Chemical Proteomics Reveals Antibiotic Targets of Oxadiazolones in MRSA

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ABSTRACT: Phenotypic screening is a powerful approach to identify novel antibiotics against methicillin-resistant *Staphylococcus aureus* (MRSA) infection, but elucidation of the targets responsible for antimicrobial activity is often challenging in the case of compounds with a polypharmacological mode-of-action. Here, we show that activity-based protein profiling maps the target interaction landscape of a series of 1,3,4-oxadiazole-3-ones, identified in a phenotypic screen to have high antibacterial potency against multidrug resistant *S. aureus*. *In situ* competitive and comparative chemical proteomics with a tailor-made activity-based probe, in combination with transposon and resistance studies, revealed several cysteine and serine hydrolases as relevant targets. Our data showcase oxadiazolones as novel antibacterial chemotype with a polypharmacological mode-of-action, in which FabH, FphC and AdhE play a central role.

The emergence of multidrug resistant bacteria in parallel with a dearth of new antibiotic drug approvals may become one of the biggest health care problems of the 21st century.[1–3] Among Gram-positive pathogens, methicillin-resistant *Staphylococcus aureus* (MRSA) continues to be the most worrisome. Recent data indicate that in 2019 drug-resistant staphylococcal infections, due predominantly to MRSA, were associated with a staggering 750,000 deaths worldwide.[4] New antibiotics with unprecedented modes-of-action (MoAs) are urgently required to counteract antimicrobial drug resistance.

Target-based screening is commonly applied to identify small molecules as chemical starting points (hits) in traditional drug discovery projects, but this strategy is less successful in antibiotic research.[5] Phenotypic screening has instead emerged as a promising approach to identify antibiotics with novel MoAs.[6–8] A challenging aspect of phenotypic drug discovery is, however, to elucidate the primary targets responsible for the antimicrobial activity observed. Recently, chemical proteomics has emerged as a powerful chemical biology technique to map target interaction landscapes of experimental drugs,[9–11] including compounds with antibacterial activity.[12,13] Inspired by these established and emerging concepts, we combined phenotypic screening with chemical proteomics to discover new MRSA antibiotics and their interacting proteins.

To this end, a focused library of 352 small molecules derived from our in-house drug discovery programs was constructed. This compound set was first screened at 100 µM for antibacterial activity against MRSA USA300 (Figure 1a). This revealed 25 compounds that prevented bacterial growth. Subsequently, the minimum inhibitory concentration (MIC), which is the lowest concentration at which bacterial growth is inhibited, was determined for each of the 25 hits. Benzyl (4-(5-methoxy-2-oxo-1,3,4-oxadiazol-3(2*H*)-yl)-2-methylphenyl)-carbamate **1** was the most potent antibacterial compound with a MIC of 6.25 µM (2.2 µg/mL). **1** contains an oxadiazolone moiety that previously has been shown to covalently react with catalytically active serine and cysteine residues in enzymes (Figure 1b),[14,15] and has antibacterial activity in Mycobacteria.[16]

