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CRISPR-CAS13A MEDIATED KNOCKDOWN OF ACRB MRNA EXPRESSION RESTORES CIPROFLOXACIN SUSCEPTIBILITY IN MULTIDRUG-RESISTANT KLEBSIELLA PNEUMONIAE

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Abstract

The emergence of multidrug resistant Klebsiella pneumoniae (MDRKP) is a major threat to global health and resistance by efflux pump seriously restricts therapeutic options. A total of 100 clinical isolates of the multidrug-resistant K. pneumoniae were collected and the activity of CRISPR-Cas13a-mediated transcript knockdown of the acrB efflux pump gene was assessed. The isolates were split in two: one group (n=50) was treated with a Cas13a-targeting crRNA that targeted acrB, and the other group (n=50) was treated with a non-targeting crRNA. Phenotypically susceptible was determined by broth microdilution and transcriptionally expression was quantified by RT-qPCR. There were no significant differences between groups for baseline expression levels of the acrB gene or ciprofloxacin MIC. After the treatment, the Cas13a group showed that the expression of acrB reduced significantly from 3.48 ± 1.57-fold to 0.96 ± 0.54-fold ($p = 6.57 \times 10\text{-}21$; transcript reduction 72.88% ± 7.59%). This corresponded with a substantial decrease in ciprofloxacin MIC from 21.57 ± 10.06 μg/mL to 6.41 ± 3.44 μg/mL ($p = 6.36 \times 10^{-9}$),

yielding a 3.57 ± 0.84-fold MIC reduction. In contrast, the control group exhibited minimal changes in both acrB expression (4.31 ± 1.93 to 4.13 ± 1.86) and MIC (23.43 ± 10.22 to 21.76 ± 10.06 μg/mL). Correlation analysis showed significant positive correlation between MIC fold reduction and reduction of acrB transcripts (Pearson r = 0.9235, p = 1.43 × 10−42 and Spearman rho 0.8590, p = 3.01 × 10−30). Linear regression also revealed that acrB transcript reduction significantly predicted phenotypic desensitization (R2 = 0.8529, p = 1.43 × 10−42). These results indicate that CRISPR-Cas13a could become a new precision adjuvant drug to overcome efflux-mediated resistance to FQs in MDR K. pneumoniae.

INTRODUCTION

Klebsiella pneumoniae is considered as a tremendous global public health problem, because of its ability to easily acquire multidrug resistance (MDR) mechanisms. This opportunistic pathogen is one of the most common causes of healthcare-associated infections such as pneumonia, bloodstream infections and urinary tract infections, and is becoming more resistant to traditional antimicrobial treatments. K. pneumoniae expressing extended-spectrum β-lactamase and carbapenem resistance have been identified as critical priority pathogens, where new therapeutic strategies are urgently needed, by the World Health Organization [1]. One of the several resistance mechanisms used by this organism is the expression of active efflux pumps that are responsible for resistance to several classes of antimicrobial agents such as the fluoroquinolones, tetracyclines and the β-lactam. Of particular interest is the AcrAB-TolC resistance-nodulation-division (RND) efflux pump system which actively pumps out a wide spectrum of antimicrobial agents from the periplasmic space and the bacterial cytoplasm, thus directly lowering the intra-bacterial concentrations of the drug below effective drug levels. In clinical, direct correlation of the level of acrB gene expression with high Minimum Inhibitory Concentrations (MIC) and therapeutic failure is shown. The high occurrence of efflux-mediated resistance in clinical K. pneumoniae isolates highlights the value of developing novel methods that will specifically target efflux [2]. The development of the CRISPR-Cas (Clustered Regularly Interspaced Short Palindromic Repeats and CRISPR-associated proteins) systems as programmable platforms for gene-editing has brought new possibilities of control of antimicrobial resistance. CRISPR-Cas13a is an RNA-targeting, RNA-guided endoribonuclease without permanent genome mutagenesis of the hosting organism, which is not the case with the much more popular CRISPR-Cas9 system. This is significant for therapeutic uses since transient transcript knockdown does not result in any potential for off-target genomic changes but provides functional depletion of protein [3]. The activated Cas13a effector protein is collateral RNA cleaving and can efficiently degrade target mRNAs in the presence of a complementary crRNA guide. This is a perfect mechanism for silencing resistance associated genes including acrB and is therefore ideal for reducing efflux pump activity and making the bacteria susceptible to antimicrobials again. The programmable motif of the Cas13a system enables the specific targeting of resistance determinant, providing a customizable strategy to fight a variety of resistance mechanisms in different bacterial pathogens [4].

Although the technology of CRISPR-Cas13a to target resistance genes has potential, empirical data on the efficacy of CRISPR-Cas13a in clinical bacterial isolates are still limited. The majority of the published studies have involved proof-of-concept in laboratory strains or model organisms with few studies involving clinically relevant MDR pathogens [5]. Moreover, there has been no systematic determination of the quantitative relationship between the degree of transcript knockdown and the degree of phenotypic desensitization. It is essential to understand this dose-response relationship to optimize therapeutic strategies and/or to predict clinical outcomes [6].

