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Chemoproteomic Study of Effect of Halogenated Hydroxynaphthalenecarboxanilides on *Staphylococcus aureus* †

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Abstract: Recently reported multihalogenated (CF_3/Cl) anilides of 1-hydroxynaphthalene-2-carboxanilides showed significant activity against both the reference strain *Staphylococcus aureus* ATCC 29213 and clinical isolates of methicillin-resistant *S. aureus* (MRSA). This fact inspired further investigation of the effect of these compounds on staphylococci. Chemoproteomics is a tool for investigating protein targets of potential drugs. It makes it possible to understand the effect of a bioactive molecule on a living system. An activity-based protein profiling (ABPP) method was employed using highly active and inactive ring-substituted 1-hydroxynaphthalene-2-carboxanilides as probes. The experiment was performed on the universally sensitive collection strain *S. aureus* ATCC 29213. Tryptic cleavage of proteins was performed prior to HPLC-MS/MS analysis. Protein profiles of control samples (*S. aureus* cells) and profiles of *S. aureus* treated with inactive/active derivatives were investigated and compared to each other. More than 1000 proteins were analyzed, with approximately 70% of the proteins was increased and 30% of the proteins was decreased after treatment with the investigated compounds. Treatment with the inactive compound mainly resulted in the expression of various proteins, so it can be assumed that the changes in the protein profile did not affect the basic biochemical pathways or that the microorganism was able to adapt by activating other pathways/expressing other proteins, and the microorganism survived. Treatment with the highly active agent resulted in much smaller proteomic changes (mainly inhibition of several proteins compared to the inactive compound), *S. aureus* failed to adapt and was killed.

Keywords: hydroxynaphthalenecarboxanilides; *Staphylococcus aureus*; ABPP; chemoproteomics; HPLC-MS/MS

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1. Introduction

At the present time, when anti-invasive (antimicrobial and antitumor) drugs are facing increasing resistance [1], one of the trends in the prevention of resistance is the development of multi-target agents [2,3]. Hydroxynaphthalenecarboxanilides, as well as their cyclic analogues salicylanilides, represent typical multi-target agents [4,5]. Hydroxynaphthalenecarboxanilides have been mainly investigated as potential anti-infective [6–8] and anticancer agents [9–12].

The effects of bioactive compounds can either depend on binding to a specific target site (structurally specific compounds) or occur independently (structurally non-specific compounds) [13,14]. A negative of multi-target agents is the fact that it is difficult to precisely determine/identify the mechanism of action. One possibility that could help reveal the mechanism of action of these compounds is the use of chemoproteomics, which has become a useful tool in modern drug discovery and preclinical research [15], providing critical insight into interactions between bioactive agents and protein targets within complex biological systems. By identifying proteins to which small molecules bind, chemoproteomics allows mapping the mechanisms of actions of potential therapeutic agents as well as identifying unintended off-target effects that could lead to toxicity [16].

The primary goal of chemoproteomics is to investigate how small bioactive molecules interact with proteins in their natural cellular environment [17]. This is typically achieved using activity-based protein profiling (ABPP), a powerful chemoproteomic technique that uses small molecule probes to label and capture proteins in a functional state [18]. The resulting labeled proteins can then be analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS), which provides a detailed proteomic profile of the cell's response to the compound [15,19].

The aim of this work is to perform a proteomic analysis to obtain the protein profile of a reference strain of *Staphylococcus aureus* treated with two selected ring-substituted 1-hydroxynaphthalene-2-carboxanilides, which could subsequently contribute to the hypothesis about the mechanisms of action of these compounds.

2. Results and Discussion

To investigate the mechanism of antistaphylococcal activity, a chemoproteomic approach was used for two isomers of the compound *N*-[bis(trifluoromethyl)phenyl]- 1-hydroxynaphthalene-2-carboxamide; derivative **1** (encoded NM64) was inactive, while isomer **2** (encoded NM33) was highly active against both the universally susceptible collection strain *Staphylococcus aureus* ATCC 29213 and several human and veterinary clinical isolates of methicillin-resistant *S. aureus* (MRSA). The compounds were prepared by recently described microwave-based synthesis [6,7,9]. The comparative ABPP technique was used to study the protein profiles of *S. aureus*. In general, *S. aureus* is a common pathogen that can cause a variety of infections, from minor skin infections to more serious conditions (MRSA infections) such as pneumonia and sepsis [20].

