SAPONINS INCREASE SUSCEPTIBILITY OF VANCOMYCIN-RESISTANT ENTEROCOCCI TO ANTIBIOTIC COMPOUNDS

Sebastian Schmidt^{1,*}, Markus M. Heimesaat², André Fischer², Stefan Bereswill^{2,**} and Matthias F. Melzig^{1,**}

¹Institute of Pharmacy, Freie Universität Berlin, Königin-Luise-Str. 2+4, 14195 Berlin, Germany

²Institute of Microbiology and Hygiene, Charité – University Medicine Berlin, Hindenburgdamm 27, 12203 Berlin, Germany

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The resistance of commensal bacteria to first and second line antibiotics has reached an alarming level in many parts of the world and endangers the effective treatment of infectious diseases. In this study, the influence of the plant-derived natural saponins glycyrrhizic acid, β -aescin, α -hederin, hederacoside C, and primulic acid 1 on the susceptibility of vancomycin-resistant enterococci (VRE) against antibiotics of clinical relevance was investigated in 20 clinical isolates. Furthermore, the antibacterial properties of saponins under study against VRE were determined *in vitro*. Results reveal that the susceptibility of VRE against gentamicin, teicoplanin, and daptomycin was enhanced in the presence of the saponin glycyrrhizic acid. Most importantly, glycyrrhizic acid (1 mg/ml) diminished the minimal inhibitory concentration (MIC) of gentamicin in gentamicin low-level intrinsic resistant VRE from 2–>8 mg/l to ≤ 0.125 –1 mg/l. The adding of β -aescin, α -hederin, hederacoside C, and primulic acid 1 to the antibiotics under study showed, compared to glycyrrhizic acid, less influence on the antibiotic potency. Only glycyrrhizic acid (1 mg/ml) and α -hederin (0.2 mg/ml) showed weak antibacterial properties against the clinical isolates. Our study points towards a therapeutic potential of saponins in the coapplication with antibiotics for bacterial infections.

Keywords: glycyrrhizic acid, vancomycin-resistant enterococci, combination effect, saponins, gentamicin, teicoplanin, daptomycin

Introduction

The effective treatment of infectious diseases is an important achievement of modern medicine. Since the introduction of antibiotic drugs into therapy, they have been regarded as the panacea to cure infections. However, the emergence of antimicrobial resistance (AMR) including multidrug resistance (MDR) endangers the treatment of infectious diseases and shows the possibility of a post-antibiotic era in which common infections and minor injuries can cause death [1].

Antibiotic resistance of bacteria is the result of a normal evolutionary process, so that the introduction of each antimicrobial drug has been followed by the detection of resistance against it as well. The expansion of AMR is accelerated by the selective pressure exerted by widespread use and misuse of antimicrobial drugs in both humans and food-producing animals. AMR is a complex global public health challenge that leads to prolonged illness and increased mortality, increases the costs for the health-care

sector, and has an impact on animal health, which probably leads to an effect on food production [1].

The antimicrobial resistance to environmental bacteria has reached alarming levels in many parts of the world [1]. In particular, bacterial species subsumed as “ESKAPE”-pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) give cause for serious concerns. They are actively involved in the generation and global dissemination of antimicrobial resistance mechanisms [2] and effectively escape effective treatment by the elimination, modification, or degradation of antibacterial drugs [3].

Enterococci are gram-positive, facultative anaerobic bacteria which represent the second to third most common cause of nosocomial infections [4]. As an opportunistic pathogen, they are part of the commensal flora. Wound infections, urinary tract infections, bloodstream infections, and endocarditis caused by enterococci occur frequently in immunocompromised patients [4]. The epidemiological

* Corresponding author: Sebastian Schmidt; Sebastian Schmidt; Königin-Luise-Str. 2+4, 14195 Berlin, Germany; Phone: +49 30 83851451; Fax: +49 30 83851461; E-mail: sebschmidt@zedat.fu-berlin.de

**Stefan Bereswill and Matthias F. Melzig contributed equally.

risk factors associated with enterococci are based on three properties of the bacteria: (1) they colonize the gut and the skin, similar to *Enterobacteriaceae* or methicillin-resistant *S. aureus*, (2) they exhibit an extraordinary environmental persistence like *Clostridium difficile* [5], and (3) all enterococci display intrinsic resistance against cephalosporins and semisynthetic penicillins, as well as aminoglycoside antibiotics [4].

