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Identification of compounds exhibiting inhibitory activity toward the *Pseudomonas tolaasii* toxin tolaasin I using in silico docking calculations, NMR binding assays, and in vitro hemolytic activity assays

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ABSTRACT

Using in silico docking calculations, NMR analysis of target–ligand binding, and hemolytic activity assays, we searched a 30,000-compound library for an effective inhibitor of tolaasin I, a *Pseudomonas tolaasii* toxin that causes virulent infection in mushrooms. Of more than 30,000 compounds screened in silico, two compounds were selected. One of these compounds, sorbitololeic acid, bound to tolaasin I and inhibited its hemolytic activity in vitro. Therefore, sorbitololeic acid can be a potential inhibitor of tolaasin I.

Tolaasin I, an extracellular lipodepsipeptide toxin produced by *Pseudomonas tolaasii*, causes brown blotch disease in mushrooms. As the major factor causing virulent infection of mushrooms by *P. tolaasii*, it forms transmembrane pores, disrupts the cell membrane, and results in tissue alteration.[1] Therefore, a tolaasin I inhibitor could be used to protect mushrooms from virulent infection. In this study, we used in silico docking calculations, NMR spectroscopy-based binding assays, and an in vitro hemolysis assay to search for an effective inhibitor of tolaasin I.

Like many other peptides found in microorganisms, tolaasin I contains D-amino acids.[2] Its specific sequence is β-hydroxyoctanoyl-ΔBut[1]-D-Pro-D-Ser-D-Leu-D-Val-D-Ser-D-Leu-D-Val-L-Val-D-Gln-L-Leu-D-Val-ΔBut-D-alloThr-L-Ile-L-Hse-D-Dab-L-Lys[18], where ΔBut, L-Hse, and D-Dab denote Z-dehydroaminobutyric acid, L-homoserine, and D-2,4-diaminobutyric acid, respectively. Several tolaasin analogues have been reported that have common structural features: a β-hydroxyoctanoic acid moiety, 7 successive D-amino acids at the N-terminus, and a lactone ring between the C-terminus and a D-alloThr hydroxyl group.[3]

Because a compound with inhibitory activity against tolaasin I must be able to bind to it, we first used in silico docking calculations to screen a compound database for compounds with tolaasin I-binding activity. For in silico binding studies, the three-dimensional (3D) structure of the target molecule must be well defined. Although Jourdan et al. reported a 3D structure of tolaasin I in solution[4], the coordinates have not been deposited in the protein databank, so we rebuilt the structure based on the reported NMR data.[5] Tolaasin I is boat-shaped, contains a left-handed α-helix between D-Pro[2] and D-alloThr[14], and a lactone ring between D-alloThr[14] and L-Lys[18]. Since the bottom of the 'boat; consists of D-Ser[3] and D-Ser[6], which face the lactone ring, it is hydrophilic.

More than 32,000 compounds contained in the LeadQuest 3D compound library Vol. 3 (Vol. 3 gold database; Tripos, St. Louis, MO) were screened.[6] The resulting docking scores ranged from −29 to +8.9. Most compounds with negative docking scores had a thiazole moiety, whereas compounds with positive scores had ester-linked sugar and carboxylic acid groups and thus had both hydrophilic and hydrophobic moieties. Not all of the compounds with good docking scores docked spatially into the target molecule. Therefore, the thiazole compounds with negative docking scores were tested for docking spatially to the target molecule, and several compounds were selected as a result. Of these compounds, 2-amino-4,5-dimethylthiazole (ADT) (Fig. 1A), which had a docking score of −7.9, was chosen on the basis of its commercial availability.

