

## RESEARCH ARTICLE

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# *N*-Aryl mercaptoacetamides as potential multi-target inhibitors of metallo- $\beta$ -lactamases (MBLs) and the virulence factor LasB from *Pseudomonas aeruginosa*<sup>†</sup>

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Increasing antimicrobial resistance is evolving to be one of the major threats to public health. To reduce the selection pressure and thus to avoid a fast development of resistance, novel approaches aim to target bacterial virulence instead of growth. Another strategy is to restore the activity of antibiotics already in clinical use. This can be achieved by the inhibition of resistance factors such as metallo- $\beta$ -lactamases (MBLs). Since MBLs can cleave almost all  $\beta$ -lactam antibiotics, including the “last resort” carbapenems, their inhibition is of utmost importance. Here, we report on the synthesis and *in vitro* evaluation of *N*-aryl mercaptoacetamides as inhibitors of both clinically relevant MBLs and the virulence factor LasB from *Pseudomonas aeruginosa*. All tested *N*-aryl mercaptoacetamides showed low micromolar to submicromolar activities on the tested enzymes IMP-7, NDM-1 and VIM-1. The two most promising compounds were further examined in NDM-1 expressing *Klebsiella pneumoniae* isolates, where they restored the full activity of imipenem. Together with their LasB-inhibitory activity in the micromolar range, this class of compounds can now serve as a starting point for a multi-target inhibitor approach against both bacterial resistance and virulence, which is unprecedented in antibacterial drug discovery.

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## Introduction

Due to the emergence of drug-resistant bacteria and the lack of new antibiotics in the pipeline, novel effective and innovative treatment options are urgently needed, in particular to cure infections with Gram-negative pathogens. One of the highly problematic Gram-negative bacteria is *Pseudomonas aeruginosa*, which has been assigned critical

priority by the World Health Organization (WHO).<sup>1</sup> This opportunistic pathogen is responsible for fatal lung infections in cystic fibrosis patients and many hospital-acquired infections.<sup>2,3</sup> *P. aeruginosa* is a versatile human pathogen that easily acquires resistance against multiple classes of antibacterial agents, including aminoglycosides, quinolones, polymyxins, and  $\beta$ -lactams.<sup>4,5</sup>

The  $\beta$ -lactams are still the most commonly used group of antibiotics.<sup>6</sup> One of the main mechanisms of resistance in Gram-negative pathogens including *P. aeruginosa* is the production of  $\beta$ -lactamases.<sup>7–9</sup> These enzymes can inactivate  $\beta$ -lactam antibiotics by the hydrolysis of their four-membered  $\beta$ -lactam ring.<sup>10,11</sup> Based on their mechanism of action, two main groups of  $\beta$ -lactamases (BLs) were identified: the serine- $\beta$ -lactamases (SBLs) and the metallo- $\beta$ -lactamases (MBLs).

SBLs act *via* a nucleophilic serine residue that covalently binds to the carbonyl unit of the  $\beta$ -lactam ring to achieve its hydrolysis. They have a variable substrate spectrum ranging from small-, broad- to extended-spectrum, and even carbapenems can be degraded by some SBLs. Additionally, several effective SBL inhibitors (*e.g.*, clavulanic acid) are already in clinical use in combination with  $\beta$ -lactam antibiotics to overcome resistance.<sup>12</sup>

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**Fig. 1** Chemical structures and reported  $K_i/IC_{50}$  values against different MBLs of QPX7728<sup>20,21</sup> and VNRX-5133 (taniborbactam),<sup>22–24</sup> two cyclic boronates that are in clinical trials.

