Research Article

In Vitro Bactericidal Activity of 4- and 5-Chloro-2-hydroxy-N-[1-oxo-1-(phenylamino)alkan-2-yl]benzamides against MRSA

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1. Introduction

The antibiotic resistance of invasive pathogens has become one of the most challenging and persistent health problems [1]. Methicillin-resistant Staphylococcus aureus (MRSA) has become the most common clinically relevant multi-resistant pathogen [2] causing both healthcare-associated and community-acquired bloodstream infections with mortality rates up to 40% [3].

The prevalence of MRSA is increasing worldwide and, according to the latest information of the European Centre for Disease Prevention and Control from 2012 [4], can be considered alarming in some European countries, especially in Portugal and Romania, where ≥50% of all S. aureus isolates from invasive infections were identified as MRSA in 2012 (although, e.g., in Romania the prevalence of MRSA was 25–50% in 2010), followed by Italy, Greece, and Poland with 25–50% isolates being MRSA in 2012 (for comparison, in Poland...
MRSA isolates constituted 10–25% from all S. aureus isolates in 2010.

The treatment failure of vancomycin, the therapeutic anti-MRSA agent of choice, due to the strains with elevated vancomycin minimum inhibitory concentration (MIC) values (i.e., the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism) within the susceptible range was described previously [5, 6]. Thus, the emergence of MRSA (and vancomycin-resistant S. aureus in the recent years as well [7]) makes the discovery of new molecular scaffolds a priority, and the current situation even necessitates the reengineering and repositioning of some old drug families to achieve adequate control of these bacteria [8]. However, for the treatment of S. aureus bloodstream infections, bactericidal antimicrobial agents are considered to be superior to bacteriostatic drugs [9]. This fact should be considered during the development of effective and safe treatment options for MRSA infections.

The history of clinical usage of salicylanilides (2-hydroxy-N-phenylbenzamides) dates back to the 1940s in therapy of tinea capitis, followed by the discovery of their anthelmintic properties in the mid 1950s [10]. Nowadays, salicylanilides (SALs) are a class of aromatic compounds possessing a wide range of interesting pharmacological activities, such as anthelmintic [11], antibacterial [12, 13], antimycobacterial [13], antifungal [14], and antiviral [15, 16], among others. Despite being studied since the 1960s, the mechanism of action responsible for biological activities of these compounds has not been explained so far. SALs have been found to inhibit the two-component regulatory systems (TCS) of bacteria [17]. The latest studies specified them also as selective inhibitors of interleukin-12p40 production that plays a specific role in initiation, expansion, and control of cellular response to tuberculosis [18]. Furthermore, salicylanilides have been recognised as inhibitors of some bacterial enzymes, such as sortase A from S. aureus [19], d-alanine-d-alanine ligase [20], or transglycosylases from S. aureus (but not from M. tuberculosis) [12]. These enzymes participate in secretion of various proteins or in biosynthesis of bacterial cell wall. Recently, salicylanilides-like derivatives were described to inhibit two enzymes essential for mycobacteria: (i) methionine aminopeptidase, catalyzing a key step of the posttranslational modification of nascent proteins, and (ii) isocitrate lyase, which is essential for the metabolism of fatty acids [21]. Thus, SALs seem to be promising candidates for development of new antibacterial agents with a novel mechanism of action. Such new agents could be a solution to the resistance challenges.

This study is a follow-up paper to a recently published article [13]. The synthesis of the series of novel derivatives of salicylamides, 4- and 5-chloro-2-hydroxy-N-[(1-oxo-1-(phenylamino)alkan-2-yl)benzamides, called diamides due to their skeleton (for general structure see Table 1), was described previously [13, 22], and their antimycobacterial and antibacterial activities against various bacterial species were reported [13]. As these compounds expressed very significant antibacterial activity with low MIC values against clinical isolates of MRSA as representatives of multidrug-resistant bacteria, we decided to extend the knowledge about the antibacterial properties of these compounds against MRSA.