In this primary chemoproteomic investigation (used ABPP approach with LC-MS/MS detection), the discussed derivatives were tested against the collection strain *S. aureus* ATCC 29213. The experiment was carried out by cultivating *S. aureus* without the addition of the tested molecules (control sample, CN), by cultivating *S. aureus* with ineffective compound **1** (NM64) and by cultivating *S. aureus* with active agent **2** (NM33). In this way, information was obtained: (*i*) on the protein representation of native bacteria (CN); (*ii*) on the change of the protein profile after the action of active substance **2** (CN vs. NM33); and (*iii*) changes in protein profile induced by the inactive agent could be filtered out without affecting viability (NM33 vs. NM64). Of approximately 1000 proteins, a statistically significant change in expression was noted for approximately 780 proteins. Individual comparisons of the control with both compounds and both isomers against each other are shown in the volcano plots below.

Figure 1 shows the changes in the protein profile that were most significant after treatment of *S. aureus* with inactive compound **1** compared to the control sample. Glyceraldehyde-3-phosphate dehydrogenase 2 (304) and alanine tRNA ligase (243) are among the proteins whose expression was increased by compound **1**. In contrast, asparagine tRNA ligase (207), 1-(5-phosphoribosyl)-5-[(5-phosphoribosylamino)methylideneamino]imidazole-4-carboxamide isomerase (92), and probable tRNA sulfurtransferase (77) were significantly inhibited by isomer **1**.

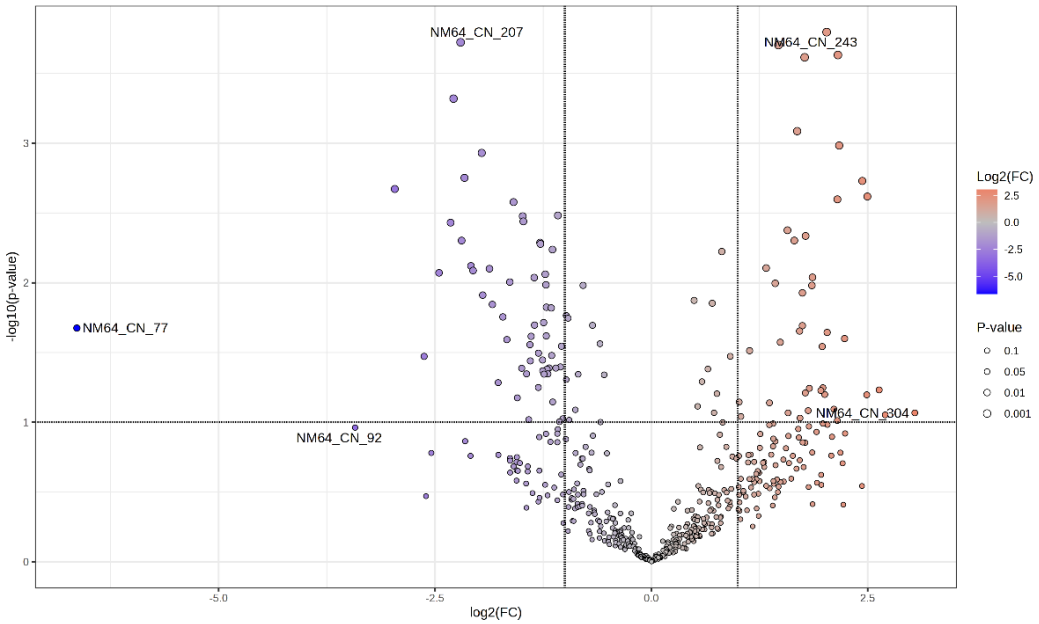

Figure 1. Volcano plot of protein changes of control *S. aureus* (CN) compared to *S. aureus* treated with inactive compound **1** (NM64). Red points = significantly increased proteins compared to control samples; blue points = significantly decreased proteins compared to control; gray points = proteins without significant changes.

The volcano plot (Figure 2) illustrates the changes in the protein profile that were most significant after treatment of *S. aureus* with effective agent **2** compared to *S. aureus* samples without the addition of the bioactive compound. In general, the effect of isomer **2** was mainly manifested by inhibition, with the most significant decrease observed in the proteins catabolite control protein A (335), 3-hexulose-6-phosphate synthase (247), NAD-specific glutamate dehydrogenase (260) 3-methyl-2-oxobutanoate hydroxymethyltransferase (409) and a 77 kDa membrane protein (541).