MBLs can be divided into classes B1 to B3 which differ in their amino acid sequence homology and, as a result, their zinc coordination.<sup>13</sup> Class B2 (e.g., CphA) has only one catalytically active zinc ion and a very narrow substrate profile,<sup>14</sup> while classes B1 (e.g., NDM) and B3 (e.g., AIM) coordinate two zinc ions and show a very broad substrate spectrum.<sup>14–16</sup> In particular the B1 class is of high clinical relevance.<sup>17–19</sup> So far, two MBL inhibitors having a cyclic boronate as core structure (see Fig. 1) are in clinical trials. QPX7728 is in phase I (NCT04380207, alone and with QPX2014) and VNRX-5133 (taniborbactam) is in phase III in combination with cefepime (NCT03840148).<sup>20–24</sup> However, no clinically approved MBL inhibitors have been reported to date.<sup>25,26</sup>

It is known that bacterial pathogenesis and infectivity are **mediated** by microbial components called virulence factors, which play pivotal roles in adhesion, biofilm formation, invasion of host tissues, and immune evasion.<sup>27,28</sup> Targeting these factors is a newly emerging approach in the field of anti-infective drug discovery. This so-called antivirulence strategy is intended to disarm the bacteria rather than killing them. Several bacterial virulence factors have been identified including toxins, extracellular enzymes needed for cell invasion and biofilm formation, cell-surface proteins that mediate bacterial attachment, secretion systems, and quorum-sensing systems that regulate bacterial cell-to-cell communication.<sup>27,29–31</sup>

One of the major virulence factors of *P. aeruginosa* is the elastase LasB. This extracellular zinc-containing protease is responsible for the invasion of host tissues, biofilm formation, and immune evasion. It has been demonstrated that LasB can cleave important components of the connective

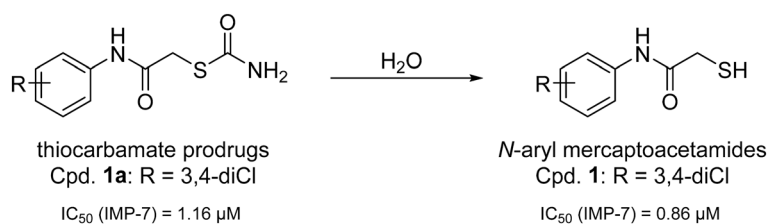
tissues such as elastin and collagen.<sup>32,33</sup> The protease was also shown to promote biofilm formation through the regulation of rhamnolipids.<sup>34</sup> Moreover, it was found to alter the human immune response by degrading host defense components such as cytokines,<sup>35</sup> antimicrobial peptides (AMP),<sup>36</sup> and surfactant proteins. These multiple roles of LasB in the infection process of *P. aeruginosa* make this protease an attractive antivirulence target.<sup>37,38</sup>

Recently, we have demonstrated the potential of thiocarbamates as prodrugs of *N*-aryl mercaptoacetamides (Scheme 1) as promising inhibitors of several bacterial proteases. Interestingly, these compounds are potent inhibitors of *P. aeruginosa* LasB and collagenases from *Clostridium* and *Bacillus* species.<sup>39,40</sup> Regarding LasB, in addition to the elucidation of the binding mode of our hit compound (**1**, Scheme 1), we demonstrated the *in vivo* efficacy of the same derivative in a *Galleria mellonella* larvae infection model. Moreover, compound **1** shows no cytotoxic effects on HepG2, HEK293 and A549 cells (see ESI†, Table S1) and its thiocarbamate prodrug **1a** (see Scheme 1) remarkably displays high selectivity over several human matrix metalloproteases (MMPs).<sup>39</sup>

It has been demonstrated that several thiol-containing drugs such as captopril (an angiotensin converting enzyme (ACE) inhibitor which is used for the treatment of hypertension), thiorphan (the active metabolite of racecadotril used for the treatment of diarrhoea), and tiopronin (which is used in patients with cystinuria, Fig. 2), display promising *in vitro* inhibitory activities toward different MBLs.<sup>41</sup> The shown three inhibitors all consist of the same core structure, that is, a 2-substituted (2-mercaptoacetyl)- or (2-mercaptopropanoyl) glycine. Substantial synthetic work has already been done on this scaffold to obtain novel MBL inhibitors, and co-crystallization experiments have been reported.<sup>42–46</sup>

Inspired by this finding, and with respect to our slightly similar mercaptoacetamide core structure, we explored the effect of our thiol-based LasB inhibitor (compound **1**) toward a class B1 MBL called imipenemase 7 (IMP-7). Interestingly, our LasB inhibitor showed a strong inhibitory effect with a sub-micromolar  $IC_{50}$  value ( $IC_{50} = 0.86 \pm 0.06 \mu$ M).<sup>39</sup>