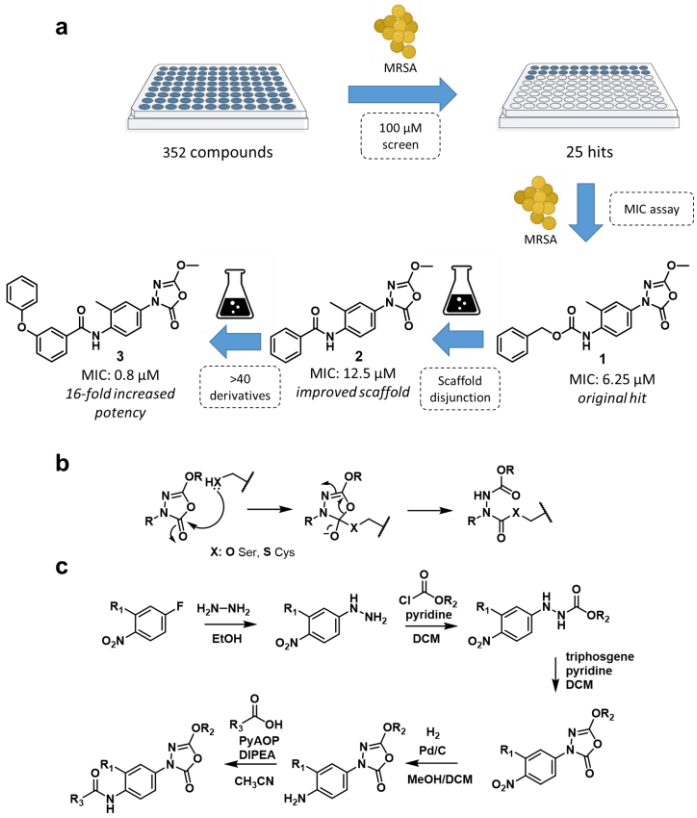

Figure 1. (a) Flow-chart from initial in-house library screen to lead compound **3**. (b) Proposed reaction mechanism of 1,3,4-oxadiazole-2-one derivatives towards reactive serine and cysteines. (c) Synthetic procedure for making **2** derivatives.

Table 1. Minimum inhibitory concentration (MIC) of initial hits, compound 3 and clinically relevant antibiotics against a panel of bacteria

Organism	Strain	MIC (μM)					
		1	**2**	**3**	meropenem	vancomycin	daptomycin
Gram-positive							
Staphylococcus aureus	**MRSA** USA300	6.25	12.5	0.8	1.1	1.4	1.2
	NY-155	3.1	12.5	0.8	9.1	0.7	1.2
	MRSA131	3.1	6.25	1.6	2.3	0.7	1.2
	COL	6.25	25	1.6	293	1.4	2.4
	MSSA ATCC 29213	25	25	1.6	≤0.1	0.7	1.2
	VISA SA MER	12.5	>50	1.6	0.3	2.8	4.9
	LIM3	6.25	12.5	0.8	≤2.3	2.8	2.4
	NRS126	25	50	3.1	293	2.8	2.4
	VRSA BR-VRSA	12.5	25	1.6	>293	88	1.2
	VRSA-1	25	50	3.1	293	88	1.2
	VRSA-2	6.25	25	0.8	293	88	≤0.6
Enterococcus faecium		>50	>50	>50	≤0.1	0.7	1.2
Gram-negative		>50	>50	>50	≤0.1 – 2.3	>88	>80

MSSA: methicillin-susceptible *S. aureus*, VISA: vancomycin-intermediate *S. aureus*, VRSA: vancomycin-resistant *S. aureus*.