Vancomycin-resistant enterococci (VRE), mostly *E. faecium*, emerged during the late 1980s [6]. Currently, the population-weighted mean percentage of vancomycin resistance in the European Union (EU) is 8.1% (2012) [7]. There are different types of vancomycin-resistance mechanisms in enterococci. VanA-type and VanB-type are the ones with the most clinical relevance. VanA-type exhibits a cross resistance against vancomycin and teicoplanin, while the VanB-type is teicoplanin susceptible [4]. Nearly all VRE are resistant to ampicillin, and some strains exhibit a high-level resistance against aminoglycosides [8]. The currently used antibiotics for VRE-related infections are linezolid, daptomycin, and tigecyclin, but data that address their efficacy are rare and the tolerability remains problematic [3]. Resistance against these last-line antibiotics occurred only at rare intervals, but do exist [9–13].

Saponins are glycosides with a hydrophilic sugar residue linked with a lipophilic triterpene, steroid, or steroidal alkaloid. The amphoteric qualities resulting from it are responsible for many effects of the saponins. In the plant kingdom, they are widespread and can be found in several plants in concentrations up to 30% [14]. On account of their antibacterial and antifungal effects, saponins presumably serve the plants for defence against infections [15, 16]. The fact that saponins can enhance the susceptibility of some bacteria against certain antibiotics *in vitro* and *in vivo* was observed in many studies. The combination of ginsenoside and kanamycin or cefotaxim enhanced the activity towards methicillin-resistant *Staphylococcus aureus* (MRSA) [17], with ampicillin, kanamycin, oxytetracycline, or chloramphenicol towards *Bacillus subtilis* and with ampicillin and cephalixin towards *S. aureus* [18]. The saponin of *Quillaja saponaria* enhanced the sensitivity of the Gram-negative bacteria *Proteus mirabilis* towards colistin [19]. Synergy between vancomycin or daptomycin and the synthetic saponin diosgenyl 2-amino-2-deoxy-D-glucopyranoside hydrochloride (HSM-1) was *in vitro* demonstrated on *S. aureus*, *Enterococcus faecalis*, *Rhodococcus equi*, and *Streptococcus pyogenes*. In a murine wound model induced by *S. aureus* or *E. faecalis* infected BALB/c mice, the coapplication of local saponin (HSM-1) and intraperitoneal treatment with vancomycin or daptomycin resulted in a higher reduction of bacteria in infected BALB/c mice as compared to the respective antibiotic treatment alone [20].

In this study, the influence of the plant-derived triterpenoid saponins glycyrrhizic acid, β -aescin, α -hederin, hederacoside C, and primulic acid 1 on the antibiotic sensitivity of vancomycin-resistant *E. faecium* was evaluated with a microbroth dilution method. We used 20 clinical

VRE isolates to estimate the combination effects of the saponins under study with the antibiotic compounds gentamicin, teicoplanin, daptomycin, vancomycin, fosfomycin, trimethoprim–sulfamethoxazole, ampicillin, flucloxacillin, linezolid, tigecyclin, penicillin, moxifloxacin, erythromycin, clindamycin, and rifampicin.

Materials and methods

Bacterial strains

We used 20 vancomycin-resistant *E. faecium* strains which were isolated from clinical stool samples in the Department of Microbiology and Hygiene at Charité — University Medicine Berlin. All strains were maintained in cryobanks (Mast Diagnostica™) as stocks at -80°C .