Based on the encouraging inhibitory activity of compound **1** against IMP-7, this study aimed to further investigate the inhibitory profile of the *N*-aryl mercaptoacetamide class against various MBLs. Aside from that, another goal was to explore the potential of using this class as multi-target



**Scheme 1** Hydrolysis of thiocarbamate prodrug **1a** to its active thiol form **1** and their corresponding  $IC_{50}$  values reported for the inhibition of IMP-7.<sup>39</sup>





**Fig. 2** Chemical structures and reported  $IC_{50}$  values of *N*-aryl mercaptoacetamides, compound **1**,<sup>39</sup> thiophan,<sup>41</sup> tiopronin<sup>41</sup> and captopril<sup>47</sup> for the inhibition of different MBLs.

inhibitors tackling both virulence and bacterial resistance. In this work, we report on the design, synthesis, and *in vitro* evaluation of 13 *N*-aryl mercaptoacetamide derivatives for inhibition of three clinically important and transferrable MBLs of the class B1: IMP-7, Verona integrin encoded metallo- $\beta$ -lactamase 1 (VIM-1) and New Delhi metallo- $\beta$ -lactamase 1 (NDM-1). Furthermore, we tested the two most active compounds in combination with the carbapenem antibiotic imipenem against a clinical NDM-1-expressing *Klebsiella pneumoniae* isolate and confirmed their activity on the bacterial level. The selectivity over nine potential human off-targets was additionally confirmed.

## Results and discussion

### Synthesis of *N*-aryl mercaptoacetamides

Here, we report on the biological evaluation of 13 *N*-aryl mercaptoacetamides bearing various polar and nonpolar substituents on the aryl moiety. We have previously demonstrated that by variation of the substitution pattern on the aryl ring (and thus the electron density and H-bonding interactions), we either obtain LasB or ColH inhibitors. Additionally, the phenyl ring seems to be crucial for the selectivity of these inhibitors over human MMPs as potential off-targets.<sup>39,40</sup> Therefore, the impact of the same structural modifications on the inhibition of MBLs should also be investigated. Eight out of the 13 compounds were newly synthesised, while five had already been published as LasB or ColH inhibitors.<sup>39,40</sup> The majority of all our previously reported *N*-aryl mercaptoacetamides were tested against LasB and ColH

in their thiocarbamate prodrug form.<sup>39</sup> However, the co-crystal structure revealed the free thiol as the active form. This finding was confirmed by stability tests and *in vitro* assays.<sup>39,40</sup> In this work, we therefore synthesised the free-thiol forms of four compounds, which had previously been tested against LasB and ColH as thiocarbamates, as well as four novel mercaptoacetamide derivatives. The synthetic access to the mercaptoacetamide derivatives is outlined in Scheme 2.

A three-step synthetic route afforded target compounds **18–25**, starting from the corresponding aniline derivatives. In a first step, an amide formation was carried out by dropwise addition of chloroacetyl chloride to a cooled solution of the corresponding aniline and DIPEA in  $CH_2Cl_2$  or THF. Second, an  $S_N2$  reaction of the alkyl chlorides **2–9** with potassium thioacetate in acetone led to the thioacetates **10–17**. Compounds **10–17** were hydrolysed with aqueous KOH in methanol to afford the thiol target molecules **18–25**. These as well as the previously synthesised compounds **1** and **26–29** (Fig. 3) were then evaluated with respect to their biological activities.

### *N*-Aryl mercaptoacetamides as broad-spectrum MBL inhibitors

To explore the potential of our thiol-based class to inhibit MBLs, we first tested the synthesised compounds **18–25** as well as the previously reported compounds **1** and **26–29** (Fig. 3) *in vitro* for their inhibitory potency against NDM-1, VIM-1 and IMP-7.<sup>39,40</sup> Based on their *in vitro* activity, we then selected several derivatives for further evaluation in bacteria in combination with imipenem.