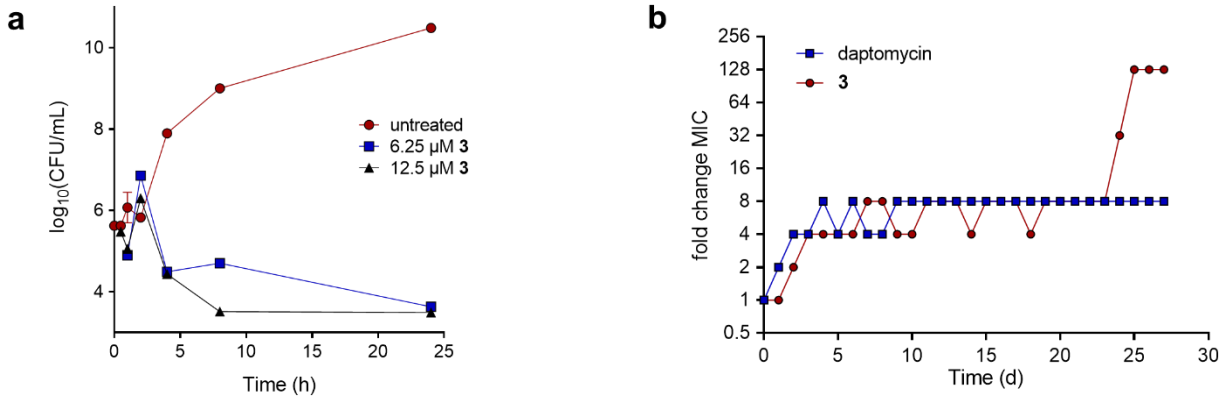

Figure 2. (a) Time-dependent killing by **3** of MRSA USA300. (b) Resistance development of MRSA USA300 against **3** and daptomycin during daily serial passaging with 0.25x MIC concentrations.

To determine the structure-activity relationship and optimize the potency of **1**, a series of 61 derivatives was synthesized and tested for antimicrobial activity against MRSA USA300 and *S. aureus* ATCC 29213 strains (Table S1-S6, Supplementary Data 1). The oxadiazolone group and the 2-methylphenyl moiety were both found to be crucial for activity. The benzylcarbamate could be changed to a phenylamide without losing activity. This led to the identification of **2** as a simplified scaffold with comparable antibacterial activity. Subsequent systematic modification of **2** (Figure 1c) resulted in the di scovery of *N*-(4-(5-methoxy-2-oxo-1,3,4-oxadiazol-3(2*H*)-yl)-2-methylphenyl)-3-phenoxybenzamide **3** as our lead compound with a 16-fold improvement in potency in both MRSA USA300 (MIC = 0.8 μM/0.3 μg/mL) and the *S. aureus* ATCC 29213 strain (MIC = 1.6 μM) compared to hit **1**.

Extended screening of **3** against other clinically relevant pathogens revealed the specific anti-staphylococcal activity of the oxadiazolones (Table 1). **3** was highly potent against a variety of *S. aureus* strains, including vancomycin-resistant strains and clinical isolates (Supplementary Data 2). Of note, **3** was generally found to be more potent against antibiotic resistant strains compared to wildtype *S. aureus*. **3** was able to time-dependently kill 99% of bacteria over the course of 24 hours, starting with a 10^6 CFU/mL inoculum (Figure 2a). Furthermore, **3** exhibits a relatively low cytotoxicity and is non-hemolytic (Table S7, Figure S1).

Next, we set out to generate strains resistant to **3** to investigate both the rate and mechanism of resistance development. MRSA USA300 was serially passaged daily in the presence of sub-MIC concentrations of compound yielding resistant mutants after 4 weeks (Figures 2b and S2). In comparison, resistance development for the control compound daptomycin, a clinically-used lipopeptide antibiotic, was found to be slower and did not exceed 8x MIC. This is commonly observed in cell membrane targeting antibiotics.[17,18] Of note, after four days the resistance towards **3** stabilized for several weeks before progressing to significantly higher values. This may indicate that multiple mutations are required to fully induce resistance, possibly suggesting a polypharmacological MoA. **3**-resistant mutant