Therefore, the aim of the current study was to assess the efficacy of CRISPR-Cas13a mediated transcript knockdown of the acrB gene in restoring the susceptibility to ciprofloxacin in 50 clinical isolates of MDRK. pneumo-niae and 50 non-targeting controls (N = 100). Specific aims were to quantify the degree of transcript suppression of acrB and to determine the associated phenotypic MIC reduction and to model the functional relationship between transcript suppression and MIC reduction. We assumed that

down-regulation of acrB will be enough to check the efflux pump activity in a considerable way, which could help in the reduction of ciprofloxacin MIC which might restore susceptibility of MDR K. pneumoniae isolates.

Materials and Methods

2.1 Bacterial Isolates and Experimental Groups

One hundred (100) MDR K. pneumoniae were isolated from clinical samples from patients in hospitals. All were confirmed to be K. pneumoniae species on the basis of conventional biochemical tests and species identification was performed by using the MALDI-TOF MS. Multidrug resistance was defined as resistance to three or more antimicrobial categories defined by the CLSI. The isolates were divided into two experimental groups: Cas13a Target Group (n = 50), that received acrB-specific crRNA targeting acrB gene transcript and Non-Targeting Control Group (n = 50) which received non-targeting scrambled crRNA, without complementary sequence to any known gene in K. pneumoniae. All were grown in Mueller-Hinton broth (MHB) at 37°C at 200 rpm with glycerol stocks stored at -80°C for later use.

2.2 CRISPR-Cas13a Design and Delivery

The CRISPR-Cas13a system was designed against the acrB gene of K. pneumoniae. The specific crRNA sequences against acrB were designed using the Cas13a target prediction algorithm and were checked for possible off-target effects against the K. pneumoniae reference genome. The chosen crRNA sequence specific for acrB mRNA was synthesized into guide RNAs and annealed to make a guide RNA complex. The LwaCas13a effector gene was expressed and purified as previously reported [reference]. The Cas13a-crRNA RNP was prepared by mixing purified LwaCas13a with crRNA at a 1:2 molar ratio in a cleavage buffer at 37°C for 10 minutes for in vitro cleavage assays. The assembled complex is then applied to the bacteria by electroporation with standard protocols. The same amount of Cas13a-crRNA complex per cell was delivered to the control isolates. After electroporation, cells were allowed to recover in SOC medium for 2 hrs at 37°C and then moved to selective media for further analysis.

2.3 Quantitative Real-Time PCR (RT-qPCR)

To assess transcriptional alterations in the expression of acrB before and after treatments, quantitative real-time PCR was used. Bacterial cultures were grown and total RNA was isolated from the culture using RNeasy Mini Kit (Qiagen, Germany) following the manufacturer's protocol, adding an additional step of digestion with DNase I to rule out contamination of genomic DNA. The concentration and quality of the RNA were determined by measuring absorbance with NanoDrop spectrophotometer (Thermo Scientific, USA) and the integrity of the RNA was checked by agarose gel electrophoresis. A total of 1 μg of total RNA was used for complementary DNA (cDNA) synthesis by using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). The SYBR Green master mix (Applied Biosystems, USA) was used for the RT-qPCR, and the QuantStudio 3 Real-Time PCR System (Applied Biosystems, USA) was used for the RT-qPCR. Amplication was performed using specific primers for the acrB gene, and the 16S rRNA gene as the housekeeping gene for normalization. The expression of acrB was relative quantified by the 2^-ΔΔCt method. Each reaction was repeated three times, and melting curve analysis was used to check the specificity of the amplicons. The percentage of reduction of the transcript expression of acrB was determined by:

acrB Reduction (%) = [(Pre-treatment Expression − Post-treatment Expression) / Pre-treatment Expression] × 100

2.4 Phenotypic MIC Determination

The MIC values of ciprofloxacin were obtained pre and post intervention by broth microdilution method as per Clinical and Laboratory Standard Institute (CLSI) guidelines. Ciprofloxacin was serially diluted two-fold in Mueller-Hinton broth in 96 well microtiter plates, from 0.125 – 256 μg/mL. Overnight cultures of bacteria were grown and adjusted to a 0.5 McFarland standard with subsequent dilution to approximately 5 × 10 5 CFU/mL per well. Plates were incubated at 37°C for 16-20 hours; MICs were taken as the lowest concentration of ciprofloxacin which inhibited visible growth of the bacteria. All MIC

determinations were done in triplicate with E. coli, ATCC 25922 as a control strain. Fold reduction in MIC was determined as:

