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Antagonism of a zinc metalloprotease using a unique metal-chelating scaffold: tropolones as inhibitors of *P. aeruginosa* elastase†

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Tropolone emerged from the screening of a chelator fragment library (CFL) as an inhibitor of the Zn^{2+}-dependent virulence factor, *Pseudomonas aeruginosa* elastase (LasB). Based on this initial hit, a series of substituted tropolone-based LasB inhibitors was prepared, and a compound displaying potent activity *in vitro* and in a bacterial swarming assay was identified. Importantly, this inhibitor was found to be specific for LasB over other metalloenzymes, validating the usage of tropolone as a viable scaffold for identifying first-in-class LasB inhibitors.

Tropone, and its hydroxylated derivative tropolone, are planar, 7-membered, unsaturated rings that are found in natural products with diverse biological activities, including compounds displaying antibacterial, antiinflammatory, antitumor, and antiviral activity.[1–3] The metal-binding capacity of tropolone is well known, using its exocyclic oxygen donor atoms to bind metal ions (*i.e.* O,O donor ligand, Fig. 1),[4] and the employment of tropolone-based units in the design of metalloprotein inhibitors has been explored.[5,6] Tropolone has been identified as an inhibitor of several Zn^{2+}-dependent metalloenzymes including carboxypeptidase A, thermolysin, matrix metalloproteases (MMP-2 and -3), and anthrax lethal factor (LF) with IC_{50} values ranging from 0.003−1.4 mM.[1,4,7] Tropolone has also been found to be a potent inhibitor of the dinuclear copper-dependent enzyme tyrosinase (IC_{50} value of ∼400 nM);[8] however, a recent crystal structure of tropolone bound to tyrosinase revealed that the natural product does not act by coordinating to the metal ion.[9]

In an effort to identify suitable metal-binding groups (MBGs) for targeting metalloprotein active sites, a fragment-based drug discovery (FBDD) approach has been applied *via* the development of chelator fragment libraries (CFLs). CFLs are specifically designed with fragments that can coordinate metal ions in the active site of metalloproteins. This approach has revealed novel scaffolds such as hydroxypyrones, hydroxypyridiones, hydroxyquinolines, and quinolone sulfonamides to be effective MBGs against a variety of metalloproteins, including MMPs, LF, and several others.[4,7,10]

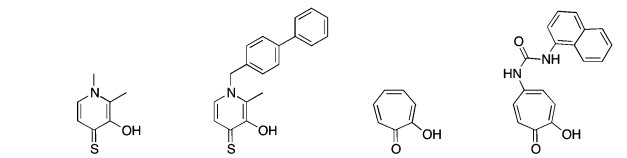

Fig. 1 Metal-binding groups (MBGs) and derived inhibitors with IC_{50} values listed for LasB inhibition.

LasB[11,12] is one of several virulence factors produced by *P. aeruginosa* to promote infection within a host.[13,14] Previous mutation-[15] and vaccine-based[16] studies have revealed that LasB plays a critical role in promoting virulence through targeted proteolysis of host tissue proteins and immune system components.[11] Moreover, LasB has also been linked to the establishment of antibiotic-resistant biofilm[17] and swarm colonies.[18,19] Because evidence exists supporting the investigation of virulence factors as promising new antibiotic targets,[20–22] the pursuit of non-peptidic, small molecule inhibitors of LasB is of interest. Recently, the screening of CFL-1.1 against *P. aeruginosa* elastase (LasB) was shown to produce several hits.[19] Among the initial hits was 3-hydroxy-1,2-dimethylpyridine-4(1*H*)-thione (3,4-HOPTO, **1**), a chelator that is also a potent inhibitor of MMPs, LF, and others metalloproteins.[10] Because 3,4-HOPTO displayed activity against LasB (IC_{50} value 81 μM, Fig. 1), an existing sublibrary of 3,4-HOPTO derivatives[10] was examined, which revealed compound **2** (Fig. 1) as a potent LasB antagonist and the first mechanism-based compound to exhibit swarming antagonism.[19] Despite its exceptional activity, the 3,4-HOPTO MBG (**1**) is known to inhibit a broad set of metalloproteins and it is likely that **2**

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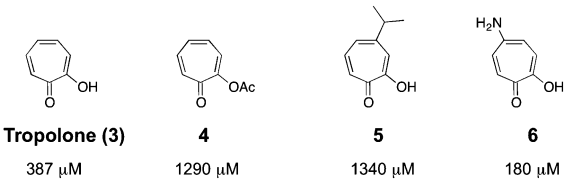

Fig. 2 Tropolone fragments with IC_{50} values listed for LasB inhibition.

and related compounds will not be highly selective for LasB (indeed compound **2** is known to inhibit MMPs and LF at concentrations of 1–4 μM).[10]

Screening of CFL-1.1 against LasB had produced other, albeit less active, MBG hits. It was hypothesized that these hits, which still showed good ligand efficiencies,[23,24] could be used to develop potent, and more selective LasB inhibitors. Herein, we report the discovery of tropolone-based inhibitors of LasB. Importantly, the identified antagonists are not only more potent than the previously reported 3,4-HOPTO-derived compound **2**, but are also highly selective for LasB over other metalloproteins.