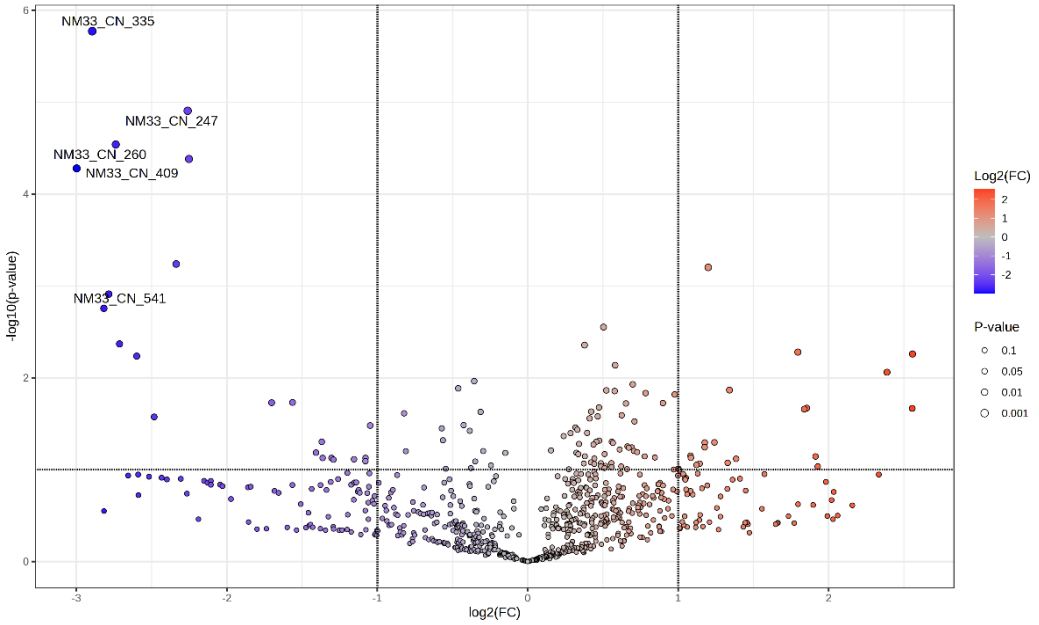

Figure 2. Volcano plot of protein changes of control *S. aureus* (CN) compared to *S. aureus* treated with effective agent **2** (NM33). Red points = significantly increased proteins compared to control samples; blue points = significantly decreased proteins compared to control; gray points = proteins without significant changes.

Figure 3 demonstrates a volcano plot of the most significant changes in the protein profile found after treatment of *S. aureus* with active isomer **2** compared to inactive derivative **1**. The graph shows that the levels of most proteins were altered (most often increased), with serine-aspartate repeat-containing proteins E (07, 08, 10) being most significantly increased. Only a few proteins were inhibited by isomer **2**, and the most significant change was observed in staphylocoagulase (197).