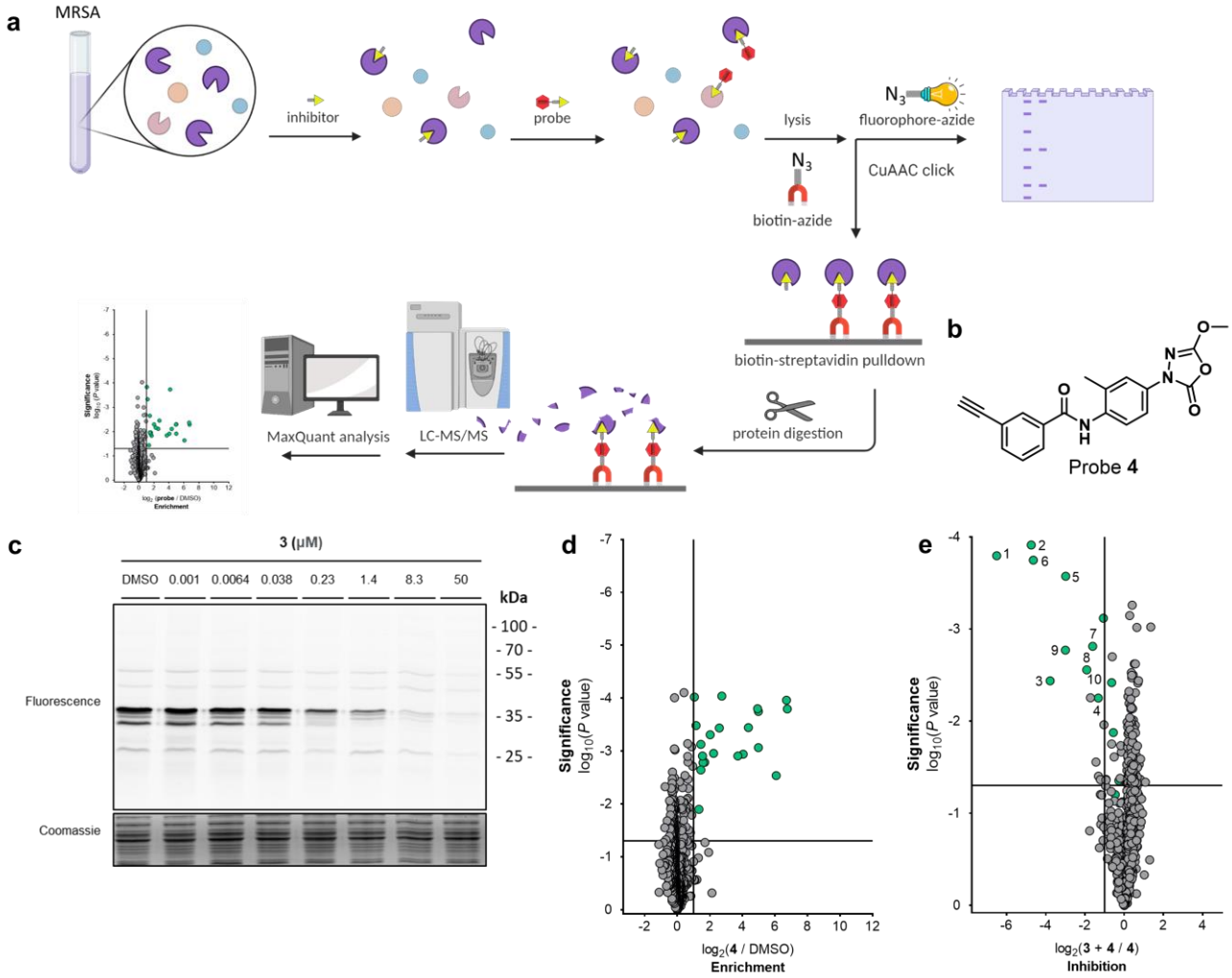

Figure (3). (a) *In situ* competitive activity-based protein profiling (ABPP) workflow on MRSA with using either SDS-PAGE or mass-spectrometry read-out. (b) Activity-based probe **4**, based on the **3** scaffold. (c) Gel-based competitive ABPP of a **3**-concentration range versus 1 μM **4** probe. (d) Mass spectrometry data enrichment plot comparing labelled proteome of 3 μM **4**-treated MRSA to **DMSO**-treated MRSA. (e) Mass spectrometry data inhibition plot comparing labelled proteome of samples preincubated with 10 μM inhibitor **3** followed by probe-labelling to solely probe-labelled samples.