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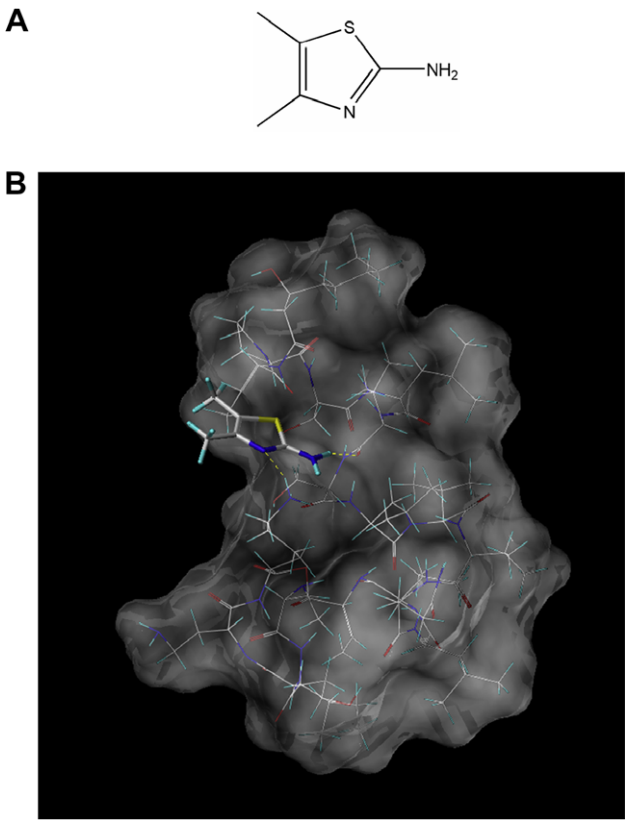

Figure 1. (A) The structure of 2-amino-4,5-dimethyl thiazole, and (B) the 3D structure of the complex of 2-amino-4,5-dimethyl thiazole and tolaasin I.

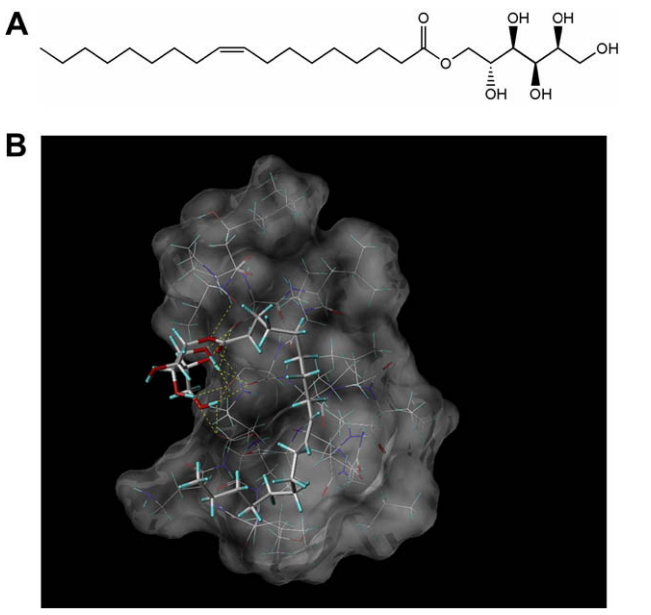

Figure 2. (A) The structure of sorbitololeic acid, and (B) the 3D structure of the complex of sorbitololeic acid and tolaasin I.

The calculated 3D structure of the ADT–tolaasin I complex is shown in Figure 1B. ADT docks into a pocket composed of the ΔBut^{1} and Lys^{18} side chains. An amine proton of ADT makes a hydrogen-bond (H-bond) with the carbonyl oxygen of tolaasin I Ser^{6}, and the nitrogen of the ADT thiazole ring makes an H-bond with an amine proton of Lys^{18} of tolaasin I.

Sorbitololeic acid (Fig. 2A), which has ester-linked sugar yielded a docking score of +8.5 and was selected by a similar process as mentioned above. The calculated 3D structure of the sorbitololeic acid–tolaasin I complex is shown in Figure 2B. The 1-hydroxyl group of the sorbitol moiety makes H-bonds with the Ser^{6} hydroxyl group and the Ser^{6} and Lys^{18} carbonyl oxygens of tolaasin I, and the 2-hydroxyl group of the sorbitol moiety makes H-bonds with the Ser^{3} and Ser^{6} hydroxyl groups and the Ser^{6} carbonyl oxygen of tolaasin I. The sorbitol 3-hydroxyl group makes H-bonds with the Ser^{6} hydroxyl group and the Lys^{18} carbonyl oxygen, and the sorbitol 4-hydroxyl group makes H-bonds with the Ser^{3} and Ser^{6} hydroxyl groups. In addition, the oxygen of the ester bond interacts with the amine group of ΔBut^{1}, and the ester carbonyl group interacts with the amine group of Lys^{18}. Thus, sorbitololeic acid has several spatial interactions with tolaasin I, unlike ADT, and it binds more tightly than ADT to tolaasin I.

