Structure-based drug design (SBDD) and *in silico* pharmacophore screening approaches combined with biochemical validation enabled the discovery of di- and tetrapeptides modulators of Y-49, TEM-1 and ampC beta lactamases

Cristina Clement[1,2] Janet Gonzalez[3], Sheuli Zakia[4], Sangeeta Tiwari[5] and Manfred Philipp[2]

[1]Pathology, Albert Einstein Coll Med CUNY, Bronx, New York, 1046; present address: Radiation/Oncology, Weill Cornell medicine, NY, 10065; USA. [2] Chemistry Department, Lehman College, Bronx, New York, USA. [3]Natural Sciences, LaGuardia Community College, Long Island City, New York, USA. [41]Laredo Community College, Laredo, Texas, United States; [5]Microbiology & Immunology, Albert Einstein College of Medicine, Bronx, NY 10461.

ABSTRACT

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, continues to be a worldwide health concern. The failure to control TB is due to the emergence of *M. tuberculosis* strains that are multiply drug resistant towards the front line antimycobacterial drugs such as isoniazid and rifampicin. One of the most effective resistance mechanisms to β-lactam antibiotics involves the production of β-lactamases which can cleave the amide bond in the target β-lactam ring. The intrinsic resistance to β-lactam antibiotics was demonstrated to be mainly due to the presence of a chromosomally-encoded gene (*blaC*) in *M. tuberculosis* for a Class A, Ambler β-lactamase (BlaC). The BlaC enzyme has been already validated as one of the lead therapeutic targets of tuberculosis therapy. In the last decade, many research studies proved that the main approach to overcome the resistance to β-lactam antibiotics the high throughput screening of new non β-lactam scaffolds, displaying inhibitory activity against β-lactamases. The spectrum of anti-TB drugs consisting of non β-lactam scaffolds has been expanded by the development of arginine and lysine-rich peptides which proved to be effective in neutralizing bacterial resistance, when administered in combination with antibiotics. Conceivably, the cationic-based peptides can be selectively enriched in natural or unnatural amino acids which can be further developed as β-lactamase inhibitors and additives of β-lactams, to help to prevent or reduce cleavage of the β-lactam ring. Following this logic strategy, we employed an original platform for molecular docking and structure-based drug design (SBDD) aimed to rational design novel tetrapeptides displaying inhibitory activity against the Y-49 enzyme, a class A β-lactamase, from *Mycobacterium tuberculosis*. The tetrapeptide pharmacophore was derived from the original sequence **RRGHYY** which was found to inhibit class A *Bacillus anthracis* Bla1, (K_i = 42 μM) and class A TEM-1 β-lactamase, (K_i = 136 μM) (*Protein Eng Des Sel* 16:853-860). The emergence of *M. tuberculosis* strains resistant to frontline antimicrobacterial drugs have greatly complicated efforts to control the spread of tuberculosis (TB). The hydrolysis of β-lactam drugs by β-lactamases is both an undesirable and highly effective resistance mechanism to β-lactam based antibiotics. Among the most promising strategies yet developed for solving this type of resistance problem was made possible by the discovery of new non β-lactam scaffolds inhibitors of β-lactamases. Herein we report the SBDD and pharmacophore-based approaches for the discovery of potential linear and cyclic peptides inhibitors of the Y-49 enzyme, TEM-1 and ampC lactamases. *In silico* docking experiments were performed with *Autodock Vina* (Scripps Institute, USA), coupled with MOE (Chemical Computing Group, Canada). The beta-lactamases 3M6B.pdb 1PZP.pdb and 1FSY.pdb were used as target proteins while tetrapeptides with the sequence space **[2HN-R-*X*-H-Y-COOH]** were docked as potential active site-directed, competitive inhibitors (where X=natural and unnatural L/D-amino acids). We have already reported that the sequence [2HN-R-*X*-H-Y-COOH] lead to the discovery of linear tetrapeptides with inhibitory activity in the lowest 30-1.0 uM range for Ki, against Y-49 β-lactamase-mediated hydrolysis of nitrocefin substrate. Herein we present the expansion of the peptide-pharmacophore space that lead to the discovery of cyclo [RRXY] tetrapeptides analogues that had at least a six-fold higher affinity for the Y-49 β-lactamase than its linear sequence, RRHY. Moreover, we further discovered novel dipeptide-pharmacophores derived from tetrahydroharman acid, D-cyclohexylalanine and fluoro-phenylglycine exhibiting promising inhibitory activity against Y-49 and ampC beta lactamases, having Ki in the lowest tens of uM range. We further tested the tetrapeptides and dipeptides lead compounds on the *in vitro* growth of mc26230, an unmarked strain of mc26030 (*M. tuberculosis* H37Rv *RD1 panCD)* and found that the lowest concentration of beta lactamase inhibitors that prevented the bacterial growth was in the order of 100-500 uM for selected dipeptide and tetrapeptides compounds.

