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In vitro activity of salicylamide derivatives against vancomycin-resistant enterococci

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ABSTRACT

A series of 13 salicylamide derivatives was assessed for antibacterial activity against three isolates of vancomycin-resistant *Enterococcus faecalis* (VRE) and *Enterococcus faecalis* ATCC 29212 as a quality standard. The minimum inhibitory concentration was determined by the broth microdilution method with subsequent subcultivation of aliquots to assess minimum bactericidal concentration. The growth kinetics was established by the time-kill assay. Ampicillin, ciprofloxacin, tetracycline and vancomycin were used as the reference antibacterial drugs. Three of the investigated compounds showed strong bacteriostatic activity against VRE (0.199–25 μM) comparable to or more potent than ampicillin and ciprofloxacin. In addition, these compounds were tested for synergistic effect with vancomycin, ciprofloxacin and tetracycline, while 5-chloro-2-hydroxy-*N*-[4-(trifluoromethyl)phenyl]benzamide showed the highest potency as well as synergistic activity with vancomycin against VRE 368. Screening of the cytotoxicity of the most effective compounds was performed using human monocytic leukemia THP-1 cells, and based on LD_{50} values, it can be stated that the compounds have insignificant toxicity against human cells.

Enterococcus is the 3rd most common nosocomial pathogen.[1] The role of enterococci in nosocomial infection increases, because they are still more resistant to clinically used antibacterial drugs.[2,3] Vancomycin-resistant *Enterococcus* (VRE) was first isolated in France, UK and USA at the end of the 1980s.[4,5] Prevalence of VRE in the world is very heterogeneous; e.g., it has tripled in Canadian hospitals between 2007 and 2013 from 1.8 to 6%.[6] The incidence of hospitalizations with infections caused by VRE increased from 4.60 to 9.48 hospitalizations per 100.000 population in the USA from 2000 to 2006.[7] On the other hand, the prevalence of VRE in Europe is not so high in general, but it is different between countries. Data from the European Antimicrobial Resistance Surveillance Network showed that the highest prevalence of VRE in Europe is in Ireland (44.0%), Portugal (23.3%), Greece (17.1%) and Germany (16.2%). On the other hand, Denmark, the Netherlands and France had very low number of VRE (0–2%).[8] Nevertheless, the increasing number of VRE is problematic not only due to complicated treatment, especially of immunocompromised patient,[9] or higher risk of mortality,[10–13] but it is also an economic problem.[14]

Problems with increasing resistance could be solved by developing new antibacterial drugs or using combinations of newly synthetized compounds with convenient antibacterial agents. Salicylamide derivatives/analogues seem to be one of the most promising groups. This group of anti-infectious agents is known by its wide range of pharmacological effects, such as antiviral,[15] antibacterial/antimycobacterial,[16–28] antifungal,[8,18] antiparasitic,[29] anthelminthic[30] and in addition, noteworthy photosynthesis-inhibiting activity.[23–26,31–34] Thus, salicylamide-based compounds can be considered as typical multi-target compounds, for which it is difficult to propose the exact mechanism of action responsible for overall biological activities. They are able to inhibit the two-component regulatory systems of bacteria, inhibit bacterial

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transglycosylase, sortase A, D-alanine–D-alanine ligase, mycobacterial isocitrate lyase and methionine aminopeptidase; they serve as uncouplers of oxidative phosphorylation, inhibitors of autophosphorylation of the KinA kinase in bacteria, inhibitors of protein kinase epidermal growth factor receptor and also as selective inhibitors of interleukin-12p40 production.[17,20,21,35]

This study is a follow-up paper to recently published results.[19,20,22] The investigated compounds, see Scheme 1 and Table 1, can be divided into three groups: starting salicylanilides-2-hydroxy-*N*-phenylbenzamides **1a–e** (group A), protected salicylanilide amino acid esters – 2-(phenylcarbamoyl)phenyl *N*-[(benzyloxy)carbonyl]-L-valinates **2a–e** (group B) and final "diamides" 2-hydroxy-*N*-[(2*S*)-3-methyl-1-oxo-1-(phenylamino) butan-2-yl]benzamides **3a–c** (group C). The synthesis and characterization of these salicylamide derivatives was described previously.[19] All the compounds were tested for their activity against vancomycin-susceptible *E. faecalis* ATCC 29212 as a reference strain and three isolates from American crows of *vanA*-carrying vancomycin-resistant *E. faecalis*.[36] Moreover, active compounds were tested to their ability to cause synergy with clinically used drugs such as vancomycin, ciprofloxacin and tetracycline. The activities of compounds, see Table 1, were expressed as minimal inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs) and were determined according to the CLSI[37] with some modification.[20] The time-kill assay was used for evaluation of bactericidal activity identified by subcultivation of the aliquots.[20] The investigation of synergistic activity was performed according to the methodology.[38] The method of fractional inhibitory concentration (FIC) was used.[39]