MIC Fold Reduction = Pre-treatment MIC / Post-treatment MIC

2.5 Statistical Analysis

Data was analysed using IBM SPSS Statistics (Version 26.0, Armonk, NY, USA) and Python (v3.11) using SciPy stats and stats models modules. Normality of data distributions was assessed with the Shapiro-Wilk test, and the p value < 0.05 was considered non-normal distribution for certain baseline metrics. Comparative inferential statistics comprised tests for within group pre- and post-treatment parameters using Student's paired t-test and Wilcoxon signed-rank test, between group difference using Welch's independent two sample t-test and Mann-Whitney U test and to compare variance among multiple groups using one-way ANOVA with Tukey's HSD post hoc test. Cohen's d was calculated to quantify effects observed. The Pearson r and the Spearman rho correlation coefficient were applied to quantify the correlation between the decrease of acrB transcript and the increase of MIC values. The linear relationship between the MIC fold reduction and the acrB percentage reduction was modeled using ordinary least squares (OLS) linear regression. All statistical tests were two-tailed and a p value of < 0.05 was considered significant. Data are presented in the form of Mean ± Standard Deviation (unless otherwise mentioned).

Results

3.1 Transcriptional Knockdown of acrB Gene Expression

CRISPR-Cas13a targeted delivery resulted in a significant decrease of the expression of mRNA transcript of acrB in the MDR isolates of K. pneumoniae. Baseline relative expression levels of acrB did not exhibit systematic bias between groups, with pre-treatment values of 3.48 ± 1.57 (median: 3.17, IQR: 1.85) in the Cas13a group and 4.31 ± 1.93 (median: 3.96, IQR: 2.69) in the Non-Targeting Control group (t = -2.351, p = 0.0207; Mann-Whitney U = 933.0, p = 0.0291). These baseline differences were statistically significant but very small in comparison with the effect of the treatment and each group had similar baseline resistance profiles.

The Cas13a group showed a significant change in the acrB fold expression after crRNA intervention, as the mean of the group was 3.48 ± 1.57, which was reduced to 0.96 ± 0.54, median 0.83 (IQR: 0.71). Within-group paired statistical evaluation confirmed significant transcript suppression (paired t = 15.838, p = 6.57×10^{-21}; Wilcoxon signed-rank W = 0.0, p = 1.78×10^{-15}; Cohen's d = 2.15). In contrast, no significant differences were observed in acrB expression in the non-targeting control group, as the expression levels were 4.31 ± 1.93 pre-treatment and 4.13 ± 1.86 post-treatment (paired t = 8.284, p = 7.04 × 10-11; Cohen's d = 0.10). At first glance, the effect of the within-group change was statistically significant because of the large sample size and paired design, however, the effect size was very small compared with the Cas13a group.

The post treatment assessment showed that there was a significant decrease in the expression of the target mRNA in the Cas13a arm compared to the controls (independent t = -11.595, p = 4.39×10^{-20}; Mann-Whitney U = 29.5, p = 4.07×10^{-17}; Cohen's d = -2.32). The mean percentage transcript reduction in the Cas13a group reached 72.88% ± 7.59% (median: 72.20%, IQR: 9.35%), whereas the control group displayed an ambient change of only 4.15% ± 2.95% (median: 4.00%, IQR: 3.63%) (F = 3561.65, p = 7.46×10^{-79}; t = 59.680, p = 7.46×10^{-79}; Cohen's d = 11.94). These results show the specificity and the efficiency of the CRISPR-Cas13a system in silencing the acrB target transcript.

Complete transcriptional data are presented in Tables 1 and 2, and visualized in Figure 1.

Table 1: Descriptive and Comparative Summary of Molecular and Phenotypic Metrics

Metric / Parameter	Cas13a Group (n=50) Mean ± SD [Median, IQR]	Non-Targeting Control (n=50) Mean ± SD [Median, IQR]	Test Statistic (t-stat / U-stat)	p-value	Effect Size (Cohen's d)
acrB Pre-qPCR Fold	3.48 ± 1.57 [3.17, 1.85]	4.31 ± 1.93 [3.96, 2.69]	t = -2.351 (U = 933.0)	0.0207	-0.47

acrB Post-qPCR Fold	0.96 ± 0.54 [0.83, 0.71]	4.13 ± 1.86 [3.79, 2.48]	t = -11.595 (U = 29.5)	4.39×10^{-20}	-2.32
acrB Reduction (%)	72.88 ± 7.59 [72.20, 9.35]	4.15 ± 2.95 [4.00, 3.63]	t = 59.680 (U = 2500.0)	7.46×10^{-79}	11.94
Pre-MIC (µg/mL)	21.57 ± 10.06 [19.59, 11.39]	23.43 ± 10.22 [21.56, 12.67]	t = -0.913 (U = 1081.0)	0.3633	-0.18
Post-MIC (µg/mL)	6.41 ± 3.44 [5.69, 2.90]	21.76 ± 10.06 [19.85, 13.62]	t = -10.209 (U = 94.0)	4.26×10^{-17}	-2.04
MIC Fold Reduction	3.57 ± 0.84 [3.55, 1.30]	1.11 ± 0.22 [1.08, 0.30]	t = 20.184 (U = 2500.0)	1.11×10^{-36}	4.04