From our prior screening of CFL-1.1 against LasB,[7,10] tropolone (**3**, Fig. 2) emerged as a "hit", with an IC_{50} value of 387 ± 1 μM.[19] As mentioned earlier, tropolone is known to inhibit Zn^{2+}-dependent thermolysin *via* metal coordination.[25,26] Importantly, acetylation of **3** to produce acetyltropolone (**4**, Fig. 2)[1] resulted in a ~3-fold reduction in potency (IC_{50} value of 1.29 ± 0.24 mM) suggesting that Zn^{2+} chelation is required for LasB inhibitory activity. Again, this parallels findings with thermolysin, where acetylation of tropolone resulted in a loss of activity.[1] Also contained in CFL-1.1 was β-thujaplicin (**5**, Fig. 2), a tropolone natural product containing an isopropyl group in the 4-position of the ring.[2,3] The IC_{50} value of **5** was 1.34 ± 0.37 mM, which is ~3-fold less potent than **3**, perhaps suggesting that steric repulsion at the 4-position is prohibiting proper Zn^{2+} chelation within the active site. However, 5-aminotropolone (**6**, Fig. 2) was found to be a more potent hit, with an IC_{50} value of 180 ± 1 μM, suggesting that selected substitutions may be tolerated on the ring system.

Encouraged by these findings, a sublibrary of compounds based on fragment **6** was constructed *via* a one-step condensation of **6** with isocyanates under microwave irradiation conditions (Fig. 3, Fig. S1†). This sublibrary was screened using a fluorescence-based assay (Fig. S2†),[19,25] and several compounds were found to have IC_{50} values of <10 μM (Table S1†). The best compounds identified from this sublibrary were more potent than the previously reported 3,4-HOPTO-based inhibitors by ~2-fold.[19] In particular, compound **7a** was found to be the most potent non-peptidic small molecule inhibitor of LasB identified to date with an IC_{50} value of 1.16 ± 0.06 μM. Moreover, compound **7a** was found to be a competitive inhibitor of the enzyme with a K_i of 336 nM (Fig. S4†). To determine the structural relevance of the urea linker used in these compounds, an analogue of **7a**, containing an amide linker was synthesized (**8**, Fig. 3) resulting in a >8-fold loss in activity. Coupling to a shorter amide linker (**9**, Fig. 3) produced a nearly 50-fold loss in potency. These compounds demonstrate that both the urea linkage and the linker length contribute to the activity of **7a** against LasB. Finally, acetylation of **7a** was performed to produce compound **10** (Fig. 3) with a blocked

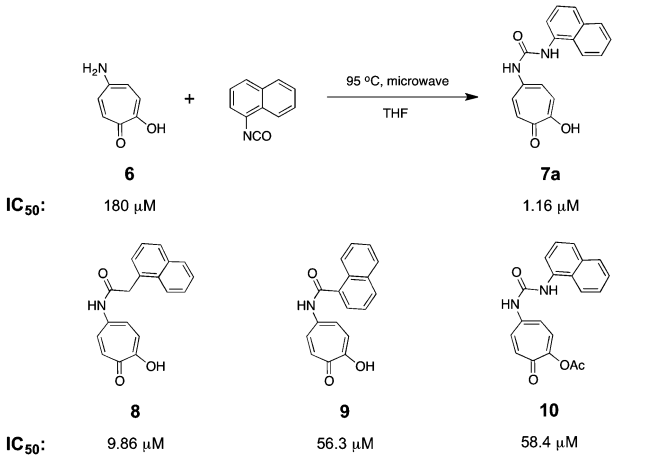

Fig. 3 Structures and IC_{50} values for tropolone sublibrary compounds.

MBG (analogous to compound **4** above). Compound **10** also showed a ~50-fold reduction in activity (IC_{50} value of 58.4 ± 0.1 μM, Fig. S3†), providing strong evidence that metal coordination is essential for LasB inhibition by tropolone compounds. This was further verified by acetylation of another potent derivative **7e** (Fig. S1, Table S1†), which resulted in an even greater loss of potency (>100-fold, data not shown).

