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Ph.D. Thesis

Integrating rational design principles to develop resistance-limiting antibiotics for Gram-negative pathogens

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“Science knows no country, because knowledge belongs to humanity, and is the torch which illuminates the world.”

Louis Pasteur, 1872

1. Introduction

Antimicrobial resistance (AMR) represents one of the most pressing global health challenges of the 21st century. Gram-negative pathogens such as *Escherichia coli*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa* are classified by the WHO as critical-priority organisms due to their high mortality burden and resistance to last-resort antibiotics. Traditional antibiotic discovery pipelines largely rely on derivatives of existing scaffolds, which often succumb rapidly to resistance mechanisms including enzymatic inactivation, target modification, efflux, and reduced permeability. This thesis builds upon recent advances in membrane-targeting strategies and proposes that combining membrane permeabilization with an additional intracellular target in a single antibiotic, so-called dual-target permeabilizers, can constrain resistance evolution.

2. Aims

The main objective of this thesis is to identify the principles that enable the development of antibiotics with a reduced risk of resistance. The focus is on compounds that both disrupt bacterial membrane integrity and inhibit an additional essential cellular process. I propose that these dual-target permeabilizers create stronger evolutionary constraints than antibiotics acting solely on a membrane-associated function or on two intracellular protein targets.

To test this hypothesis:

- Resistance evolution was examined in clinically relevant Gram-negative pathogens (*E. coli, K. pneumoniae, A. baumannii,* and *P. aeruginosa*) exposed to 16 antibiotics, to assess whether dual-target permeabilizers limit resistance emergence.
- The genetic and mechanistic basis of resistance were investigated, including *de novo* target-site or efflux mutations, gene amplification, and horizontally acquired resistance genes.
- The environmental prevalence and transfer potential of resistance determinants were evaluated using functional metagenomic screens from human, soil, and clinical microbiomes.
- Bactericidal activity was characterized by quantifying population dynamics under lethal antibiotic exposure to

determine whether rapid killing contributes to limiting resistance development.

Together, these approaches integrate laboratory evolution, genomic analysis, functional metagenomics, and time-kill experiments to determine whether dual-target permeabilizers can overcome common evolutionary trajectories to resistance and to provide guidance for designing antibiotics with improved long-term efficacy.

3. Methods and Materials

Strains, antibiotics, and media

Antibiotic-sensitive and multidrug-resistant strains of *E. coli, K. pneumoniae, A. baumannii,* and *P. aeruginosa* were used. Sixteen antibiotics representing four modes of action were tested. Experiments were conducted primarily in Mueller–Hinton broth, with modified media where required.

Membrane permeabilization assay

Outer membrane disruption was quantified in *E. coli* using the NPN fluorescence assay, comparing antibiotic-treated cells to untreated and polymyxin B controls.

Frequency-of-Resistance (FoR) assay

High-density bacterial populations were plated on supra-MIC antibiotic concentrations to determine spontaneous resistance frequencies. Resistant colonies were further characterized by MIC testing and whole-genome sequencing.

High-throughput laboratory evolution (ALE)

Parallel bacterial populations were evolved under gradually increasing antibiotic concentrations for 20 passages, generating 728 evolved lines to assess maximum resistance potential.

High-throughput MIC measurements

MICs of ancestral and evolved strains were determined by automated broth microdilution following CLSI guidelines. Resistance level was calculated compared to ancestral strains.

In vitro growth measurements

Growth curves were recorded for selected lines, and area under the curve (AUC) was used as a proxy for relative fitness.

Whole-genome sequencing and variant analysis
Resistant lines were sequenced using Illumina technology. Mutations were identified through read mapping and variant calling, with strict filtering to exclude artifacts and hypermutator strains.

Comparative mutational analysis
Mutated genes were analyzed for pathway enrichment using KEGG and COG annotations to identify common resistance-associated functions.

AcrAB efflux system screening
Efflux-deficient and overexpression strains were tested to assess the contribution of the AcrAB efflux pump to antibiotic susceptibility.

Genome-wide overexpression library screening
The *E. coli* ASKA overexpression library was screened to identify genes whose increased expression confers resistance. Resistant clones were sequenced to identify responsible ORFs.

ORF identification from overexpression library
Sequencing reads were mapped to the reference genome to determine which overexpressed genes were enriched under antibiotic selection.

Functional metagenomic screens
Environmental and clinical metagenomic libraries were screened in *E. coli* and *K. pneumoniae* to identify mobile resistance genes.

Annotation of antibiotic resistance genes (ARGs)

Metagenomic inserts were sequenced, ORFs predicted, and resistance genes identified using CARD and ResFinder databases, followed by redundancy filtering and taxonomic assignment.