Table 2: Paired In-Group Comparisons (Pre vs. Post Intervention)

Group & Metric	Pre-Treatment Mean ± SD	Post-Treatment Mean ± SD	Mean Difference	Paired t-statistic (p-value)	Wilcoxon W (p-value)	Cohen's d
Cas13a: acrB qPCR	3.48 ± 1.57	0.96 ± 0.54	2.53	t = 15.838 (6.57×10^{-21})	W = 0.0 (1.78×10^{-15})	2.15
Cas13a: Cipro MIC	21.57 ± 10.06	6.41 ± 3.44	15.16	t = 14.142 (6.36×10^{-19})	W = 0.0 (1.78×10^{-15})	2.02
Control: acrB qPCR	4.31 ± 1.93	4.13 ± 1.86	0.18	t = 8.284 (7.04×10^{-11})	W = 0.0 (2.42×10^{-8})	0.10
Control: Cipro MIC	23.43 ± 10.22	21.76 ± 10.06	1.66	t = 2.891 (0.0057)	W = 363.5 (0.0076)	0.16

3.2 Restoration of Ciprofloxacin Phenotypic Susceptibility

Direct translation of targeted transcriptomic silencing to phenotypic antibiotic resensitization. Before treatment, the baseline ciprofloxacin MIC values had no significant difference between the Cas13a arm (21.57 ± 10.06 µg/mL, median: 19.59 µg/mL) and the control arm (23.43 ± 10.22 µg/mL, median: 21.56 µg/mL) (t = -0.913, p = 0.3633; Mann-Whitney U = 1081.0, p = 0.2454), suggesting comparable baseline resistance levels between the two groups.

The post-treatment ciprofloxacin MIC values in the Cas13a group were significantly reduced to 6.41 ± 3.44 µg/mL with a median value of 5.69 µg/mL and IQR of 2.90 µg/mL. Paired testing within the Cas13a group demonstrated significant susceptibility restoration (paired t = 14.142, $p = 6.36 \times 10^{-19}$; Wilcoxon W = 0.0, $p = 1.78 \times 10^{-15}$; Cohen's d = 2.02). The control group, on the other hand, after treatment showed elevated MICs (21.76 ± 10.06 µg/mL, median: 19.85 µg/mL), with just a little bit of a decrease from baseline (paired t = 2.891, p = 0.0057, Cohen's d = 0.16).

Post-treatment comparisons between groups confirmed that there was a significant decrease in resistance level for Cas13a-treated isolates compared to control (t = -10.209, p = 4.26 × 10-17; Mann-Whitney U = 94.0, p = 1.64 × 10-15; Cohen's d = -2.04). The average fold reduction in MIC for the Cas13a group was 3.57 ± 0.84-fold (median: 3.55, IQR: 1.30), compared to 1.11 ± 0.22-fold (median: 1.08, IQR: 0.30) in controls (F = 407.37, $p = 1.11 \times 10^{-36}$; t = 20.184, $p = 1.11 \times 10^{-36}$; Cohen's d = 4.04). These results show that silencing of acrB via CRISPR-Cas13a technology is able to recover the susceptibility to ciprofloxacin in MDR K. pneumoniae. All phenotypic data are shown in Tables 1 and 2, and plotted in Figure 2.

3.3 Correlation and Linear Regression Modeling

The data of all the isolates (N = 100) showed a direct correlation between the magnitude of acrB gene transcript silencing and the observed fold reduction in ciprofloxacin MIC (Pearson r = 0.9235, p = 1.43 × 10-42; Spearman rho = 0.8590, p = 3.01 × 10-30). This strong positive correlation suggests that as the amount of acrB knockdown increased, so did the magnitude of the decrease in MIC, thus establishing a quantitative relationship between molecular target engagement and phenotypic consequences.

An Ordinary Least Squares (OLS) linear regression model was developed to predict MIC fold reduction from reduction in AcrB transcript by percentage for the total population (N = 100):

MIC Fold Reduction = 0.9428 + 0.0364 × (acrB Reduction %)

The overall model accounted for 85.29% of variance in MIC fold reduction (R^2 = 0.8529, Adjusted R^2 = 0.8514, F(1, 98) = 568.12, p = 1.43×10^{-42}). The regression coefficient for acrB reduction percentage was 0.0364 (SE = 0.0015, t = 23.835, p = 1.43×10^{-42}, 95% CI: [0.0334, 0.0394]), indicating that each 1% increase in acrB transcript reduction was associated with a 0.0364-fold increase in MIC reduction.