For all the wells of the microtitration plates that corresponded to a MIC value, the sum of the FICs ($\sum$FIC) was calculated for each well, using the equation $\sum FIC = FIC_A + FIC_B = (C_A/MIC_A) + (C_B/MIC_B)$, where MIC_A and MIC_B are the MICs of drugs A and B alone, respectively, and C_A and C_B are the concentrations of the drugs in combination, respectively.[39,40] Synergy was defined as a $\sum FIC \leq 0.5$; additivity was defined as $0.5 < \sum FIC < 1$; indifference was defined as $1 < \sum FIC < 4$; and antagonism was defined as $\sum FIC > 4$.[38] As the FIC index was evaluated for every single wall corresponding to the MIC value, the results are presented as a range. Only the MIC values of vancomycin and tetracycline against resistant strains were determined, because only these were needed for tests of synergism.

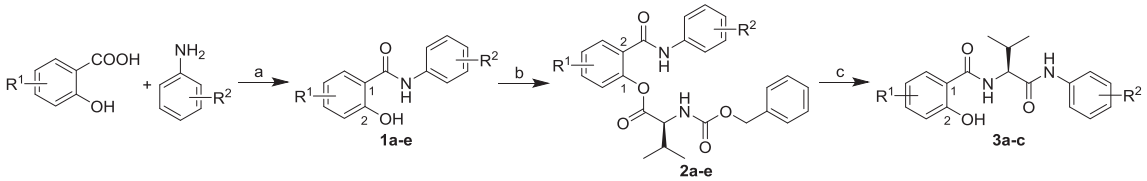
Based on the results, see Table 1, it is evident that the compounds of group A expressed the highest potency among all the investigated compounds. Compounds **1a–c** showed strong bacteriostatic activity against VRE; even they were more potent than the used standards – ampicillin and ciprofloxacin. On the other hand, salicylanilide esters (group B) showed antibacterial activities against neither VRE nor vancomycin-susceptible strain *E. faecalis*, although in a previous study, these compounds demonstrated only little weaker antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) than salicylanilides of group A.[19] Within group C, only compound **3a** showed bactericidal effect against vancomycin-susceptible strain *E. faecalis*. No antibacterial activity against VRE was observed.

All the compounds were substituted by R^1 substituent in the *para*- or *meta*-position of the salicylic ring, while chlorine is preferred for high potency. Replacing chlorine in the salicylic ring by a nitro moiety caused the loss of antibacterial activity, as it is shown in case of compound **1d**. It is more advantageous to substitute the anilide ring by a lipophilic and electron-withdrawing group, such as CF_3 (compound **1a**) or 3,4-Cl (compound **1c**), rather than only by an electron-withdrawing nitro moiety (compound **1b**). On the other hand, already the substitution by only an electron-withdrawing moiety significantly increased potency against all three VRE strains; compare compound **1b** (R^2 = 4-NO_2) and compound **1e** (R^2 = H). The activity is negatively influenced by the substitution of the anilide ring by electron-donating moieties.

4-Chloro-*N*-(3,4-dichlorophenyl)-2-hydroxybenzamide (**1c**) was the most active compound against VRE. Only this compound is substituted by Cl in position $C_{(4)}$ of the salicylic ring. The rest of compounds (except **1d**) are substituted by Cl in position $C_{(5)}$. Similar results of antimicrobial investigations of activity against different bacteria were reported also by Pauk et al.[19]

It was surprising that, based on the MICs values, bactericidal activity was found only for derivative 5-chloro-2-hydroxy-*N*-(4-nitrophenyl)benzamide (**1b**), the least effective compound among three active derivatives **1a–c** of group A. The time-kill curve assay was used for testing the bactericidal effect of the compounds, which indicated the possibility of bactericidal activity in the pre-test subcultivation aliquots on agar. If in this test MBC was $\leq 4 \times$ MIC, a compound was considered as potentially bactericidal and consequently tested using the time-kill curve assay.[38,41] Unfortunately, despite compound **1b** demonstrated bactericidal activity against VRE 342B and VRE 725B when the method of subcultivation aliquot on agar was used, the subsequent time-kill curve assay showed only bacteriostatic effect, see Fig. 1A and 1B. The same situation was discovered with MRSA in the previous study.[20] It could be caused by the difference between microtiter broth dilution and broth macrodilution.[20]

