Clavulanic acid binds with a second target of nonclassical peptidoglycan synthase (3→3 linkage) L, D -transpeptidase of *Mycobacterium tuberculosis*

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ABSTRACT

Bacterial penicillin binding proteins (PBPs) catalyze synthesis of peptidoglycan layer in cell wall, which provides shape, strength and survival capability of microbe. β-lactam antibiotics inactivate D, D-transpeptidase; however, L, D-transpeptidase leads to resistance for several β-lactam antibiotics. Clavulanic acid is an FDA approved drug that inhibits the β-lactamase, which indirectly enhances the efficacy of β-lactams. Here we report for the first time that 2 Å high resolution crystal structure of L, D- transpeptidase binds with clavulanic acid and captures propionaldehyde adduct with the catalytic residue Cys354. This observation has profound clinical implications as it shows clavulanic acid is effective in binding with L, D-transpeptidase in addition to β-lactamase. We provide biochemical data for acylation reaction, and accumulation of L, D-transpeptidase in the cell membrane to support the concept that peptidoglycan modification leads to resistance to several β-lactams. The findings of this study demonstrate that clavulanic acid can inhibit non-classical peptidoglycan synthesis, which is highly abundant in non-replicating that are drug resistant bacteria and implicated in resistance to several β-lactam antibiotics. We conclude that as a clinical perspective for treatment with therapeutic drugs formulation to multi-drug resistant (MDR) and extensively-drug resistant (XDR-TB) strains, clavulanic acid should be considered as an effective first-line drug in combination with amoxicillin or carbapenems.

Importance: The present structural study provides the evidence that clavulanic acid binds with a second target, which is a cell wall synthase enzyme L, D-TP of drug resistant bacteria. The structural information provides that clavulanic acid binding and adduct formation is very similar to β-lactamase enzyme. Clavulanic acid antibacterial potency increased in the presence of either carbapenems or amoxicillin. Clavulanic acid from a clinical point of view can target two enzymes (L, D-TP and β-lactamase) simultaneously, which could be a potential candidate to treat drug, multi-drug resistant bacteria and other bacterial infections as combination therapy.

INTRODUCTION

The peptidoglycan (PG) layer is a vital component of the bacterial cell wall that ensures mechanical strength, shape and maintains the bacterial cytosolic osmotic pressure. The chemical compositions of PG layers are very similar in both gram positive and Gram-negative bacteria. The final step of PG polymerization is the formation of peptide cross-linkages between two adjacent peptide stems, resulting in interconnected PG multi-layers. In all bacteria, peptide cross-linkages are generated by transpeptidase (TP) enzymes that are anchored in the cell wall membrane. The extent of peptide cross-linkages varies in different bacterial species. For example, the total percentage of peptide cross-linkages in E. coli, E. coli KN126, and Mycobacterium tuberculosis are 50% , 43 % and about 70-80 %, respectively.

The bacterial PG layer contains two types of peptide cross linkages 4→3 (classical) and 3→3(non-classical) linkages. The 4→3 peptide cross-linkage is catalyzed by D,D-TP. In contrast, the 3→3 peptide cross linkage is catalyzed by L,D-TP. Drug resistant and multi-drug resistant bacteria have increased levels of non-classical PG cross-linkages. *In vitro* studies have shown that genetic manipulation of L,D-TP results in a reduction in bacterial size, altered cell surface, and reduced bacterial virulence. To clear persistent and drug resistant bacteria, one promising approach includes targeting L,D-TP, along with D,D-TP, by blocking its function on PG polymerization.

Here, we provide the first structural evidence that clavulanic acid reacts with L, D-TP and traps propionaldehyde as an adduct with the catalytic residue Cys354. The covalent adduct formation is identical to the earlier reported β-lactamase-adduct; propionaldehyde of M. tb

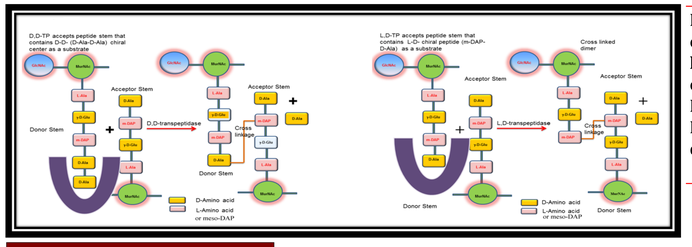

Figure 1: A) D,D-transpeptidase catalyzes 4→3 cross linkages in PG layer of bacterial cell wall during exponential growth phase; and B) L,D-TP catalyzes 3→3 linkages in PG layer of bacterial cell wall during persistence phase

HYPOTHESIS

We hypothesize that over expression of L, D-TP should accumulate in the membrane fraction that would modify the cell wall by increasing the non-classical PG synthesis.

We propose that clavulanic acid has structural similarities with carbapenems, and it can acylate with L,D-TP like meropenem or imipenem .