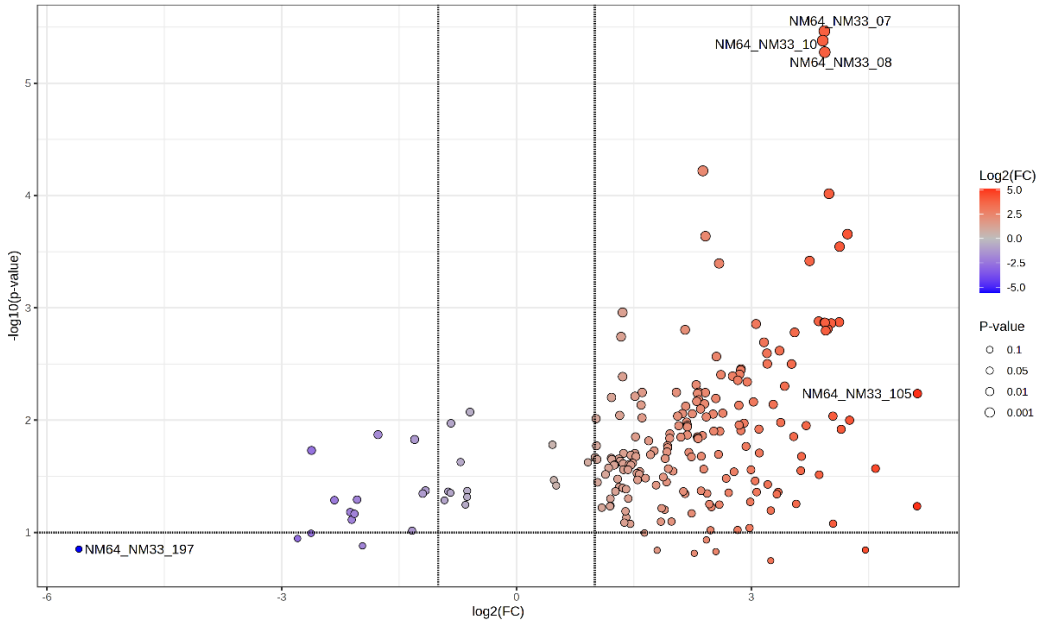

Figure 3. Volcano plot of protein changes of *S. aureus* treated with active isomer **2** (NM33) compared to *S. aureus* treated with inactive isomer **1** (NM64). Red points = significantly increased proteins compared to inactive compound; blue points = significantly decreased proteins compared to inactive compound; gray points = proteins without significant changes.

From the heatmap in Figure 4, it is clear that the greatest changes in *S. aureus* were manifested in the levels of proteins such as arginine tRNA ligase (47), putative aldehyde dehydrogenases (49, 48), inosine-5′-monophosphate dehydrogenase (55), when treatment with the active agent **1** resulted in a significant inhibition of these proteins compared to control. Conversely, in the case of proteins such as arginase (80), dihydrolipoyllysine- residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex (85) and phosphoglycerate kinase (64), the most significant increase in these proteins occurred after exposure to the active compound. When comparing control with *S. aureus* samples treated with inactive isomer **2**, a greater number of more significant changes can be observed. Inhibition due to the inactive compound was more pronounced for the proteins serine-aspartate repeat-containing protein E (07), alcohol dehydrogenase (38), phosphoglycerate kinase (64), serine-aspartate repeat-containing protein C (09) and bone sialoprotein-binding protein (12). Conversely, an increase in the level during treatment with the ineffective isomer **1** occurred in the case of proteins arginine deiminase (32), thiol peroxidase (32), serine-aspartate repeat-containing protein D (04), D-lactate dehydrogenase (18), small ribosomal subunit protein (90), deoxyribose-phosphate aldolase (69), putative dipeptidase SAR1836 (60) and uncharacterized protein SAV1875. It is worth noting that for most of the proteins that were increased by the influence of ineffective compound **1**, there was almost no change in the case of treatment with active agent **2** compared to the control (see proteins 17, 58, 42, 03, 34).