In addition, synergistic activity with ciprofloxacin, tetracycline and vancomycin was tested for three most active derivatives **1a–c**, see Table 2. No antagonism was detected. Synergistic effect of ciprofloxacin was tested only against isolate VRE 725B, because other isolates were ciprofloxacin-susceptible.[37] The additivity of combinations with compounds **1a–c** was investigated. Combinations of tetracycline showed only additive effect with **1b** against VRE 342B and VRE 368 and with **1a** against VRE 368. The best result was demonstrated by the combination of vancomycin and **1a**. This combination had synergistic effect against VRE 368 with $\sum$FIC = 0.375–1.064 and additive effect against VRE 342B with low $\sum$FIC = 0.750–1.250. Vancomycin also showed additivity



Scheme 1. Synthesis of 2-hydroxy-*N*-phenylbenzamides **1a–e** (group A), 2-(phenylcarbamoyl)phenyl *N*-[(benzyloxy)carbonyl]-L-valinates **2a–e** (group B) and 2-hydroxy-*N*-[(2*S*)-3-methyl-1-oxo-1-(phenylamino)butan-2-yl]benzamides **3a–c** (group C). Individual R^1 and R^2 substituents are mentioned in Table 1. Reagents and conditions: (a) PCl_3, chlorobenzene, microwave irradiation; (b) *N*,*N*-dicyclohexylcarbodiimide, DMF; (c) HBr/AcOH, triethylamine, $CHCl_3$.[19]

Table 1
Discussed 2-hydroxy-*N*-phenylbenzamides **1a–e** (group A), 2-(phenylcarbamoyl)phenyl *N*-[(benzyloxy)carbonyl]-L-valinates **2a–e** (group B) and 2-hydroxy-*N*-[(2*S*)-3-methyl-1-oxo-1-(phenylamino)butan-2-yl]benzamides **3a–c** (group C), *in vitro* antibacterial activity (MIC, MBC) of compounds and *in vitro* cytotoxicity assay (LD_{50}) of chosen compounds compared to ampicillin (AMP), ciprofloxacin (CPX), vancomycin (VAN), tetracycline (TET) and camptothecin (CMT) standards. ND = not determined. * = MIC values used for calculation of selectivity index (SI).

Comp.	R^1	R^2	[μg/ml] ([μM])						[μM]	SI (LD_{50}/ MIC*)
			E. faecalis ATCC 29212		VRE 342B		VRE 368	VRE 725B	THP-1	
			MIC	MBC	MIC	MBC	MIC	MIC	MBC	LD_{50}	
1a	5-Cl	4-CF_3	0.063 (0.199)	2 (6.34)	0.125 (0.396)*	8 (25.34)	1 (3.16)	0.25 (0.792)	8 (25.34)	39.66 ± 0.77	100
1b	5-Cl	4-NO_2	0.25 (0.850)	2 (6.83)	0.5 (1.710)*	2 (6.83)	4 (13.67)	1 (3.42)	2 (6.83)	23.95 ± 0.64	14
1c	4-Cl	3,4-Cl	0.125 (0.395)	0.5 (1.579)	0.063 (0.199)*	8 (25.27)	0.25 (0.790)	0.063 (0.199)	8 (25.27)	25.56 ± 0.21	128
1d	5-NO_2	4-Cl	>256 (>874)	>256 (>874)	>256 (>874)	>256 (>874)	>256 (>874)	>256 (>874)	>256 (>874)	>40	–
1e	5-Cl	H	0.125 (0.50)	0.25 (1.01)	16 (64.60)	>256 (>1033)	32 (129)	256 (1033)	32 (129)	128 (516)	>40	–
2a	4-Cl	4-CF_3	>256 (>466)	>256 (>466)	>256 (>466)	>256 (>466)	>256 (>466)	>256 (>466)	>256 (>466)	>256 (>466)	ND	–
2b	4-Cl	4-NO_2	>256 (>486)	>256 (>486)	>256 (>486)	>256 (>486)	>256 (>486)	>256 (>486)	>256 (>486)	>256 (>486)	ND	–
2c	5-Cl	3,4-Cl	>256 (>465)	>256 (>465)	>256 (>465)	>256 (>465)	>256 (>465)	>256 (>465)	>256 (>465)	>256 (>465)	ND	–
2d	4-NO_2	4-Cl	>256 (>466)	>256 (>466)	>256 (>466)	>256 (>466)	>256 (>466)	>256 (>466)	>256 (>466)	>256 (>466)	ND	–
2e	4-Cl	4-OCH_3	>256 (>499)	>256 (>499)	>256 (>499)	>256 (>499)	>256 (>499)	>256 (>499)	>256 (>499)	>256 (>499)	ND	–
3a	5-Cl	4-CF_3	8 (19.29)	8 (19.29)	>256 (>617)	>256 (>617)	>256 (>617)	>256 (>617)	>256 (>617)	>256 (>617)	ND	–
3b	5-Cl	4-CH_3	>256 (>709)	>256 (>709)	>256 (>709)	>256 (>709)	>256 (>709)	>256 (>709)	>256 (>709)	>256 (>709)	ND	–
3c	4-Cl	4-Br	>256 (>601)	>256 (>601)	>256 (>601)	>256 (>601)	>256 (>601)	>256 (>601)	>256 (>601)	>256 (>601)	ND	–
AMP	–	–	4 (11.45)	1 (3.02)	4 (11.45)	1 (3.02)	4 (11.45)	2 (5.72)	2 (5.72)	ND	–
CPX	–	–	1 (3.02)	1 (3.02)	1 (3.02)	1 (3.02)	1 (3.02)	64 (193)	64 (193)	ND	–
VAN	–	–	ND	ND	512 (353)	ND	512 (353)	ND	1024 (706)	ND	–
TET	–	–	ND	ND	64 (144)	ND	64 (144)	ND	64 (144)	ND	–
CMT	–	–	–	–	–	–	–	–	–	0.161 ± 0.071	–

