Competitive Profiling of Ligandable Cysteines in *Staphylococcus aureus* with an Organogold Compound

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With the idea of exploiting metal-templated reactions to achieve selective modification of cysteines in proteins for antibacterial applications, an organometallic cyclometalated Au(III) compound was explored in a competitive chemoproteomic approach based on the isoDTB-ABPP (isotopically labelled desthiobiotin azide-activity-based protein profiling) technology in *S. aureus* cell extracts. In this way, more than 100 ligandable cysteines where identified, of which 10 were close to functional sites of proteins encoded by essential genes indicating potential for antibiotic development. Interestingly, more than 50% of the identified ligandable sites were not engaged by organic α-chloroacetamides in a previous study, indicating that organometallic compounds expand the ligandable space in bacteria. A selected interaction identified by isoDTB-ABPP was validated using an enzyme activity assay, and intact protein mass spectrometry showed that cysteine arylation of an unprecedented target occurs with the studied compound. The obtained results constitute the proof-of-concept that this family of organogold compounds has potential for therapeutic protein targeting *via* selective, covalent modification of cysteine residues in bacteria. Looking more broadly, our study demonstrates that the targets of cyclometalated gold compounds can be studied proteome-wide with competitive residue-specific chemoproteomics enabling the expansion of the known ligandable proteome to sites that can be addressed with this compound class.

Introduction

Bacterial antimicrobial resistance is one of the leading causes of death world-wide and is estimated to have directly caused more than 1.2 million deaths in 2019.[1] One way to overcome this global health challenge will be the identification of novel antibacterial targets that can be liganded with small molecules.[2] While recent successes with reversible, organic compounds show that there is still a lot of potential to address new targets with known concepts,[3-6] systematically exploring other compound classes for antibacterial activity could open up entirely new target families. Using the isoDTB-ABPP (isotopically labelled desthiobiotin azide-activity-based protein profiling) technology,[7] which is based on the isoTOP-ABPP (isotopic tandem orthogonal proteolysis–ABPP) platform,[8,9] we have recently demonstrated that irreversible, cysteine-directed covalent inhibitors in combination with competitive, residue-specific chemoproteomic approaches allow efficient identification of many new binding sites with ligandable cysteines in bacteria in parallel.[7]

In this context, in recent years, it has become evident that metal complexes possess many attractive properties that make them good therapeutic candidates for addressing elusive target proteins especially for antibiotic applications.[10-16] This is based on unique 3D structures that are inaccessible to organic molecules, but can be attained with metal-based compounds facilitating their binding to specific target sites,[10, 17] as well as by the ability of metal complexes to undergo activation by redox process and ligand exchange reactions in cells.[18, 19] Notably, organometallic compounds featuring a direct metal-carbon bond have attracted increasing attention as therapeutic agents, since they promote previously unattainable chemical transformations in biological environment,[20, 21] including cross-coupling reactions,[22-24] cycloadditions,[25] hydrogenation and transfer hydrogenation reactions,[26, 27] ester amidation[28] or functional group deprotection (uncaging) reactions[29].

Recently, some of us started working on organometallic Au(III) complexes of different families, which were shown to possess anticancer and antibacterial properties.[30-35] Specifically, we focused on cyclometalated Au(III) C^N complexes and on the identification of their protein targets and respective binding modes.[18, 35, 36] It was observed that organogold compounds in this family can template the formation of covalent C^N-peptide adducts *via* C–S cross-coupling at cysteine residues.[37-39] Among the selected Au(III) complexes, the [Au($C^{CO}N$)Cl_2] compound (**1**, $C^{CO}N$ = 2-benzoylpyridine, Fig. 1a) was identified as the most reactive and prone to cysteine arylation in buffered aqueous solution (pH 7.4) at 37 °C.[37] Furthermore, compound **1** showed moderate antibacterial effects (MIC approx. 12.5-50 μM, unpublished data and ref. [35]) in a number of bacterial strains. The chemoselectivity with respect to cysteine arylation was assessed by reacting **1** with different model peptides.[40] Interestingly, the compound was not able to promote other types of C–X (X = N or O) cross-coupling reactions and did not arylate cysteines at the N-terminal side. Based on the obtained results, a general reaction mechanism for cysteine arylation was proposed whereby a cysteinate residue first binds Au(III) *trans* to the N of the C^N ligand *via* a ligand exchange reaction. Afterwards, a second amino acid residue or backbone nucleophile coordinates to Au(III), favouring the bond breakage between the nitrogen and the metal centre (Fig. 1b).[41] Formation of the latter intermediate is crucial to promote the subsequent C–S cross-coupling *via* reductive elimination.