Phylogenetic and geographic analysis of ARGs

Over 16,000 *E. coli* genomes were analyzed to determine the prevalence, phylogenetic distribution, and geographic spread of identified resistance genes.

Quantifying bacterial survival under antibiotic exposure

Killing kinetics were measured by exposing bacteria to varying antibiotic concentrations and quantifying survival via CFU counts and fluorescence-based assays.

4. Results

Antibiotic classification

16 antibiotics were classified according to (i) the number of cellular targets and (ii) their membrane-permeabilizing activity. Target number was assigned based on literature, while membrane disruption was tested using the NPN uptake assay, where increased fluorescence indicated outer membrane permeabilization.

This resulted in four categories:

1. *Dual-target (DT) permeabilizers* (tridecaptin M152-P3, POL7306, SCH79797) – preclinical compounds combining membrane disruption with an additional intracellular target; these formed the primary focus of the study.

2. *Single-target (ST) permeabilizers* (e.g., polymyxin B, SPR206) – membrane permeabilizing peptides.

3. *ST non-permeabilizers* – conventional antibiotics with one intracellular target.

4. *DT non-permeabilizers* – dual-target drugs without membrane activity, mainly topoisomerase inhibitors.

This framework enabled systematic comparison of how target number and membrane disruption influence resistance evolution.

Short-term resistance (FoR assays)

Using multidrug-resistant and sensitive strains of *E. coli, K. pneumoniae, A. baumannii,* and *P. aeruginosa*, we measured spontaneous resistance frequencies. Combinations with reduced baseline susceptibility (MIC > 4 μg/mL) were excluded.

Resistance readily emerged against ST permeabilizers, DT non-permeabilizers, and ST non-permeabilizers. In contrast, resistance to DT permeabilizers was rare. Although polymyxin B resistance occasionally increased dramatically (>128-fold), no mutants arose in several strain–drug combinations. As FoR assays may miss extremely rare events, long-term evolution experiments were performed.

Adaptive laboratory evolution (ALE)

Prolonged evolution (60 days) generated higher resistance levels overall, yet DT permeabilizers consistently showed significantly lower maximal resistance compared to other classes. For example, resistance to SCH79797 increased only fourfold in certain strains, whereas polymyxin B resistance exceeded 1,024-fold.

Across comparisons, DT permeabilizers displayed lower median resistance than ST permeabilizers in 69% of cases, with no instance where an ST permeabilizer performed better. Resistance evolution varied among species but overall remained significantly limited for DT permeabilizers ($p < 0.01$).

Fitness costs and mutational landscape

We measured growth of 385 evolved lines in antibiotic-free conditions. For SCH79797 and tridecaptin M152-P3, resistance correlated negatively with fitness (R = –0.79 and –0.55), indicating evolutionary constraints. Similar trade-offs were observed for some ST non-permeabilizers.

Genomic analysis revealed largely antibiotic-specific mutational patterns: 80% of mutated genes were drug-specific, and no mutation was shared across all antibiotics. DT permeabilizer–adapted lines showed distinct mutation profiles compared to ST permeabilizers.

Membrane-targeting drugs frequently selected mutations in cell envelope regulatory systems (e.g., BasRS, PhoPQ) and LPS-related genes. SCH79797 adaptation primarily involved mutations in *acrR*, implicating the AcrAB-TolC efflux system. Functional assays confirmed that efflux modulation affected SCH79797 susceptibility but not POL7306, tridecaptin, or polymyxin B.

Cross-resistance patterns

Polymyxin B–resistant lines displayed cross-resistance to SPR206 and colistin but not to SCH79797 or tridecaptin M152-P3. A subset showed modestly reduced susceptibility to POL7306. Overall, resistance mechanisms against DT permeabilizers exhibited limited overlap with other membrane-targeting antibiotics.

Gene amplification

Screening the ASKA overexpression library showed that gene amplification could increase resistance (up to eightfold) for several antibiotics, often via transcriptional regulators such as *marA* and *soxS*.

However, no overexpressed gene conferred resistance to DT or ST permeabilizers, suggesting that increased gene dosage does not effectively protect against membrane-targeting agents.

Environmental reservoir of resistance

Functional metagenomic screens from soil, gut, and clinical microbiomes identified 1,045 resistance-associated contigs. DT permeabilizers yielded significantly fewer resistance fragments than other classes ($p < 0.05$). Notably, no resistance contigs were detected for tridecaptin M152-P3.

Among identified ORFs, only a few resembled known ARGs, mostly linked to efflux regulation. Screening over 16,000 natural *E. coli* genomes revealed that none of the 14 candidate ARGs associated with DT permeabilizers were present in environmental isolates, whereas resistance genes against other classes were detected in up to 8.4% of genomes.