**Scheme 2** Synthesis of the thiols **18–25** as potential multi-target inhibitors of LasB and metallo- $\beta$ -lactamases.





Fig. 3 Previously reported thiols 26–29 as potential multi-target inhibitors of bacterial virulence and resistance.<sup>39,40</sup>

### MBL activity assays

We used an activity assay based on kinetic fluorescence using fluorocillin as a substrate<sup>41</sup> to evaluate the inhibitory activity of the mercaptoacetamide derivatives against NDM-1, VIM-1 and IMP-7. The results can be found in Table 1.

Remarkably, all derivatives were active against all three MBL enzymes and consequently displayed broad-spectrum effects *in vitro* (Table 1). Compound 1 showed a sub-micromolar activity against both IMP-7 ( $IC_{50} = 0.86 \pm 0.06 \mu M$ )<sup>39</sup> and NDM-1 ( $IC_{50} = 0.65 \pm 0.04 \mu M$ ) and an  $IC_{50}$  of  $2.2 \pm 0.3 \mu M$  against VIM-1. All the other derivatives showed low micromolar activities against the three tested MBLs. Regarding many previously reported MBL inhibitors (a selection can be found in Fig. 2 and 4),<sup>41–43,48–50</sup> our compounds can be considered equipotent. Only the class of cyclic boronates (e.g. 34 (Fig. 4), QPX7728, and VNRX-5133 (Fig. 1)) was significantly more active against MBLs (with  $IC_{50}$  values in the low nanomolar range) and has already two candidates in clinical trials.<sup>20,23,50,51</sup>

In comparison to compound 1, all structural modifications of the aryl moiety resulted in a moderate loss of activity against all MBLs. Regarding IMP-7, the  $IC_{50}$  values range from  $1.9 \mu M$  (compound 26) to  $11 \mu M$  (compound 24) with better tolerance for the nonpolar substituents. The most potent derivative against NDM-1 was compound 28 ( $1.5 \mu M$ ),

and as observed for IMP-7, the nonpolar groups were slightly preferred. Concerning VIM-1, all compounds showed comparable activities with  $IC_{50}$  values ranging between  $2.3 \mu M$  (26) and  $5.9 \mu M$  (24). Additionally, for mono-substituted compounds featuring a chlorine or methyl group, the position of the substituent did not have a significant effect on the biological response.

### Growth-inhibition assays

Encouraged by the promising *in vitro* activities of our *N*-aryl mercaptoacetamides, we investigated the effects of compounds 1 and 26 to restore the antibacterial effect of imipenem (ESI,† Fig. S1) in a growth inhibition assay. As we wanted to investigate MBL activity without the influence of LasB inhibition, and as our compounds were the most active on NDM-1, we selected a clinical imipenem-resistant *K. pneumoniae* isolate (strain T2301) that is able to express NDM-1 but not LasB. *K. pneumoniae* is one of the known ESKAPE pathogens and is rated critical by the WHO on their global priority list of antibiotic-resistant bacteria.<sup>52,53</sup> Nosocomial infections with *K. pneumoniae* have caused several outbreaks in multiple locations with very high mortality rates, *i.e.*, up to 72%.<sup>54–56</sup> NDM-1 (and other antibiotic resistance genes) spread between several pathogens *via* horizontal transfer,<sup>57–59</sup> and NDM-1-expressing *K.*

Table 1 Chemical structures, MBL and LasB inhibition of *N*-aryl mercaptoacetamides and their thiocarbamate prodrug forms

