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Cell permeable vanX inhibitors as vancomycin re-sensitizing agents

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ABSTRACT

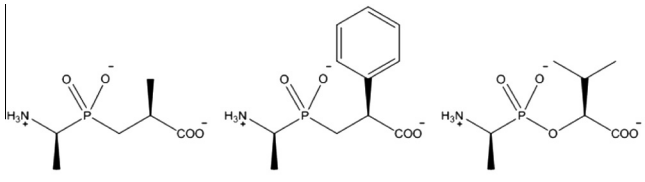
VanX is an induced zinc metallo D-Ala-D-Ala dipeptidase involved in the viable remodeling of bacterial cell wall that is essential for the development of VREF. Here we report two cyclic thiohydroxamic acid-based peptide analogs that were designed, synthesized and investigated as vancomycin re-sensitizing agents. These compounds exhibit low micromolar inhibitory activity against vanX, with low cytotoxicity and were shown to increase vancomycin sensitivity against VREF. The improved pharmacological properties of these novel inhibitors over previous transition state mimics should provide an enhanced platform for designing potent vanX inhibitors for overcoming vancomycin resistance.

Vancomycin is the last line of antibiotic defense against serious Gram-positive infections when all other therapeutic agents have failed. Continual emergence of vancomycin resistant bacterial pathogens such as vancomycin resistant *Enterococcus faecium* (VREF) and *Staphylococcus aureus* (VRSA) poses a major health threat to children, immune deficient and post-surgical patients in hospitals.[1–3] There is an urgent need to develop alternative therapy for combating vancomycin resistant pathogens.

Peptidoglycan (PG) is an essential component of bacterial cell wall and has been widely exploited in the development of effective antibacterial agents.[4] Vancomycin inhibits PG biosynthesis by binding to the terminal amino acid residues, D-alanyl-D-alanine (D-Ala-D-Ala) of the NAM/NAG-peptide subunits. The formation of a tightly bound complex prevents *trans*-peptidation and cross-linkages among glycan strands, leading to the collapse of the compromised bacterial cell wall and ultimately cell death.[5]

High-level vancomycin resistance is conferred by the acquisition and over expression of a five cassette gene (*vanR*, *vanS*, *vanH*, *vanA* and *vanX*)[6] that results in the accumulation of D-alanyl-D-lactate (D-Ala-D-Lac) as an alternate peptidoglycan precursor for bacterial cell wall remodeling. Removal of the endogenous D-Ala-D-Ala by the *vanX* gene product, vanX enzyme allows predominant incorporation of D-Ala-D-Lac into the PG layer with a thousand-fold decrease in affinity of vancomycin for D-Ala-D-Lac over D-Ala-D-Ala.[7] This variation circumvents vancomycin inhibitory action against the bacterial PG biosynthesis pathway and renders vancomycin inactive as an antibacterial agent.[8]

Over the past two decades, vanX has been viewed as an attractive drug target for re-establishing vancomycin sensitivity.[9] VanX is a zinc metallo D-Ala-D-Ala dipeptidase that catalyzes the hydrolytic cleavage of the dipeptide amide bond.[9,10] Transition state analogs, tetrahedral intermediates, and other mechanism-based irreversible inhibitors[11] have been investigated to understand its catalytic mechanism.[10] Subsequently, several classes of phosphinates,[12] phosphothioate,[13] phosphonates,[12] and phosphoramidates[13] transition state mimics (TSM) with low micromolar inhibition biochemical activities have been reported as vanX inhibitors (Fig. 1).



1 1.5 μM **2** 2.9 μM **3** 6.1 μM

Figure 1. Transition state vanX inhibitors.

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Unfortunately, these inhibitors exhibit no cellular penetration properties required for cell based inhibition activity, rendering them unattractive for further therapeutic development. In this study, we explore the design of novel cyclic thiohydroxamic acid peptide analogues that, for the first time, exhibit both low micromolar biochemical inhibitory activity against vanX and vancomycin re-sensitizing activity against VREF in cell-based assay.