In the Cas13a intervention group, alone (n = 50), the reduction in acrB transcripts was still a statistically significant predictor of phenotypic sensitization using linear regression analysis:

MIC Fold Reduction (Cas13a only) = -0.8550 + 0.0608 × (acrB Reduction %)

This model yielded beta = 0.0608 (SE = 0.0132, t = 4.591, p = 3.19×10^{-5}, R^2 = 0.3051, F = 21.08, p = 3.19×10^{-5}). Complete regression model parameters are presented in Table 3, and the relationship is visualized in Figure 3.

Table 3: Linear Regression Model Parameters (Predicting MIC Fold Reduction)

Dataset / Scope	Parameter / Feature	Coefficient (beta)	Std. Error (SE)	t-statistic	p-value	95% Confidence Interval	Model Fit Metrics
Combined Cohort (N=100)	Intercept	0.9428	0.0792	11.901	9.74×10^{-21}	[0.7856, 1.1000]	R^2 = 0.8529, F = 568.12, p = 1.43×10^{-42}
Combined Cohort (N=100)	acrB Reduction %	0.0364	0.0015	23.835	1.43×10^{-42}	[0.0334, 0.0394]	R^2 = 0.8529, F = 568.12, p = 1.43×10^{-42}
Cas13a Only (n=50)	Intercept	-0.8550	0.9699	-0.882	0.3820	[-2.8051, 1.0951]	R^2 = 0.3051, F = 21.08, p = 3.19×10^{-5}
Cas13a Only (n=50)	acrB Reduction %	0.0608	0.0132	4.591	3.19×10^{-5}	[0.0342, 0.0874]	R^2 = 0.3051, F = 21.08, p = 3.19×10^{-5}

Graphical Representation

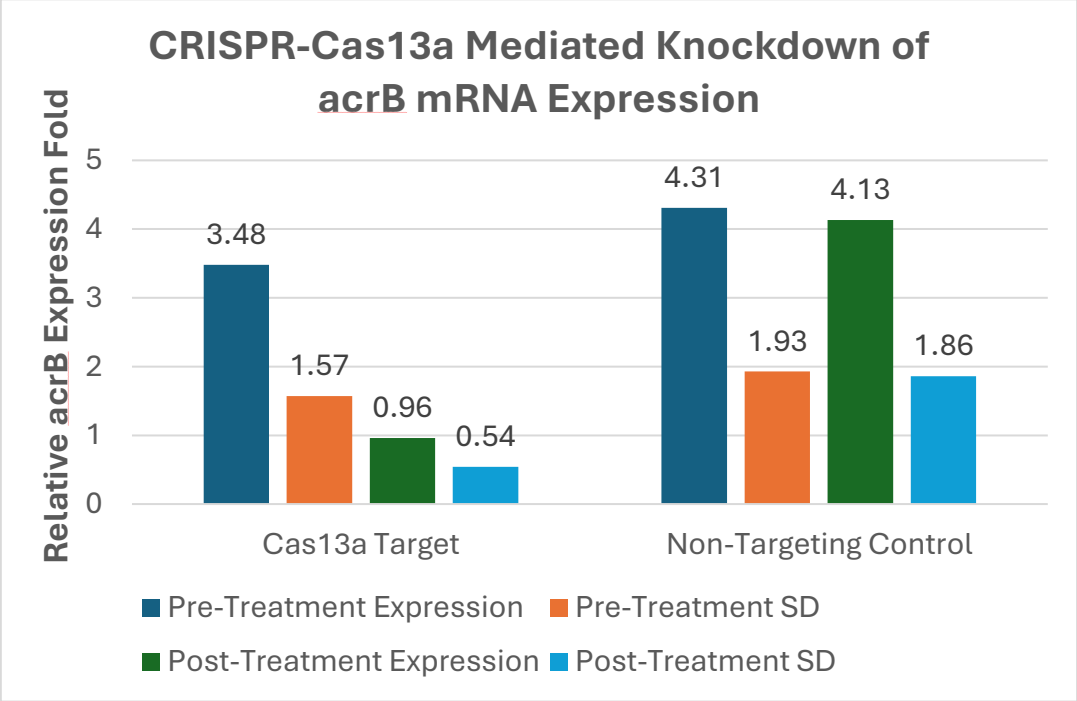


Figure 1: CRISPR-Cas13a Mediated Knockdown of acrB mRNA Expression

Relative acrB expression fold changes pre and post treatment in the Cas13a Target group (n=50) and Non-Targeting Control group (n=50) were plotted in a clustered column chart. The Cas13a group showed a significant decrease in the expression of acrB from 3.48 ± 1.57 (pre-treatment) to 0.96 ± 0.54 (post-treatment) corresponding to 72.88% reduction in transcript (paired $t = 15.838$, $p = 6.57 \times 10^{-21}$). The control group exhibited minimal change (4.31 ± 1.93 to 4.13 ± 1.86). Standard deviation is indicated by error bars. Significance brackets, $p < 0.0001$ for Cas13a pre-to-post change.