with other salicylanilides, specifically with **1b** against VRE 368 and with **1a** against VRE 342B. It is important to note that synergistic effect with ampicillin was not tested, because all strains are susceptible to ampicillin.[36]

The cytotoxicity of chosen compounds **1a–e** was determined using a LDH assay kit as described previously.[42] For the discussed compounds, the cytotoxicity was evaluated as the LD_{50} value (LD_{50} – lethal dose causing death in at least 50% of the cell population) using the human monocytic leukaemia THP-1 cells. The toxicity values of the tested compounds were compared with camptothecin (LD_{50} = 0.161 ± 0.071 μM) as a standard. Selectivity indexes were calculated as a rate of LD_{50} and MIC [μM] values of VRE 342B as a representative of resistant isolates. Results are shown in Table 1. Based on these observations, it can be concluded that all tested compounds **1a–e** can be considered as insignificant toxic agents for mammalian cells in subsequent design of novel therapeutic agents.

In the previous study[20], diamides **3a** and **3c** showed strong bactericidal activity against MRSA. In general, the antibacterial activity of salicylanilides is predominantly bacteriostatic; nevertheless, it can be hypothesized that the bactericidal activity of both diamides is caused by the presence of two amide bonds. In fact the whole structure of these diamides mimics structural features of "three-cycles salicylanilides" that were described by Cheng et al. as agents able to inhibit transglycosylation in cell wall synthesis of *S. aureus*.[17] As the inhibition of cell wall biosynthesis is characteristic for bactericidal agents, it is possible that the inhibition of transglycosylation is responsible for the bactericidal activity of salicylic diamides against MRSA.[14,20]

The difference in activity against MRSA and VRE can be explained just by the resistance to vancomycin. Vancomycin is a glycopeptide antibiotic that inhibits transglycosylation. Enterococci carrying gen *vanA* synthetize D-ala-D-lac instead of D-ala-D-ala, to which vancomycin has 100× lower affinity. All VRE strains in this study carried gen *vanA*, and their MICs for vancomycin were 512–2046 μg/ml, although MRSA strains used in the previous study[19,20] were vancomycin-susceptible. It is possible that salicylic diamides interact with a step of the enzymatic reaction participating in the synthesis of peptidoglycan, and their interaction with this target is disabled by the presence of *vanA* in VRE. This theory could be supported by the activity of salicylic diamides against the reference vancomycin-susceptible strain *E. faecalis* ATCC 29212.

The difference between the activities of compounds **3a** and **3c** against *E. faecalis* could be caused by the different position of Cl on the salicylic ring and the presence of the CF_3 group on the anilide ring of compound **3a**. Concerning antimicrobial activity, it was observed that the substitution of $C_{(4)}'$ by the CF_3 moiety is preferable to that by Br.[16,18–20,22–29] Simultaneously it can be expected that the mechanism of action of salicylanilides is different from that of salicylic diamides, because salicylanilides were active against VRE.

