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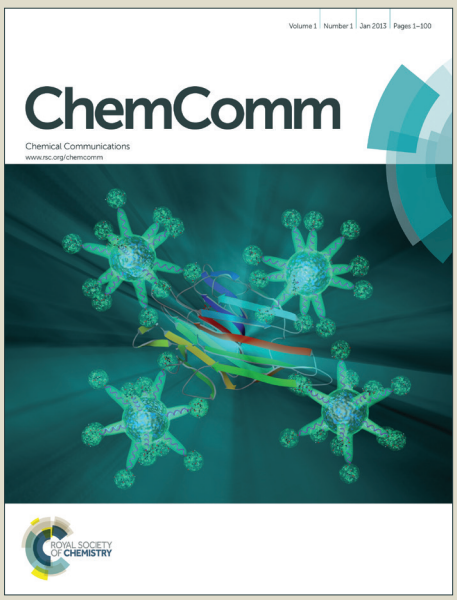

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ARTICLE TYPE

Simulating the Inhibition Reaction of *Mycobacterium tuberculosis* L,D-Transpeptidase 2 by Carbapenems

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A theoretical free energy study describes the inactivation of a new Tuberculosis target, the L,D-transpeptidase 2 enzyme. A new reaction mechanism of two carbapenem inhibitors is proposed and molecular features are determined from QM/MM and PMF approaches. The theoretical findings with the new proposed mechanism agree in principle with experimental data.

The *Mycobacterium tuberculosis* (*Mtb*) peptidoglycan layer is maintained mainly by cross-link bonds built by transpeptidase enzymes. They cross-link the glycan chain by forming (D,D) 4→3 and (L,D) 3→3 peptide linkages, which are formed by D,D and L,D-transpeptidases, respectively.[1] Since 80% of the peptidoglycan cross-links are of the 3→3 linkages, the L,D-Transpeptidase (LDT) enzyme may have a major role in peptidoglycan biosynthesis.[2] Among the five LDTs paralogues encoded by the chromosome *Mtb* strain, the LDT_{Mt2} show to be essential for virulence of acute infection in a mouse model.[3]

The LDTs enzymes are efficiently inhibited by carbapenems, such as meropenem, imipenem, doripenem and ertapenem.[4] Carbapenems form an extremely important class of antibiotic β-lactam compounds. Among hundreds of different β-lactams, carbapenems exhibit a wide spectrum of activity with the strongest potency against Gram-negative and Gram-positive bacteria.[5] The development of carbapenems in treating of tuberculosis disease has raised wide interest since these compounds, in association with clavulanic acid as a β-lactamase inhibitor, are evenly active against extensively drug-resistant *Mtb* (XDR-TB).[6] These drugs have been shown to kill both dormant and growing forms of the bacilli.[6b]

The inhibition reaction of LDTs by carbapenems is similar to the first step of the LDT catalytic mechanism, which leads to enzyme acylation by the donor stem peptide[7], where a covalent adduct is formed by the LDT active-site residue (cysteine) and the carbapenem β-lactam ring.[8] Triboulet and co-workers have proposed that LDT inactivation by carbapanems is carried out in a two-step reaction.[9] In the first part, a (reversible) tetrahedral intermediate is formed (EI^{ox}), the oxyanion. Whereas, the second step (irreversible) leads to formation of the acyl enzyme (EI*), the adduct form. However, their proposal from experimental observations does not take into account the probable protonation states for the catalytic residues.

Our recent theoretical study described that the acylation step is preceded by thiol-imidazole pair formation (Cys and His catalytic residues) in its zwitterionic form,[10] which will potentially be involved in the mechanism of LDT enzymes reacting with carbapenems.

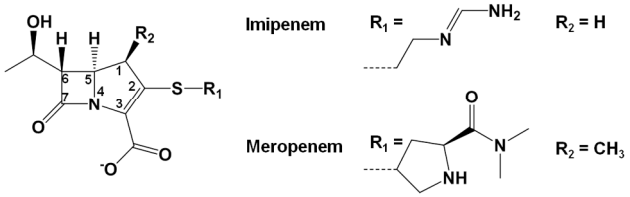
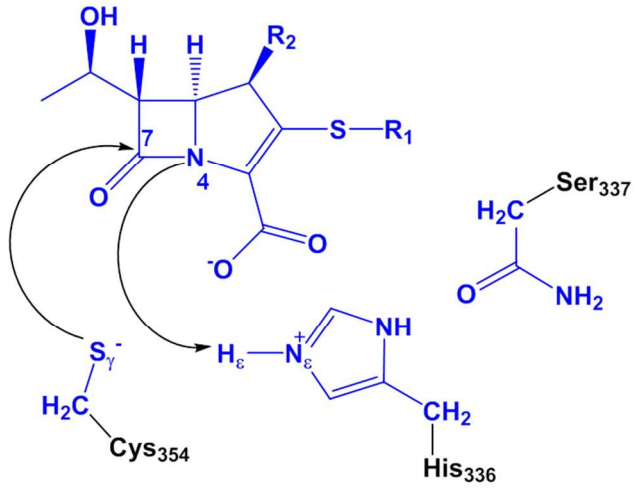


Fig. 1 Carbapenem inhibitors of LDT employed in the present report.