VanX is a zinc dependent dipeptidase that can only hydrolyze D,D-dipeptide substrates, and does not exhibit any activity against tripeptides, terminally capped dipeptides and specific stereoisomeric dipeptides.[10] Many designs of metallo enzyme inhibitors incorporate a hydroxamic acid as the bidentating group of choice, but we purposely avoided this moiety because of its potential liabilities in their selectivity toward other divalent metals that can lead to off-target cytotoxicity. While simple acyclic hydroxamic acids are not suitable as drug candidates, cyclic hydroxamic acids or their isosteres such as cyclic α-hydroxy ketones and non-toxic thioketones constitute viable alternatives.[14] Heterocyclic hydroxamic acid-containing compounds have been studied extensively as antiviral and antibacterial agents with favorable pharmacological properties.[15] Hydroxy pyridones such as deferiprone have been used clinically to treat β-thalassaemia.

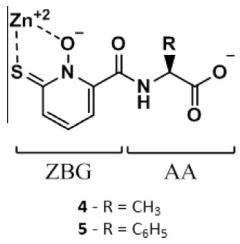


Figure 2. Design of ZBG-AA. Expected mode of chelation by ionized **4** and **5** with vanX Zn^{+2} cofactor.

Based on established low molecular weight of previous TSM vanX inhibitors, our earlier designs of metallo binding inhibitors[16,17] and docking studies, we hypothesized that an effective vanX inhibitor should consist only of a zinc binding group (ZBG) and a single D-amino acid group (AA) in order to bind within the catalytic site of vanX (Fig. 2). Our design incorporates pyrithione, a heterocyclic thiohydroxamic acid moiety as the zinc specific binding group with a single D-amino acid. Pyrithione is a monoanionic zinc specific bidentating chelator that forms a five-membered complex with Zn^{2+} via its oxygen and sulfur atomic centers. Zinc pyrithione (ZPT) has been isolated from the *Polyalthia nemoralis* roots used in Chinese herbal remedy[18] and shown to possess antimalarial properties.[19] Pyrithiones was first synthesized in 1950's as an analogue to aspergillic acid, a naturally occurring antibiotic.[20] Today, pyrithiones (sodium, zinc and copper) have been well established as active antidandruff[21] and antifouling[22] agents. More recently, pyrithiones have been shown to exhibit both anticancer[23] and antiviral activities.[24,25] Furthermore, they possess desired non-toxic[14], ionophoric properties to overcome the cellular penetration problems experienced by TSM compounds **1**–**3** (Fig. 1), making the design ideal for the last line of antibiotic combination therapy.

Previous structural study has revealed details of the vanX active site.[26] Its substrate specificity can be attributed to the divalent zinc cofactor, the overall compactness of the binding pocket, and the pre-organized amino acid sidechains inside the active site for the stabilizing the zwitterionic termini of the dipeptide substrates (Fig. 3A). Docking of the stereoisomeric dipeptides into the active site reveals zwitterionic D,D-dipeptides are structurally preferred over stereoisomeric dipeptides (not shown). The negatively

