M, Pál C, Papp B. (2019). Chemical-genetic profiling reveals cross-resistance and collateral sensitivity between antimicrobial peptides. BioRxiv. doi: 10.1101/542548 **([#]equal first authors).** IF:NA

*Lázár V, Martins A, Spohn R, Daruka L, Grézal G, Fekete G, Számel M, **Jangir PK,** Kintses B, Csörgő B, Nyerges Á, Györkei Á, Kincses A, Dér A, Walter FR, Deli MA, Urbán E, Hegedűs Z, Olajos G, Méhi O, Bálint B, Nagy I, Martinek TA, Papp B, Pál C. (2018). Antibiotic-resistant bacteria show widespread collateral sensitivity to antimicrobial peptides. Nature Microbiology 3, 718–731. https://doi.org/10.1038/s41564-018-0164-0.
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*These publications serve as the basis of this PhD dissertation

Full papers (as a first or co-author) not included in the thesis:

Kintses B, Méhi O, Ari E, Számel M, Györkei Á, **Jangir PK,** Nagy I, Pál F, Fekete G, Tengölics R, Nyerges Á, Lik I, Bálint A, Molnár T, Bálint B, Vásárhelyi BM, Bustamante M, Papp B, Pál C. (2019). Phylogenetic barriers to horizontal transfer of antimicrobial peptide resistance genes in the human gut microbiota. Nature Microbiology 4, 447–458. IF: 14.174

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Declaration

I declare that the data used in the thesis written by Pramod Kumar Jangir reflect the contribution of the doctoral candidate to the article: "*Kintses B[#], **Jangir PK**[#], Fekete G[#], Számel M, Méhi O, Spohn R, Daruka L, Martins A, Hosseinnia A, Gagarinova A, Kim S, Phanse S, Csörgő B, Györkei Á, Ari E, Lázár V, Faragó A, Bodai L, Nagy I, Babu M, Pál C, Papp B. (2019). Chemical-genetic profiling reveals cross-resistance and collateral sensitivity between antimicrobial peptides. BioRxiv. doi: 10.1101/542548 ([#]equal first authors)".

The results reported in the PhD dissertation and the publication were not used to acquire any PhD degree previously. I further declare that the candidate has made a significant contribution to the creation of the abovementioned publication.

Szeged, August 23, 2019

Csaba Pál, Ph.D.