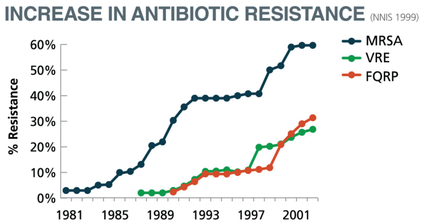
Introduction

Problem:

- According to the World Health Organization, antibiotic resistance is one of the top ten global public health threats facing humanity
- 2.8 million antibiotic resistant infections occur in the United States every year



"National Nosocomial Infections Surveillance (NNIS) System Report, Data Summary from January 1990-May 1999, Issued June 1999," 1999

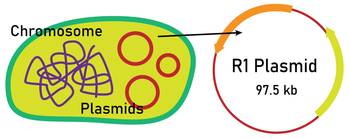
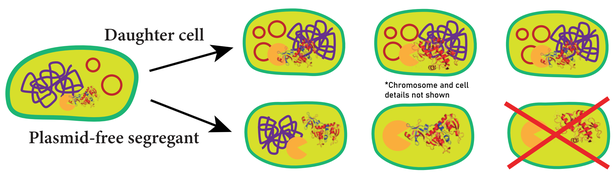


Figure 1: The R1 Plasmid is a conjugative, multi-drug resistance plasmid found in *Enterobacteriaceae* (de la Cueva-Méndez & Pimentel 2007)

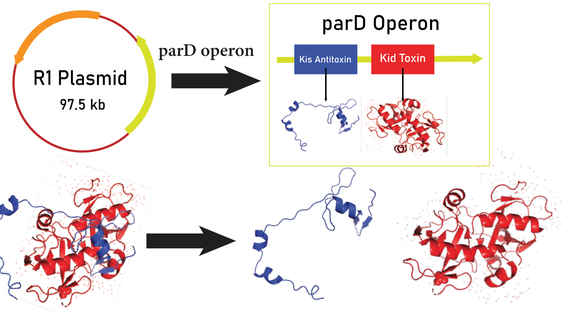
Plasmids are a major carrier of antibiotic resistance through horizontal gene transfer.

The R1 plasmid encodes for the Kid-Kis toxin-antitoxin system



- In the absence of a selective pressure, bacteria containing plasmids have a reproductive disadvantage as plasmids impose a metabolic burden on the cell

- To prevent plasmid loss, many plasmids have evolved toxin-antitoxin systems to kill through post-segregational killing in cells that fail to inherit the plasmid

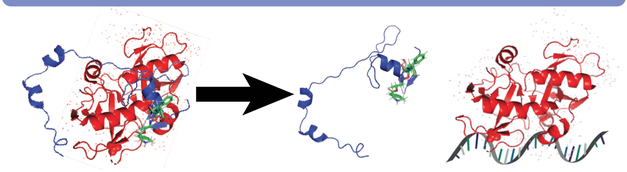


Protein-protein interactions between the Kid toxin and Kis antitoxin proteins regulate the Kid-Kis system

Disruptions to the protein-protein interaction causes cell death through the degradation of RNA.

Research Question:

How can small-molecule inhibitors be designed to artificially activate toxin-antitoxin systems contained in antibiotic resistance plasmids as a novel antibiotic strategy through an internal cell death mechanism?

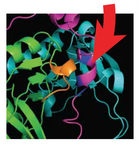


By developing a small-molecule inhibitor to target the binding site between the toxin and the antitoxin, this could potentially disrupt the protein-protein interaction to free the toxin from the system to act on the cellular target.

Hypothesis:

The region of the toxin-antitoxin system with the highest efficacy and affinity as a target for a small-molecule inhibitor to artificially activate the Kid-Kis toxin-antitoxin system will be the interacting residues on the Kis antitoxin responsible for the binding to the Kid toxin.