The aim of the current study was to assess the overall in vitro bactericidal activity of nine newly synthesized diamides in dependence on time and concentration against clinical isolates of MRSA as representatives of multidrug-resistant bacteria. To the best of our knowledge, this is the first study dealing with the evaluation of novel microbiological characteristics of SAL analogues and revealing their bactericidal effect.

2. Materials and Methods

2.1. Synthesis of Compounds. The synthetic pathway of the series of novel diamides was described recently [13, 22], and their structures (see Table 1) were confirmed by IR, NMR, and MS spectrometry, and the purity of the compounds was checked by CHN analysis [13, 22].

2.2. Culture Media and Antibiotics. All media were prepared from dehydrated powders (Oxoid, Basingstoke, UK) according to manufacturer's instructions. Ampicillin (AMP), ciprofloxacin (CPX), and vancomycin (VAN) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions were prepared by dissolving the antibiotic in sterile deionized water [26].

2.3. Bacterial Strains. In vitro antibacterial activity of the synthesized compounds was evaluated against representatives of multidrug-resistant bacteria, three clinical isolates of MRSA: clinical isolate of animal origin MRSA 63718 (Department of Infectious Diseases and Microbiology, Faculty of Veterinary Medicine, University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic) carrying mecA gene; MRSA SA 630 [27]; and MRSA SA 3202 [27] (National Institute of Public Health, Prague, Czech Republic) both of human origin. Suspected colonies were confirmed by PCR; a 108bp fragment specific for S. aureus was detected [28]. All isolates were tested for the presence of the mecA gene encoding methicillin resistance [29]. These three clinical isolates were classified as vancomycin-susceptible (but with higher MIC of vancomycin equal to 2 μg/mL (VA2-MRSA) within the susceptible range for MRSA 63718) methicillin-resistant S. aureus (VS-MRSA). For the MICs of vancomycin, see Table 1. Vancomycin-susceptible methicillin-susceptible Staphylococcus aureus (VS-MSSA) ATCC 29213, obtained from the American Type Culture Collection, was used as the reference and quality control strain. The bacteria were stored at −80°C and were kept on blood agar plates (Columbia agar base with 5% ovine blood) between experiments.

2.4. Determination of Minimum Bactericidal Concentrations (MBCs). The MBCs (i.e., the lowest concentrations of antibacterial agents required to kill a particular bacterium) were determined by subculturing aliquots (20 μL) from wells with no visible bacterial growth and from control wells of MIC determination onto substance-free Mueller-Hinton agar (MHA) plates. The plates were incubated aerobically at 37°C for 24 h for colony count. The MBC was defined as the lowest concentration of substance, which produced ≥99.9% killing.
after 24 h of incubation as compared to the colony count of the starting inoculum [30]. To ensure reproducibility, each MBC assay was performed in at least triplicate on separate occasions.

2.5. Time-Kill Assays. Time-kill assays were performed by the broth macrodilution method according to previously described methodology [30] with some modifications. Briefly, flasks containing sterile fresh Mueller-Hinton broth (MHb) with the appropriate antimicrobial agent were inoculated with the test organism in logarithmic growth phase (MHB) with the appropriate antimicrobial agent were inoculated with the test organism in logarithmic growth phase. The plates were incubated at 37°C for 16 to 48 h, and the number of colonies was determined. To ensure reproducibility, each time-kill experiment was carried out in duplicate on separate occasions with results presented as the mean of all experiments. The growth control without the addition of antimicrobial agents and the control containing DMSO without any antimicrobial agent to exclude antibacterial activity of this solvent were included. Time-kill curves were constructed by plotting the log$_{10}$ CFU per millilitre versus time (over 24 h), and the change in bacterial concentration was determined. The results were analysed by evaluating the numbers of strains that yielded $\Delta$(log$_{10}$ CFU/mL) values of $-1$ (corresponding to 90% killing), $-2$ (99% killing), and $-3$ (99.9% killing) at 4, 6, 8, and 24 h compared to counts at 0 h. Bactericidal activity was defined as a reduction of at least 99.9% ($\geq 3$ log$_{10}$) of the total count of CFU/mL in the original inoculum.

