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Inhibitors of anthrax lethal factor

Brandon D. Gaddis,[a] Larisa V. Avramova[b] and Jean Chmielewski[a,b,*]

[a]*Department of Chemistry, Purdue University, West Lafayette, IN 47907, USA*
[b]*Bindley Bioscience Center, Purdue University, West Lafayette, IN 47907, USA*

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Abstract—An inhibitor of anthrax lethal toxin mediated cell death (**1**) was identified by a medium throughput cell-based screen. This compound was determined to specifically inhibit anthrax lethal factor (LF), and subsequent SAR studies produced an even more potent inhibitor (**4**). Mechanistic studies identified these agents as uncompetitive inhibitors of LF with K_i values of 3.0 and 1.7 μM, respectively, with good cell potency and low cytotoxicity.

Anthrax lethal factor (LF) is a 776 residue, Zn^{2+}-dependent metalloprotease which functions to cleave members of the mitogen-activated protein kinase (MAPK)- kinase (MEK) family.[1–3] Cleavage of MEKs occurs within the N-terminal proline-rich region which lies directly before the kinase domain.[4] This cleavage disrupts the ability of MEKs to phosphorylate and activate MAPKs, and disrupts modulation of cellular responses to various stimuli including pathogens.[5–7] LF has been shown to act as the key virulence factor for *Bacillus anthracis* since mouse death is seen in the absence of edema factor, but not in the absence of lethal factor.[8]

Because of its vital role in anthrax pathogenesis, much work has focused on finding potent inhibitors of LF. For instance, a library of peptide hydroxamates based on metalloprotease peptide substrates was screened and one was found to inhibit LF with a K_i value of 1 nM.[9] A library of known Zn-metalloprotease inhibitors was screened against LF, and a subsequent structure–activity study produced a small molecule hydroxamate with IC_{50} values of 54 nM and 210 nM for the in vitro and cell-based assays, respectively.[10] Polyphenolic compounds in green tea were tested for inhibition of LF, and a catechin gallate was found to be a potent inhibitor of LF with IC_{50} values of 97 nM and <1 μM for in vitro and cell-based assays, respectively.[11] These studies led to the identification of several non-competitive LF inhibitors with galloyl moieties that were generated via a Pictet–Spengler reaction followed by in situ screening (K_i values of 2–124 μM).[12]

High-throughput screening (HTS) has also been used extensively to identify inhibitors of LF, including small molecule quinoline-containing compounds (K_i values of 0.5–5 μM),[13] rhodanine derivatives (IC_{50} values of 0.2–80 μM following an SAR study),[14] a small molecule catechol (K_i value of 1.1 μM),[15] a series of mixed-type inhibitors (IC_{50} values of 1–11 μM),[16] and aminoglycosides.[17] Further work with aminoglycosides and cationic derivatives produced additional mixed-type LF inhibitors.[18–21]

Currently, in the literature, there are several potent LF inhibitors that work in vitro. However, there are significantly fewer inhibitors with cell-based activity, an important point since LF functions in the cytosol.[3] Therefore, it is essential that agents not only inhibit LF cleavage of substrate, but are also active in a lethal toxin challenge of macrophages. Identifying compounds that inhibit LF, yet are able to enter and remain active in cells, constitutes a major challenge in developing effective anti-lethal factor therapeutics.

Toward the goal of identifying compounds which are effective at rescuing cells from lethal toxin-induced death, the LOPAC 1280 library of compounds from Sigma was screened using a medium throughput, lethal toxin challenge, cell-based assay.[22] Several compounds were found that rescued cells from lethal toxin mediated cell

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* Corresponding author at present address: Department of Chemistry, Purdue University, West Lafayette, IN 47907, USA; e-mail: chml@purdue.edu

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death. A secondary in vitro lethal factor (FRET) assay[9,23] was used with this subset of compounds, and *N*-oleoyldopamine (OLDA) (Fig. 1, **1**) was identified as an inhibitor of LF with an IC_{50} value of 15 ± 2 μM.[24]

OLDA is a known agonist of the transient receptor potential vanilloid type 1 (TRPV1) protein and is also a weak CB1 cannabinoid receptor ligand,[25] but no activity against LF has been described. Importantly, OLDA was found to have activity in the cell-based assay with an IC_{50} value of 5.0 ± 0.2 μM for rescuing cells from lethal toxin induced death. OLDA was also found to have low cytotoxicity itself, with complete cell viability up to a concentration of 30 μM. It is interesting that OLDA is more potent in the cell-based assay as compared to the in vitro assay with LF. This difference may simply be due to the differences in LF concentration in the two experiments (50 nM vs 0.6 nM, respectively), however other reported inhibitors using similar conditions demonstrated a drop in efficacy in the cell assay. Therefore, it is possible that OLDA may play additional roles in cell rescue.