Interaction Site



Methods and Procedures

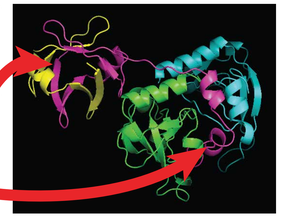
Construct the structure of the Kid-Kis toxin-antitoxin system through homology modeling → Determine target binding sites by calculating the residues that contribute the majority of the binding energy → Compare binding site residues to published literature and structural data to corroborate results → Construct inhibitors using de-novo design to target binding site residues → Perform computational analyses of molecular properties for drug-likeness and activity predictions

Construction of the Kid-Kis Toxin-Antitoxin System Protein Structure

Homology model of the Kid-Kis toxin-antitoxin system constructed with SwissModel

N-terminus of Kis antitoxin protein: Blocks lethal activity of the toxin

C-terminus of Kis antitoxin protein: Binds to Kid toxin to repress activity



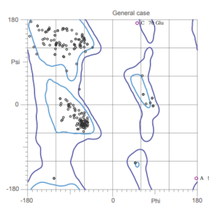


Figure 2: Ramachandran plot to assess the quality of the homology model structure of the Kid-Kis toxin-antitoxin system. The structure was ranked in the 60th percentile out of all structures with the same resolution.

Identification of Kid-Kis Interacting Region for Drug Target

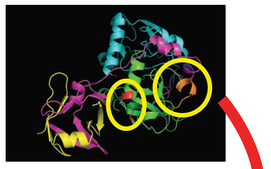
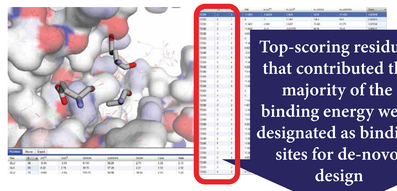


Figure 3: Top-scoring binding sites identified in PocketQuery, highlighted in red and dark blue

Figure 4 : Ser47 to Arg53 were identified as the active sites on the Kid toxin based on previous structural studies (Hargreaves et al. 2002)

The top-scoring residue cluster on Glu66-Arg72 highly corresponds with previous structural data on active sites on the Kid toxin



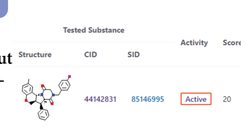
Top-scoring residues that contributed the majority of the binding energy were designated as binding sites for de-novo design

Residue Name	Residue Number	Chain
GLU	66	C
ILE	67	C
ALA	69	C
GLU	71	C
ARG	72	C
LEU	109	B
ARG	92	A
GLU	95	A
THR	29	C

Construction of QSAR Model to Predict Activity

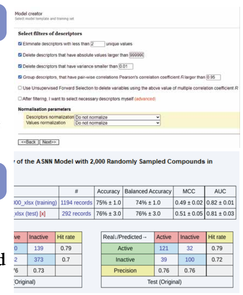
Data Preparation:

- Data set derived from a publicly available PubChem high-throughput screening bioassay on mazEF toxin-antitoxin system activators
- 2,000 compounds (50% active and 50% inactive)



Model Construction:

- Constructed model using RFR, ASNN, KNN, and MLR
- Descriptors calculated include ChemAxon, ALogPS, and OEState descriptors

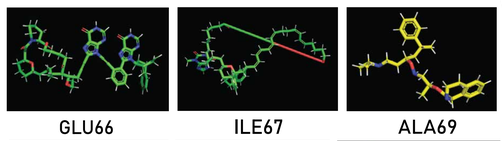


Model Validation:

- 80% training data and 20% testing data
- Calculated for accuracy, balanced accuracy, and AUC

Results

Active:



Inactive:

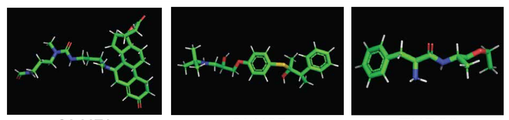


Figure 5: Nine small-molecule inhibitors were designed to target top-scoring residues located on Chain C of the Kis antitoxin and Chain A and B of the Kid toxin. Based on the constructed QSAR model, six out of nine inhibitors were predicted to be active compounds.