Cp.	R	thiol		thiocarbamate		
		Thiol $IC_{50}^a$ [ $\mu M$ ] IMP-7	Thiol $IC_{50}^a$ [ $\mu M$ ] NDM-1	Thiol $IC_{50}^a$ [ $\mu M$ ] VIM-1	Thiol $IC_{50}^a$ [ $\mu M$ ] LasB	Thiocarbamate $IC_{50}^a$ [ $\mu M$ ] LasB
1	3,4-DiCl	$0.86 \pm 0.06$	$0.65 \pm 0.04$	$2.2 \pm 0.3$	$6.6 \pm 0.3$ (ref. 39)	$6.2 \pm 0.3$
18	H	$4.7 \pm 0.7$	$3.6 \pm 0.9$	$5.1 \pm 1.3$	$39 \pm 3$	—
26	2-Cl	$1.9 \pm 0.5$	$1.8 \pm 0.1$	$2.3 \pm 0.2$	$11 \pm 1$	$14 \pm 1$
19	3-Cl	$4.1 \pm 1.6$	$2.7 \pm 0.4$	$3.6 \pm 1.2$	$14 \pm 2$	$19 \pm 1$
27	4-Cl	$2.4 \pm 0.3$	$2.1 \pm 0.1$	$4.7 \pm 1.8$	$21 \pm 1$ (ref. 39)	$16 \pm 1$
20	2-Me	$6.4 \pm 2.0$	$6.0 \pm 0.5$	$4.9 \pm 1.3$	$14 \pm 1$	—
21	3-Me	$4.4 \pm 1.5$	$3.4 \pm 0.2$	$4.1 \pm 0.8$	$38 \pm 3$	$48 \pm 2$
22	4-Me	$5.4 \pm 1.5$	$3.4 \pm 0.3$	$5.0 \pm 1.6$	$19 \pm 1$	$36 \pm 1$
23	4-OMe	$8.6 \pm 1.8$	$5.0 \pm 2.1$	$5.9 \pm 1.3$	$24 \pm 1$	$48 \pm 1$
24	4-C <sub>2</sub> H <sub>4</sub> OH	$11 \pm 4$	$6.7 \pm 3.0$	$5.9 \pm 2.0$	$52 \pm 3$	—
25	4-SO <sub>2</sub> NH <sub>2</sub>	$7.7 \pm 1.2$	$6.3 \pm 1.5$	$4.1 \pm 0.9$	$54 \pm 3$	—
28	4-Ac	$5.4 \pm 2.5$	$1.5 \pm 0.2$	$3.5 \pm 0.8$	$72 \pm 5$	$73 \pm 3$
29	3-Cl-2,6-diMe	$2.7 \pm 0.7$	$8.2 \pm 2.6$	$4.0 \pm 0.7$	$12 \pm 1$ (ref. 39)	—

<sup>a</sup> Means of at least two independent measurements  $\pm$  standard deviation.







**Fig. 4** A selection of the structural variations of reported MBL inhibitors and their corresponding inhibitory activities *in vitro* (**30**,<sup>48</sup> **31**,<sup>43</sup> **32**,<sup>49</sup> **33**,<sup>42</sup> and **34**<sup>50</sup>).

*pneumoniae* have caused several outbreaks in hospitals in the last decade.<sup>60–62</sup> Growth curves of the bacterial isolate with imipenem were determined in the presence and absence of the named compounds (two separate experiments, see Fig. 5A and B). Compounds **1** and **26** were selected based on their promising activities against MBLs.

As shown in the growth curves in Fig. 5, the presence of imipenem (at 8  $\mu$ g mL<sup>-1</sup>, *i.e.*, 0.5 $\times$  MIC) delayed the initiation of bacterial growth by a few hours, but after 24 hours, no relevant differences between the growth of imipenem-treated bacteria and the imipenem-free control were observed. Remarkably, when MBL inhibitors **1** (Fig. 5A, at 50  $\mu$ g mL<sup>-1</sup> (= 21  $\mu$ M)) or **26** (Fig. 5B, at 50  $\mu$ g mL<sup>-1</sup> (= 25  $\mu$ M)) were combined with imipenem (at 8  $\mu$ g mL<sup>-1</sup>), the inhibitory potential of imipenem was fully restored, and no growth of bacteria occurred over 24 hours. MBL inhibitors alone did not exhibit any intrinsic antibacterial activity at 50  $\mu$ g mL<sup>-1</sup>. As an additional control experiment, we employed EDTA as an MBL inhibitor, with MBL inhibition being caused by the metal-chelating properties of EDTA (ESI,† Fig. S2). The resultant growth curves were rather similar to those obtained with MBL inhibitors **1** and **26**, respectively. Overall, this highlights the potency of the reported MBL inhibitors not just on the enzymatic, but also on the cellular level, making them attractive candidates for further development.