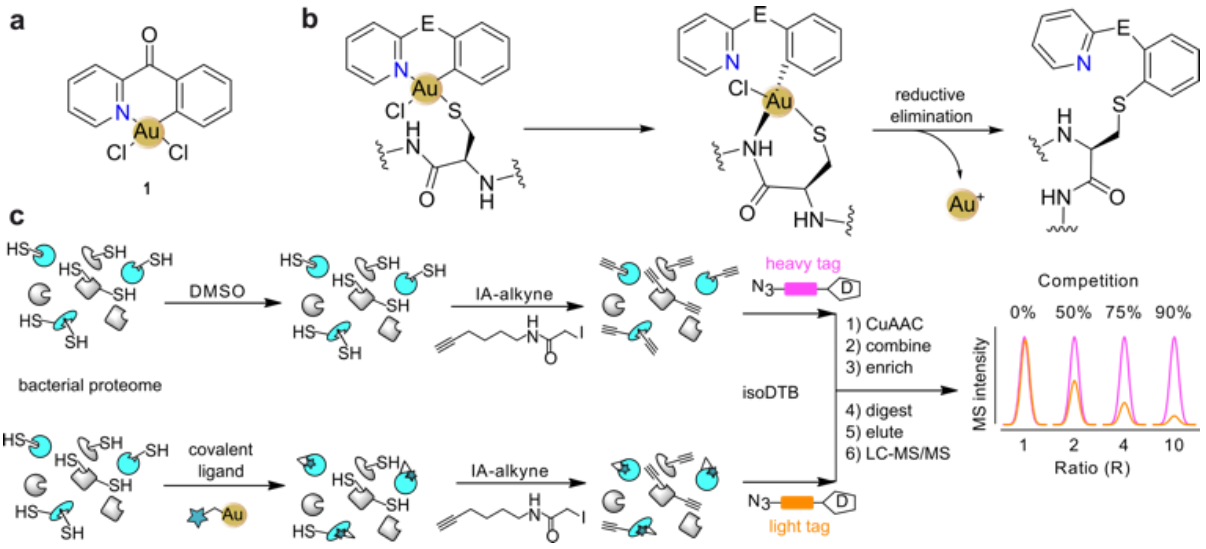


Figure 1. (a) Structure of the cyclometalated complex [Au(C^CO^N)Cl$_2$] (**1**, C^CO^N = 2 benzoylpyridine). **(b)** Proposed mechanism of cysteine arylation templated by compound **1** *via* reductive elimination. **(c)** Workflow of isoDTB-ABPP experiments. isoDTB: isotopically labeled affinity tags, D: desthiobiotin.