Chemicals

The following drugs and media from the indicated commercial sources were used: ampicillin (Aldrich), gentamicin (Serva), moxifloxacin (Health Care AG), trimethoprim–sulfamethoxazole (Roche Diagnostics GmbH), tigecyclin (Wyeth), penicillin (Grünenthal GmbH), erythromycin (Serva), clindamycin (Pfizer), teicoplanin (sanofi-aventis), vancomycin (Cell Pharm), fosfomycin (InfectoPharm), rifampicin (Grünenthal GmbH), linezolid (Pfizer), mupirocin (SmithKline Beecham), Mueller-Hinton II broth (Becton, Dickinson and Company), glycyrrhizic acid (Roth), β -aescin (Merck), α -hederin (Roth), hederacoside C (Roth), and primulic acid 1 (WKGP Melzig).

Minimal inhibitory concentration determination

The combinatory effects of antibiotics and saponins were determined with a microdilution method which is used for the standardized determination of the minimal inhibitory concentrations (MICs) of antimicrobial compound in routine laboratories. Therefore, defined concentrations of saponins were added to serial dilutions of antibiotics prepared in 96-well microplates to a volume of 200 μL . Antibiotics and saponins were dissolved in cation-adjusted Mueller-Hinton broth. The concentrations of saponins ranged from 0.2 to 1 mg/ml and, for the antibiotics, from 0.0075 to 250 mg/l. The prepared microplates with antibiotic test concentrations were stored at -20°C for a maximum of 4 months. The inoculum contained 2×10^6 colony forming units (CFU)/ml of bacteria, and the microplates were aerobically incubated at 37°C for 24 h. The MIC was rated as the lowest concentration of the antibiotic compound that inhibited the growth of bacteria, which was detected by reading the absorbance at 620 nm. A similar technique was used to determine the antibacterial effect of saponins. Here, the influence on the growth of bacteria was determined by the number of bacteria or the absor-

bance and compared to the growth in media. Ampicillin (10 mg/ml) and peracetic acid 0.4% (v/v) were used as positive controls.

Results

Antibacterial properties of saponins

We determined the antibacterial potency of the saponins under study against 18 clinical isolates of vancomycin-resistant *E. faecium* via a microdilution method. Glycyrrhizic acid, β -aescin, and hederacoside C were tested in concentrations of 1 mg/ml and, in addition, β -aescin in concentrations of 0.5 mg/ml. We chose a saponin concentration of 1 mg/ml as the highest concentration we used in the combination studies to preclude that changes in the MIC are only based on antibacterial effects from the saponins alone. Due to problems with the solubility, we have tested α -hederin and primulic acid 1 in concentrations of 0.2 mg/ml. The number of assays of the saponin concentrations is shown in Table 1. In concentrations of 1 mg/ml, β -aescin and hederacoside C showed no antibacterial activity against *E. faecium*, likewise β -aescin in con-

centrations of 0.5 mg/ml and primulic acid 1 in 0.2 mg/ml. However, glycyrrhizic acid (1 mg/ml) and α -hederin (0.2 mg/ml) displayed a weak antibacterial effect on the clinical isolates. The influence of the saponins on cultures of *E. faecium* is presented in Fig. 1.