#### *N*-Aryl mercaptoacetamides as multi-target inhibitors of LasB and MBLs

The promising antibacterial effects of *N*-aryl mercaptoacetamides in combination with imipenem (Fig. 5) prompted us to further investigate the potential of this class as multi-target inhibitors targeting both virulence and resistance. Therefore, all synthesised compounds were evaluated for their potential inhibition of LasB as a bacterial virulence factor.

Inhibitory activity towards ColH was not determined, as the best two compounds on MBLs (**1** and **26**) had already been tested in their thiocarbamate form against ColH and did just show micromolar activities, which is more than three orders of magnitude less potent than the ColH-inhibiting hit compound **28**.<sup>40</sup>

The evaluation of compounds **1**, **19**, **21**, **26**, and **27** in a functional LasB inhibition assay expectedly<sup>39,40</sup> showed similar activities compared to their thiocarbamate prodrug derivatives (Table 1). However, compounds **22** and **23** were two-fold more active than their thiocarbamate congeners. Regarding the novel mercaptoacetamides, the unsubstituted derivative **18** as well as the 2-methyl compound **20** showed decreased  $IC_{50}$  values relative to compound **1**. Moreover, as expected based on our previous studies,<sup>39</sup> compounds **24** and **25** were the least active derivatives in the series due to the presence of polar substituents that are unfavourable for LasB inhibition.

#### Selectivity over other proteases

As most of our compounds all show activity on LasB and the three tested MBLs, and as it is known that **28** additionally is a nanomolar collagenase H (ColH) inhibitor,<sup>40</sup> it is crucial to exclude that our compounds are just non-selective zinc-chelators. Thiocarbamates **1a** and **28a** had already been evaluated for their selectivity over a range of six MMPs which had been chosen with respect to their structural variations in their S1' binding pockets.<sup>39,40</sup> We decided to test our hit compound **1** accordingly and, additionally, expand the selectivity panel with two histone deacetylases (HDACs) and the tumour necrosis factor  $\alpha$  converting enzyme (TACE). Those three enzymes are all zinc-dependent proteases.<sup>63–65</sup> HDACs play a crucial role in epigenetic regulation by modification of gene expression and thus in cell proliferation.<sup>66,67</sup> TACE, also known as “a disintegrin and metalloprotease 17” (ADAM17), is



A



B



Fig. 5 Growth-inhibition assay. Growth curves of NDM-1-expressing *K. pneumoniae* (T2301) over time (h) in the absence and presence of imipenem at  $8 \mu\text{g mL}^{-1}$  (i.e.,  $0.5 \times \text{MIC}$ )  $\pm$  MBL inhibitors at  $50 \mu\text{g mL}^{-1}$  ( $= 21 \mu\text{M}$  (1) or  $25 \mu\text{M}$  (26)); compound 1 (A) and compound 26 (B).

important for a functional immune system, for the nervous system and tissue generation.<sup>68–71</sup> Therefore, these enzymes represent important antitargets. As controls, batimastat (ESI,† Fig. S3) as unselective MMP inhibitor, trichostatin A (ESI,† Fig.

S4), a known inhibitor of HDACs, and ilomastat as TACE inhibitor (ESI,† Fig. S5) were chosen. Compound 1 was tested at a concentration of  $100 \mu\text{M}$  for its inhibitory activity on those nine enzymes. The results are reported in Table 2.