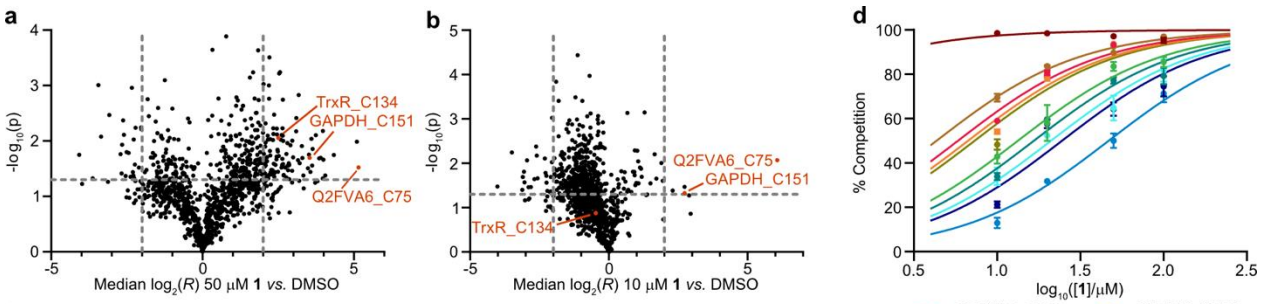
Following these promising results, we envisaged the application of compounds of this family as *i)* tools for competitive residue-specific chemoproteomic technologies to enable profiling of unprecedented ligandable cysteine residues in bacterial cells,[8, 9] and/or *ii*) as promising novel cysteine-targeted antibacterial agents. Therefore, we started investigating in an unbiased fashion, which cysteines can be engaged with this organogold compound class in the entire *S. aureus* proteome. To avoid laborious synthesis of Au(III) C^N complexes with suitable affinity handles, we applied a competitive approach based on the isoDTB-ABPP technology (Fig 1c).[7] In brief, in this strategy, the *S. aureus* proteome is split into two samples, one of which is treated with compound **1** and the other one with DMSO as a solvent control. In the next step, iodoacetamide alkyne (IA-alkyne)[8] is used to label many cysteine residues with alkynes. At the sites, at which compound **1** is already bound, this reactivity is blocked leading to a difference in alkyne modification between the inhibitor-treated and control-treated sample at these specific sites. This difference is read out using isotopically labelled (light and heavy) desthiobiotin azide (isoDTB) tags that are appended by cupper-catalyzed azide-alkyne cycloaddition (CuAAC)[42] and after combination of the two samples used for enrichment. Following digestion of the enriched proteins, the modified peptides are eluted and analysed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).

Afterwards, the abundance of the alkynylated peptides can be relatively quantified between the two samples using the light and heavy isotopic label allowing quantification of the degree of binding of compound **1**. While cysteines that are not bound by **1** will show ratios between the heavy and light channel close to $R = 1$, the specific targets of **1** will show high ratios ($R >> 1$). As the modified peptides are directly detected, this technology not only allows determination of the target proteins, but also of the exact interaction sites. In this way, a global understanding of the binding sites that are ligandable by compound **1** is obtained in the entire *S. aureus* proteome.

Following this chemoproteomic approach, *S. aureus* cell extracts were treated with compound **1** at room temperature for 1 hour at concentrations ranging from 10 μM to 100 μM. It should be noted that **1** is only moderately active as antibacterial agent on *S. aureus* culture (MIC values 12.5 – 50 μM).[35] Therefore, we deprioritized the identification of targets in living cells at low concentrations and rather focused on the broad mapping of cysteines that are ligandable with this compound class in lysates at higher concentrations; thus, avoiding potential issues with compound's uptake.

Using the isoDTB-ABPP platform, we obtained data on a total of 1486 cysteines in the *S. aureus* proteome (Table S1). While at 100 μM broad competition at many targets was observed (see Fig. S1), we detected a much narrower window of specifically competed proteins at 50 μM and below (Fig. 2a,b and Fig. S1). For all further analysis, we therefore focussed on the proteins that were significantly engaged ($\log_2(R) > 2$, $p < 0.05$) at 50 μM or below. In this way, we identified 108 cysteines that are ligandable by compound **1**.

Interestingly, 27 of these cysteines were found in proteins encoded by essential genes[43] and, of those, 10 were assigned to be close to the respective functional protein sites (Fig. 2c).[44] The latter include catalytic nucleophiles (Cys-151 in gapA1, Cys-2 in glmS and Cys-102 of mnmA), other active site residues (Cys-134 in trxB and Cys-112 in fabH), residues of metal binding sites (Cys-24 of rpsZ, Cys-100 of glmU, Cys-829 in secA1 and Cys-38 in tarJ) and residues of nucleotide binding sites (Cys-44 and Cys-275 in metK).[44] In line with what we observed for organic molecules as competitors,[45] we detected concentration-dependent competition for many cysteines (Fig. 2d) that could be fit with a dose response model, indicating that the isoDTB-ABPP method gives quantitative, residue-specific engagement data also for organometallic compounds. Moreover, of the 108 cysteines liganded by compound **1**, 59 were not liganded by any member of a