INTRODUCTION

➢Beta-lactamase enzymes. The simplest classification for these enzymes is by protein sequence, whereby the beta-lactamases are classified into four molecular classes: A,B,C and D, based on conserved and distinguishing amino acid motifs (1-7). Classes A, C, and D include enzymes that hydrolyze their substrates by forming an acyl enzyme through an active site serine, whereas class B β-lactamases are metalloenzymes that utilize at least one active-site zinc ion to facilitate β-lactam hydrolysis. The production of β-lactamases in both Gram-negative and Gram-positive bacteria is one of the most efficient and prevalent mechanisms of resistance to β-lactam antibiotics hydrolyzing the drugs before they can reach their target and exert the desired effect. All of these resistance mechanisms are important, and each bacterium can create a combination of defenses depending on the selective pressures placed on it (1-7).

➢Discovery of peptides inhibitors of beta-lactamase. This research focused on discovery of novel peptides scaffolds, with improved drug-like properties as potential inhibitors of Y-49, and TEM-1 and Amp-C beta-lactamases using a combination of molecular docking and SBDD platforms and biochemical assays of enzyme inhibition (Figure 1).

We decided to start the search for potential inhibitors using tetrapeptides derived from a pharmacophore represented by a 6-mer sequence discovered by W. Huang *et al.* during a phage display screening assay. The 6-mer linear peptide RRGHYY inhibited class A *Bacillus anthracis* Bla1, (Ki = 42 μM) and class A TEM-1 β-lactamase, (Ki = 136 μM) (Figure 1).

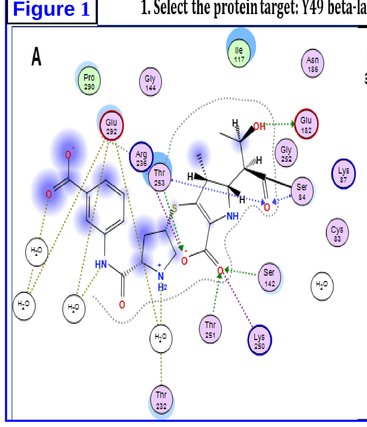
1. Selection of the pdb target of interest:
Beta-lactamase (BlaC) in complex with *ERTAPENEM (3M6B.pdb)*

Extract the x,y,z coordinates (x=-6.619; y=-6.952; z=2.546) of the original 1RG ligand from the complex 3M6B.pdb and perform directed rigid docking using Autodock/Vina and MOE

Discovery of *novel tetrapeptides* inhibitors of Y-49 (Class A, β-lactamase) using SBDD approaches

3M6B.pdb was used as a protein template in docking experiments involving the private collection of tetrapeptides derived from the sequence 2HN-RXHY-COOH. *VINA-Autodock* software was used to assess the ΔG (free energy) of interaction between the tetrapeptides and the beta-lactamase target, generating the structure-activity relationship (SAR) for the potential inhibitors of Y-49-β-lactamase (which has the same amino acid sequence as 3M6B.pdb). Additional docking experiments were performed using TEM-1 and ampC protein targets: 1FSY AMP-C, 4 and 1PZP TEM-1.

Figure 1 1. Select the protein target: Y49 beta-lactamase in complex with *ERTAPENEM (3M6B.pdb).*



Ligand RG1	Receptor		Chain	Interaction	Distance	E (kcal/mol)
N 2	O	HOH 358	(A)	H-donor	2.75	-4.6
OAG 28	OE1	GLU 182	(A)	H-donor	2.66	-3.2
OAC 19	N	SER 84	(A)	H-acceptor	2.85	-1.7
OAC 19	N	THR 253	(A)	H-acceptor	2.79	-5.2
OAT 42	OG1	THR 253	(A)	H-acceptor	2.56	-0.8
OAU 43	OG	SER 142	(A)	H-acceptor	2.58	-3.2
OAU 43	OG1	THR 251	(A)	H-acceptor	2.61	-3.7
OAT 42	NH1	ARG 236	(A)	ionic	3.88	-0.8
OAU 43	NZ	LYS 250	(A)	ionic	3.88	-0.8
6-ring	O	HOH 360	(A)	pi-H	3.46	-0.5