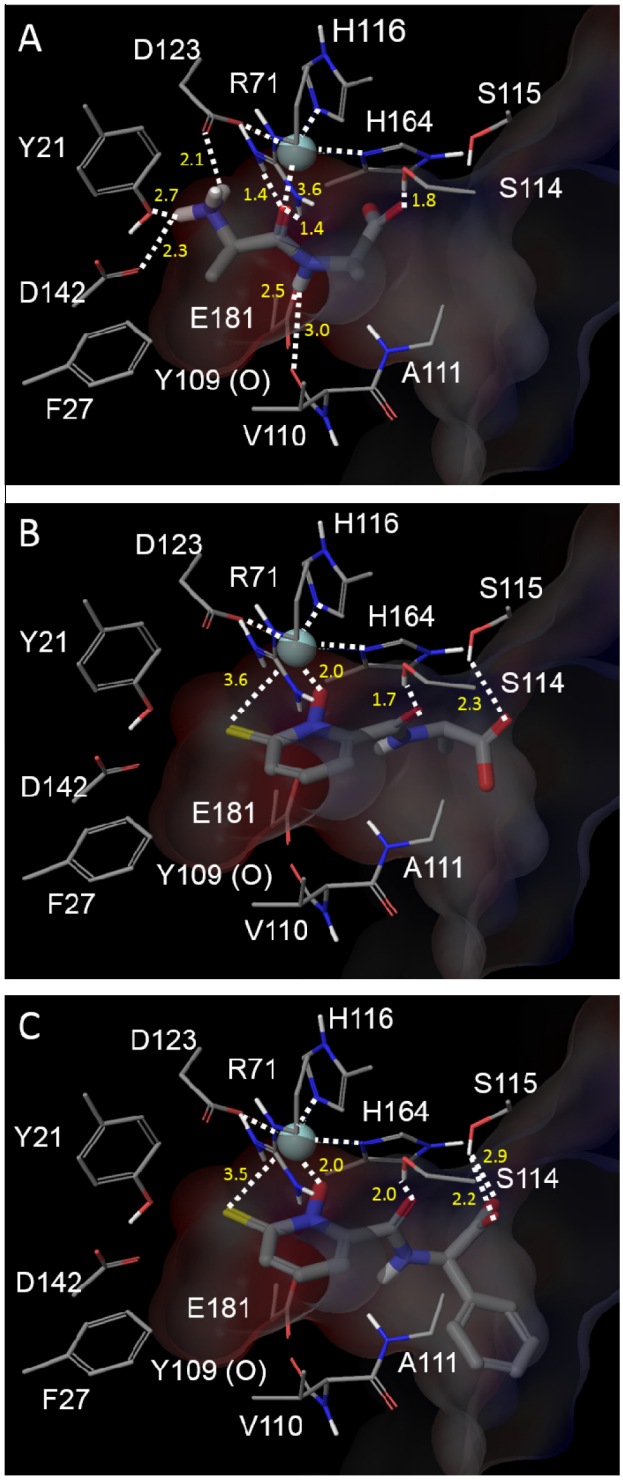
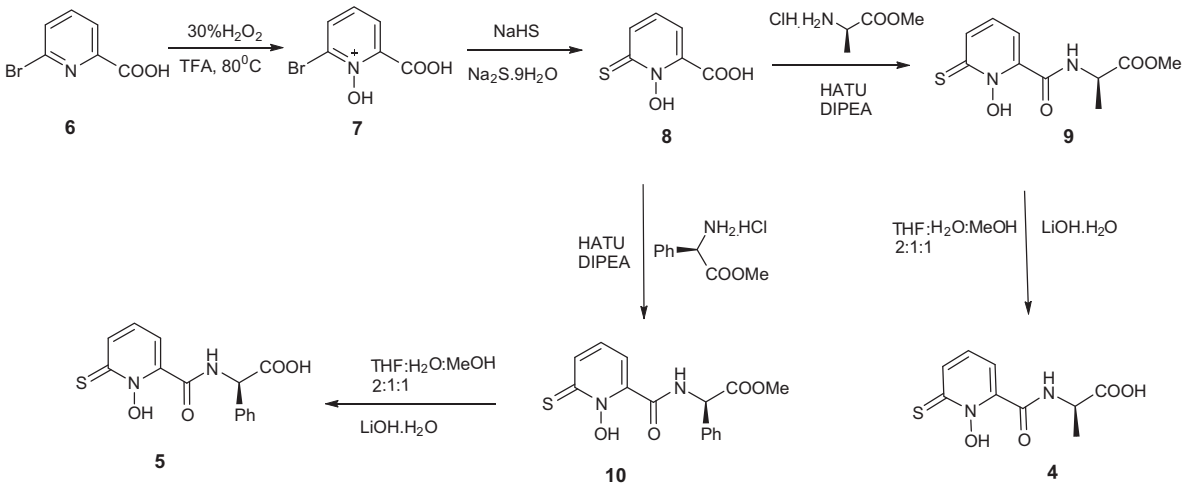


Figure 3. Model of the vanX active site with (A) D-Ala-D-Ala substrate, (B) **4** and (C) **5**. The translucent molecular surface colored by electrostatic potential surface highlights the stereoselectivity and the diminutive size of the binding pocket. The measured distances in angstroms are shown in yellow. Both S114 and S115 are essential is stabilizing the amide oxygen and carboxylic C-terminus of compounds **4** and **5**.

