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Evaluation and Binding-Mode Prediction of Thiopyrone-Based Inhibitors of Anthrax Lethal Factor

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Anthrax lethal factor (LF) is one of three proteins involved in anthrax pathogenesis and lethality. Inactivation of the LF gene in *B. anthracis* leads to a decrease in virulence by 1000-fold or greater, which suggests that anthrax pathology is highly dependent on LF.[1] Herein, we report an effective inhibitor of anthrax lethal factor based on a heterocyclic chelator scaffold. We also present computational predictions of the binding mode for this inhibitor and evidence that accurate prediction of binding modes requires use of a molecular surface-like boundary between solute and solvent.

Anthrax LF is a zinc(II)-dependent, hydrolytic enzyme that cleaves the N terminus of the D domain of mitogen-activated protein kinase kinases (MAPKKs). This cleavage impairs essential signal transduction pathways and results in macrophage apoptosis along with other harmful consequences for the host.[2–4] The potential for bioterrorism and inadequate treatments for anthrax, especially at late stages of infection, have amplified interest in finding effective anthrax lethal factor inhibitors (LFi). Several approaches have led to the identification of a variety of LFi,[5–8] including library screening and optimization,[9,10] fragment-based NMR screening (BI-11B3, Figure 1),[11] mass-spectrometry-based screening,[12] and re-examination of inhibitors of other metalloproteinases and related hydroxamate-based compounds.[9,13–15] An example of the latter class is the broad-spectrum matrix metalloproteinase inhibitor (MMPi) GM6001 (Figure 1), which was found to be an effective inhibitor of LF in vitro and in cell culture.[9] Structural characterization of GM6001 in the LF active site shows that the hydroxamate group of the inhibitor chelates to the catalytic zinc(II) ion.[9] Indeed, the direct binding of the active-site zinc(II) ion is proposed to be important in the majority of LFi described to date.[7,9–11] From the use of hydroxamate-based inhibitors, we were prompted to apply a bioinorganic approach to the design of LFi. Our strategy focuses on the metal–ligand interactions of a metalloprotein inhibitor[16–18] which appear to be central to the inhibition of LF. We report the effectiveness of several previously described ligands as zinc-binding groups (ZBGs)[19] for incorporation into LFi. The ZBGs, shown in Figure 1, were selected based on their inhibition of MMPs, as well as their potential to overcome the limitations associated with hydroxamate-based inhibitors.[16] We also report on the potency and binding of a novel, complete LFi based on a thiopyrone chelator (AM-2S, Figure 1).

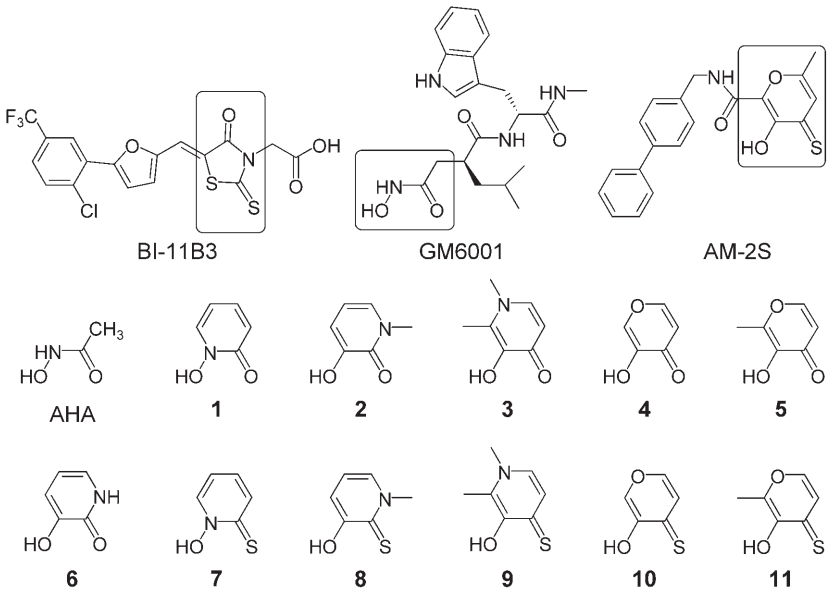

Figure 1. Previously described LF inhibitors BI-11B3 and GM6001; the new LF inhibitor AM-2S is based on one of the heterocyclic zinc-binding groups (ZBGs, **1**–**11**) examined herein. The ZBG of each full-length inhibitor is boxed.