3. Results and Discussion

Diamides seem to be promising candidates for antibacterial agents with very strong anti-MRSA activity, as it was published recently [13]. In the present study the series of nine newly synthesized diamides was evaluated as prospective bactericidal agents against representatives of multidrug-resistant bacteria, three clinical isolates of MRSA, and Staphylococcus aureus ATCC 29213 (methicillin-susceptible) as the reference and quality control strain. Since SALs and their analogues are known as compounds with bacteriostatic activity, each time-kill experiment was carried out in duplicate without any antimicrobial agent to exclude antibacterial activity of this solvent were included. Time-kill curves were constructed by plotting the log$_{10}$ CFU per millilitre versus time (over 24 h), and the change in bacterial concentration was determined. The results were analysed by evaluating the numbers of strains that yielded $\Delta$(log$_{10}$ CFU/mL) values of $-1$ (corresponding to 90% killing), $-2$ (99% killing), and $-3$ (99.9% killing) at 4, 6, 8, and 24 h compared to counts at 0 h. Bactericidal activity was defined as a reduction of at least 99.9% ($\geq 3$ log$_{10}$) of the total count of CFU/mL in the original inoculum.

### Table 1: Chemical structures and in vitro MIC and MBC [µg/mL] values of tested 5- and 4-chloro-2-hydroxy-N-[1-oxo-1-(phenylamino)alkan-2-yl]benzamides (bactericidal effect of individual compounds against particular strains marked in bold).

| Comp. | $R^1$ | $R^2$ | $R^3$ | MIC [µg/mL] | MBC [µg/mL] |
|-------|-------|-------|-------|-------------|-------------|
|      |       |       |       | 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4 | |
| 1a    | 5-Cl  | 4-CH$_3$ | (S)-CH$_3$ | $>256$ | $>256$ | $>256$ | $>256$ | $>256$ | $>256$ | $>256$ |
| 1b    | 5-Cl  | 4-CH$_3$ | (S)-CH(CH$_3$)$_2$ | $>256$ | $>256$ | $32$ | $32$ | $>256$ | $128$ | $>256$ |
| 1c    | 5-Cl  | 4-CH$_3$ | (S)-benzyl | $>256$ | $>256$ | $>256$ | $>256$ | $>256$ | $>256$ |
| 1d    | 5-Cl  | 4-CH$_3$ | (R)-CH$_2$-indolyl | $>256$ | $>256$ | $>256$ | $>256$ | $>256$ | $>256$ |
| 1e    | 5-Cl  | 4-OCH$_3$ | (S)-CH(CH$_3$)$_2$ | $>256$ | $>256$ | $>256$ | $>256$ | $>256$ | $>256$ |
| 1f    | 5-Cl  | 4-ClF$_3$ | (S)-CH(CH$_3$)$_2$ | 4 | 2 | 2 | 2 | 4 | 4 | 8 | 4 |
| 1g    | 4-Cl  | 4-Br | (S)-CH(CH$_3$)$_2$ | 8 | 4 | 4 | 4 | 16 | 8 | 8 | 8 |
| 1h    | 4-Cl  | 3,4-CI | (S)-CH(CH$_3$)$_2$ | 2 | 1 | 1 | 1 | 4 | 1 | 4 | 2 |
| 1i    | 4-Cl  | 3,4-Cl | (S)-benzyl | 1 | 1 | 0.5 | 0.5 | 8 | 1 | 8 | 1 |
| AMP   | —     | —     | —     | $>16$ | $>16$ | $>16$ | $0.25$ | $>16$ | $>16$ | $>16$ | $0.25$ |
| CPX   | —     | —     | —     | $>16$ | $>16$ | $>16$ | $0.5$ | $>16$ | $>16$ | $>16$ | $0.5$ |
| VAN   | —     | —     | —     | 2 | 1 | 1 | 1 | 2 | 1 | 1 | 1 |

Staphylococcal strains: 1: MRSA 63718; 2: MRSA SA 630; 3: MRSA SA 3202; 4: Staphylococcus aureus ATCC 29213. AMP: ampicillin; CPX: ciprofloxacin; VAN: vancomycin. MIC breakpoints for S. aureus ATCC 29213 [µg/mL]: AMP > 2, CPX > 1, VAN > 2 [23–25].
ciprofloxacin, and vancomycin. Potential bactericidal activity of diamides was assessed using MBC assay [26]. MBC values of all tested compounds are recorded in Table 1 as well.