AIMS

Aim 1: Co-crystallize L-D, transpeptidase with clavulanic acid and to trap covalent adduct with catalytic Cys354

Aim 2: To demonstrate the acylation reaction between L-D-transpeptidase and clavulanic acid upon incubation

Aim 3: Assess the accumulation of L-D, transpeptidase in bacterial membrane fraction

Aim 4: Evaluate antibacterial property of clavulanic acid with and without carbapenems or Augmentin

METHODS

- The His-tagged L,D-TP was purified using Nickel column and crystallized with clavulanic acid or 6-aminopencillic acid (16 mg/ml protein was incubated with ligand at 4° C before crystallization [protein and ligand molar ratio is 1:5])
- The structures of L, D-TP-Clavulanic complex and L, D-TP-6-aminopenecillic acid complex were determined using molecular replacement
- The enzyme kinetics was performed by incubating L, D-TP with clavulanic acid 1:1.25 M ratio at 22 ^{0}C
- Antimicrobial property of clavulanic acid or in combination with carbapenem or augmentin was performed using BL21-DE3 cells with and without induction of IPTG

RESULTS

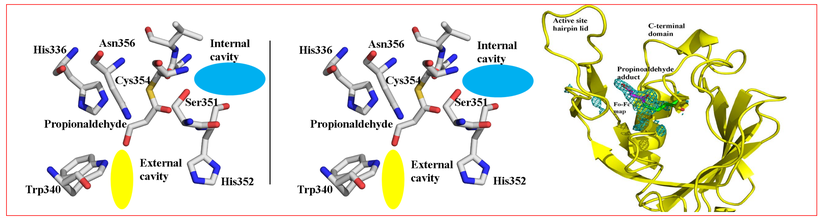

Figure 2. A) Substrate binding pocket contains two active site cavities; B) Catalytic cysteine-354 traps the propionaldehyde adduct at the C-terminal domain C) Fo-Fc electron density for the covalent-adduct, and D)The bound adduct induces the conformational change on the active site lid.

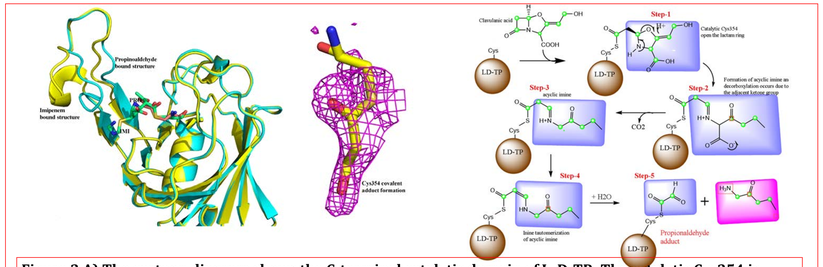

Figure 3 A) The cartoon diagram shows the C-terminal catalytic domain of L, D-TP. The catalytic Cys354 is shown in stick model (green color) and bound adduct is shown in violet color. The bound adduct induces the conformational change on the active site lid. B) Fo-Fc electron density for the covalent-adduct; and C) The proposed mechanism of acylation reaction between L, D-TP and clavulanic acid. The acyl intermediate undergoes chemical cleavage and forms covalent adduct with catalytic

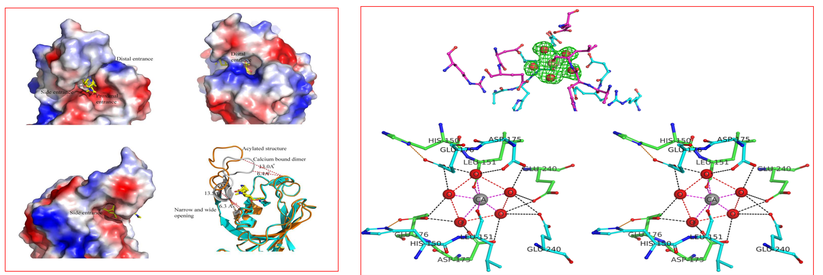

Figure 4: A) Electropotential surface of C-terminal domain with proximal cavity; B) surface diagram shows the distal cavity; C) shows the very narrow side entrance, D) shows the difference in the movement of catalytic lid between inhibitor bound vs calcium bound dimeric structure, and E) Electron density for bound calcium

ACKNOWLEDGEMENTS

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Disclaimer

The finding/opinion presented here express the view of the presenter. It does not reflect the views of US-Food and Drug Administration.

Over expression of L, D-TP causes resistance to several β-lactam antibiotics

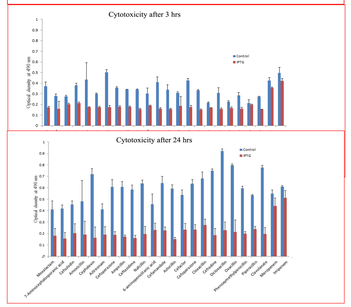

Figure 5A . Bacterial whole cell cytotoxic assay. . Blue color indicates cells were without induction of IPTG and treated with 5 μM of panel of antibiotics. Red indicates cells induced with IPTG and treated with 50 μM of panel of antibiotics. The bacterial killing is highly significant in control wells without induction of IPTG.