The additional goals of this work were to determine the mode of inhibition used by OLDA and to elucidate what structural elements on the aromatic ring were important for inhibition of LF. In order to elucidate OLDA's mechanism of inhibition, kinetic studies were undertaken.[26] A Lineweaver–Burk analysis of OLDA with LF (Fig. 2) produced data whose lines were parallel, indicative of uncompetitive inhibition (as did analysis

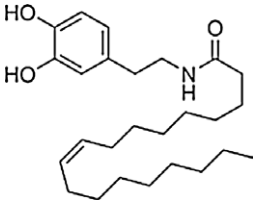


Figure 1. Structure of OLDA (**1**).

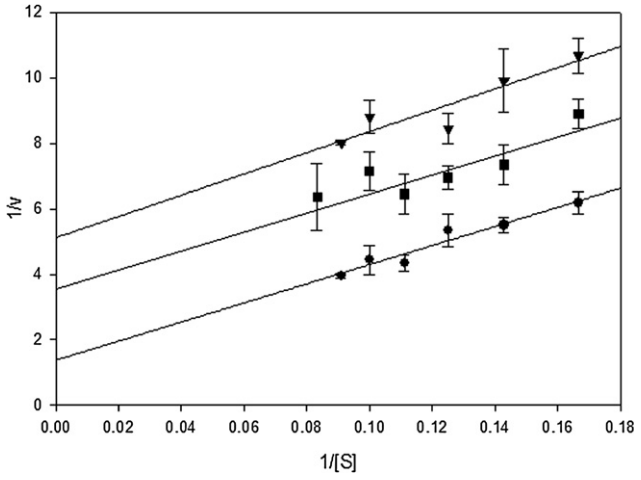


Figure 2. Lineweaver–Burk analysis of OLDA (**1**) with LF (12.5 nM): (●) 0 μM OLDA, (■) 4.1 μM OLDA, (▼) 8.2 μM OLDA; pH 7.4, Hepes buffer, 25 °C.

of the non-double reciprocal kinetics data). These data provided a K_i value for OLDA of 3.0 ± 0.2 μM. An uncompetitive mechanism of inhibition indicates that OLDA and substrate may co-occupy LF, producing an inactive ESI complex, a fact that seems at first glance to be at odds with possible Zn^{2+}-binding of the catechol moiety of OLDA. It has been demonstrated that Zn^{2+}, however, is not essential for substrate binding to LF; indeed a co-crystal structure of LF (without zinc) and substrate has been obtained.[27] The true nature of this co-occupation awaits structural elucidation.

Because catechols are known in the literature to bind Zn^{2+},[28] it was hypothesized that the catechol moiety in OLDA binds the Zn^{2+} in LF, preventing substrate cleavage. Therefore, proposed modifications to OLDA focused on the catechol moiety (Fig. 3). If the catechol is binding the Zn^{2+} in the LF active site, removal of a hydroxyl group (**2**) or methylation of the hydroxyl groups (**3**) should produce compounds which are no longer inhibitors of LF. Compounds **2** and **3** were synthesized in one step by treating the necessary phenethylamines with the *N*-hydroxysuccinimide (NHS) ester of oleic acid (Scheme 1a).[29] Agents **2** and **3** were found to not inhibit LF up to a concentration of 100 μM, lending support to the Zn^{2+}-binding hypothesis.

The next proposed change to OLDA involved the addition of hydroxyl groups to the catechol moiety (**4** and **5**). These agents were synthesized as described for compounds **2** and **3**.[30] Addition of a hydroxyl group in the meta position to the OLDA chain (**4**) had little effect on LF inhibition as compared to OLDA with an IC_{50} value of 13 ± 2 μM. Interestingly, the addition of a hydroxyl group in the ortho position to the OLDA chain (**5**) was found to decrease the potency significantly (IC_{50} value of 70 ± 3 μM). It is hypothesized that a hydroxyl substituent in this position may prevent proper inhibitor binding due to steric or electronic factors.