charged side chains of D123 and D142 and the phenol group of Y21 provide the key stabilization interactions for the positively charged N-terminus while the S114 hydroxyl group is essential for stabilizing the negatively charged C-terminal carboxylate group. The small binding pocket can accommodate only low

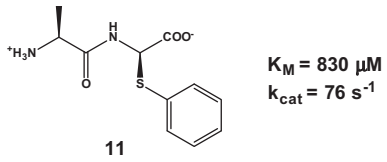


Scheme 1. Synthesis of **4** and **5**.

molecular weight inhibitors, similar to that of the D-Ala-D-Ala dipeptide substrate.

To examine the design of our vanX inhibitors, docking studies of **4** and **5** were carried out using the Schrodinger's modeling suite package.[27] Our study began with the model of the D-Ala-D-Ala vanX complex (Fig. 3A) based on the reported apo X-ray structure of vanX (PDB: 1R44) and structural details of the transition state analogue described by Park and co-workers.[26] The Docking study has been extensively described elsewhere.[28,29] Briefly, energy minimization with an implicit generalized solvent model was used to account for consistent structural relaxation of the final complex model. Based on this model, docking of **4** and **5** were carried out using Glide[27] at Standard Precision (SP) with Zn^{2+} defined as the required constraint. For comparison, docking of the D-Ala-D-Ala was carried out to reproduce the expected mode of substrate binding (Fig. 3A). Subsequent docking of the design inhibitors further revealed the preferred mode of binding for **4** and **5** involving the expected ionized pyrithione within the expected distances necessary for forming bidentating chelate with the zinc cofactor (Fig. 3B and C). The potential mode of binding for both **4** and **5** is observed to be slightly shifted toward the opening of the active site as compared to the D-Ala-D-Ala natural substrate. This is likely due to the placement of the ZBG in the terminal end of the molecule. This mode of zinc chelation lead to the removal of the hydrogen bond between Y109(O) and the amide hydrogen observed in the dipeptide substrate and a shift in the hydrogen bonding network involving S114 and S115 for stabilizing the amide oxygen and the carboxylic C-terminus of compounds **4** and **5**. This compensating effect could explain the observed G-scores for **4** and **5** (−7.6 and, −8.3, respectively) as compared to D-Ala-D-Ala (−8.8).

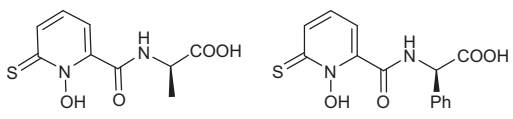
To test the validity of our design framework, we have synthesized **4** and **5** as model compounds (see Scheme 1). 6-Thio *N*-hydroxy pyridine 2-carboxylic acid, **8**, was synthesized from 2-bromo pyridine 6-carboxylic acid, **6**, according to literature procedures.[30] Initially, a variety of usual peptide coupling agents, for example EDCI or CDI, or benzotriazole in combination with various of bases and conditions were attempted for coupling of *N*-benzyloxy **8** with amino acids such as D-alanine methyl esters or D-phenyl alanine methyl esters. In all cases the coupling reaction gave unacceptable low yields and debenzylation of coupled product also became troublesome. Peptide coupling of **8** was best accomplished (without protection of *N*-hydroxy group) with HATU (1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo [4,5-*b*]pyridinium-3-oxido hexafluoro phosphate) to give good yields. The coupling of **8** (no need to protect N-OH group) with D-alanine methyl ester or phenyl alanine methyl ester followed by hydrolysis with lithium hydroxide in methanol–THF–water-mixture, gave **4** or **5** in 40%, and 62% yield respectively. Extended periods of hydrolysis at room temperature gave similar yields but racemization occurred.



11

Figure 4. Reported kinetic parameters for D-alanyl-α-R-phenylthioglycine as the reporting substrate for the biochemical assay (see Ref. 31).