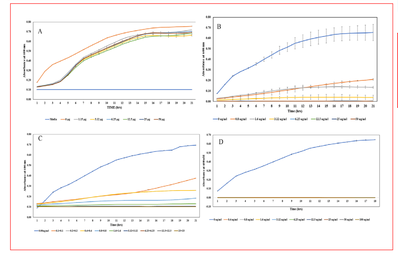

Figure 6B. The graph shows the inhibition of bacterial growth upon incubation with various concentrations of clavulanic acid. The growth curve shows clavulanic acid decreases bacterial growth significantly for the initial 6 h. The growth curve also shows that all concentrations of clavulanic acid had a similar effect. 8B) The effect of doripenem on bacterial growth. The graph shows a dose-dependent response and MIC value was 3.2 μg/ml. 8C) This figure shows the bacterial growth upon incubation in a combination of doripenem and clavulanic acid (1:1 molar ratio). The MIC value is 1.6 μg/ml (i.e., 0.8 μg/ml + 0.8 μg/ml of doripenem and clavulanic acid). 8D) The MIC value for meropenem was 0.4 μg/ml. Combination of meropenem and clavulanic acid completely inhibited the bacterial growth even at 0.2 μg/ml concentration (1:1 molar ratio). The bacterial growth curve data are average of three independent experiments.

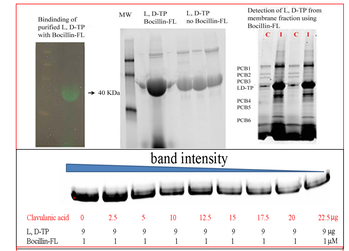

Figure 7A This figure shows binding of BocillinTM FL penicillin with purified L, D-TP from the his-tag column and the fluorescent band indicates the binding.; Blanes 5,6 and 7, which are eluted fractions that were not incubated with fluorescent compound; and 6C) this figure shows the presence of L, D-TP in the membrane fraction, lanes 1 and 3 are the membrane fractions of uninduced E.coli BL21-DE3 cells; Lane 2 and 4 are membrane fractions of E.coli BL21-DE3 cells induced with IPTG.

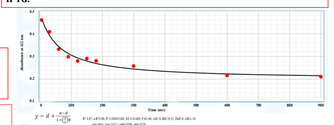

Figure 7B

Figure 7B. Kinetic analysis of L, D-TP binding with clavulanic acid. The volume of enzyme assay mixture is 1 ml that contains 100 μm of L, D-TP enzyme 120 μm of clavulanic acid (1:1.2 molar ratio) incubated as reported elsewhere. 100 μl of reaction volume was aspirated at each time point and free enzyme was measured by incubating with 10 mM DTNB and measured at 412.

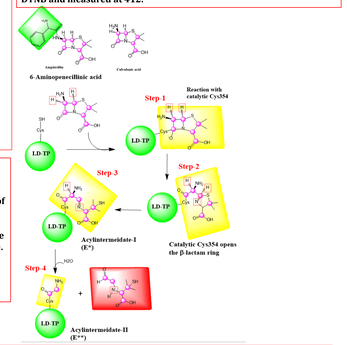

Figure 7: C Structural similarity and difference between ampicillin and 6-aminopenecillic acid. Ampicillin has an additional amino-phenylacetaldehyde side chain, which is shown in green color box. The acyl reaction between L, D-TP with 6-aminopenecillic acid should be identical to ampicillin.

CONCLUSIONS

- Accumulation of L, D-TP in the cell membrane can modify PG layer from classical to non-classical cross-linkages
- Structural study provides evidence that clavulanic acid binds with a second target, which is a cell wall synthase enzyme L, D-TP
- Biochemical data provides evidence for acylation reaction between L, D-TP with clavulanic acid
- Clavulanic acid antibacterial potency increased in the presence of either carbapenems or Augmentin
- Clavulanic acid can target two enzymes (L, D-TP and β-lactamase) simultaneously
- Clavulanic acid (in combination with first line drugs and β-lactams) could serve as a repurposed drug to treat MDR and XDR bacteria

FDA RELEVANCE

- Antibiotic resistance is a growing public health issue; hence, antibacterial agents that can target drug resistant bacteria is urgently needed. FDA Reform Act, H.R. 5651 recently has reported that shortage of drugs to treat infections. To overcome this crisis is to alleviate shortages of drugs by modifying currently existing drugs and expediting the approval drugs.
- From a clinical perspective to treat infections and cure MDR, XDR-TB , clavulanic acid should be considered as an effective drug with first line drugs and potentially used in combination with amoxicillin or carbapenems. Clavulanic acid (in combination with first line drugs and β-lactams) could serve as a repurposed drug to treat MDR and XDR bacteria