Communications to the Editor

Dithiol Compounds: Potent, Time-Dependent Inhibitors of VanX, a Zinc-Dependent D,D-Dipeptidase Required for Vancomycin Resistance in *Enterococcus faecium*

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VanX is a novel Zn-dependent D,D-dipeptidase which is essential for vancomycin resistance in *Enterococcus faecium*.[1,2] Contrary to most proteases and peptidases, it prefers to hydrolyze the amide substrate D-Ala-D-Ala but not the related kinetically and thermodynamically more favorable ester substrate D-Ala-D-lactate, with more than a 10^5-fold differential in catalytic efficiency.[2] In order to gain insight into the dipeptidase catalytic mechanism with the prospect of drug design to reverse clinical vancomycin resistance, inhibition studies of VanX have been carried out. One strategy is based on the ability of phosphorus-containing amino acid analogs to mimic the unstable tetrahedral intermediates in peptide hydrolysis,[3] and indeed phosphinate analogs of D-Ala-D-Ala are potent slow-binding inhibitors for VanX.[4]

For zinc peptidases, another effective strategy to design a potent inhibitor is to incorporate zinc-binding ligands, especially a mercaptan moiety.[5] Spectroscopic and structural studies of an inhibitor−thermolysin complex have confirmed that a mercaptan inhibitor, as predicted, displaces a bound water molecule and binds to enzyme with the sulfur, presumably in the anionic form, tetrahedrally coordinated to the zinc in the active site.[6] We have tested several compounds with a monothiol moiety as inhibitors for VanX and observed a range of potency (Table 1); D-cysteine is most potent, with a K_i of 13 μM. Considering that K_m for substrate D-Ala-D-Ala is 1.4 mM, D-cysteine appears to bind with VanX about 100 times tighter than substrate with the assumption that K_m is equal to K_d. Since D-serine is not a good inhibitor for VanX, the free thiol group of D-cysteine is likely to play a key role as a tight binder to VanX. Among these compounds, a β-thiol propionic acid motif forms the basic inhibitory structure.

Table 1. Inhibition Constants of Monothiol Compounds for VanX[a]

inhibitor	K_i (μM)
D-cysteine	13
L-cysteine	79
thiosalicylic acid	122
mercaptosuccinic acid	430

[a] All compounds were purchased from Aldrich, and K_i was estimated by Dixon plot[11] at 37 °C in 50 mM Hepes buffer, pH 8.0, with D-Ala-D-Ala as substrate (10 mM).

Table 2. Kinetic Parameters of Dithiol Compounds as Time-Dependent Inhibitors[a]

inhibitor	k_{inact} (min^{-1})	$t_{1/2}$ (min)	K_i (μM)	K_i* [b] (μM)
1,2-ethanedithiol	0.23	3.0	410	1.8
2,3-dimercapto-1-propanol	0.18	3.9	36	0.32
2,3-dimercapto-1-propanesulfonic acid	0.44	1.6	240	0.19
1,3-propanedithiol	0.12	5.8	980	17
dithiothreitol	0.23	3.0	1200	7.3

[a] All compounds were purchased from Aldrich and used without further purification. Stock solutions of these compounds were prepared freshly before each assay. [b] The final inhibition constant K_i* was estimated by Dixon plot[11] of steady-state kinetics at 37 °C in 50 mM Hepes buffer, pH 8.0, with D-Ala-D-Ala as substrate (10 mM). K_i* for 2,3-dimercapto-1-propanol was determined by slow-binding inhibition kinetic analysis as described in the caption below Figure 1.

Further investigation found that a dithiol compound, dithiothreitol (DTT), which is an antioxidation reagent normally included in the protein purification buffer, not only is a potent inhibitor for VanX with an apparent K_i of 7.3 μM (Table 2) but also demonstrates time-dependent inhibition kinetics (Figure 1). It has been reported that enzymes can be gradually inactivated and degraded in a time-dependent manner by reactive oxygen species generated by iron contamination in buffers through Fenton chemistry.[7] A reducing reagent, such as DTT, mercaptoethanol, or ascorbic acid, helps to recycle the oxidized metalion and exacerbates the problem. In this case, VanX cannot be inactivated by either mercaptoethanol or ascorbic acid. SDS−PAGE analysis of VanX after incubation with DTT also did not reveal any protein degradation. Addition of the chelating agent EDTA or treatment of buffer with metal-chelating resin had no effect on time-dependent inhibition of VanX by DTT. Kinetic analysis showed that inactivation of VanX by DTT is at least 10 times faster than any reported nonenzymatic oxidative degradation of protein in the presence of the contaminating iron and DTT (data not shown). A second possible explanation for DTT to inactivate VanX is that DTT might act as a chelating reagent to remove the zinc ion from the VanX active site. However, metal content analysis of VanX showed that zinc content in VanX remains at the same stoichiometric level after treatment of VanX with DTT (data not shown). Also of note, we have previously reported that the two cysteine residues of VanX were not essential for enzymatic activity.[4]

Steady-state inhibition kinetic analysis of additional dithiol compounds showed that they are much more potent than the monothiol compounds in Table 1. The apparent K_i*s estimated from Dixon plots range from 17 μM for 1,3-propanedithiol to 0.19 μM for 2,3-dimercapto-1-propanesulfonic acid (Table 2). Like DTT, they all show time-dependent inhibition kinetics (Figure 1) which could be caused by either an irreversible