To confirm the in silico docking result, assays were performed in vitro using NMR spectroscopy to detect binding of ADT and sorbitololeic acid to tolaasin I. Because the structure of a small molecule is perturbed upon binding to its target molecule, the binding can be monitored by NMR spectroscopy. A large molecule shows faster relaxation and slower diffusion than a small molecule, and fast relaxation causes the NMR signal to broaden. While a large molecule produces a broad NMR line width, a small molecule gives a sharp line width. In fact, the ideal ligand gives a single sharp line well removed from any other signal. When a ligand binds its target molecule, the ligand–target complex behaves like the target.[7,8] Therefore, if a ligand binds the target, the NMR signals of the ligand–target complex are similar to those of the target.[8]

For the NMR experiments, ADT hydrochloric acid (ADTH) and sorbitololeic acid were purchased from Sigma (St. Louis, MO), dissolved in methanol-d_4 (MeOH-d_4) (500 μL) at 1 mM[9], and placed in 5-mm NMR sample tubes. Tolaasin I was isolated from the fermentation broth of *P. tolaasii* according to the methods previously described by Shirata et al. and Kim and co-workers[10,11] The tolaasin I obtained from the broth[12] was dissolved in MeOH-d_4 at a final concentration of 1.5 mM, and various amounts (20–100 μL) of tolaasin I solution were added into the 1-mM ligand solution in the NMR tube. ^{1}H NMR data were collected after every 20 μL addition of tolaasin I solution.

The ^{1}H NMR spectra of ADTH before and after the addition of tolaasin I solution are shown in Figure 3A. The ^{1}H peaks at 2.12 ppm and 2.18 ppm denote 4-methyl and 5-methyl protons, respectively, of ADTH. After the addition of tolaasin I, the peak at 2.12 ppm was shifted upfield to 2.05 ppm, and the peak at 2.18 ppm was shifted upfield to 2.15 ppm. However, when MeOH-d_4 solvent alone was added into the ADTH solution, the resulting NMR spectrum was the same as that shown in Figure 3A[13], indicating that the chemical shifts were probably not caused by formation of the target–ligand complex. Instead, they may have been caused by the exchange of protons from hydrochloric acid with methanol on the ADT amine group.

The ^{1}H NMR spectra of sorbitololeic acid before and after the addition of tolaasin I solution are shown in Figure 3B. Unlike ADTH, the sorbitololeic acid peaks did not shift upon the addition of tolaasin I. Instead, the areas under the peaks changed. The integrated peak area ratio of the 4.57 ppm peak relative to the 0.9-ppm peak increased by 38% after 100 μL of tolaasin I solution was added, and the integrated peak area ratio of the 1.31 ppm peak relative to the 0.9 ppm peak decreased by 32%. The peaks observed at 0.9 ppm, 1.31 ppm, and 4.57 ppm were assigned as the oleic acid methyl