Table 2. List of probe targets significantly outcompeted by 3

#	Uniprot ID	Protein	Description	Sequence length (aa)	Gene	Essentiality	References
1	Q2FDS6	FphE	Uncharacterized hydrolase	276	SAUSA300_2518	No	21, 22
2	Q2FI93	FabH	3-oxoacyl-[acyl-carrier-protein] synthase 3	313	*fabH*	Yes	24 – 27
3	A0A0H2XJL0	FphH	Carboxylesterase	246	*est*	No	21
4	A0A0H2XHZ1	HZ1	Putative lysophospholipase	271	SAUSA300_0070	No	
5	A0A0H2XHD0	FphC	Hydrolase. alpha/beta hydrolase fold family	304	SAUSA300_1194	No	21
6	A0A0H2XHH9	HH9	Putative lipase/esterase	347	SAUSA300_0641	No	
7	A0A0H2XJG5	FphB	Uncharacterized protein	322	SAUSA300_2473	No	21
8	A0A0H2XFI2	FI2	Uncharacterized protein	275	SAUSA300_0321	No	
9	A0A0H2XIB7	IB7	Acetyl-CoA c-acetyltransferase	379	*vraB*	No	23
10	A0A0H2XG10	AdhE	Aldehyde-alcohol dehydrogenase	869	*adhE*	No	28 – 30

strains did not show cross-resistance with commonly administered antibiotics (Supplementary Table 8). Together with the high activity of **3** against multidrug resistant *S. aureus* strains, these observation point to a unique MoA.

Having established that the oxadiazolones are potent antibiotics against various pathogenic *S. aureus* strains, we set out to identify interaction partners using activity-based protein profiling (ABPP). The oxadiazolone moiety covalently reacts to catalytically active amino acids in enzymes, therefore we hypothesized that a strategically positioned alkyne ligation handle on the scaffold of **3** could be used to introduce a fluorescent or affinity tag (e.g., biotin) to visualize small molecule–protein interactions in living systems (Figure 3a).

To this end, the meta-phenoxy group of **3** was substituted with an alkyne, resulting in activity-based probe **4** (Figure 3b). The antibacterial activity of **4** was confirmed in MRSA (MIC = 3.1 μM). The probe was subsequently used in an *in*

situ competitive ABPP workflow (Figure 3a).[19] Briefly, MRSA USA300 was cultured until exponential phase (OD_{600} = 0.7) and treated with competitor **3** or DMSO, followed by labeling with probe **4**. Bacteria were lysed and the probe-labeled proteins were conjugated to a reporter tag (fluorophore-azide or biotin-azide) via copper-catalyzed azide–alkyne cycloaddition (CuAAC) ("click") chemistry. When coupled to a fluorescent Cy5 reporter group, ABPP enables visualization of probe-labeled proteins by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and in-gel fluorescence scanning. This resulted in clear labelling by **4** of several proteins, of which most were dose-dependently outcompeted by **3** (Figure 3c).

To identify the probe-labeled proteins, we coupled the probe **4**-labelled proteins to a biotin reporter group, which allows affinity enrichment and identification of probe-labeled proteins by mass spectrometry (MS)-based proteomics.[20] Around 30 proteins were found to be significantly enriched (p < 0.05, > 2-fold enrichment) by probe treatment (Figure 3d, Supplementary Data 3). Pretreatment with **3** significantly inhibited (p < 0.05, > 2-fold inhibition) the labeling of 10 proteins by probe **4** (Figure 3e), suggesting that these proteins are interaction partners of oxadiazolone **3**.

The Fph proteins (B, C, E, H) were recently discovered and annotated in MRSA as fluorophosphonate binding hydrolases.[21] FphB was found to be a fatty acid metabolizing virulence factor, while FphE activity has been used to phenotypically characterize MRSA through imaging.[22] Target proteins HZ1 and HH9 are reported to have hydrolase activity (Table 2), but their biological function has not been extensively studied. IB7 is a putative acetyl-CoA c-acetyltransferase with thiolase activity,[23] while FI2 is an uncharacterized protein. FabH also known as 3-oxoacyl-[acyl-carrier-protein] synthase 3 is an essential enzyme that initiates bacterial fatty acid synthesis[24], and has recently been explored as a drug target.[25–27] AdhE is an aldehyde alcohol dehydrogenase, essential in facultative anaerobic organisms in anaerobic conditions.[28,29] Both FabH and AdhE are known to metabolize substrates using an active site cysteine.