A previously described coupled colorimetric assay using D-alanyl-α-R-phenyl thioglycine substrate **11**[31] was employed to determine the inhibition constant, K_i, of **4** and **5**. The colorimetric substrate needed for enzymatic biochemical assay, α-phenylthio containing peptide (H-d-Ala-Psg-OH, **11**) and the corresponding diastereomer, H-d-Ala-dl-Psg-OH were prepared according to the literature procedure.[31] The expression and purification of vanX was carried out according to earlier studies of Walsh and co-workers.[12] The initial rates were calculated for the first 300 s, and absorbance changes were converted to concentration change using the molar extinction coefficient for the liberated thionitrobenzoic acid. The kinetic parameters for the colorimetric substrate **11** (see Fig. 4) were in accord with the earlier reported K_M value of 0.83 ± 0.08 mM,[31] which was also comparable to the kinetic parameters of the natural D-Ala-D-Ala substrate (K_M = 0.11 ± 0.01 - mM). In contrast, the diastereomer of colorimetric substrate **11** was found to be enzymatically inactive—a fact that supports the



4 5

Figure 5. Low μM inhibition constants for **4** and **5** against vanX.

stereo selectivity of vanX enzyme for certain dipeptides. After reproducing these kinetic parameters, the K_i for **4** and **5** were determined by least-squares fit to the Michaelis–Menten equation. The determined K_i's were 6.84 and 2.74 μM for compounds **4** and **5**, respectively (Fig. 5). These are comparable to the low micromolar inhibitory activities of the earlier reported transition state mimics **1**–**3** (Fig. 1).

Based on these findings, we determined the ability of compounds **4** and **5** to re-sensitize VREF (E12-2) to vancomycin. A checkerboard MIC analysis was performed to determine their additive or synergistic activities with vancomycin. Individually, **4** and **5** did not inhibit bacterial growth of VREF (not shown). The MIC of vancomycin against VREF was reduced from 256 μg/ml to 125 μg/ml in the presence of **4** (212 μg/ml) and **5** (72 μg/ml) (Table 1). This corroborate our design strategy that both compounds were capable of penetrating the bacterial cell wall, inhibit vanX, and, thereby, reduce vancomycin MICs. VanX inhibitor **4** was 3 times more potent than **5** in parallel to their observed biochemical K_i values.

Table 1
Checkerboard MIC analysis of E12-2 against vancomycin

Compound	MIC (μg/ml)	MIC (μM)
4	212	875
5	72	236

We next assessed the cytotoxicity of compounds **4** and **5** against human embryonic kidney HEK 293 cell lines by MTT assay. Culturing and maintenance of HEK293 cells were performed as described by ATCC. The CC_{50}'s for both compounds **4** and **5** were 76 μM and 64 μM after 72 h treatment, comparable to existing drugs such as amoxicillin (Table 2). Interestingly, vancomycin was shown to be non-toxic against the HEK293 cells. The observed cytotoxicity of these compounds further support our design strategy of using a more specific zinc binding group to limit inhibition of off-targets proteins that can lead to unwanted cytotoxicity.

Table 2
MTT cytotoxicity study against human embryonic kidney HEK 293 cells

Compound	CC_{50} (μM)
Vancomycin	>100
Amoxicillin	24
4	76
5	64

We have synthesized two pyrithione amino acid compounds using a novel vanX inhibitor design frame-work. These compounds exhibit low micromolar biochemical inhibitory activity that were comparable to previously reported transition state mimic inhibitors, low cytotoxicity against human cells and the ability to penetrate and re-establish vancomycin sensitivity against VREF cell culture. Since neither compounds **4** nor **5** exhibits any antibacterial activity by themselves, they act as true re-sensitizing agents of vancomycin. We recognize a one to two fold reduction in vancomycin concentration may not appear to be significant; however, this is the first proof of concept that a cell permeable vanX inhibitor can re-sensitize vancomycin against a vancomycin resistant bacterial pathogens. To the best of our knowledge, we have overcome a major drug design problem over the last two decades by utilizing heterocyclic thiohydroxamates in our design. Finally, these data strongly support and demonstrate the feasibility toward further explore the biochemical and biological properties of vanX inhibitors **4** and **5** as lead candidates for rational modification to further improve their efficacy.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.03.097.

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