Synergistic effects of saponins with antibiotics

The combination effect of antibiotics and saponins were assessed by the change of the MIC of the antibiotic in the presence of glycyrrhizic acid, β -aescin, α -hederin, hederacoside C, and primulic acid 1 at concentrations of 0.2 mg/ml to 1 mg/ml. First, we determined the susceptibility of the 20 clinical isolates against the antibiotics under study. The clinical isolates of VRE were resistant to ampicillin, trimethoprim-sulfamethoxazole, and vancomycin. They showed a low-level intrinsic resistance against gentamicin, three of them even a high-level resistance. Seven isolates were resistant against teicoplanin, probably from the VRE Van-A type. The isolates were susceptible against linezolid, tigecyclin (MIC: ≤ 0.0625 mg/l to 0.125 mg/l) and showed a MIC of mupirocin of ≤ 1 mg/l. In Tables 2–5, the MIC of the antibiotics under study without saponin is shown. Furthermore, the clinical breakpoints for phenotypic antimicrobial susceptibility testing of the used antibiotics against *Enterococcus* spp. are shown in Table 6. In combination with glycyrrhizic acid and β -aescin, only gentamicin, teicoplanin, daptomycin, vancomycin, fosfomycin, and trimethoprim-sulfamethoxazole showed changes in their MIC (Tables 2 and 3). In the presence of α -hederin, hederacoside C, and primulic acid 1, no change in the MIC of the tested antibiotics occurred (Tables 2–5). The following antibiotics displayed no changes in the MIC in combination with the saponins under study: ampicillin, flucloxacillin, linezolid, tigecyclin, penicillin, moxifloxacin, erythromycin, clindamycin, and rifampicin (Tables 4

Table 1. Number of assays (*n*) of the saponin concentrations in Fig. 1

Saponin concentrations	<i>n</i>
Glycyrrhizic acid 1 mg/ml	27
β -Aescin 1 mg/ml	21
β -Aescin 0.5 mg/ml	8
α -Hederin 0.2 mg/ml	9
Hederacoside C 1 mg/ml	6
Primulic acid 1 0.2 mg/ml	8

Fig. 1. Effect of some saponins in different concentrations on the growth of 18 clinical isolates with vancomycin-resistant *Enterococcus faecium* (VRE) ($\pm$ range)

Table 2. Influence of glycyrrhizic acid, β -aescin, α -hederin, hederacoside C and primulic acid 1 on the minimal inhibitory concentration (MIC) of gentamicin against two groups of clinical isolates with vancomycin-resistant *Enterococcus faecium* (VRE). Numbers of isolates showing the respective results out of the total number of isolates are indicated in parentheses. (n/a: not available, n/c: no change)

Saponin concentrations (mg/ml)	MIC (mg/l)			
	High-level resistant (3 isolates)	<i>n</i>	Low-level intrinsic resistant (17 isolates)	<i>n</i>
0	>1000		2–>8	
Glycyrrhizic acid				
1.0	≤ 250 (2/2)	4	≤ 0.125 –1 (16/16)	29
0.95	≤ 250 (1/1)	2	0.25–1 (3/3)	7
0.9	≤ 250 –500 (3/3)	3	0.25–0.5 (2/2)	6
0.85	≤ 250 –500 (3/3)	6	0.25–1 (3/3)	5
0.8	≤ 250 –500 (3/3)	6	0.5–1 (2/2)	3
0.75	≤ 250 –500 (2/2)	3	0.25–0.5 (3/3)	3
0.5	n/c (1/1)	1	1 (2/2)	3
0.2	n/a		2 (1/2)	2
β -Aescin				
1.0	n/c (2/2)	3	1–4 (14/15)	21
0.9	n/a		2 (1/1)	1
0.75	n/a		1 (1/1)	4
0.5	n/c (2/2)	3	0.5–2 (15/15)	16
α -Hederin				
0.4	n/a		1 (1/2)	2
0.2	n/c (2/2)	2	2 (2/4)	7
Hederacoside C				
1.0	n/c (1/1)	2	2 (1/3)	5
0.75	n/a		n/c (1/1)	1
0.5	n/a		n/c (1/1)	1
Primulic acid 1				
0.4	n/a		n/c (1/1)	2
0.2	n/c (2/2)	3	n/c (3/3)	5

and 5). Moreover, no antagonism was observed for any combination tested.