Kinetic studies were undertaken to determine the mechanism of inhibition for compound **4**. As with OLDA, compound **4** was found to be an uncompetitive inhibitor of LF with a K_i value of 1.7 ± 0.1 μM, a twofold increase in potency as compared to OLDA. Compound **4** was also evaluated in the anthrax lethal toxin challenge, cell-based assay. In this assay compound **4** demonstrated the ability to rescue cells from lethal toxin-mediated cell death. Cell rescue by **4** reached a maximum at a concentration of 20 μM (46–49%), but addition of increasing amounts of inhibitor beyond 20 μM did not lead to additional cell rescue up to a concentration of 85 μM. Inhibitor **4** was found to have low cytotoxicity with >85% cell viability at 30 μM.[31] These data may suggest that cell rescue by **4** could be limited by its ability to cross the cell membrane and enter the cytosol where LF functions.

The final proposed alteration involved substitution of the catechol for another known Zn^{2+} binding moiety, a hydroxamate (**6**). The synthesis of compound **6** (Scheme 1b) proceeded by coupling hydroxylamine hydrochloride to 4-(tert-butoxycarbonyl)ethylbenzoic

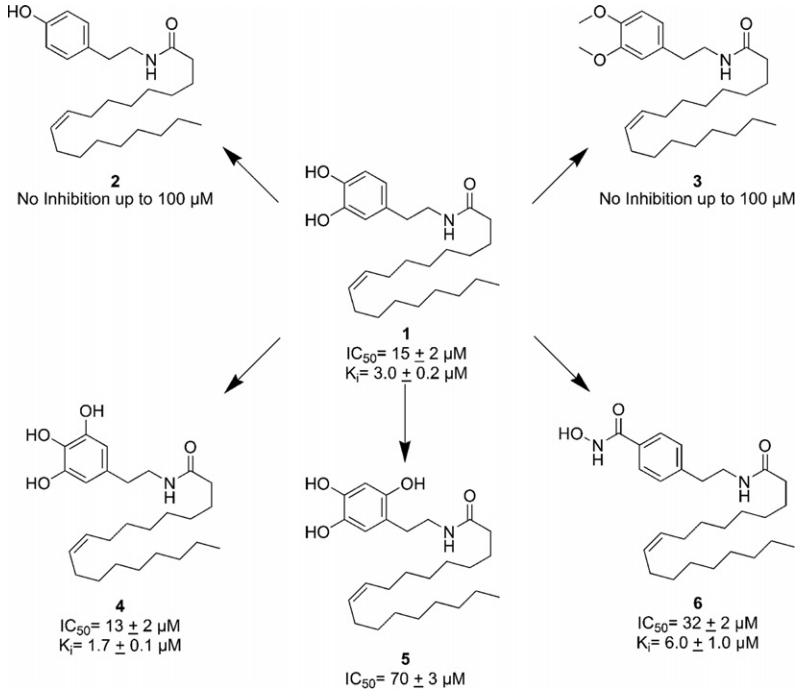
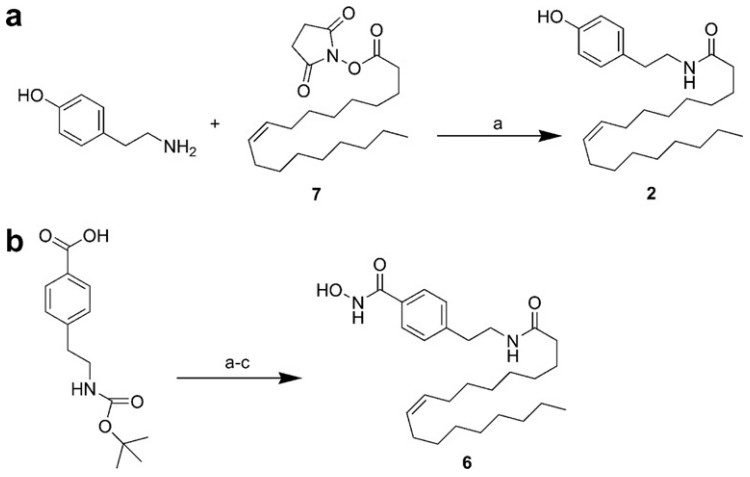


Figure 3. Structures of synthetic agents containing modifications to the phenyl-moiety of OLDA.



Scheme 1. (a) A representative synthesis of compound **2**; a—3 equiv of DIEA, DMSO; (b) synthesis of compound **6**; a—2 equiv of hydroxylamine hydrochloride, 2 equiv of EDCI, 0.2 equiv of DMAP, 2 equiv of DIEA, DCM, rt; b—TFA-DCM (1:1), 0 °C; c—1 equiv of **7**, 3 equiv of DIEA, DMSO, rt.

acid, followed by Boc-deprotection, and subsequent reaction of the free amine with NHS-activated oleic acid (**7**).[32] Incorporation of a hydroxamate group para to the OLDA chain produced an LF inhibitor with an IC_{50} value of 32 ± 2 μM. Lineweaver–Burk analysis of **6** with LF provided a K_i value of 6.0 μM and indicated that **6** also acted by an uncompetitive inhibition mechanism. From these results, we can conclude that alternative metal-binding moieties can be incorporated into OLDA-like molecules to produce LF inhibitors.