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Gene name	Protein name	Cysteine residue	Type of functional site
gapA1	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Cys-151	Catalytic nucleophile
glmS	Glutamine-fructose-6-phosphate aminotransferase	Cys-2	Catalytic nucleophile
rpsZ	30S ribosomal protein S14 type Z	Cys-24	Zinc binding site
mnmA	tRNA-specific 2-thiouridylase MnmA	Cys-102	Catalytic nucleophile
glmU	Bifunctional protein GlmU	Cys-100	Magnesium binding site
metK	S-adenosylmethionine synthase	Cys-44, Cys-275	Potassium binging site, ATP binding site
secA1	Protein translocase subunit SecA 1	Cys-829	Zinc binding site
tarJ	Ribulose-5-phosphate reductase 1	Cys-38	Zinc binding site
fabH	3-oxoacyl-[acyl-carrier-protein] synthase 3	Cys-112	Active site
trxB	Thioredoxin reductase (TrxR)	Cys-134	Redox-active site*

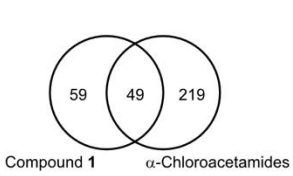

Figure 2. (a,b) Volcano plots of the isoDTB-ABPP experiments that show the median $\log_2(R)$ of the ratio between the heavy (compound-treated) and light (DMSO-treated) channels and the $-\log_{10}(p)$ of the statistical significance in a one-sample t-test for all quantified cysteines for compound **1** at 50 μM **(a)** and 10 μM **(b)**. Grey dotted lines indicate the cut-offs of $\log_2(R) = \pm 2$ and $p < 0.05$ that were used for hit selection. Selected proteins discussed in the text are highlighted in orange. **(c)** Cysteines liganded by **1** at 50 μM or below that are in annotated functional sites of proteins encoded by essential genes. *: Functional site inferred from the 100% identical gene with Uniprot code P66011. **(d)** Concentration dependence of the degree of competition determined using isoDTB-ABPP for a selection of cysteines. Data points represent the median, error bars the standard deviation and lines a dose-response curve fit. All experiments were performed in duplicate. **(e)** Venn diagram of the ligandable cysteines identified in this study with compound **1** and in a previous study with a library of α-chloroacetamides.[7]

previously screened α-chloroacetamide library (Fig. 2e, Table S1).[7] These unique targets include Cys-44 of metK, Cys-24 of rpsZ and Cys-829 in secA1, located in functional sites of proteins encoded by essential genes (Fig. 2c). These results indicate that organometallic compounds like **1** indeed access a different portion of the proteome and can, therefore, be very beneficial to target binding sites that are hard to address with organic compounds.

Of note, one of the competed cysteines was located in the functional site of the protein encoded by gapA1, corresponding to Glyceraldehyde-3-phosphate dehydrogenase (GADPH) an enzyme recently unveiled as a target of antibacterial Ag^+ ions in *E. coli*, and inhibited by Cu^+ in *S. aureus*.[46, 47] Moreover, the results highlighted Cys-134 of the bacterial thioredoxin reductase (TrxR, Fig. 2a,c) as one targeted residue. In bacteria, TrxR belongs to the antioxidant thioredoxin system[48, 49] and features a redox active site disulfide/dithiol couple consisting of Cys-134 and Cys-137. Interestingly, TrxR has already been proposed as pharmacological target for antibacterial gold-based compounds.[50-53]

To assess the relevance of compound **1** binding to cysteine residues with respect to enzyme activity, we conducted enzyme inhibition studies on the bacterial target TrxR using a DTNB-based plate reader assay (see Experimental for details).[50] Thus, purified *S. aureus* TrxR was treated with **1** at different concentrations and incubated for 75 min at 25 °C. In accordance with the isoDTB-ABPP data, compound **1** efficiently inhibits the TrxR activity (IC_{50} = 0.258 ± 0.052 μM, Fig. S2).