Based on the obtained results, all compounds assessed as active according to MIC values in our previous study (If–i) showed low or moderate MBC values against all four strains. The MBC values of these compounds did not exceed the highest tested drug concentration and ranged from 1 to 16 μg/mL. In all cases, there were comparable MBC values for the clinical isolates of MRSA and the S. aureus reference strain.

Bactericidal activity is defined as a ratio of MBC to MIC of ≤4 [32]. Comparison of the MIC and MBC values of the discussed compounds for each isolate indicates that the effect of diamides was bactericidal for all active compounds. Compound 4-chloro-N’-(2S)-1-[(3,4-dichlorophenyl)amino]-1-oxo-3-phenylpropan-2-yl]-2-hydroxybenzamide (ii) with bacteriostatic effect against clinical isolates of MRSA 63718 and MRSA SA 3202 was the only exception from this rule. In Table 1 bactericidal activity is expressed in bold.

As mentioned above, SALs are known to exhibit a bacteriostatic effect [31], so it was very interesting to discover that diamides possess bactericidal activity. The amide bond (–CONH–) can cause interactions with a variety of enzymes [33]; therefore the presence of two amide bonds could be responsible for the bactericidal effect of diamides against MRSA. The activity of SALs and their analogues results from multiple mechanisms, which are still under investigation; for example, it was found that SALs are capable of inhibiting transglycosylases in later stages of S. aureus (including MRSA) cell wall biosynthesis [12]. These enzymes catalyse the step prior to the transpeptidation in the peptidoglycan biosynthesis and are responsible for polymerization of lipid II, which occurs at the outer face of the membrane [12]. Since antibacterial agents targeting cell wall biosynthesis act as bactericidal agents [30, 34], the failure in the cell wall biosynthesis due to the inhibition of transglycosylases could be responsible for bactericidal activity of diamides against MRSA.

Based on these findings, antibacterial active diamides with bactericidal effect against all four tested strains as prospective bactericidal agents were chosen for subsequent time-kill curve studies to determine the real dependence of bactericidal effect on concentration over time.

Compounds 5-chloro-2-hydroxy-N’-(2S)-3-methyl-1-oxo-1-[(4-(trifluoromethyl)-phenyl)amino]butan-2-yl]benzamide (If), N’-(2S)-1-[(4-bromophenyl)amino]-3-methyl-1-oxobutan-2-yl]-4-chloro-2-hydroxybenzamide (lg) and 4-chloro-N’-(2S)-1-[(3,4-dichlorophenyl)amino]-3-methyl-1-oxobutan-2-yl]-2-hydroxybenzamide (ih) were tested in time-kill studies at 1x, 2x, and 4x MIC against all MRSA isolates and the S. aureus reference strain. The antibacterial effect of DMSO [35] used as the solvent of the tested compounds was excluded in this assay, as time-kill curves of this solvent were identical or very similar to those of the growth control. The extent of bacterial killing was estimated by the number of these strains showing a decrease ranging from 1 to 3 log₁₀ CFU/mL in viable cell count at different times after incubation. A summary of these data is presented in Table 2. Based on these data it can be concluded that the bactericidal potency of tested diamides against all four strains decreased as follows: If > lh > lg. No bactericidal activity (i.e., ≥3 log₁₀ CFU/mL decrease) was observed at 1x MIC for any strain and time after incubation tested. At 4x MIC from the four strains, compounds If, lg, and lh killed 2, 1, and 2 strains, respectively, at 8h after incubation and 4, 2, and 2 strains, respectively, at 24h after incubation.

The findings of time-kill studies for each of the four staphylococci strains at exposure to compounds If, lg, and lh are summarized in Table 3. Bactericidal activity (i.e., ≥3 log₁₀ CFU/mL decrease) is expressed in bold.