Selection of De-Novo Design Inhibitors

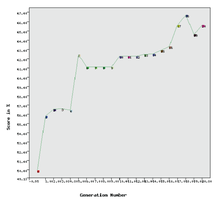


Figure 6: Example of de-novo design convergence plot for one run, scored using a user-defined scoring function, based on binding site specificity and drug-likeness rules. The final inhibitor was selected from the top-scoring generation in each run.

QSAR Model Predictive Statistics

MLR Model = 70% Accuracy
KNN Model = 72% Accuracy
RFR Model = 73% Accuracy
ASNN Model = 76% Accuracy

Data Set	#	Accuracy	Balanced Accuracy	MCC	AUC
Training set: DATASET_2000_xlsx (training)	1194 records	75% ± 1.0	74% ± 1.0	0.49 ± 0.02	0.82 ± 0.01
Test set: DATASET_2000_xlsx (test) [x]	292 records	76% ± 2.0	76% ± 2.0	0.51 ± 0.05	0.81 ± 0.03

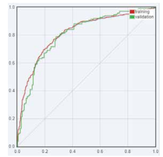
Real:/Predicted→	Active	Inactive	Hit rate
Active	520	139	0.79
Inactive	162	373	0.7
Precision	0.76	0.73	

Training (Original)

Real:/Predicted→	Active	Inactive	Hit rate
Active	121	32	0.79
Inactive	39	100	0.72
Precision	0.76	0.76	

Test (Original)

Figure 7: The ROC curve demonstrates the high accuracy of the ASNN model in distinguishing between active and inactive compounds with an AUC of 0.81



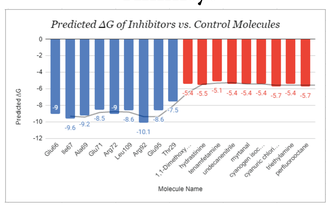
Efficacy:

Predicted Activity	Active	Inactive	Total
Interacting Region (Glu66-Arg72)	4	1	5
Non-Interacting Region	2	13	15
Total	6	14	20

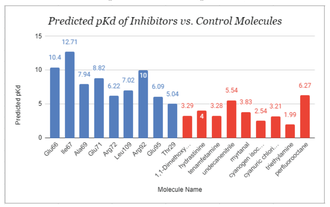
$\chi^2 = 0.00485$ ($p \leq 0.05$)

Figure 8: A Chi-squared test of statistical significance on the predicted activity of inhibitors targeting the critical interaction region from Glu66 to Arg72 compared to all other inhibitors and controls found statistically significant results ($p \leq 0.05$)

Affinity:



p=0.000000252 ($p \leq 0.05$)



p=0.000459 ($p \leq 0.05$)

Figure 9: Calculations for binding affinity were conducted with PRODIGY-LIGAND in HADDOCK for change in Gibbs free energy and KDeep by PlayMolecule for pKd, a form of the dissociation constant. Inhibitors were statistically significant in comparison to nine control molecules with no known affinity to the target.

Lipinski's Rule of Five / **Veber et al. (2002)**

Inhibitor Name	Mass (< 500)	LogP (< 5.0)	H-Bond Donors (< 5)	H-Bond Acceptors (< 10)	Topological Polar Surface Area (< 140)	Rotatable Bonds (< 15)
Glu66	869.56 Da	13.79	0	7	235.05 Å	11
Ile72	907.21 Da	13.03	1	9	121.26 Å	3
Ala69	499.64 Da	2.22	5	8	112.08 Å	15
Glu71	542.71 Da	2.89	2	9	108.05 Å	9
Arg72	463.52 Da	2.05	3	10	168.35 Å	21
Leu109	474.59 Da	2.84	5	7	121.10 Å	5
Arg92	743.95 Da	4.46	5	9	121.95 Å	19
Glu95	403.57 Da	4.42	3	4	61.72 Å	10
Thr29	264.32 Da	0.62	3	5	81.43 Å	7

Figure 10: Calculated Lipinski's Rule of Five and Veber et al. molecular properties for drug-likeness. All rule violations are shown in red. Five out of nine inhibitors fell near or under suitable drug delivery properties with < 2 rule violations.