As competition of IA-alkyne labelling in isoDTB-ABPP experiments, as well as TrxR inhibition, is in principle possible by either coordination of the Au(III) centre to the target cysteine or by the aforementioned C–S cross-coupling arylation reaction, we set out to validate the actual mechanisms-of-action on one of our identified target proteins, namely the GCN5-like putative *N*-acetyltransferase (Uniprot code Q2FVA6). While this protein is not encoded by an essential gene, it stood out by its high engagement even at 10 μM of compound **1** (R = 67 at 10 μM corresponding to >98% competition). At this concentration, **1** showed high selectivity for competition of Cys-75 of this protein (Fig. 2b) having only four other targets with significantly lower R values. It should be noted that Cys-75 is not competed by any α-chloroacetamide in our earlier study (Table S1),[7] although it was quantified for all of these compounds, showing that this is an interaction that is specific to the gold complex **1**. Additionally, in a published NMR structure,[54] Cys-75 is close to the thioester of the cofactor acetyl CoA (3.3 Å, Fig. 3a) indicating that blocking it with compound **1** has the potential to inhibit the enzyme activity.

The relatively small size of this protein (approx. 11.9 kDa) allowed us to perform intact protein high-resolution electro-spray ionization mass spectrometry studies (HR-ESI-MS) following previously reported procedures.[37, 55, 56] In detail, the wild type GCN5-like putative *N*-acetyltransferase (WT) and its mutant (Mu, C75A) were incubated at a concentration of 5 μM with compound **1** in 1:3 ratio in H_2O/ACN (2:1) for 1 hour at room temperature. Figure 3 shows the mass spectra for the wild-type protein before and after treatment with **1**. The obtained results demonstrate the formation of the Au(III) coordination adduct $[WT\text{-}Met+Au^{III}(C^{CO}N)]^{n+}$ obtained upon exchange of the two chlorido ligands of **1**, as well as the cysteine arylation product $[WT\text{-}Met+(C^{CO}N)]^{n+}$ (Fig. 3, Table S2-S3). These results are in line with previously reported reactivity studies of

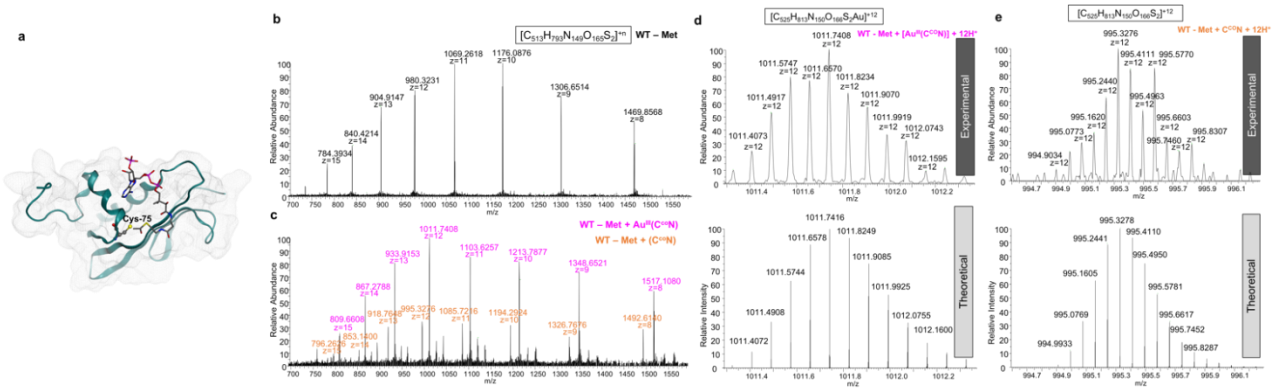

Figure 3. (a) Structure of the GCN5-like putative N-acetyltransferase (PDB: 2H5M). The ligandable Cys-75 is highlighted. The cofactor acetyl CoA is shown as sticks. **(b,c)** Reference spectra of WT GCN5-like putative N-acetyltransferase before **(b)** and after **(c)** addition of $[Au^{III}(C^{CO}N)Cl_2]$ incubated for 1 h at room temperature. **(d,e)** Comparisons of experimental *vs.* theoretical isotopic pattern distributions of the formed coordination (WT-Met-$[Au^{III}(C^{CO}N)]$) **(d)** and covalent (WT-Met-$[(C^{CO}N)]$, cysteine arylation product) **(e)** adducts.

compound **1** and derivatives with model peptides.[36, 37, 40] Of note, the unbound WT protein species could not be identified in the gold-treated sample indicating the protein's marked reactivity with compound **1**. Interestingly, in the case of the C75A mutant, only formation of species featuring a bound gold fragment of general formula $[Mu\text{-}Met+Au^{III}(C^{CO}N)]^{n+}$ were identified, while the signals of the unbound protein were still detected (Fig. S3, Table S2-S3). As expected, the cysteine arylation product could not be detected in this experiment.