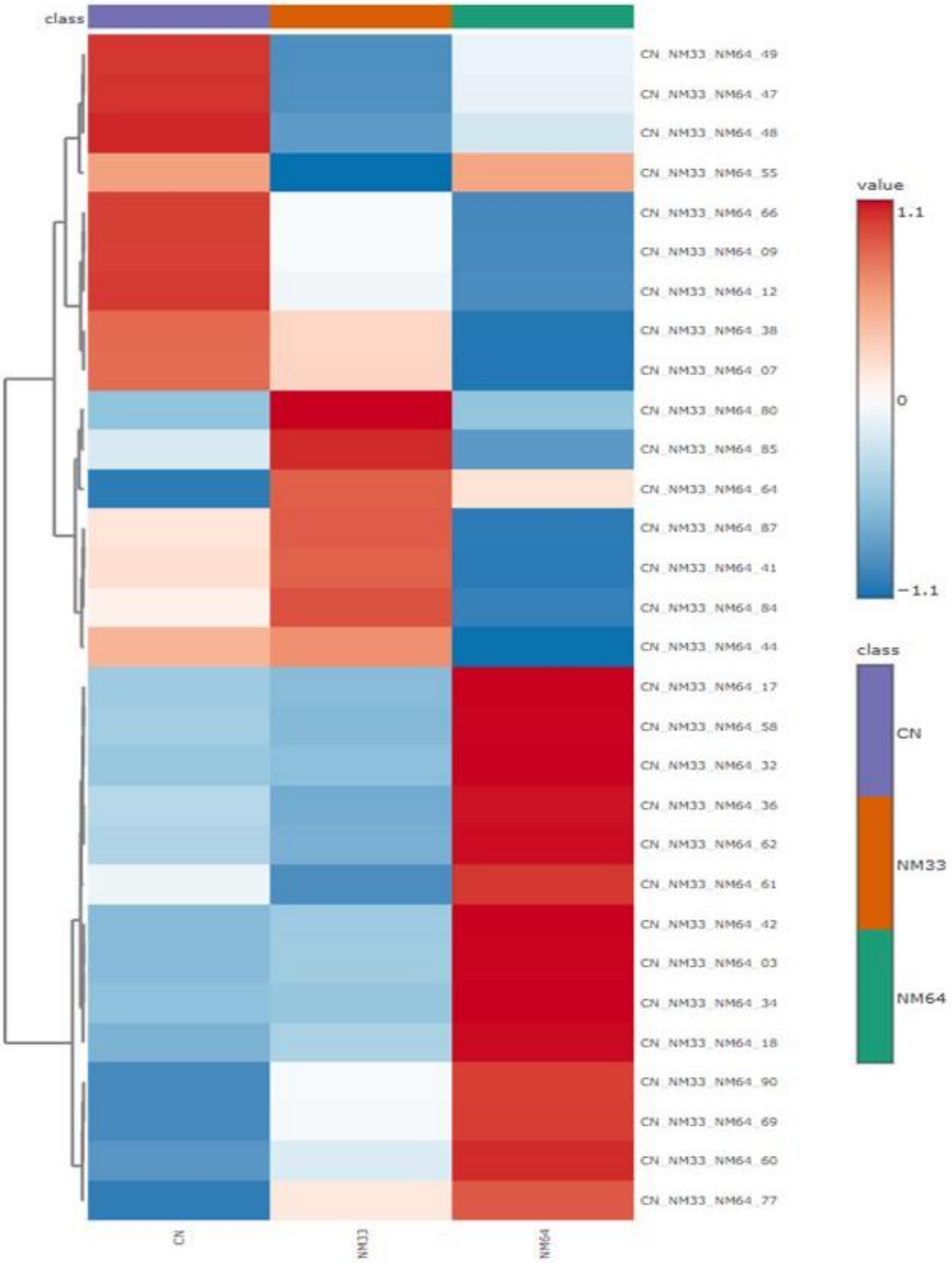

Figure 4. Heatmap of protein changes based on treatment of *S. aureus* with inactive isomer **1** (NM64), active isomer **2** (NM33) compared to control (CN). Dark blue color = lowest protein level, dark red = highest protein level. Purple color = control samples of *S. aureus*, orange color = *S. aureus* treated with isomer **2**, green color = *S. aureus* treated with isomer **1**.

Although inactive isomer **1** affected significantly more proteins (90%) than effective agents **2**, these changes did not kill *S. aureus*. It can be assumed that the changes in the protein profile did not affect the basic biochemical pathways or the microorganism was able to adapt by activating other pathways/expressing other proteins, and thus the treatment of *S. aureus* with compound **1** resulted in stress in the cells, but the microorganism survived. Treatment with the highly active agent **2** resulted in much smaller proteomic changes compared to the inactive agent **1**, *S. aureus* failed to adapt and was killed.

3. Experimental Section

This study utilized activity-based protein profiling (ABPP), specifically its comparative technique, to identify protein targets within the proteome of cell lysates. The experiments were conducted on the universally sensitive collection strain *S. aureus* ATCC 29213. *S. aureus* was treated as previously described, e.g., [4,5,7,8]. Prior to MS detection, proteins underwent tryptic digestion. Protein identification and statistical analysis were conducted using Proteome Discoverer Software (Thermo Fisher Scientific, West Palm Beach, FL, USA), utilizing the *Staphylococcus aureus* Uniprot database [21].

Aliquots of purified complex peptide mixtures of 100 ng were separated using Acquity M-Class UHPLC (Waters, Milford, MA, USA). Samples were loaded onto the nanoEase Symmetry C18 trap column (25 mm length, 180 μm diameter, 5 μm particles size). After 2 min of desalting/concentration by 1% acetonitrile containing 0.1% formic acid at a flow rate of 8 μL/min, peptides were introduced to the nanoEase HSS T3 C18 analytical column (100 mm length, 75 μm diameter, 1.8 μm particle size). For the thorough separation, a 90 min gradient of 5–35% acetonitrile with 0.1% formic acid was applied at a flow rate of 300 nL/min. The samples were sprayed (3.1 kV capillary voltage) to the quadrupole time-of-flight mass spectrometer Synapt G2-Si with an ion mobility option (Waters). Spectra were recorded in a data-independent manner in high definition MSE mode. Ions with 50–2000 m/z were detected in both channels, with a 1 s spectral acquisition scan rate. Data processing was done in Progenesis QI 4.0 (Waters). For peak picking, the following thresholds were applied: Low energy 320 counts and high energy 40 counts. Precursors and fragment ions were coupled, using correlations of chromatographic elution profiles in low/high energy traces. Then, peak retention times were aligned across all chromatograms. Peak intensities were normalized to the median distribution of all ions, assuming the majority of signals are unaffected by experimental conditions.

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