METHODS AND RESULTS

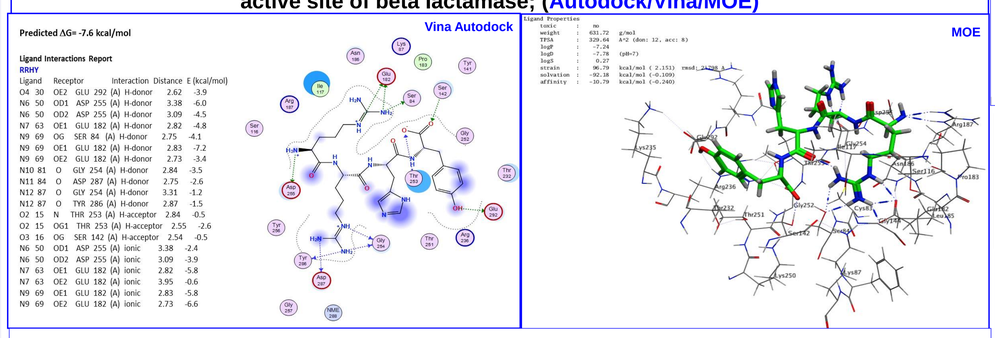
Figure 2

Key structural features of the sequence space 2HN-RXHY-COOH determine the free energy (ΔG) of interaction between the *tetrapeptide inhibitors* and the target beta-lactamase Y-49

1. **The guanidine group of Arg in most of the designed peptides interacts with either Asn between distances of 2.6 to 3.9 Å, or with Asp from 2.9 to 4.7 Å within the active site of the class A beta-lactamase. The bond distances between the guanidino group of Arg and Asp are within salt-bridge distances. Furthermore the N-terminal Arg residue positions the N-terminal main chain nitrogen to within donating hydrogen-binding distance of Ser237 or Asn104 in the active site.**
2. **The interaction of the Tyr hydroxyl group with Met, Asn, and/or Thr and Ser within the active site contributes to the free energy of binding.**
3. **The main chain carboxylate group of the tetrapeptides is within binding distance of Ser/Thr, Arg, and Asn adding to the binding affinity of these 4-mers.**
4. **The substitution with Ala for Arg at the N-terminus of the tetrapeptides decreases the predicted binding affinities, supporting the structural basis for having these interactions important for stabilizing the binding of the substrate in the active site.**
5. **The hydroxyl group of Tyr at the C-terminus binds either Asn, or Thr (only one structure binds Ser). We substitiuted Phe for Tyr and the ΔG values were more positive, showing a lost in the predicted binding affinities for the tetrapeptides inhibitors.**

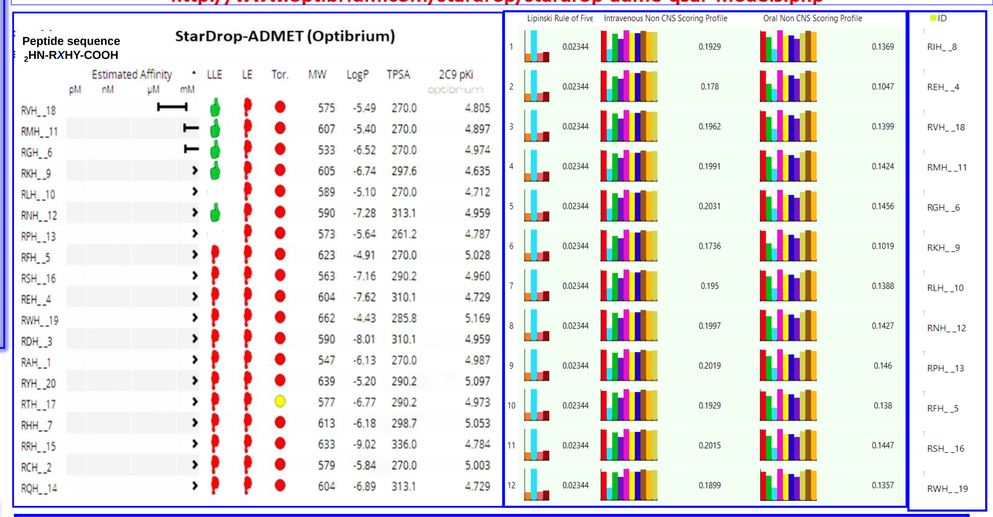
Figure 3

***Poseview* of the RRHY tetrapeptide in the active site of the3M6B highlighting the steric, hydrogen bonds and the electrostatic interactions established with selected residues in the active site of beta lactamase; (Autodock/Vina/MOE)**