The combinatory effect between gentamicin and saponins was determined in 20 isolates; three of them were high-level gentamicin resistant (MIC: >1000 mg/l) and 17 were low-level intrinsic resistant (MIC: 2 mg/l to >8 mg/l). The combination with glycyrrhizic acid was superior to that of the other saponins under study. In the presence of 1 mg/ml glycyrrhizic acid, the MIC of the 17 isolates with low-level intrinsic resistance changed to ≤ 0.125 mg/l to 1 mg/l. At lower glycyrrhizic acid concentrations, the MIC reduced in a dose-dependent manner. The three high-level gentamicin resistant isolates showed a diminishment of the MIC to concentrations of ≤ 250 mg/l to 500 mg/l by adding 0.8 mg/ml to 0.9 mg/ml glycyrrhizic acid. Two of three isolates were tested with concentrations of 1 mg/ml glycyrrhizic acid, and the MIC of gentamicin changed from >1000 mg/l to ≤ 250 mg/l (Table 2). Given that a MIC of gentamicin ≤ 250 mg/l indicates a minimal inhibitory con-

centration between >4 mg/l and ≤ 250 mg/l, it is not clear whether the threshold of the high-level gentamicin resistance (>128 mg/l) was reached. β -Aescin exhibited less potent activity compared to glycyrrhizic acid in combination with gentamicin. In the presence of 1 mg/ml β -aescin, 14 of 15 tested low-level intrinsic resistant isolates showed changes in the MIC of gentamicin to values from 1 mg/l to 4 mg/l. No change in the MIC, however, was observed in the isolates with gentamicin high-level resistance by adding β -aescin (0.5 mg/ml and 1 mg/ml) (Table 2).

The impact of saponins on the minimal inhibitory concentration of teicoplanin was further investigated in seven teicoplanin-resistant isolates (MIC: 32 mg/l to 48 mg/l). Like with gentamicin, the combination of teicoplanin with glycyrrhizic acid resulted in the highest reduction of the MIC. In the presence of 1 mg/ml glycyrrhizic acid, the MIC of teicoplanin changed to values between 0.5 mg/l and 16 mg/l. Three of the seven isolates, in combination with 1 mg/ml glycyrrhizic acid, showed a MIC of teico-

Table 3. Influence of glycyrrhizic acid, β -aescin, α -hederin, hederacoside C and primulic acid 1 on the minimal inhibitory concentration (MIC) of teicoplanin, daptomycin, vancomycin, fosfomycin and trimethoprim/sulfamethoxazole against clinical isolates with vancomycin-resistant *Enterococcus faecium* (VRE). Numbers of isolates showing the respective results out of the total number of isolates are indicated in parentheses. (n/a: not available, n/c: no change)