For compound If rapid concentration-dependent antibacterial effect was recorded against clinical isolate of MRSA 63718. Time was not the predictive factor influencing the antibacterial activity because log₁₀ differences in CFU/mL from the starting inoculum were the same for 4x MIC (with the highest efficiency with a reduction in bacterial count of 5.30 log₁₀ CFU/mL) or very similar for 2x MIC (with a moderate regrowth after 24h causing a loss of bactericidal activity) over 24h. The bactericidal effect was maintained even at 2x MIC at 4h after incubation for this strain (reduction of 3.08 log₁₀ CFU/mL). For the remaining strains, clinical isolates of MRSA SA 630, MRSA SA 3202, and S. aureus ATCC 29213, reliable bactericidal effect was recorded at 4x MIC at 24h after incubation for all these strains with a reduction in bacterial count of 3.22, 3.30, and 3.65 log₁₀ CFU/mL, respectively.

For compound lg bactericidal effect against MRSA 63718 was noticed at 2x MIC at 6 and 8h after incubation and at 4x MIC at 4, 6, and 8h after incubation with a reduction in bacterial count ranging from 3.10 to 3.58 log₁₀ CFU/mL. The most effective killing was achieved at 6h for both concentrations. As in the case of compound If, a regrowth was observed after 24h after incubation. For the remaining isolates of MRSA, SA 630 and SA 3202, bactericidal effect occurred only at 4x MIC at 24h after incubation with a reduction in bacterial count of 3.38 and 4.01 log₁₀ CFU/mL, respectively. The highest bactericidal effect was recorded for MRSA SA 3202 at 4x MIC at 24h after incubation. A reduction consistent with bacteriostatic effect (0.03 to 2.37 log₁₀ CFU/mL) was observed at other concentrations over time for both isolates. No bactericidal effect was observed for the S. aureus reference strain; compound lg demonstrated a pattern of bacteriostatic activity against this strain with a reduction in bacterial count ranging from 0.07 to 2.33 log₁₀ CFU/mL at 4x MIC over time. In other cases, a slight increase in bacterial counts (i.e., overgrowth) compared with the starting inoculum was observed with values ranging from 0.10 to 1.57 log₁₀ CFU/mL for this reference strain.

For compound lh bactericidal effect against MRSA 63718 was maintained at 4x MIC at 6 and 8h after incubation with a reduction in bacterial count of 3.54 and 3.31 log₁₀ CFU/mL, respectively. The same as for lg, the most potent bactericidal effect was maintained at 6h after incubation. Regrowth at 24h after incubation causing a loss of bactericidal activity was recorded similarly as with previous compounds. The reason for regrowth of the test organism at 24h in the experiment is unknown. Most probably, selection of resistant mutants is responsible for this phenomenon [30]; degradation of the drug in the growth medium is not assumed, as regrowth was
not observed for any other tested strain. For MRSA SA 630 concentration-dependent killing was recorded at 4x MIC at 6, 8, and 24 h after incubation with log10 differences in CFU/mL from the starting inoculum being very similar over time (ranging from 3.18 to 3.39 log10 CFU/mL). For MRSA SA 3202 reliable bactericidal effect was maintained only at 4x MIC at 24 h after incubation with a reduction in bacterial count of 3.02 log10 CFU/mL. As for compound Ig, bacteriostatic activity against S. aureus reference strain was observed with a reduction in bacterial count ranging from 0.34 to 2.62 log10 CFU/mL at 2x and 4x MIC. Overgrowth (values ranging from 0.04 to 1.43 log10 CFU/mL) was recorded at 1x MIC for this strain.

It is of note that in all staphylococci strains with similar MICs and MBCs for compounds Ig and Ih the responsiveness to antibacterial activity of these compounds varied with clinical strains of MRSA being effectively killed and the reference strain remaining unaffected at 4x MIC.

There is a discrepancy between bactericidal results of MBC assay compared with time-kill kinetics. This difference could be caused by comparing microtiter (MBC assay) to macrobroth (time-kill assay) dilutions [36]. Moreover, although time-kill assays are more labour intensive and time consuming than MBC assays, they are recognised to provide a greater degree of characterisation of the cell eradication potential of antibacterial agents [37].