These findings indicate that mobile resistance determinants against DT permeabilizers are rare in nature.

Killing kinetics

Under 10× MIC exposure for 4 hours, DT permeabilizers ranked among the most potent bactericidal agents. SCH79797

completely eradicated all tested species, whereas colistin achieved full killing only in *P. aeruginosa*.

A significant positive correlation was observed between evolved resistance levels and survival under treatment ($\rho = 0.37$, $p = 0.0019$), suggesting that drugs limiting resistance also exhibit stronger killing. Dose–response analyses showed steep killing curves for both DT and ST permeabilizers.

Overall, dual-target permeabilizers combine rapid bactericidal activity with limited resistance evolution, highlighting their promise as next-generation antibiotics.

5. Discussion

Antibiotic resistance represents a major global health crisis, reversing decades of medical progress and increasing morbidity, mortality, and healthcare costs. As resistance continues to emerge even against newly developed agents and pharmaceutical pipelines decline, identifying the fundamental principles that ensure long-term antibiotic efficacy has become urgent. My PhD research addresses this challenge by proposing and experimentally validating a rational design framework for *"dual-target permeabilizers"*, a novel antibiotic strategy that combines membrane disruption with a second, distinct intracellular mechanism to limit resistance evolution.

To evaluate this concept, we applied two complementary laboratory evolution approaches across four clinically important Gram-negative pathogens (*Escherichia coli, Klebsiella pneumoniae, Acinetobacter baumannii,* and *Pseudomonas aeruginosa*). Short-term experiments selected single-step resistance mutations under high antibiotic concentrations, while long-term adaptive evolution gradually increased drug exposure to promote high-level resistance. In parallel, functional metagenomic screens of environmental and clinical microbiomes were performed to detect mobile resistance genes capable of horizontal transfer. Together, these approaches enabled a comprehensive comparison of resistance trajectories and dissemination risks.

Three preclinical compounds: SCH79797, tridecaptin M152-P3, and POL7306 emerged as strong examples of the dual-target permeabilizer strategy. Each disrupts membrane integrity while engaging a distinct secondary target: SCH79797 activates

and binds the mechanosensitive channel MscL and inhibits folate biosynthesis; tridecaptin M152-P3 binds lipid II; and POL7306 targets the outer membrane protein BamA. Notably, bacterial populations evolved under these drugs exhibited only modest increases in minimum inhibitory concentrations (MICs), with few or no mutations detected in the genes encoding their primary targets. Cross-resistance to conventional antibiotics was rare. For example, polymyxin B–adapted strains remained fully susceptible to dual-target permeabilizers.

When resistance mutations did arise, they often imposed significant fitness costs, reflected in reduced growth rates that may limit their persistence. Unlike many intracellular antibiotics, dual-target permeabilizers were also resilient to resistance via gene amplification, as membrane perturbations are intrinsically deleterious. Functional metagenomic analyses further supported these findings, revealing a striking scarcity of mobile resistance genes against SCH79797, tridecaptin M152-P3, and POL7306 in both environmental and clinical microbiomes.

Dose–response experiments demonstrated steep, bactericidal killing within a narrow concentration window. Although polymyxin B and colistin also exhibit rapid killing, they remain susceptible to resistance via lipopolysaccharide (LPS) modification. In contrast, the additional intracellular targets of dual-target permeabilizers preserve efficacy even when membrane composition changes. Importantly, no target-site mutations were observed in *mscL*, folate pathway genes, lipid II biosynthesis genes, or *bamA*. Efflux-mediated resistance was

minimal, with only modest AcrAB-TolC upregulation detected in SCH79797-adapted lines. By comparison, dual-target topoisomerase inhibitors readily evolved high-level resistance (median 128-fold MIC increase) through target mutations and efflux activation.

Overall, these results establish key principles for designing resistance-evading antibiotics: integrating membrane permeabilization with a second essential target constrains evolutionary pathways, imposes fitness costs on resistant mutants, and reduces the likelihood of horizontal gene transfer. The mechanosensitive MscL channel represents a particularly promising target due to its conservation in bacteria, absence in mammals, and activity in both growing and dormant cells. Overall, this work provides both mechanistic insight and a practical framework for next-generation antibiotic development, offering a rational path toward more sustainable therapies against multidrug-resistant pathogens.

Összefoglaló

A több sejtfunkciót célzó antibiotikumok várhatóan kevésbé hajlamosak a bakteriális rezisztencia kialakulására. Feltételezésünk szerint a kettős célzás elengedhetetlen, de önmagában nem elegendő a rezisztencia megelőzéséhez. Csak azok az antibiotikumok esetében figyelhető meg csökkent mértékű rezisztencia-kialakulás, amelyek egyszerre célozzák a membrán integritását, és egy másik sejtútvonalat is blokkolnak. A hipotézis teszteléséhez három antibiotikum-jelöltre koncentrálunk: a POL7306-ra, a Tridecaptin M152-P3-ra és a SCH79797-re, amelyek mindegyike megfelel a fenti kritériumoknak.