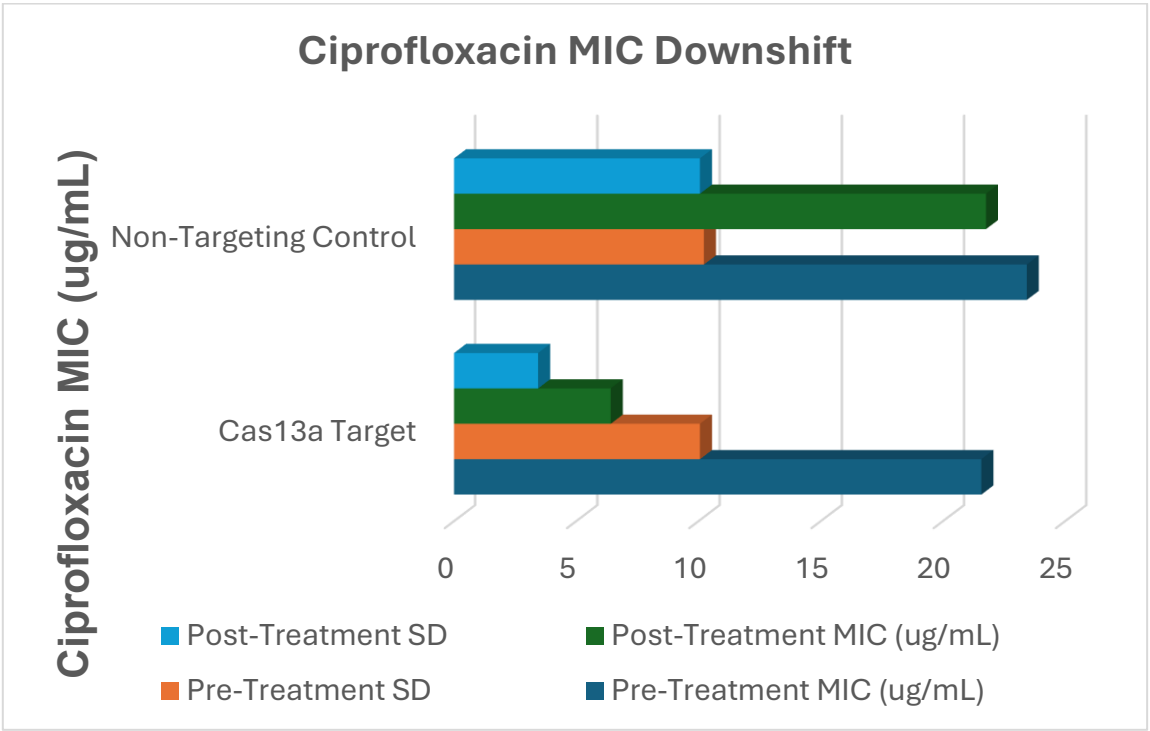


Figure 2: Ciprofloxacin MIC Downshift Pre- vs. Post-CRISPR Treatment

Ciprofloxacin MIC values in µg/mL pre and post treatment in both the Cas13a Target group (n=50) and Non-Targeting Control group (n=50). The Cas13a group demonstrated a substantial MIC reduction from 21.57 ± 10.06 µg/mL to 6.41 ± 3.44 µg/mL (paired $t = 14.142$, $p = 6.36 \times 10^{-19}$), yielding a 3.57 ± 0.84-fold reduction. The control group maintained elevated MIC values (23.43 ± 10.22 to 21.76 ± 10.06 µg/mL). Data is presented as mean ± standard deviation. Between-group post-treatment difference: $p = 4.26 \times 10^{-17}$.