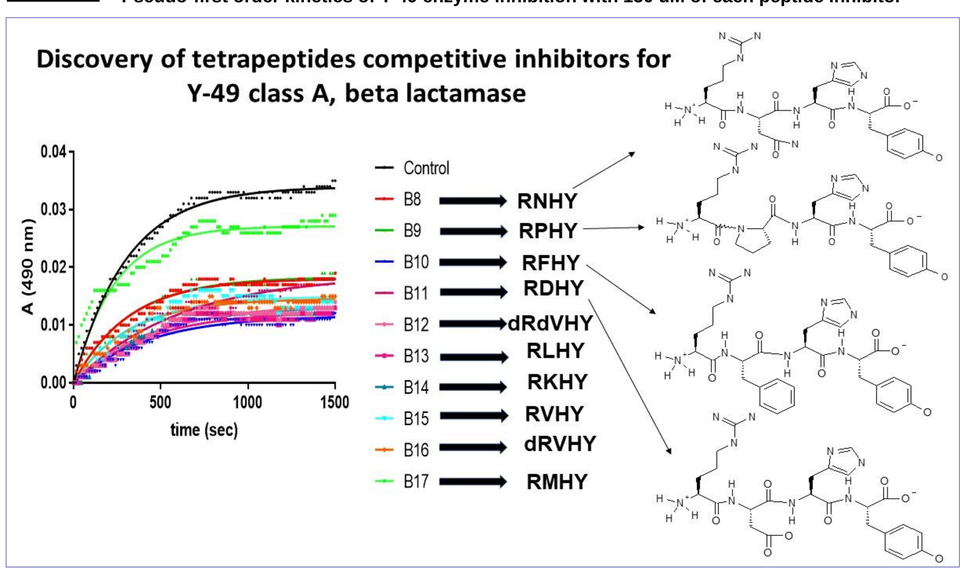
Y-49 Beta lactamase tetrapeptides inhibitors: assessment of ADMET properties using OPTIBRIUM SeeSAR and StarDrop software

http://www.optibrium.com/stardrop/stardrop-adme-qsar-models.php



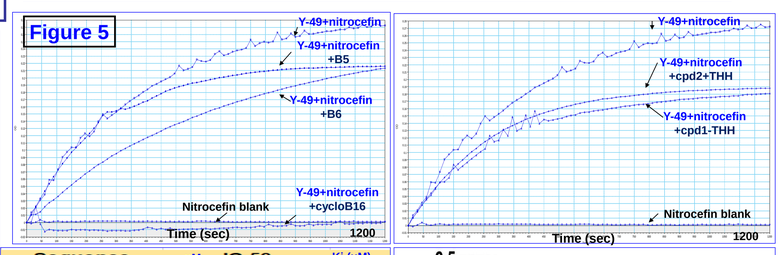
Structure-activity relationship (SAR) of selected lead tetrapeptides inhibitors of Y-49 beta-lactamase: Enzyme kinetics with recombinant beta-lactamases and nitrocefin substrate

Figure 4 **Pseudo-first order kinetics of Y-49 enzyme inhibition with 150 uM of each peptide inhibitor**

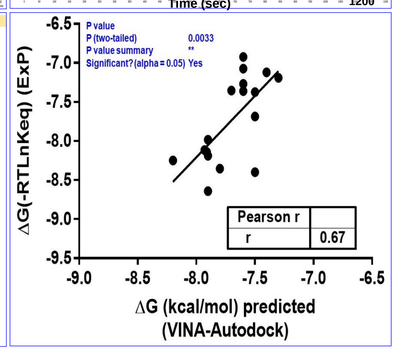


Structure-activity relationship (SAR) of selected lead tetrapeptides inhibitors of Y-49 beta-lactamase: real-time kinetics