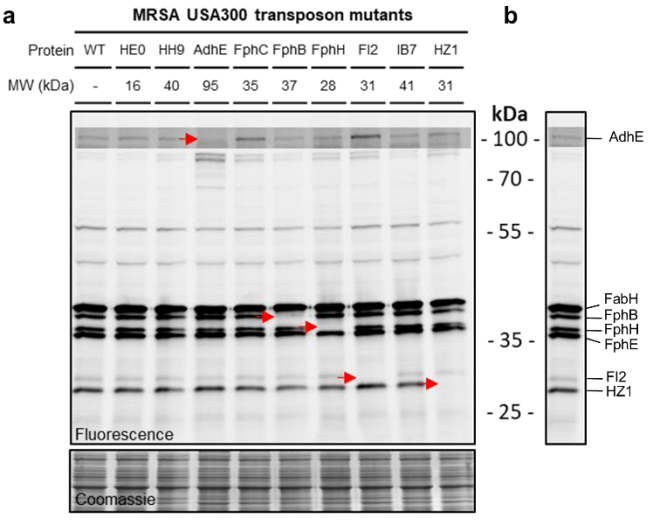

Figure 4. (a) 4 labelling of MRSA USA300 mutant strains, each with a transposon sequence inserted in genes of identified 4 targets. (b) Wild type MRSA USA300 4 probe labelling bands annotated with corresponding proteins.

To confirm the identity of the probe targets with gel-based ABPP, we screened the probe-labeled proteome of nine transposon mutants of MRSA that lack the gene encoding one of the identified target proteins of **3** (Figure 4a). The labeling of AdhE, FphB, FphH, FI2 and HZ1, but not FphC and HH9, could be attributed to specific fluorescent bands on SDS-PAGE (Figure 4b). The lower resolution of gel-based ABPP (overlapping bands) or insufficient sensitivity compared to MS-based ABPP may explain why FphC and HH9 were not identified on gel. Since FabH is essential for MRSA viability, no transposon mutant is available for this protein. Instead, we confirmed the identity of FabH on gel by competitive ABPP using the selective FabH inhibitor Oxa2 (Figure S3).

To assess which target proteins were responsible for the antibiotic effect, we hypothesized that the protein inhibition profile of potent oxadiazolones (MIC ≤ 12.5 µM) would be different compared to the interaction profile of their close analogues with no activity (MIC > 50 µM). In a competitive chemical proteomics set-up, we, therefore, compared the interaction profile of three inactive derivatives (**5**-**7**) with three active compounds (**1**-**3)** (Figures 5a and S4).

Strong FphB inhibition was seen in the samples pretreated with **1**, but not by the other compounds F12, IB7, HH9 and HZ1 were not significantly inhibited by the bioactive oxadiazolone **3**, but did show engagement by the inactive compounds **5, 6** or **7**. FphE and FphH were strongly inhibited by all compounds. These observations in combination with the viability of the transposon mutants suggest that FphB, IB7, HH9, F12, FphE, FphH and HZ1 are not essential for the antimicrobial activity of **3**. FabH was significantly engaged, but not fully, by all compounds. Since the transposon mutant of FabH is not viable, this implies that partial inhibition of FabH activity could contribute to the bioactivity of the oxadiazolones, however, it was not sufficient to kill MRSA by itself.