In this report, we have applied hybrid quantum mechanics/molecular mechanics (QM/MM)[11] molecular dynamics (MD) simulations and umbrella sampling[12] approaches to investigate the inactivation mechanism of LDT_{Mt2} by meropenem and imipenem, respectively. The calculations were carried out with the self-consistent charge density functional tight binding (SCC-DFTB) semi-empirical method[13] in the QM part of the system, using *ff99SB* classical force field[14] for the protein and the TIP3P water model[15] for the explicit solvent. The atom link method[16] was used to satisfy the QM-MM boundary conditions. The system building, theory level and energy minimization protocols are described in the ESI file. They are similar to the protocols applied previously on the LDT_{Mt2} catalytic mechanism.[10] The SCC-DFTB method was successfully applied to β-lactam systems.[17] Besides, to provide an independent vision on the QM/MM systems described above, a model for the reaction in water was also simulated using same conditions (see ESI file for details).

The QM/MM umbrella sampling approach was performed in two steps (Scheme 1). (a) In the first one, two reaction coordinates were selected for the sampling: $RC_X = d(S_\gamma - C_7)$ (nucleophilic attack from S_γ of Cys354 side chain to C_7 of carbonyl group in the β-lactam ring of the inhibitor, sampled from 1.60 to 3.40 Å) and $RC_Y = d(C_7 - N_4)$ (breaking of $C_7 - N_4$ bond in the β-lactam ring of the inhibitor, sampled from 1.30 to 2.80 Å), to simulate the acylation step. (b) In the second, just, one reaction coordinate was used for the sampling: $RC = d(N_\varepsilon - H_\varepsilon) - d(N_4 - H_\varepsilon)$ (proton transfer from N_ε of His336 side chain to N_4 atom of carbonyl group in the β-lactam ring of the inhibitor, sampled from -2.50 to 1.50 Å). For each simulation window, 20

ps of MD production were preceded by 5 ps of equilibration with a time step of 1.0 fs. All the scans were executed in steps of 0.20 Å. This procedure follows the protocol applied previously for the catalytic mechanism.[10] The QM/MM umbrella sampling simulations were performed within the Amber12 MD package.[18] The 2D and 1D free energy surface (FES) for the acylation and proton transfer reaction steps, respectively, were calculated using the variational free energy profile (VFEP)[19] (see ESI for details).[10] Finally, the PMFs were corrected by means of M06-2X-D3/MM single-point energies for the minimum free energy path (MFEP) (see ESI for details).



Scheme 1 The inactivation mechanism proposal for the LDT_{Mt2} by carbapenem taking into account previous theoretical findings for the catalytic mechanism.[10] The blue atoms are in QM part of simulation models. The details about cutted bond on the QM/MM frontier are included on the ESI.

The 2D FES for the first step of the inactivation mechanism of LDT_{Mt2} by meropenem inhibitor is presented in Fig. 2. According to the results, the tetrahedral intermediate (EI^{ox}) proposed by Triboulet and co-workers,[9] will not be formed during the reaction studied, since this step would be thermodynamically unfavourable (ΔG^{o}_{calc}> 20.00 kcal mol^{-1}). Moreover, the ΔG^{o}_{calc} for reaction involving this inhibitor and LDT_{Mt2} is –13.08 kcal mol^{-1} making the nucleophilic attack of thiol group of Cys354 catalytic residue to carbonyl group in the β-lactam ring of the meropenem inhibitor thermodynamically favourable. These results are taking into account that the Cys-thiolate/His-imidazolium pair is in its zwitterionic form, as described previously.[10] In addition, the transition state (TS_1) and anionic INT found on the MFEP (Fig. 2) are similar to structures proposed for the mechanism of carbapenem hydrolysis by CphA,[17c] and subclass B2 metallo-β-lactamase.[20] Similar results were found for the imipenem (see ESI, Fig. S1). Graphical presentations for TS_1 and TS_2 are also presented in the ESI (Fig. S4).