Table 2 Inhibition values of six MMPs, TACE and two HDACs in the presence of  $100 \mu\text{M}$  1,  $0.5 \mu\text{M}$  trichostatin A and  $\text{IC}_{50}$  values of batimastat.<sup>40,72</sup> Ilomastat resulted in  $91 \pm 4\%$  inhibition of TACE activity at  $1 \mu\text{M}$

	1 inhibition at $100 \mu\text{M}^a$ [%]	Batimastat $\text{IC}_{50}^a$ [nM]	Trichostatin A inhibition at $0.5 \mu\text{M}^a$ [%]
MMP-1	n.i.	$2.2 \pm 0.1$	n.d.
MMP-2	n.i.	$1.8 \pm 0.1$	n.d.
MMP-3	n.i.	$5.6 \pm 0.9$	n.d.
MMP-7	n.i.	$7.0 \pm 0.2$	n.d.
MMP-8	n.i.	$0.7 \pm 0.2$	n.d.
MMP-14	n.i.	$2.8 \pm 0.2$	n.d.
TACE	$40 \pm 4$	n.d.	n.d.
HDAC-3	$9 \pm 7$	n.d.	$94 \pm 3$
HDAC-8	$11 \pm 5$	n.d.	$95 \pm 2$

<sup>a</sup> Means and SD of at least two independent measurements are displayed; n.d. = not determined; n.i. = no inhibition (if  $\leq 10\%$ ).



Compound **1** showed no inhibition of the six tested MMPs and the two HDACs. A slight inhibition of TACE ( $40 \pm 4\%$ ) at a rather high concentration of  $100 \mu\text{M}$  was found, but this still corresponds to a selectivity factor of more than five, which should be considered sufficient in particular in this early phase of development.

### Cytotoxicity assays

It had already been demonstrated in our previous studies that compound **1** did not show any cytotoxicity against the three tested cell lines HepG2, HEK293 and A549 at a concentration of  $100 \mu\text{M}$ . The results can be found in the ESI,† Table S1.<sup>39,73</sup>

Additionally, compound **1** already had been subjected to a more complex *in vivo* toxicity model in zebrafish embryos (ESI,† Table S2).<sup>73</sup> Here the maximum tolerated concentration (MTC) was  $10 \mu\text{M}$ . At higher concentrations (e.g.  $30 \mu\text{M}$ ), the compound precipitated in this assay and the zebrafish embryos died.<sup>73</sup> Further studies to determine whether the toxicity derived from the compound's *in vivo* effect itself or from the formation of crystals will be performed in the course of future hit optimisation.

## Conclusions

In summary, based on the encouraging inhibitory activity of a previously reported LasB inhibitor (compound **1**) against IMP-7, we designed, synthesised and evaluated a series of *N*-aryl mercaptoacetamide derivatives against the three metallo- $\beta$ -lactamases IMP-7, VIM-1 and NDM-1. Interestingly, all tested compounds showed promising inhibition of all three MBLs. Compound **1** displayed the strongest inhibitory effects with submicromolar  $\text{IC}_{50}$  values toward both IMP-7 and NDM-1, and low micromolar activity against VIM-1.

To confirm the potential of our mercaptoacetamide derivatives as MBL inhibitors, compounds **1** and **26** were tested in combination with imipenem against a clinical NDM-1-expressing *K. pneumoniae* isolate. Remarkably, at  $50 \mu\text{g mL}^{-1}$  ( $\sim 20 \mu\text{M}$ ), both compounds were able to restore the activity of imipenem. Additionally, compound **1** showed high selectivity over a total of nine potential off-targets (six MMPs, TACE and two HDACs) at a concentration of  $100 \mu\text{M}$ , at which it already previously had demonstrated to also have no cytotoxic effects on human cell lines.<sup>39,73</sup> These promising effects of our *N*-aryl mercaptoacetamides against MBLs, their previously confirmed anti-LasB properties and their demonstrated high selectivity over human off-targets inspires the use of this class as multi-target inhibitors tackling both virulence and resistance of pathogens. This is a new approach that is unprecedented in antibacterial drug discovery.

Although further investigations will be needed to improve the inhibitory potencies of our inhibitors, our results demonstrate an attractive new multi-targeting approach, which can enhance the efficacy of currently used antibiotics against resistant MBL-expressing pathogens. In addition to restoring the sensitivity to antimicrobial agents, the effect of

the reported compounds on virulence factors can make bacteria less pathogenic and more vulnerable towards immune response and/or low doses of antibiotics. Even though no improvement of the *in vitro* inhibitory activities of compound **1** has been achieved so far, the reported results indicate that some structural variations in the aryl moiety appear to be feasible. This opens the way to a future optimisation of this compound class, also with respect to ADME-T properties.