Concerning antibacterial effect, it is not generally important if the antibacterial agent is also bactericidal at higher concentrations, because the inhibition of bacterial proliferation usually achieves a therapeutic effect; the patient’s immune system is capable of coping with the infection then [34]. However, bactericidal therapy could produce a better treatment result by rapid reduction of the bacterial load [38]. Moreover, in the case of an immune system disorder (e.g., immunosuppressive therapy, AIDS patients, etc.) bactericidal agents are unequivocally indicated. Considering steadily escalating numbers of immunocompromised patients with endocarditis, meningitis, or osteomyelitis in recent years, it is necessary to achieve bacterial killing and broaden the spectrum of antimicrobial agents with bactericidal active compounds [30].

The clinical outcome of MRSA bacteraemia is significantly influenced by vancomycin MIC. Treatment failure exceeding 60% for S. aureus with vancomycin MIC of 4 μg/mL resulted in the change of susceptibility breakpoint from 4 μg/mL to 2 μg/mL by the Clinical and Laboratory Standards Institute (CLSI) in 2006 [23] as well as by the US Food and Drug Administration (FDA) in 2008 [39]. It has been recommended that for infections caused by MRSA strains with elevated vancomycin MICs (2 μg/mL), alternative therapy should be considered [40]. It is of note that based on time-kill assays in the present study, all tested diamides (particularly compound If exhibiting rapid bactericidal concentration-dependent effect even at 2x MIC) were most effective against isolate MRSA 63713, which is the strain with elevated vancomycin MIC of 2 μg/mL. The activity against the remaining isolates with vancomycin MIC of 1 μg/mL was lower.

Concerning the emergence of decreasing vancomycin susceptibility of MRSA isolates and thus the therapeutic efficacy of vancomycin therapy, our aim was to determine the potential bactericidal role of novel antibacterial compounds against MRSA in vitro. Based on the obtained results, diamides can be suitable candidates for such novel bactericidal active compounds presenting a promising starting point for further investigations to ascertain real in vivo activity and the exact mechanism of action.
Table 3: Change in viable counts (log_{10} CFU/mL) of MRSA and *S. aureus* strains following incubation for 24h with 5-chloro-2-hydroxy-N-[(2S)-3-methyl-1-oxo-1-[[4-(trifluoromethyl)-phenyl]amino]butan-2-yl]benzamide (If), N-([2S]-1-[[4-bromophenyl]amino]-3-methyl-1-oxobutan-2-yl]-4-chloro-2-hydroxybenzamide (Ig), and 4-chloro-N-[(2S)-1-[[3,4-dichlorophenyl]amino]-3-methyl-1-oxobutan-2-yl]-2-hydroxybenzamide (Ih) (bactericidal effect is expressed in bold).