The in vitro potency of compounds **1**–**11** (Figure 1) against LF was evaluated in an assay based on established procedures by using a fluorescent peptide substrate (Table 1).[20] Compounds **1**–**11** were compared with the hydroxamate group used in many LFi and metalloproteinase inhibitors, as represented by acetohydroxamic acid (AHA, Figure 1). It is important to recognize that compounds **1**–**11** represent only the ZBG portion of a metalloproteinase inhibitor. These ZBGs can be used as platforms to which backbone substituents can be

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Table 1. IC_{50} values for ZBGs and AM-2S.

ZBG	IC_{50} [μM][a]	Potency[b]
AHA	11400 ± 1000	n.d.
1	6570 ± 160	1.7
2	32000 ± 3000	0.36
4	27000 ± 3000	0.42
6	6100 ± 500	1.9
7	3900 ± 200	2.9
8	690 ± 70	16
9	1460 ± 60	7.8
10	204 ± 16	56
11	260 ± 30	43
AM-2S	13.9 ± 0.3	820

[a] Values determined in a fluorescence-based assay against LF (± SD) obtained from at least three independent experiments. [b] Reported in fold relative to AHA.

added to provide additional potency and selectivity, resulting in complete LFi. The O,O donor ligands **1**, **2**, **4**, and **6**, on average, showed inhibition similar to that of AHA. Compound **3** had very poor water solubility, and compound **5** showed no inhibition up to its solubility limit of ~6 mM. This suggests that inhibitors based on **1**, **2**, **4**, and **6** will have similar potency to that of hydroxamate-based LFi, but may avoid some of the clinical shortcomings of hydroxamate-based metalloproteinase inhibitors.[16] Furthermore, O,S donor ligands **7**–**11** showed improved LF inhibition over AHA (Table 1). This is consistent with our earlier findings, which show that sulfur-containing ligands inhibit zinc-dependent metalloproteinases more effectively than their O,O analogues.[19]

To obtain potent, selective metalloproteinase inhibitors, the ZBG must be appended to a backbone moiety to target the protein of interest. GM6001 (Figure 1) has a hydroxamate ZBG that is attached to a complex backbone with a hydrophobic leucine mimetic at the P1′ position;[9] the result is that GM6001 is a very potent, broad spectrum MMPi as well as a potent LFi. This motif, a ZBG appended with a hydrophobic P1′ substituent, has been suggested as a general strategy for obtaining potent LFi.[9] Based on this construct, we attached a biphenyl backbone to a thiopyrone ZBG (compounds **10** and **11**) to obtain the inhibitor AM-2S (Supporting Information). Evaluation of AM-2S in the LF assay gave an IC_{50} value of ~14 μM against LF (Table 1). This value is similar to those of several reported LFi,[21] including GM6001, which has been found to have an IC_{50} value of 2–20 μM.[9,11]

Binding modes of AM-2S within the LF active site were investigated computationally using AMBER.[22] A complete set of conformers was generated for AM-2S, and each conformer was aligned with the crystal structure of LF (PDB code: 1PWQ) based on two possible positions for the ZBG (Supporting Information). ZBG positions were based on model compound crystal structures.[17,19] Each alignment of every conformer was energy-minimized under generalized Born (GB) solvation while harmonically restraining the coordinates of the protein, zinc(II) ion, and ZBG.

Two different GB models were employed, OBC GB[23] and GBn.[24] These models differ in how they define the dielectric boundary between the solute and solvent. Their boundaries are related to two of the most commonly used surface definitions, the van der Waals surface (vdWS) and the molecular surface (MS). With a vdWS, any point not inside a solute atom is defined as solvent; with an MS, only points outside the surface defined by rolling a solvent sphere over the solute are defined as solvent. The MS has the attractive property of excluding any space smaller than a water molecule from the solvent region, whereas the vdWS defines these small crevices between solute atoms as containing water. vdWS (or related smoothed surfaces) are commonly used in implicit solvent models because they are computationally more tractable than MS, but recent results have shown that the nonphysical solvent pockets allowed by vdWS can cause errors in protein solvation free energies and hydrogen bonding potentials.[25] Both the OBC GB and GBn models are based on a vdWS with correction terms that attempt to emulate the properties of an MS. OBC GB employs a geometry-independent correction that makes corrections on an averaged basis and has little effect on surface atoms, whereas GBn uses a geometrically based pairwise correction that corrects for solvent pockets near the surface as well as within the core of the protein.