Instead, the MFEP on this reaction shows a concerted mechanism to form an anionic intermediate state (INT, see Fig S4 in ESI for a graphical presentation) for the whole reaction, which is in accordance with experimental kinetics data.[21] In energetic terms, considering this MFEP (Fig 2), the calculated activation free energy ($\Delta G^{\ddagger}_{calc}$= 7.27 kcal/mol) is underestimating the experimental value of activation free energy ($\Delta G^{\ddagger}_{exp}$ = 15.74 kcal mol^{-1}).[21] In order to fix inaccuracies in estimating the experimental absolute value by DFTB/MM potential, we have corrected the activation barrier using single-point at M06-2X-D3/MM level, where we have obtained $\Delta G^{\ddagger}_{calc}$= 17.41 kcal mol^{-1} at this potential, which is in remarkable agreement with the experimental value. Despite the DFTB/MM potential have failed in describe the absolute value of $\Delta G^{\ddagger}_{exp}$, it is important to point out that the theoretical energetics obtained for imipenem and meropenem have the same trend of reported experimental data[20], which support the mechanism described by our DFTB/MM free energy surface (see Table S2 and S3).

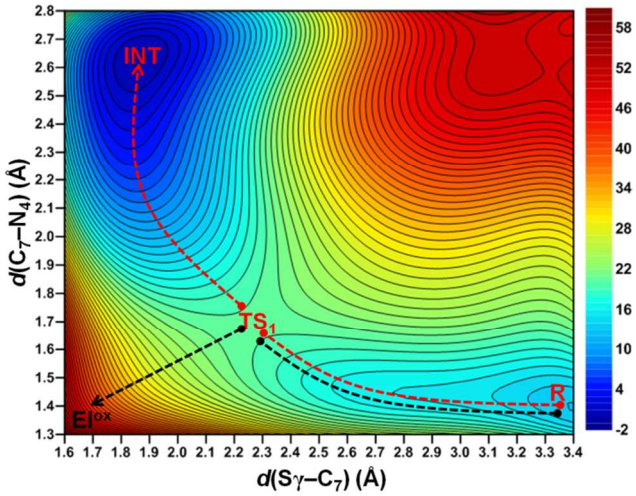


Fig. 2 2D Free energy surface (FES) obtained by DFTB/MM for the first step of meropenem in LDT_{Mt2}. R: reactant; TS_1: transition state; INT: intermediate; EI^{ox}: oxyanion state. The energy values are report in the kcal mol^{-1}. The red dashed line present the minimum energy path (MFEP), while the black dashed line present the experimental proposal.

For the second step, the value for $\Delta G^{\ddagger}_{calc}$ at M06-2X-D3/MM level is 20.00 kcal mol^{-1} (~2.60 kcal mol^{-1} higher than the preceding step) and shows a concerted mechanism similar to the first step (see ESI, Fig. S2). This result agrees with the experimental results.[21] A crystal structure of the LDT_{Mt2}-meropenem complex adopts two different adduct forms for the drug side chain after covalent bond linkage,[22] referred as state I and II. Our computational study shows that the P state found on the second step is in good accordance to the experimental state I (see ESI, Fig. S3), which is not the ground state for the meropenem inhibitor covalently linked to Cys catalytic residue.[8, 22]

More thorough investigation of this reaction mechanism (covalent bond formation between the inhibitor and the catalytic site) is necessary to better understand the main features that contribute to the inactivation of LDTs by carbapanems and for others β-lactam compounds. The relevant calculated parameters (bond distances) found for the chemical species upon inactivation of the active site by these carbapenem inhibitors are summarized in the ESI file (Table S1).

As highlighted previously, the experimental proposal of inactivation of LDTs by carbapenems does not consider the zwitterionic form of the catalytic residues as the most stable protonation state in the enzyme active site. In this report, the theoretical results provided from the MFEP about the inhibition

mechanism of LDT_{Mt2} agree in principle with experimental values. The complete reaction mechanisms including the experimental and theoretical values are summarized in the ESI (Fig. S4 and Table S2, respectively). The $\Delta G^{\ddagger}_{calc}$ values obtained for the whole reaction at M06-2X-D3/MM level are 17.41 and 20.00 kcal mol^{-1} for the first and second step, respectively, which is suitable to experimental evidences. Finally, Our QM/MM simulations may be useful for the design of more potent carbapenem LDT inhibitors, leading to the development of new antibiotics against tuberculosis and other bacilli infections.

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Notes and references

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† Electronic Supplementary Information (ESI) available: [major details of materials and methods, including the PDB code for the crystal structure used. Free energy surfaces, relevant parameters and theoretical findings for the all reactions calculated]. See DOI: 10.1039/b000000x/

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