A jelen doktori dolgozat bemutatja, hogy az ESKAPE kórokozók, köztük az *Escherichia coli*, a *Klebsiella pneumoniae*, az *Acinetobacter baumannii* és a *Pseudomonas aeruginosa*, esetében korlátozott az antibiotikumokkal szembeni rezisztencia kialakulása, míg a kettős célpontú topoizomeráz-antibiotikumok esetében gyakoribb a rezisztencia megjelenése. Számos sejtmechanizmust azonosítottunk, amelyek gátat szabhatnak a rezisztencia kialakulásának.

Először is, a *de novo* mutációk csak korlátozott mértékben növelik a rezisztenciát, beleértve azokat is, amelyek az adott antibiotikum molekuláris célpontját vagy az effluxpumpákat érintik. Másodszor, a rezisztencia génamplifikáció révén nem érhető el. Harmadszor, funkcionális metagenomikai módszerekkel kimutattuk, hogy a mobil rezisztenciagének ritkák az emberi bélben, a talajban és a klinikai mikrobiomokban. Végül, a membráncélzó antibiotikumok

magas koncentrációjának való kitettség a baktériumpopulációk gyors kiirtásához vezetett. Összességében ez a munka mechanisztikus betekintést és gyakorlati keretrendszert nyújt a következő generációs antibiotikumok fejlesztéséhez, és ésszerű utat kínál a több gyógyszerrel szemben rezisztens kórokozók elleni fenntarthatóbb terápiák felé.

List of publications

Number of scientific publications: 4 (+ 2 pre-print)

Number of citations: 132

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Peer-reviewed publications

* **Maharramov, E**., Czikkely, M. S., Szili, P., Farkas, Z., Grézal, G., Daruka, L., Kurkó, E., Mészáros, L., Daraba, A., Kovács, T., Bognár, B., Juhász, S., Papp, B., Lázár, V., & Pál, C. *Exploring the principles behind antibiotics with limited resistance.* Nat. Commun. 16, 1842 (2025). IF: 17.2

Daruka, L., Czikkely, M. S., Szili, P., Farkas, Z., Balogh, D., Grézal, G., **Maharramov, E.**, Vu, T.-H., Sipos, L., Juhász, S., Dunai, A., Daraba, A., Számel, M., Sári, T., Stirling, T., Vásárhelyi, B. M., Ari, E., Christodoulou, C., Manczinger, M., Enyedi, M. Z., Jaksa, G., Kovács, K., van Houte, S., Pursey, E., Pintér, L., Haracska, L., Kintses, B., Papp, B., & Pál, C. *ESKAPE pathogens rapidly develop resistance against antibiotics in development in vitro.* Nat. Microbiol. 10, 313–331 (2025). IF: 20.7

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***** *This publication serves as the basis of this PhD dissertation.*

Preprints

Szili, P., Daruka, L., Spohn, R., Vu, T.-H., **Maharramov, E.**, Grézal, G., Czikkely, M., Csernyák, M., Vonyó, A., Benedek, B., Daraba, A., Judák, F., Martins, A., Balogh, D., Csörgő, B., Dunai, A., Kovács, T., Ábrahám, A., Lázár, V., Stirling, T., Vásárhelyi, B., Kintses, B., Réthi-Nagy, Z., Juhász, S., Papp, B., & Pál, C. *A comprehensive analysis reveals biocides with limited resistance in ESKAPE pathogens.* (2025). Under review in Nature Microbiology.

Kalapis, D., Kovács, K., Balogh, D., **Maharramov, E.**, Ajibola, W., Silander, O., Fehér, T., Pál, C., & Papp, B. *Gene loss promotes the evolution of metabolic innovations through transcriptional rewiring.* (2025). Under review in PLOS Biology.

Declaration

I declare that the data used in the thesis written by Elvin Maharramov reflect the contribution of the doctoral candidate to the article: "***Maharramov, E.**[#], Czikkely, M. S.[#], Szili, P., Farkas, Z., Grézal, G., Daruka, L., Kurkó, E., Mészáros, L., Daraba, A., Kovács, T., Bognár, B., Juhász, S., Papp, B., Lázár, V., & Pál, C. Exploring the principles behind antibiotics with limited resistance. Nat. Commun. 16, 1842 (2025). (#equal first authors)".

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

Szeged, February 12, 2026

Supervisor:

Csaba Pál, Ph.D.