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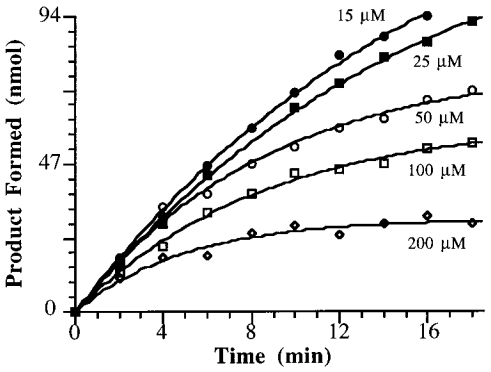
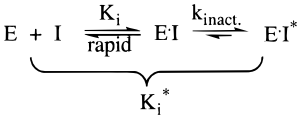


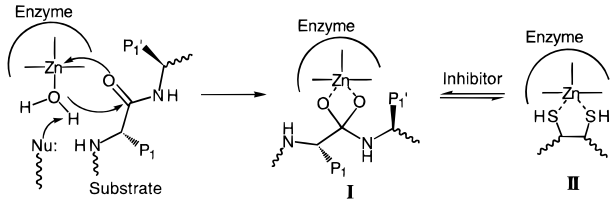
Figure 1. Time-dependent inactivation of VanX by dithiol compounds. Assay mixture contains 50 mM Hepes, pH 8.0, and 10 mM D-Ala-D-Ala in a final volume of 150 μL at 37 °C. The assays were started by addition of VanX (77 nM). Aliquots (10 μL) were removed at time intervals, and the amount of D-alanine formed was determined by a modified Cd−ninhydrin assay.[4] Only the data of 2,3-dimercapto-1-propanol are shown here, and its concentration is marked on each curve in the figure. The concentrations of other dithiol compounds for inhibition kinetic analyses were as follows: 1,2-ethanedithiol (150, 300, 450, 600, and 1000 μM), 2,3-dimercapto-1-propanesulfonic acid (8, 20, 50, 100, and 200 μM), 1,3-propanedithiol (0.5, 1, 2, 4, and 6 mM), dithiothreitol (1, 3, 6, and 10 mM). The collected data points were directly fitted to the integrated inactivation rate equation $P = V_0(1 - e^{-k_{obs}t})/k_{obs}$ to generate a progress curve, and the observed inactivation rate constant k_{obs} was determined. Then the apparent inactivation rate constant k_{inact} and the initial inhibition constant K_i were determined by the Lineweaver−Burk plot of $1/k_{obs}$ vs $1/I$.[9] The half-time to fully turn on the inactivation, $t_{1/2}$, was determined by the relationship $t_{1/2} = 0.693/k_{inact}$. For 2,3-dimercapto-1-propanol, the data points were also fitted into the integrated rate equation for slowing-binding inhibition $P = V_s t + (V_0 - V_s)(1 - e^{-kt})/k$ to determine the initial inhibition constant K_i and the final inhibition constant K_i* as described before.[4]

inactivation or a slow-binding inhibition.[8] Since there was always some VanX residual activity after a prolonged incubation with a dithiol compound, it is likely that the observed time-dependent inhibition can be described by a slow-binding inhibition model (Scheme 1):[8] enzyme forms an initial complex E·I with inhibitor in a rapid step, and then the initial complex slowly converts to a more stable complex, E·I*, which can be characterized by an initial inhibition constant K_i and an overall inhibition constant K_i*. The apparent inactivation rate constant k_{inact} and the initial inhibition constant K_i were readily determined from progress curves as in Figure 1. The overall inhibition constant K_i* was estimated by steady-state kinetics through a Dixon plot or determined by slow-binding inhibition

Scheme 1



Scheme 2



kinetic analysis as previously reported.[4] Kinetic parameters are summarized in Table 2.

Among the dithiol compounds tested, 2,3-dimercapto-1-propanesulfonic acid and 2,3-dimercapto-1-propanol are the most potent ones, with K_i*s up to 10^4-fold tighter than substrate K_m, and in the same magnitude as the phosphinate analog of the tetrahedral intermediate of D-Ala-D-Ala hydrolysis.[4] The inhibition studies with several analogs of dithiol compounds showed that the two thiol groups are imperative for potent inhibition with a preferred two-carbon spacer between two thiol groups. Noticeably, these two dithiol compounds are all racemic mixtures of four diastereomers and it is possible that each one of them has a different inhibition activity for VanX. Further inhibitor specificity studies of these dithiol compounds should help us to understand more of the slow-binding inhibition and to design more specific inhibitors for VanX.

The catalytic mechanism of zinc proteases has been proposed to go through a four-membered ring reaction intermediate **I** (Scheme 2) with biscoordination of both oxygen atoms of the tetrahedral adduct to the zinc. It is possible that, in the dithiol inhibitions of VanX, the two thiol groups, as in the case of mercaptan−thermolysin complex, act as two ligands to the zinc ion in the active site with a five-membered ring transition-state analog **II** (Scheme 2). While this proposed geometry in inhibited dithiol−VanX complexes has to be proved by structural studies, there is a precendent for such possible bidentate ligation of DTT at the active site of the metalloenzyme phosphotriesterase as assessed by cadmium NMR analysis.[10]

Incorporation of a dithiol moeity into D-Ala-D-Ala substrate scaffolding may generate a more potent and specific inhibitor for VanX, which would have the prospect of reversing phenotypic Gram-positive bacterial resistance to vancomycin.

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