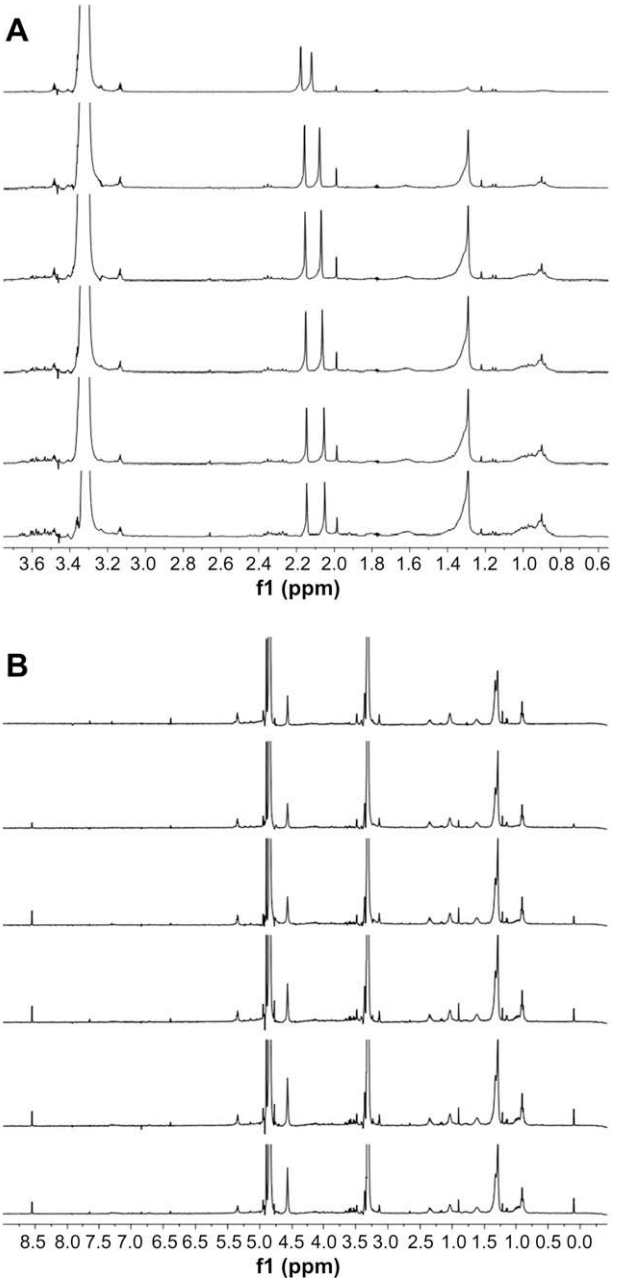

Figure 3. Stacked ^{1}H NMR spectra obtained from successive additions of tolaasin I solution into a solution of ADTH (A) or sorbitololeic acid (B).

group, the oleic acid methylene group, and the sorbitololeic acid hydroxyl group, respectively.

To confirm that these changes in peak area ratios were caused by formation of the tolaasin I–sorbitololeic acid complex, the MeOH-d_4 solvent alone was added into the sorbitololeic acid ligand solution. The peak area ratios did not change[13], indicating that sorbitololeic acid does bind to tolaasin I, whereas ADTH does not. In these experiments, the addition of the tolaasin I solution into the ligand solutions resulted in the dilution of the ligand solution from 1 mM to 0.83 mM.

Although NMR experiments can demonstrate binding, other experiments are needed to demonstrate inhibition of toxicity. As described by Kim and co-workers[11] tolaasin I disrupts cell membranes at low concentration, and this disruption can be detected as hemolysis.[14] One hemolytic unit (HU) of tolaasin is defined as the amount that completely hemolyzes a 1% erythrocyte suspension within 30 min.

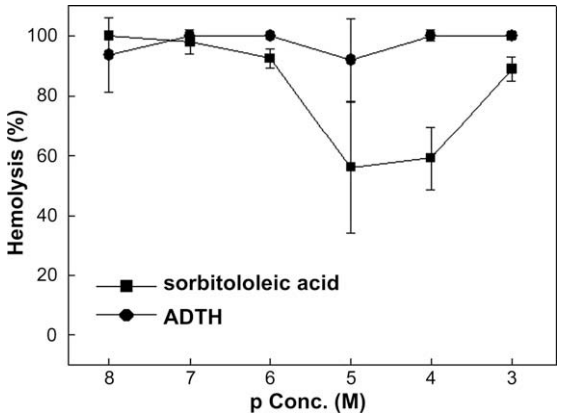

Figure 4. Effects of sorbitololeic acid and ADTH on tolaasin-induced hemolysis. Sorbitololeic acid and ADTH were added at the indicated concentrations.

We measured the effects of sorbitololeic acid and ADTH on tolaasin-induced hemolysis of rat erythrocytes. As shown in Figure 4, in the presence of one HU of tolaasin, sorbitololeic acid at 10–100 μM suppressed hemolysis by 40%. At the concentrations higher than 1 mM, sorbitololeic acid itself acted as a hemolytic agent and increased hemolysis regardless of the presence of tolaasin. Nonetheless, these data demonstrate that sorbitololeic acid binds to tolaasin I and suppresses its hemolytic activity. ADTH, on the other hand, did not affect hemolysis by tolaasin I, in agreement with the NMR results.

Sorbitololeic acid binding may influence any of the toxic processes of tolaasin, including multimerization of tolaasin molecules (which is a prerequisite for toxicity), membrane binding, ion channel formation, and massive ion movement through the tolaasin channel. Since sorbitololeic acid is a detergent, it probably interrupts the multimerization of tolaasin molecules.