Finally, significant inhibition of FphC and AdhE labeling was only found by the bioactive compounds, but not by the inactive compounds **5**-**7** (Figure 5b). Next, we tested whether antibiotic activity of the inactive compounds **5**-**7** could be induced in the FphC and AdhE transposon mutants. Gratifyingly, it was observed that both **6** and **7** showed increased antimicrobial activity in both transposon mutants (Table 3, Table S10), but not in a FphB transposon mutant, which was taken along as a negative control. Of interest, compounds **6** and **7** did not become as active as **3**. Compound **5**, which has no activity on AdhE and very weak activity on FphC (< 20%), remained inactive in all individual transposon mutants. Although we cannot exclude that other proteins also play a role, we interpret these data to mean that combined engagement of FphC and AdhE is required for antimicrobial activity of the oxadiazolones.

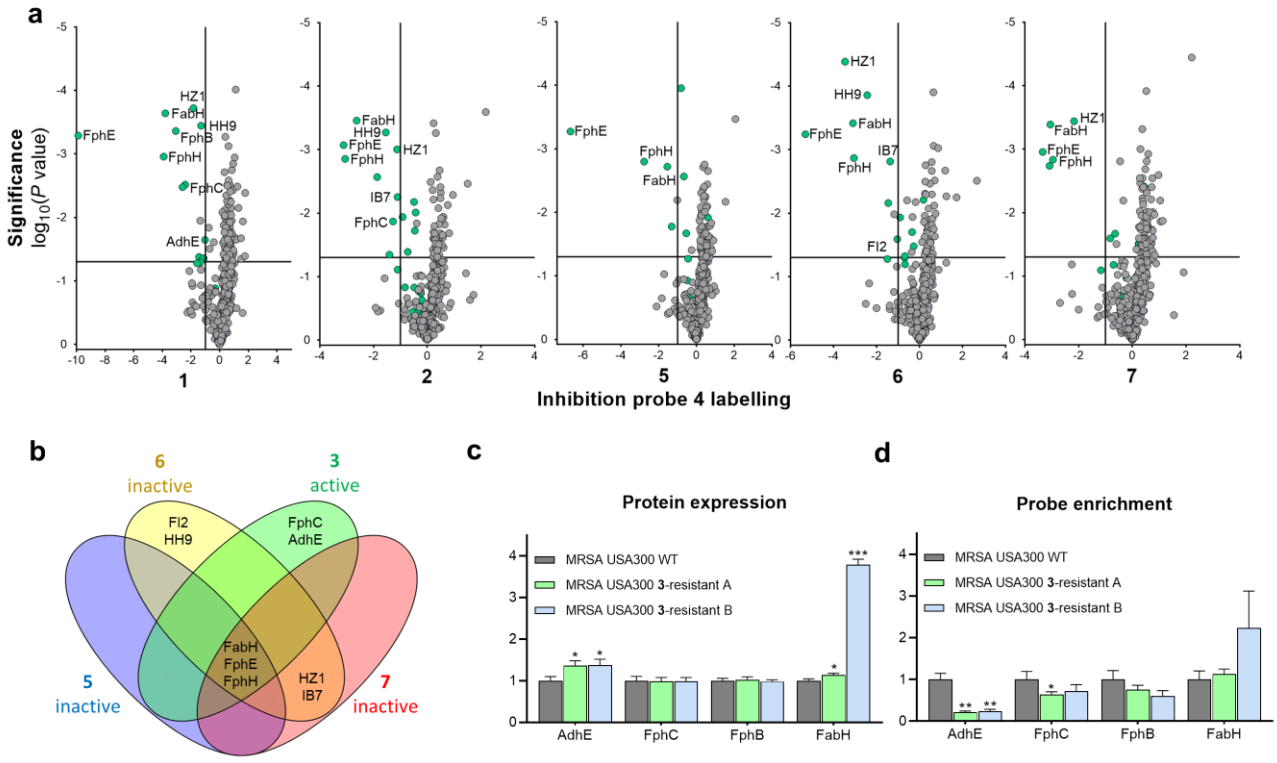

Figure 5. (a) Individual inhibition plots of a selection of active and inactive compounds. **3** was dosed at 1 μM, while the inactive inhibitors were dosed at 10 μM. (b) Venn diagram showing overlap of >50% inhibited proteins between active compound **3** and three inactive compounds **5**-**7**. (c) Relative general protein levels in **3**-resistant strains compared to wild-type. (d) Relative protein levels enriched by **4** in **3**-resistant strains (Figure 2b) compared to wild-type.