Overall, inhibition of the enzymatic activity of TrxR as well as arylation of Cys-75 of GCN5-like putative *N*-acetyltransferase validate the data that we obtained in the isoDTB-ABPP study and verify that arylation with compound **1** can proceed on unprecedented proteins identified using this technology. Thus, our data delivers a map of ligandable cysteines in *S. aureus* that can be addressed with organogold compounds such as **1**, which might constitute the basis for a new family of antibacterials acting as covalent binders of cysteine residues.

Conclusions

Transition-metal-based strategies to selectively modify proteins hold promise in addressing challenges in chemical biology and in the development of novel therapeutic approaches for different diseases. Here, we have successfully profiled cysteines in the pathogenic bacterium *S. aureus* that can be liganded with the organogold compound **1** using the isoDTB-ABPP technology. In this way, more than 100 cysteines that are liganded by this compound were identified, of which more than 50 could not be liganded by any member of a previously investigated library of cysteine-directed α-chloroacetamides.

Notably, we identified 10 ligandable cysteines in functional sites of proteins encoded by essential genes, which include proteins relevant to metal and redox homeostasis in bacteria and which are prime candidates to be explored further for potential pharmacological relevance. Further target validation studies showed potent inhibition of bacterial TrxR by compound **1** *in vitro*. In addition, the GCN5-like putative N-acetyltransferase, that stood out through very high engagement by compound **1** in the isoDTB-ABPP studies, was shown to undergo cysteine arylation by **1**, whereas the C75A mutant is not subjected to this covalent modification. Therefore, these results demonstrate the high cysteine selectivity of the arylation reaction templated by the Au(III) complex and further validate the isoDTB-ABPP technology for cysteine profiling in complex bacterial lysates.

Overall, our study shows that Au(III) cyclometalated compounds have a high potential to address unique binding sites in the proteome, where they can promote selective metal-templated bioorthogonal reactions and thereby elicit biological effects that are not attainable with organic molecules in a straightforward fashion. Despite these promising results in bacterial lysates, compound **1** shows only moderate antibacterial activity against *S. aureus.* This could be due to different factors, including its extracellular deactivation caused by competitive binding to extracellular components, low uptake into bacterial cells or by an insufficiently high engagement of the relevant cysteines in the complex cell environment. Therefore, further optimization of the compound's scaffold to improve the antibacterial activity will be necessary in the future, for example including targeting functionalities in the C^N backbone.[38, 57] It is worth mentioning that, in a recent study, some of us reported on a cyclometalated Au(III) C^N analogue of **1** with promising antibacterial effects, that is now undergoing target engagement studies at relevant concentrations in living bacteria.[35]

In summary, we demonstrate for the first time that residue-specific chemoproteomic experiments with the isoDTB-ABPP platform allow the competitive profiling of the targets of organometallic compounds in the complex environment of the cellular lysate. As many metal complexes that could potentially bind to or react selectively with cysteine residues have been reported to have antibacterial activity,[10, 20] this study opens up the field of quantitatively studying their cellular interactions using residue-specific chemoproteomics, provided that

the bonding interactions will be sufficiently stable. Through performance of such experiments in bacteria to identify relevant targets, as well as in human cell lines to identify potential off-targets, we envision that this technology will make significant contributions to realising the potential of metal complexes as antibacterial drugs in the near future.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

S.M.H. acknowledges funding by the Fonds der Chemischen Industrie through a Liebig Fellowship and by the TUM Junior Fellow Fund. C.S. thanks the Dr. Ing. Leonhard Lorenz-Stiftung for their financial support. M.Z. gratefully acknowledges funding by the Studienstiftung des Deutschen Volkes through a Ph.D. fellowship and by the Marianne-Plehn-Programm. We gratefully acknowledge Prof. Stephan A. Sieber and his group for their generous support. Dr. Darren Wragg is gratefully acknowledged for designing Figure 3a.

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