Summarizing, it can be concluded that tested compounds 4-chloro-*N*-(3,4-dichlorophenyl)-2-hydroxybenzamide (**1c**), 5-chloro-2-hydroxy-*N*-[4-(trifluoromethyl)phenyl]benzamide (**1a**) and 5-chloro-2-hydroxy-*N*-(4-nitrophenyl)benzamide (**1b**) showed strong bacteriostatic activity against VRE, and at the same time, compound **1a** showed synergistic effect with vancomycin against VRE 368. The anti-VRE activity significantly depends on compound lipophilicity and strong electron-withdrawing properties of the substituent on the anilide ring. Moreover, the most potent compounds can be considered as insignificant toxic agents. Based on the obtained results and the previous study, it can be stated that salicylanilides and their derivatives are promising candidates for the study of novel antibacterial drugs against multiresistant strains.

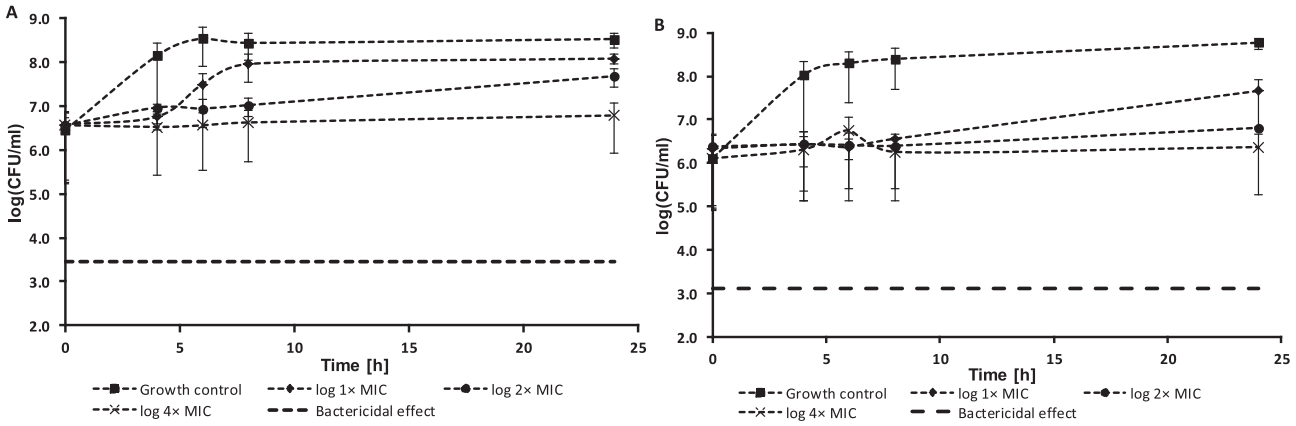


Fig. 1. Time-kill curve of compound **1b** against VRE 342B (**A**) and against VRE 725B (**B**).

Table 2
Combined effect of most potent salicylamide derivatives and ciprofloxacin (CPX), tetracycline (TET) and vancomycin (VAN).

Isolate	Combination of compounds	Separate MIC [μg/ml]	FIC index	Concentration [μg/ml] causing synergistic effect	Concentration [μg/ml] causing additive effect
VRE 725B	**1a**/CPX	0.25/128	0.532–1.125	–	0.125/32 0.008/64
	1b/CPX	1.00/64	1.000–1.250	–	–
	1c/CPX	0.125/64	0.756–1.250	–	0.032/32
VRE 342B	**1a**/TET	0.5/64	1.008–1.5	–	–
	1b/TET	1.00/64	0.75–1.25	–	0.5/16
	1c/TET	0.063/64	1.016–1.508	–	–
VRE 368	**1a**/TET	0.5/64	0.625–1.25	–	0.25/8
	1b/TET	2.00/64	0.75–1.25	–	1.00/16
	1c/TET	0.125/64	1.016–1.504	–	–
VRE 725B	**1a**/TET	0.5/64	1.032–1.500	–	–
	1b/TET	1.00/64	1.032–2.125	–	–
	1c/TET	0.125/64	1.016–1.504	–	–
VRE 342B	**1a**/VAN	1.00/1024	0.750–1.250	–	0.5/256
	1b/VAN	1.00/512	1.00–2.250	–	–
	1c/VAN	0.125/512	0.629–1.128	–	0.063/64 0.032/256
VRE 368	**1a**/VAN	0.5/1024	0.375–1.064	0.125/128	0.25/32 0.063/512
	1b/VAN	2/512	0.625–1.250	–	0.25/256
	1c/VAN	0.5/512	1.00–1.25	–	–
VRE 725B	**1a**/VAN	0.25/1024	1.000–1.250	–	–
	1b/VAN	1.00/1024	1.063–1.500	–	–
	1c/VAN	0.063/1024	1.031–2.234	–	–

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