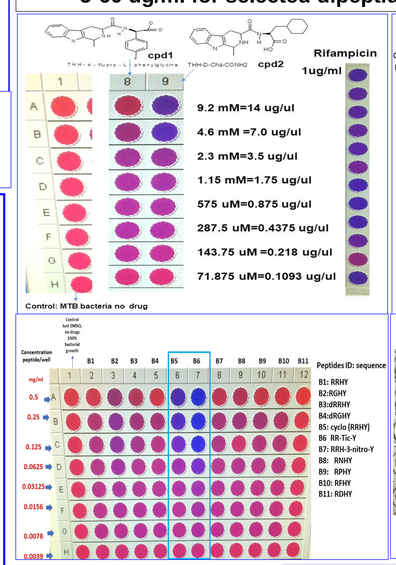
Figure 5

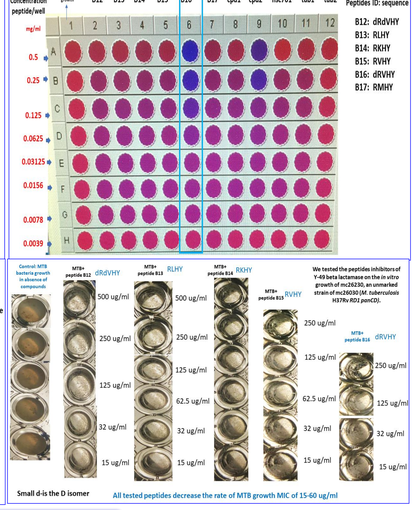


Sequence	uM	IC-50	Ki (uM)
RVHY	7.97		6.33
RMHY	1.94		1.53
RLHY	3.89		0.81
RPHY	9.14		1.91
RFHY	9.14		1.9
RNHY	5.76		1.2
RKHY	18.32		3.81
RFHY	11.27		2.35
RDHY	8.07		1.68
dRVHY	3.72		0.77
dRdVHY	18.52		3.85
RRHY	15.14		13.12
dRRHY	4.12		3.26
RGHY	1.63		1.29
dRGHY	0.97		0.76
RAHY	7.10		6.54
REHY	8.76		1.82
RIHY	10.30		10.3
RQHY	6.47		6.47
RSHY	8.50		8.5
RTHY	9.52		9.52
RWHY	7.50		7.5
cyclo[RRHY]	2.52		1.9



We tested the tetrapeptides and dipeptides lead compounds on the *in vitro* growth of mc26230, an unmarked strain of mc26030 (*M. tuberculosis* H37Rv *RD1 panCD)* and found that the lowest concentration of beta lactamase inhibitors that prevented the bacterial growth was in the order of 5-60 ug/ml for selected dipeptide and tetrapeptides compounds.





CONCLUSIONS

➢A tetrapeptide library (2HN-RXHY-COOH) with arginine in position 1, histidine in position 3, ending with tyrosine in position 4, and having both free NH3 and COOH ends, was constructed by performing SAR where X was replaced with all 20 L-amino acids. New lead tetrapeptides were discovered: RLHY has Ki of 0.81 uM while the tetrapeptides dRGHY and dRVHY have Ki of 760 nM and 770 nM, respectively. In all cases the replacement of L-isomer of Arg at the N-terminus with the D-isomer (dR) resulted in at least two-fold enhanced inhibitory activity. Moreover, the cyclic analogue cyclo [RRHY] increased at least six-fold the affinity for the Y-49 beta lactamase, from Ki of 13.12 uM characterizing the linear RRHY peptide to a Ki of 1.9 uM for the cyclo [RRHY].

➢Recently, Caitlyn M. Rotondo et al. (2015, (7)) discovered novel nanomolar peptide inhibitors of metallo-beta-lactamases (MBL class). All the lead peptides inhibitors of MBL are poly-Arginine based sequences(derived from one of the lead sequence, Ac-Cys-Tyr-βAla-$(Arg)_8$-Val-Leu-Arg-OH). This recent report support our findings from SBDD approach where the arginine based tetrapeptides are low uM inhibitors of Y-49 beta-lactamase.

➢Predicted ADMET properties of peptides from 2HN-RXHY-COOH series suggests that tetrapeptides have better drug-like properties than the original hexamer peptide RRGHYY pharmacophore from which were originally developed; thus the linear and cylized tetrapeptides could be used as new scaffolds for developing potent anti-Y49 beta lactamase inhibitors.

➢THH-dipeptide derivatives proved to have antimicrobial activity characterized by a minimal inhibitory concentration of 5-0.8 ug/ml against the growth of *M. tuberculosis* H37Rv *RD1 panCD.*

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