| Strain  | MIC/MBC | Conc. | Log_{10} difference in CFU/mL from inoculum |
|---------|---------|-------|------------------------------------------|
|         |         | 4h    | 6h    | 8h    | 24h   |
| Comp. If |         |       |       |       |       |
| MRSA 63718 | 4/4   | 1× MIC | 0.34  | 0.56  | 0.66  | 1.68  |
|          |       | 2× MIC | −3.08 | −3.33 | −3.75 | −2.40 |
|          |       | 4× MIC | −5.30 | −5.30 | −5.30 | −2.40 |
| MRSA SA 630 | 2/4  | 1× MIC | 0.65  | 1.16  | 1.36  | 0.65  |
|          |       | 2× MIC | −0.26 | −0.77 | −1.40 | −2.07 |
|          |       | 4× MIC | −0.83 | −3.26 | −2.52 | −3.22 |
| MRSA SA 3202 | 2/8  | 1× MIC | 1.21  | 1.56  | 1.75  | 1.57  |
|          |       | 2× MIC | 0.06  | −0.05 | −0.65 | −1.59 |
|          |       | 4× MIC | −0.07 | −1.05 | −2.70 | −3.30 |
| S.a.    | 2/4    | 2× MIC | −0.25 | −0.74 | −1.52 | −2.52 |
|          |       | 4× MIC | −1.17 | −2.85 | −3.88 | −3.65 |
| Comp. Ig |         |       |       |       |       |
| MRSA 63718 | 8/16  | 1× MIC | 0.43  | 0.65  | 0.75  | 0.95  |
|          |       | 2× MIC | −2.54 | −3.23 | −3.15 | −0.76 |
|          |       | 4× MIC | −3.18 | −3.58 | −3.10 | −2.24 |
| MRSA SA 630 | 4/8   | 1× MIC | 0.98  | 1.42  | 1.57  | 0.55  |
|          |       | 2× MIC | −0.12 | −0.73 | −1.50 | −0.28 |
|          |       | 4× MIC | −1.00 | −1.54 | −2.37 | −3.38 |
| MRSA SA 3202 | 4/8  | 1× MIC | 0.56  | 1.47  | 1.70  | 2.14  |
|          |       | 2× MIC | −0.03 | −0.07 | −0.82 | −0.69 |
|          |       | 4× MIC | −0.35 | −0.56 | −1.49 | −4.01 |
| S.a.    | 4/8    | 2× MIC | 0.16  | 0.10  | 0.80  | −0.34 |
|          |       | 4× MIC | −0.07 | −0.11 | −1.65 | −2.33 |
| Comp. Ih |         |       |       |       |       |
| MRSA 63718 | 2/4   | 1× MIC | −0.76 | −1.09 | −0.71 | 0.89  |
|          |       | 2× MIC | −1.77 | −2.07 | −1.97 | 0.47  |
|          |       | 4× MIC | −2.90 | −3.54 | −3.31 | −2.65 |
| MRSA SA 630 | 1/1   | 2× MIC | 0.19  | −1.19 | −1.39 | −0.30 |
|          |       | 4× MIC | −2.72 | −3.21 | −3.39 | −3.18 |
| MRSA SA 3202 | 1/4  | 1× MIC | 0.27  | 0.82  | 0.96  | 1.12  |
|          |       | 2× MIC | 0.17  | −0.27 | −0.53 | −0.09 |
| S.a.    | 1/2    | 1× MIC | 0.27  | 0.06  | 0.00  | 1.43  |
|          |       | 2× MIC | 0.04  | −0.35 | −0.94 | −1.20 |

CFU: colony-forming units; Conc.: concentration (multiplicity of MIC).

* ≥3 log_{10} reduction in CFU implies a bactericidal effect.

* b < 3 log_{10} reduction in CFU implies a bacteriostatic effect.
4. Conclusions

The present study is the first evidence of bactericidal effect of SAL analogues. Compound 5-chloro-2-hydroxy-N-{[2S]-3-methyl-1-oxo-1-{[4-(trifluoromethyl)-phenyl]amino}butan-2-yl]benzamide (If) exhibiting remarkable rapid concentration-dependent bactericidal effect at 2x MIC at 4, 6, and 8 h (with a reduction in bacterial count ranging from 3.08 to 3.75 \log_{10} \text{CFU/mL}) and at 4x MIC at 4, 6, 8, and 24 h (5.30 \log_{10} \text{CFU/mL} reduction in bacterial count) after incubation against MRSA 63718 was the most potent agent. Reliable bactericidal effect against other strains was maintained at 4x MIC at 24 h. For compounds N-{[2S]-1-{[(4-bromophenyl)amino]-3-methyl-1-oxobutan-2-yl]-4-chloro-2-hydroxybenzamide (Ig) and 4-chloro-N-{[2S]-1-{[(3,4-dichlorophenyl)amino]-3-methyl-1-oxobutan-2-yl]-2-hydroxybenzamide (Ih), a pattern of bacteriostatic effect was observed for \textit{S. aureus} ATCC 29213, and the most potent bactericidal effect against MRSA 63718 was recorded at 4x MIC at 6 h after incubation for both compounds. Against other strains, reliable bactericidal effect was maintained at 4x MIC at 24 h after incubation. Considering the necessity to broaden the spectrum of bactericidal agents, diamides from the current study with a novel mechanism of action could present a very promising and interesting solution to this challenge for the future.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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