Both GB models identified the structure shown in Figure 2 as having the lowest free energy. This structure is 3.4 kcal mol^{-1} lower in energy than the next-best conformer if calculated by

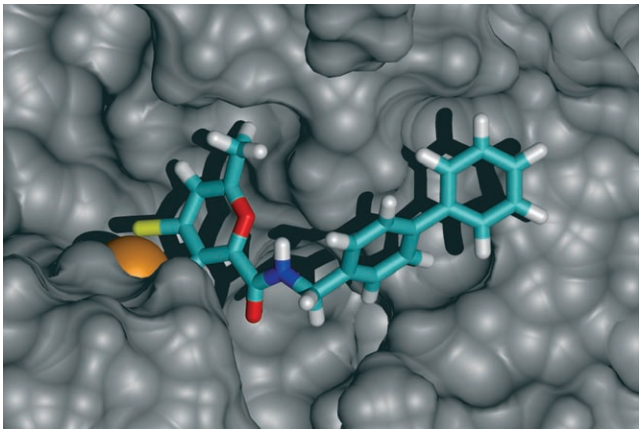

Figure 2. Lowest-energy configuration of AM-2S in the LF active site, as identified by both GB models. AM-2S is colored by atom type, and the active-site zinc(II) ion is represented by an orange sphere.

the OBC GB model and 2.5 kcal mol^{-1} lower if calculated by GBn. The biphenyl group is found in the substrate-binding groove of LF near the binding location of that observed for the LF20 peptide.[9]

In contrast, for the second alignment of the ZBG, the inhibitor conformation with the lowest free energy was dependent on which dielectric boundary was used. The lowest-energy conformers calculated by GBn and OBC GB are shown in Figure 3. The lowest-energy conformer calculated with OBC GB places the biphenyl group into a narrow groove, which is highlighted in Figure 4.

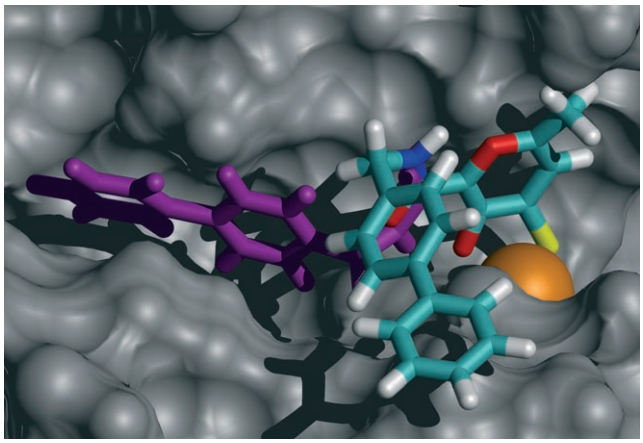

Figure 3. Lowest-energy configurations of AM-2S in the LF active site for the second ZBG orientation. Configuration I (purple) appears to be lower in energy only with use of a vdWS-like solvent–solute boundary such as that employed by OBC GB; configuration II (colored by atom) is the correct configuration as identified by GBn.