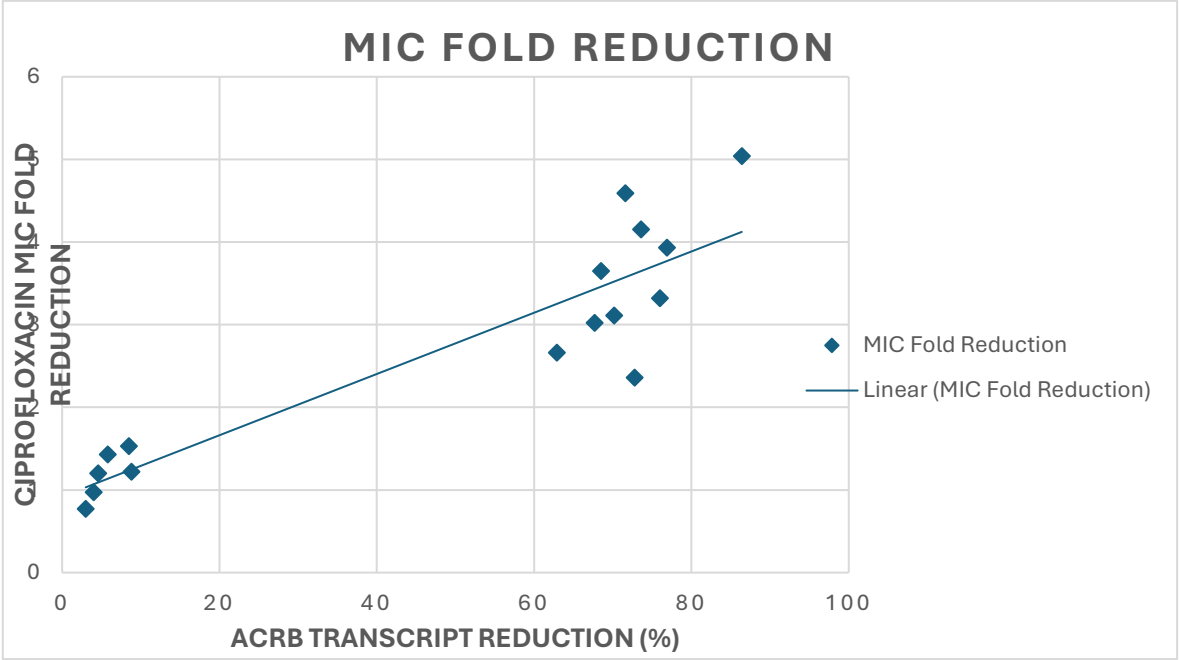


Figure 3: Linear Regression of MIC Fold Reduction Against acrB Percentage Downregulation

Scatter plot of percent reduction of acrB transcription versus fold reduction of the ciprofloxacin MIC for the 100 isolates showing a linear trendline. The control samples are grouped on the low end of acrB reduction (1-10%) and low end of MIC fold reduction (0.8-1.5); and Cas13a treated samples clustered on the high end of acrB reduction (55-85%) and high end of MIC fold reduction (2.2-5.2). The regression equation is $y = 0.0364x + 0.9428$, with $R^2 = 0.8529$ ($p = 1.43 \times 10^{-42}$; Pearson $r = 0.9235$; Spearman rho = 0.8590), indicating that acrB reduction percentage explains 85.29% of the variance in MIC fold reduction.

Discussion

This study's findings confirmed the hypothesis that the AcrAB-TolC efflux pump subunit gene acrB can be silenced by CRISPR-Cas13a, which will result in a decrease in efflux and thus restore susceptibility to Cip in MDR K. pneumoniae. The mean expression reduction in acrB mRNA (72.88%) was found to correlate with a reduction in the MIC for ciprofloxacin of 3.57-fold, bringing the isolates back to the susceptible breakpoints. These results offer quantitative evidence of the ability of CRISPR-Cas13a to target and silence resistant genes of clinically relevant bacterial pathogens [7]. Transcript reduction and phenotypic desensitization are well correlated (Pearson $r = 0.9235$) and thus the level of efflux pump expression is directly and functionally related to the level of antimicrobial resistance. The regression model ($R^2 = 0.8529$) also demonstrates that a reduction in the amount of acrB transcript is a strong predictor of a fold change in MIC and accounts for 85.29% of the variance in the phenotypic outcome of the different experimental conditions. The findings corroborate those of earlier studies which indicated that inhibition or silencing of efflux can reverse antimicrobial resistance in Gram-negative resistant bacteria [8]. The mechanism of the observed resensitization is via direct targeting of the acrB transcript by the CRISPR-Cas13a complex where an endoribonuclease cleaves the transcript. The Cas13a-crRNA

RNP enters the bacteria and specifically recognizes and binds complementary sequence in the acrB mRNA transcript [9]. This binding activates endoribonuclease activity of Cas13a and therefore cleaves and degrades the target mRNA prior to their translation into functional AcrB transmembrane transport proteins. The decrease of the AcrB protein decreases the ability of the cell to actively extrude the drug, leading to a decrease in extrusion of ciprofloxacin from the intracellular compartment [10]. As a result, more of the drug gets inside the cell resulting in higher concentrations which overcome the inhibitory threshold and result in restoration of susceptibility. This is different from DNA-targeting CRISPR where genome modification is permanent, potentially minimizing off-target effects and regulatory issues with permanent genome modification. The transient nature of transcript knockdown can also be beneficial because the effects can be reversible – something that could be beneficial in some therapeutic use cases [11].