Significant Findings

4 out of the 5 inhibitors targeting Glu66 - Arg72 region of the Kis antitoxin = Active

Two-tailed t-test assuming unequal variances calculated the p-value of ΔG and pKd under 0.05

p=0.00485 ($p \leq 0.05$)

Prediction Accuracy of the ASNN Model with 2,000 Randomly Sampled Compounds in Data Set

Data Set	#	Accuracy	Balanced Accuracy	MCC	AUC
Training set: DATASET_2000_xlsx (training)	1194 records	75% ± 1.0	74% ± 1.0	0.49 ± 0.02	0.82 ± 0.01
Test set: DATASET_2000_xlsx (test) [x]	292 records	76% ± 3.0	76% ± 3.0	0.51 ± 0.05	0.81 ± 0.03

Real:/Predicted→	Active	Inactive	Hit rate
Active	121	32	0.79
Inactive	39	100	0.72
Precision	0.76	0.76	

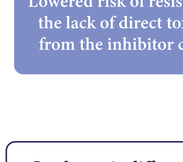
Test (Original)

The ASNN classification model can classify compounds as 'Active' or 'Inactive' with high predictive accuracy

The null hypothesis predicting no significant difference in binding affinity and activity in inhibitors targeting the interacting region for the artificial activation of the Kid-Kis toxin-antitoxin system can be rejected.

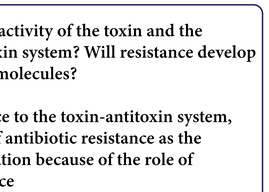
Conclusion

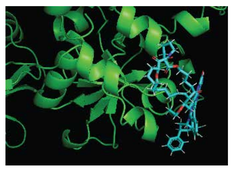
Due to the role of plasmids in the spread of antibiotic resistance through horizontal gene transfer, plasmids are critical targets to limit the rise of resistance



The R1 plasmid responsible for conferring multi-drug resistance in *Enterobacteriaceae* species encodes for the Kid-Kis toxin-antitoxin system that acts lethally as an internal cell death mechanism

To artificially activate the Kid-Kis toxin-antitoxin system, the regions identified as critical interaction sites for the development of small-molecule inhibitors were primarily found in the Glu66 to Arg72 region of the Kis antitoxin





The QSAR classification model predicted six out of nine inhibitors as active and binding affinity calculations found favorable binding energy and strong affinity towards the target; Activity and binding affinity were statistically significant in comparison to control molecules

Therefore, the construction of several active inhibitors to disrupt the protein-protein interaction responsible for regulating the lethal activity of the Kid-Kis toxin-antitoxin system may be a promising target for exploiting an internal cell death mechanism as a novel antibiotic strategy

Significance

1. Exploit an existing cell death mechanisms within plasmids as novel target for antibiotics
2. Specific targeting of bacteria containing resistance plasmids without harming commensal bacteria
3. Lowered risk of resistance due to the lack of direct toxic activity from the inhibitor compounds
4. No toxin-antitoxin system homologs contained within eukaryotic cells; lowered risk of toxicity towards human cells

In future research...

- Can bacteria differentiate between the toxic activity of the toxin and the inhibitor molecule activating the toxin-antitoxin system? Will resistance develop to the toxin-antitoxin system or the inhibitor molecules?
- In the case that the bacteria evolves resistance to the toxin-antitoxin system, this may be beneficial to limiting the spread of antibiotic resistance as the resistance plasmid would be lost in the population because of the role of toxin-antitoxin systems in plasmid maintenance
- Will active inhibitors of one toxin-antitoxin system activate another system with structural similarities, or across toxin-antitoxin families?