Table 3. MIC values MRSA USA300 transposon mutants of target proteins

	MIC (μM)		
	Inactive **5**	Inactive **6**	Inactive **7**
MRSA USA300 WT	>50	>50	>50
AdhE transposon mutant	>50	**25**	**12.5**
FphC transposon mutant	>50	**50**	**25**
FphB transposon mutant	>50	>50	>50

Principal component analysis of the chemical proteomics data confirmed that inhibition of AdhE and FphC activity was associated to a large extent with the antibacterial activity (Table S9, Figure S7). Thus, our chemical proteomics data reveal that multiple targets (in particular, FphC, AdhE and FabH) play a role in the observed antimicrobial activity of the oxadiazolones.

To further test our hypothesis, we investigated whether FphC and AdhE activity was changed in two of the **3**-resistant MRSA strains we generated compared to WT MRSA (Figures 5c, 5d and S5, Supplementary Data 3c, 3d and 4). Using chemical and global proteomics it was observed that AdhE activity was significantly decreased, while its protein abundance was upregulated in the two resistant strains. FphC-activity was also reduced, but to a lower extent. Interestingly, FabH protein levels were significantly increased in the resistant strains, which was accompanied by cross-resistance of these strains to the FabH inhibitor Oxa2 (8x increase in MIC). Taken together, these data suggest that combined inhibition of FabH, FphC and AdhE contributes to the antimicrobial activity of compound **3**.

To summarize, a phenotypic screen of a focused library led to the identification of oxadiazolones as a new chemotype with antibiotic activity against pathogenic, multidrug resistant *S. aureus* strains and clinical isolates. A medicinal chemistry program combined with chemical proteomics led to the identification of compound **3** as the most potent antibiotic capable of interacting with multiple bacterial cysteine and serine hydrolases in a covalent manner. Three complementary lines of investigation point to FabH, FphC and AdhE as playing central roles in the antimicrobial activity of **3** and structurally similar oxadiazolones. i) comparative chemical proteomics, ii) gain of function in transposon mutants, and iii) resistance-induced proteomic changes. FabH has previously been identified as a drug target, whereas the function of AdhE and FphC has been less well explored. Recent studies implicate AdhE as a virulence factor in *E. coli*.[30] FphC is a membrane-bound serine hydrolase with unknown function. Of note, we cannot rule out that other factors, not detected by our chemical proteomics approach, may also contribute to the antibacterial effect of **3**, such as non-covalent interactions with proteins or other classes of

biomolecules.

To conclude, our findings further highlight the value of synthetic compound libraries as an excellent source for antibiotic drug discovery complementary to natural products. By applying comparative and competitive chemical proteomics, using a new tailor-made activity-based probe with a strategically positioned ligation tag, we successfully elucidated the polypharmacological mode-of-action of the oxadiazolones and identified their targets in MRSA. Notably, a target-based approach alone would have not been able to uncover the mode-of-action of the oxadiazolones, thereby showcasing the power of chemical proteomics as a valuable chemical biology technique for antibiotic drug discovery. Future experiments are directed towards understanding the biological role of these targets and further optimization of the compounds as viable drug candidates.

Supporting Information

Contains Supplementary Table 1 – 9, Supplementary Figure 1 – 5, Materials and Methods, Synthetic Procedures, and NMR data of key compounds (PDF format).

Supplementary Data

Contains Supplementary Data 1 – 4 (Excel format).

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