## Experimental section

### Chemistry

Compounds **1**,<sup>39</sup> **26**,<sup>40</sup> **27**,<sup>39,40</sup> **28**<sup>40</sup> and **29**<sup>39</sup> were previously reported. All other compounds were synthesised according to Scheme 1 as described in detail in the ESI.†

### IMP-7, NDM-1 and VIM-1 *in vitro* inhibition assays

The assays were performed according to a previously described procedure.<sup>41</sup> They were carried out at room temperature in black polystyrol 96-well plates (Corning) using fluorocillin (prepared as described by Rukavishnikov *et al.*<sup>74</sup>), as substrate. The fluorescence emitted by the fluorescent product difluorofluorescein was kinetically monitored using a Tecan fluorescent plate reader Infinite F200.  $\text{IC}_{50}$  values were calculated using data obtained from measurements with at least six different inhibitor concentrations, applying a sigmoidal dose-response (variable slope with four parameters) equation using GraphPad Prism 5 (GraphPad software, La Jolla, CA, USA) software.

### Growth inhibition assay

A computerised incubator (Tecan Infinite M200 pro, Crailsheim, Germany) was used to obtain the growth curves over a time course. Optical density ( $\text{OD}_{600}$ ) was measured for evaluating bacterial (i.e., *K. pneumoniae* NDM-1) density in suspension. Prior to each experiment, bacteria were cultured to the exponential phase ( $\text{OD}_{600} = 0.6\text{--}0.8$ ) in cation-adjusted Mueller Hinton broth (Becton Dickinson, Heidelberg, Germany).  $5 \mu\text{L}$  of diluted bacterial suspension and  $100 \mu\text{L}$  of cation-adjusted Mueller Hinton broth either with or without imipenem (Arcos Organics, Schwerte, Germany) and/or the respective MBL inhibitor were added into wells of a 96 well plate to give a final bacterial inoculum of approx.  $5 \times 10^5 \text{ CFU mL}^{-1}$ . The temperature was adjusted to  $37^\circ\text{C}$ . A permanent shaking speed of 450 rpm was used and only interrupted before  $\text{OD}_{600}$  measurement. The optical densities of samples were recorded on-line every 10 minutes for 24 hours. Experiments were determined in triplicate. Representative growth curves are shown. For quality control, bacterial strain *E. coli* ATCC 25922 was used.

### LasB *in vitro* inhibition assay

The purification of LasB as well as the performance, measurement and evaluation of the FRET-based *in vitro*



inhibition assay were carried out as previously described by Kany *et al.*<sup>39</sup>

### MMP *in vitro* inhibition assay

The assays were performed as previously described.<sup>39,40</sup>

### TACE *in vitro* inhibition assay

ADAM-17 (TACE) inhibitor screening assay kit was purchased from Sigma-Aldrich. The assay was performed according to the guidelines of the manufacturer. Fluorescence signals were measured in a CLARIOstar plate reader (BMG Labtech).

### HDAC *in vitro* inhibition assay

HDAC3 and HDAC8 inhibitor screening kits were purchased from Sigma-Aldrich. The assay was performed according to the guidelines of the manufacturer.

## Author contributions

Samir Yahiaoui, investigation, writing – original draft; Katrin Voos, investigation, validation, visualization, writing – original draft; Jörg Haupenthal, conceptualization, project administration, validation, writing – review & editing; Thomas A. Wichelhaus, project administration, resources, supervision, validation, visualization, writing – review & editing; Denia Frank, investigation; Lilia Weizel, investigation; Marco Rotter, investigation; Steffen Brunst, investigation; Jan S. Kramer, investigation; Ewgenij Proschak, project administration, supervision, validation, writing – review & editing; Christian Ducho, conceptualization, project administration, supervision, validation, writing – review & editing; Anna K. H. Hirsch, conceptualization, funding acquisition, project administration, supervision, validation, writing – review & editing.

## Conflicts of interest

There are no conflicts to declare.

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