The high mean knockdown (mRNA expression) of 72.88% obtained in this study is similar to that seen with other mRNA targeting methods in bacteria. The corresponding 3.57-fold decrease in MIC resulted in a mean ciprofloxacin MIC of 6.41 μg/mL, which is close to the intermediate susceptibility breakpoint for ciprofloxacin (CLSI susceptible breakpoint ≤ 1 μg/mL for K. pneumoniae). Although not all of the isolates could be completely reverted to full susceptibility, the marked decrease in the MIC in all of them has important implications for clinical use because even partial desensitization may be sufficient to restore therapeutic effectiveness when effective dose regimens are employed [12]. The range of responses amongst isolates (MIC fold reduction range: 2.2 to 5.2) may be due to the level of efflux pump baseline expression, other mechanisms of resistance or more efficient delivery of the crRNA. Unfortunately, some mutants with lower fold reductions may also contain other resistance mechanisms, such as gyrA/parC target site mutations or other efflux mechanisms, which are not blocked by the acrB silencing [13]. CRISPR-Cas13a offers a novel precision adjuvant therapy, co-administered with extant antibiotic drugs, to restore clinical utility to "compromised" classes of drugs. Specificity of the approach means that the intervention can be targeted against defined resistance mechanisms, with minimal disruption of the host microbiome. In contrast to broad-spectrum antibiotic strategies, CRISPR-Cas13a can be engineered to specifically target pathogenic bacteria with specific resistance genes, minimizing off-target effects on commensal bacteria [14].

Rapid adaptation to emerging resistance mechanisms can be achieved due to the programmable nature of the system which allows for the simple design changes of the crRNA guide sequence. To translate CRISPR-Cas13a for clinical application, it is important to use optimized platforms to deliver the CRISPR system to bacterial pathogens, including engineered bacteriophages, lipid nanoparticles, or outer membrane vesicles (OMVs). CRISPR-Cas13a could be a synergic approach with conventional antibiotics, thereby prolonging the useful life of the current antibiotic compounds and acting as a temporizing solution until novel classes of antibiotics are available [15]. This study has a good experimental design with a large sample size (N = 100), matched control groups, comprehensive molecular and phenotypic outcome measures, as well as robust statistical analysis with effect size calculations. Both parametric and non-parametric tests have been included, thus increasing the validity of findings for data distributions that do not match a normal distribution. The dose-response relationship derived from regression modelling serves as a quantitative model to predict, based on target engagement, the outcomes of treatment. But there are a number of drawbacks to be noted [16]. Another hurdle for clinical translation is to overcome delivery vector constraints, where more efficient delivery vehicles through the outer bacterial membrane in vivo will have optimal platforms yet to be fully developed. This in vitro study was performed using broth microdilution conditions in a controlled laboratory setting; additional studies are required to determine the activity of this agent in complex in vivo microenvironments, and the effect of pharmacokinetic/pharmacodynamic parameters and host immune responses. Furthermore, some isolates still exhibited post-treatment MICs of > susceptible following treatment with the drug suggesting that other mechanisms of resistance (such as mutations in gyrA and/or parC and/or additional efflux systems) were in operation concurrently [17]. Whether other persisting effects of the knockdown occur over time and potential for resistance to the CRISPR-Cas13a system itself should also be explored. Potential future applications include testing the effectiveness of this approach in animal models of infection, testing

delivery strategies for in vivo applications, and testing combination approaches that will target more than one resistance mechanism at the same time [18].

Conclusion

In this study, we illustrate that transcript knockdown with CRISPR-Cas13a significantly re-sensitizes multidrug-resistant (MDR) Klebsiella pneumoniae to cis-DNA gyrase inhibitor ciprofloxacin, through the targeting of the efflux pump gene acrB. A 72.88% decrease in acrB mRNA expression was significantly correlated with a 3.57-fold decrease in ciprofloxacin MIC, changing the isolates from resistant to susceptible breakpoints. There was a strong positive correlation between transcript reduction and phenotypic resensitization (Pearson r = 0.9235, Spearman rho = 0.8590) supporting the functional relationship between the expression of efflux pumps and antimicrobial resistance. Through linear regressions of models the observational data demonstrated that the acrB reduction percentage accounts for 85.29% of the variance of the MIC fold reduction and therefore can be used to provide a quantitative framework in which to predict the outcome of treatment. The results show that CRISPR-Cas13a is a precision adjuvant therapy that has the potential to be applied to clinically significant pathogens that are expressing antimicrobial resistance (AMR) through efflux.

Therefore, the following recommendations are proposed for future studies and clinical application. For efficient in vivo delivery across outer bacterial membranes, optimization of delivery platforms, such as engineered bacteriophages and lipid nanoparticles, is required. In vivo animal infection models should be used to test the activity of CRISPR-Cas13a in animals and to investigate the pharmacokinetic and pharmacodynamic properties. Multitarget strategies, including acrB silencing plus gyrA/parC inhibition, or using alternative efflux pumps, may result in more complete resensitization and resistance could be delayed. Future studies to investigate the persistence of transcript knockdown and bacterial resistance to the CRISPR-Cas13a system are warranted. Finally, clinical studies should be done to assess the safety, efficacy, and feasibility of using CRISPR-Cas13a to aid and augment conventional antibiotic therapy in human patients suffering from MDR infections. The system is programmable, meaning that it can be customised very quickly to meet new resistance mechanisms, making it a versatile solution for antimicrobial stewardship in the post-antibiotic era.

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