Because the 3,4-HOPTO chelating motif found in **2** (Fig. 1) was known to inhibit other Zn^{2+} metalloproteins, including MMPs,[7,10] the specificity of **2** *versus* tropolone **7a** was examined against a panel of metalloenzymes. Three Zn^{2+}-dependent metalloproteins, MMP-2, MMP-9, and human carbonic anhydrase II (hCAII), and one Cu^{2+}-dependent metalloenzyme, tyrosinase, were examined as a gauge of selectivity. Specificity was determined by treating each enzyme with 50 μM of either **2** or **7a** and monitoring percent enzymatic activity. Both **2** and **7a** had a modest effect on hCAII, each reducing activity by ~25% at 50 μM (Fig. S5†). In contrast, **2** was found to completely suppress the enzymatic activity of MMP-2 and MMP-9 at 50 μM, while **7a** showed only weak inhibition (<40% at 50 μM, Fig. 4). Finally, neither **2** nor **7a** showed any inhibition against tyrosinase. The loss in activity of **7a** against tyrosinase is a key observation, as unsubstituted tropolone (**3**) is a very potent inhibitor of this enzyme (IC_{50} value ~400 nM, Fig. S5†). Similar results were obtained with several other tropolone-derived inhibitors (data not shown). Therefore, by derivatizing tropolone into a more advanced lead compound (**7a**), a highly selective LasB inhibitor has been obtained that, unlike HOPTO-derived inhibitors, is selective for LasB over other Zn^{2+}-dependent metalloproteins, and is also inactive against the Cu-dependent tyrosinase, for which the tropolone MBG has an inherently high affinity. This illustrates that by judicious selection of both MBG and backbone target-specific metalloprotein inhibitors can be designed.

Based on the enhanced potency and specificity of compound **7a**, its activity was further characterized in a cellular assay. As stated above, LasB activity is required for the induction of swarming in *P. aeruginosa*.[18,19] Importantly, the ability of *P. aeruginosa* to form swarm colonies has been linked to the development of antibiotic resistance,[27,28] indicating that small molecule inhibitors of LasB could be used as adjuvants with traditional antibiotics

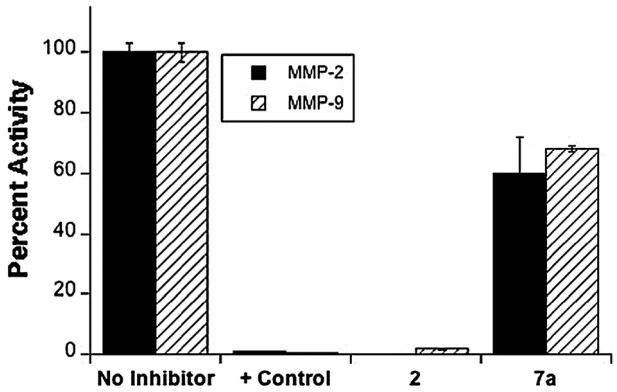

Fig. 4 Inhibition of Zn^{2+}-dependent MMP-2 (solid) and MMP-9 (lines) by a broad-spectrum MMP inhibitor (+ control†), **2**, and **7a**.

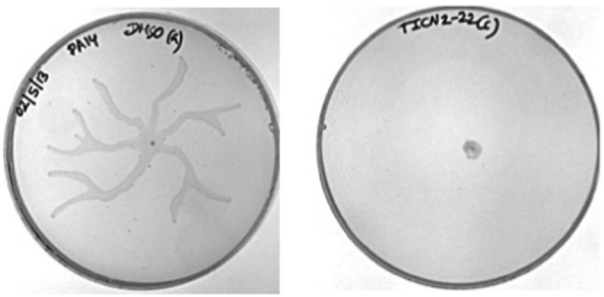

Fig. 5 Swarming of *P. aeruginosa* strain PA14 in the absence (left, DMSO control) or presence of **7a** (right, 25 μM).

to enhance the susceptibility of antibiotic-resistant *P. aeruginosa* to these drugs.[29] To examine the anti-swarming activity of compound **7a**, *P. aeruginosa* strain PA14 was grown on swarm agar plates containing either DMSO (control) or 25 μM of **7a**. As shown in Fig. 5, this tropolone-based inhibitor was able to completely inhibit the swarming phenotype at this concentration, exhibiting swarming inhibitory properties comparable to **2**.[19] Importantly, **7a** was found to be non-cytotoxic to PA14 at a concentration of 25 μM (Fig. S6†). Finally, compound **10**, which has an acetylated tropolone MBG, was found to be much less effective at inhibiting swarming (Fig. S7†). Thus, these results demonstrate the potential of this natural product-based chelating moiety for the design of antimicrobial metalloprotease inhibitors.

In conclusion, tropolone-based metalloprotein inhibitors have been developed by a chelator-focused FBDD approach. These compounds are the most potent non-peptidic small-molecule inhibitors of LasB reported to date and show excellent activity in a cell-based swarming assay. Importantly, the tropolone MBG-derived inhibitors are more active and more selective than the previously identified HOPTO-based compounds. The work presented here is consistent with earlier studies on tropolone-based metalloprotein inhibitors. While the majority of the previous tropolone-based inhibitors were identified by screening of natural products, this study demonstrates how use of chelator fragment libraries and sublibraries can rapidly identify leads for the development of such inhibitors. The present findings clearly suggest that identification of privileged chelating scaffolds for a given metalloenzyme can lead to the realization of both *potent and selective* metalloprotein inhibitors.

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