In conclusion, we have identified two heretofore undisclosed inhibitors of anthrax LF. Interestingly, these agents function as inhibitors in an uncompetitive mode. One compound, OLDA (**1**), also demonstrated good activity in the anthrax lethal toxin challenge of macrophages with low cytotoxicity. Modifications of **1** have shown that the catechol moiety is essential for activity, and that there is the potential for producing inhibitors with alternate metal-binding moieties. Future work will focus on identifying inhibitors with enhanced metal-binding properties as well as determining the importance of the hydrophobic tail of OLDA for inhibition.

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References and notes

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29. *Characterization data for compounds* **2** *and* **3**. Compound **2**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.06 (d, J = 8.1 Hz, 2H), 6.80 (d, J = 8.1 Hz, 2H), 5.42 (s, 1H), 5.36–5.30 (m, 2H), 3.48 (q, J = 6.3 Hz, 2H), 2.65 (t, J = 6.3 Hz, 2H), 2.16 (t, J = 7.5 Hz, 2H), 2.05–1.99 (m, 4H), 1.59–1.55 (m, 2H), 1.29–1.27 (m, 20H), 0.88 (t, J = 6.3 Hz, 3H); MALDI MS: m/z 402.53 ($M+H^+$), 424.45 ($M+Na^+$). Compound **3**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.80 (d, J = 6.3 Hz, 1H), 6.74 (d, J = 6.3 Hz, 1H), 6.72 (s, 1H), 5.38 (s, 1H), 5.36–5.30 (m, 2H), 3.88 (s, 6H), 3.48 (q, J = 6.3 Hz, 2H), 2.65 (t, J = 6.3 Hz, 2H), 2.16 (t, J = 7.5 Hz, 2H), 2.05–1.99 (m, 4H), 1.59–1.55 (m, 2H), 1.29–1.27 (m, 20H), 0.88 (t, J = 6.3, 3H); MALDI MS: m/z 446.03 ($M+H^+$), 468.92 ($M+Na^+$).
30. *Characterization data for compounds* **4** *and* **5**. Compound **4**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.33 (s, 2H), 5.72 (s, 1H), 5.36–5.30 (m, 2H), 3.48 (q, J = 6.3 Hz, 2H), 2.65 (t, J = 6.3 Hz, 2H), 2.16 (t, J = 7.5 Hz, 2H), 2.05–1.99 (m, 4H), 1.59–1.55 (m, 2H), 1.29–1.27 (m, 20H), 0.88 (t, J = 6.3 Hz, 3H); MALDI MS: m/z 434.62 ($M+H^+$); 456.20 ($M+Na^+$). Compound **5**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.53 (s, 1H), 6.45 (s, 1H), 6.35 (s, 1H), 5.36–5.30 (m, 2H), 3.48 (q, J = 6.3 Hz, 2H), 2.65 (t, J = 6.3 Hz, 2H), 2.16 (t, J = 7.5 Hz, 2H), 2.05–1.99 (m, 4H), 1.59–1.55 (m, 2H), 1.29–1.27 (m, 20H), 0.88 (t, J = 6.3 Hz, 3H); MALDI MS: m/z 434.44 ($M+H^+$).
31. Cytotoxicity study: J774A.1 cells were allowed to grow to confluency in 96-well plates before 0–100 μM compound in media (0.6% DMSO) was added to the cells. Cells were allowed to incubate with the compound mixture for 4 h at 37 °C before MTT was added. Cells were allowed to incubate for an additional 1.5 h at 37 °C before cell viability was determined.
32. Characterization data for compound **6**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.07 (d, J = 8.1 Hz, 2H), 7.32 (d, J = 8.1 Hz, 2H), 5.45 (s, 1H), 5.36–5.30 (m, 2H), 3.57 (q, J = 6.3 Hz, 2H), 2.91 (t, J = 6.3 Hz, 2H), 2.16 (t, J = 7.5 Hz, 2H), 2.05–1.99 (m, 4H), 1.59–1.55 (m, 2H), 1.29–1.27 (m, 20H), 0.88 (t, J = 6.3 Hz, 3H); MALDI MS: m/z 445.07 ($M+H^+$).