In summary, a compound library was searched for compounds with inhibitory activity against tolaasin I using in silico docking experiments and in vitro NMR binding assays. In addition, the ability of the candidate inhibitors to inhibit the hemolytic activity of tolaasin I against rat erythrocytes in vitro was measured. The in silico experiments yielded hundreds of compounds with good docking scores; from these compounds, two were selected based on their spatial docking conditions and commercial availability. When the 3D structures of the ligand–target complexes were elucidated, ADT showed relatively few interactions with tolaasin I, but sorbitololeic acid interacted with it at several points. As expected, NMR experiments revealed that sorbitololeic acid, but not ADTH, bound to tolaasin I. This information about the target–ligand bonding was supplemented with hemolytic activity measurements to examine inhibitory activity. ADT did not inhibit hemolytic activity, but sorbitololeic acid inhibited hemolysis. Therefore, sorbitololeic acid can act as an inhibitor of tolaasin I, which causes virulent infection of mushrooms.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.05.068.

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5. The 3D structure of tolaasin I was recreated using the NMR data obtained from the paper published by Jourdan et al.[4] and which give information about the sequential map. The initial structure of tolaasin I was built using Sybyl (Tripos, St. Louis, MO). The constraints and the dihedral angles of phi were provided based on the sequential map and the data shown in the table of the Letter by Jourdan et al., respectively. Because the paper did not provide information about the lipid moiety, it was assumed to be a flexible chain. Molecular modeling calculations and graphical representation were performed using Sybyl 7.3 on a Linux workstation. Energy minimization was carried out using AmberFF02 force field and Gasteiger–Huckel charges. After energy minimization, simulated annealing was carried out by raising the temperature from 300 K to 1000 K over 1000 fs and then dropping it from 1000 K to 300 K over 5000 fs. The conformer with the lowest energy was chosen for further experiments.
6. The docking calculations were carried out on an Intel Core 2 Quad Q6600 (2.4 GHz) computer running a Linux operating system (CentOs 5.0 WS) using FlexX (Tripos). To select the most biologically active ligand conformation, the maximum number of positions per ligand was set to five. The compounds used for the docking in this experiment were obtained from the LeadQuest 3D compound library Vol. 3: gold type database (Tripos), which contains about 32,000 compounds. Any part of the tolaasin I surface was considered to be a potential docking site. The selection radius for docking was 6.5 Å. The docking process was iterated 30 times for the ligand. The docking scores obtained from the docking process for tolaasin I ranged from −29 to +8.9.
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9. Six mg of tolaasin I was dissolved in methanol-d_4 (MeOH-d_4) at a final concentration of 1.5 mM. ADTH and sorbitololeic acid were dissolved in 500 μL of MeOH-d_4 in 5-mm NMR tubes, and their concentrations were adjusted to 1 mM. All NMR experiments were performed on a Bruker Avance 400 NMR spectrometer (Bruker, Karlsruhe, Germany). The experimental details were the same as those described in Lee et al.[8]
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12. *Pseudomonas tolaasii* was fermented in 1 L of Pseudomonas Agar F (Difco, Detroit, MI) at 25 °C for 3 days. It was extracted with *n*-butanol (1 L), and the extracted solution was freeze–dried for 1 day. The dried material (420 mg) was dissolved in 50% aqueous ethyl acetate (20 mL) and centrifuged at 3200 rpm for 10 min, resulting in a water fraction, an ethyl acetate fraction, and a membrane fraction. Mass spectrometry showed that tolaasin I was in the membrane fraction.[13] The procedure yielded 6 mg of tolaasin I.
13. Data not shown in the text can be found in the Supplementary data.
14. The hemolytic activity of tolaasin I was measured with rat erythrocytes. Defibrinated rat erythrocytes were washed three times with HEPES-buffered saline (5 mM HEPES and 150 mM NaCl, pH 7.4) and then diluted in the same buffer to create a 10% solution of erythrocytes. Tolaasin diluted in the same buffer was added to the erythrocyte suspension, and the mixture was incubated for 30 min at 37 °C. Hemolysis was monitored by observing the change in absorbance at 600 nm using a UV–vis spectrophotometer (U-2000, Hitachi, Tokyo). One unit of tolaasin was defined as the amount of tolaasin that induces complete hemolysis of a 1% erythrocyte suspension within 30 min.