Considering only the nonsolvation components of the calculated energy, the configuration identified by OBC GB (henceforth referred to as configuration I, Figure 3, purple) is lower in energy than the configuration calculated with GBn (configuration II, Figure 3, colored by atom). This difference in energy is due mostly to more favorable van der Waals interactions between the biphenyl group and the protein. However, configuration I, highlighted in Figure 4, entails a significant energetic

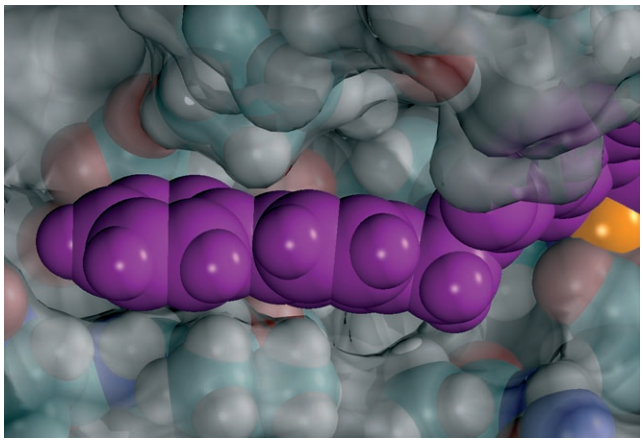

Figure 4. Space-filling view of the biphenyl group (purple) for configuration I from Figure 3. LF is colored by atom type, with transparent gray molecular surface superimposed to aid visualization. Note the small crevices between the biphenyl group and the LF binding groove and the partially obscured oxygen atoms near the bottom of the groove.

penalty for desolvating the polar groups in the groove by displacing water with the nonpolar biphenyl group. In the more vdWS-like OBC GB model, this penalty is underestimated as a result of solvent-filled crevices between the biphenyl group and protein (Figure 4), and configuration I is calculated to have a solvation energy only 2.8 kcal mol^{-1} higher than that of configuration II, such that configuration I is incorrectly identified as having the lowest total energy. In the more MS-like GBn model, solvent is excluded from these crevices, leading to a greater difference in solvation energies of 5.8 kcal mol^{-1}, which is sufficient to identify configuration II as having the lowest energy.

Because GB models involve approximations of both the dielectric boundary and electrostatic interactions, the results were confirmed by using the less efficient but more physically rigorous Poisson–Boltzmann (PB) method implemented in APBS.[26] PB calculations with an uncorrected vdWS show little difference in solvation energy, with configuration I more favorable than II by 0.4 kcal mol^{-1}. When the PB calculation employed a true MS, the solvation energy of configuration II is 36.6 kcal mol^{-1} lower than that of I, confirming the selection of configuration II by GBn as the correct result. Although the solvation energies calculated by GBn show substantial improvement over OBC GB in terms of agreement with MS PB results, GBn does yield a significantly smaller difference in solvation energies between the two configurations than found in the MS PB results. This suggests that the MS approximation employed by GBn does not completely correct the underestimation of desolvation of polar groups in the groove for configuration I.

The configuration illustrated in Figure 2 is approximately 28 kcal mol^{-1} lower in energy than the best alternative in the second ZBG orientation illustrated in Figure 3. Nevertheless, Figure 3 may represent a relevant configuration, as there are unfavorable steric interactions between the ZBG and protein for both ZBG orientations, but they are considerably worse for the conformers in Figure 3. It was necessary to keep the protein fairly rigid so that the minimizations would be computationally tractable, which precluded these unfavorable interactions from being relaxed in the computational modeling. Although it seems likely that the protein would be sufficiently flexible to decrease or eliminate these interactions, there is no straightforward way to calculate the resulting conformations or energies. Therefore, while it is reasonable to compare the relative energies of different poses with the same ZBG orientation, no fair comparison can be made between poses having different ZBG orientations on the basis of the results presented herein. Until further studies have been conducted that incorporate greater protein flexibility or alternate protein conformations that do not clash with the ZBG, the poses illustrated in Figures 2 and 3 (configuration II) should both be considered valid possibilities.

The results presented herein illustrate the importance of a physically realistic dielectric boundary in docking and binding studies. Earlier work on this topic with small-molecule hydrogen bonding and salt bridge models had shown that errors due to solvent pockets occurred mostly for high-energy configurations (transition states),[25] but these results show that for small-molecule–protein interactions, even minimum-energy conformations can be sufficiently affected to produce erroneous ranking of binding modes. It may be noted that a nonpolar small molecule and a polar, concave protein surface, as found in the system examined herein, are likely to produce the greatest disparity between MS and vdWS results. However,

these properties are sufficiently common in systems of interest that the problems caused by nonphysically small solvent pockets cannot be safely ignored.

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