Saponin concentrations (mg/ml)	MIC (mg/l)									
	Teicoplanin-resistant (7 isolates)	<i>n</i>	Daptomycin (20 isolates)	<i>n</i>	Vancomycin-resistant (20 isolates)	<i>n</i>	Fosfomycin (20 isolates)	<i>n</i>	Trimethoprim/Sulfamethoxazol (20 isolates)	<i>n</i>
0	32–48 (> 32)		2–4 (> 2)		16– $\geq$ 256		16–64		16– $\geq$ 320	
Glycyrrhizic acid										
1.0	0.5–16 (7/7)	16	0.25–2 (18/18)	33	1–16 (5/18)	33	8–16 (6/18)	33	$\leq$ 4–64 (7/18)	33
0.95	1–8 (2/2)	6	0.5–2 (4/4)	9	8 (1/4)	9	8–16 (3/4)	9	n/c (4/4)	9
0.9	4–16 (2/3)	8	0.5–2 (4/4)	9	n/c (4/4)	9	16 (1/4)	9	n/c (4/4)	9
0.85	8 (2/4)	9	1–2 (6/6)	11	n/c (6/6)	11	16–32 (4/6)	11	n/c (6/6)	11
0.8	8–16 (1/3)	6	1–2 (5/5)	10	n/c (5/5)	10	16–32 (2/5)	10	n/c (5/5)	10
0.75	8 (1/2)	3	1–2 (5/5)	6	n/c (5/5)	6	32 (1/5)	6	64 (1/5)	6
0.5	n/c (2/2)	3	2 (2/3)	4	n/c (3/3)	4	n/c (3/3)	4	n/c (3/3)	4
0.2	n/a		n/c (2/2)	2	n/c (2/2)	2	n/c (2/2)	2	n/c (2/2)	2
β -Aescin										
1.0	1 (1/7)	10	1–2 (4/17)	25	1–16 (8/17)	25	$\leq$ 4–16 (2/17)	25	8–128 (10/17)	25
0.9	n/a		n/c (1/1)	1	n/c (1/1)	1	n/c (1/1)	1	16 (1/1)	1
0.75	n/a		n/c (1/1)	4	n/c (1/1)	4	n/c (1/1)	4	8–32 (1/1)	4
0.5	8–16 (2/6)	13	2 (3/14)	21	4–16 (4/14)	21	n/c (14/14)	21	$\leq$ 4–32 (10/14)	21
α -Hederin										
0.4	n/c (2/2)	2	n/c (2/2)	2	n/c (2/2)	2	n/c (2/2)	2	8 (1/2)	2
0.2	n/c (4/4)	6	n/c (6/6)	9	16 (1/6)	9	n/c (6/6)	9	32 (1/6)	9
Hederacoside C										
1.0	n/c (3/3)	5	n/c (4/4)	7	n/c (4/4)	7	n/c (4/4)	7	n/c (4/4)	7
0.75	n/a		n/c (1/1)	1	16 (1/1)	1	n/c (1/1)	1	n/c (1/1)	1
0.5	n/c (1/1)	1	n/c (1/1)	1	n/c (1/1)	1	n/c (1/1)	1	n/c (1/1)	1
Primulic acid 1										
0.4	n/c (1/1)	2	n/c (1/1)	2	n/c (1/1)	2	n/c (1/1)	2	n/c (1/1)	2
0.2	n/c (3/3)	4	n/c (5/5)	8	n/c (5/5)	8	n/c (5/5)	8	n/c (5/5)	8

planin in the susceptible range (≤ 2 mg/l). β -Aescin, however, did not display enhanced antimicrobial activity when combined with teicoplanin (Table 3).

The *in vitro* interactions between daptomycin and saponins were tested in 20 isolates with a MIC of daptomycin between 2 mg/l and 4 mg/l. Glycyrrhizic acid concentrations of 0.5 mg/ml led to a lower MIC of daptomycin. Notably, with the highest glycyrrhizic acid concentration of 1 mg/ml, the MIC of daptomycin against all investigated isolates could be reduced to values between 0.25 mg/l and 2 mg/l. In combination with β -aescin, however, a change in the MIC of daptomycin occurred only in very few isolates (Table 3).

Only for some isolates, the combination of saponins with vancomycin, fosfomycin, or trimethoprim–sulfamethoxazole led to changes in respective MICs (Table 3). The combinatory effect of saponins and vancomycin was tested in 18 vancomycin-resistant isolates. Only in

two isolates, however, the MIC of vancomycin changed to the susceptible range (MIC: ≤ 4 mg/l) when 1 mg/ml glycyrrhizic acid or 1 mg/ml β -aescin was added. Fourteen isolates were resistant against the combination of trimethoprim–sulfamethoxazole, whereas in combination with saponins, only in one isolate the MIC dropped to ≤ 4 mg/l (resistant: > 1 mg/l).

Discussion

Antimicrobial resistance is a growing threat to the effective treatment of infections in general and, particularly, in wound infections or the prevention of postoperative surgical site infections [1]. The current lack of novel antibiotic drugs worsens the situation of multidrug resistance given that, in the latest 50 years, only a few new classes of antibiotics were discovered [21]. There are some options to

Table 4. Influence of glycyrrhizic acid, β -aescin, α -hederin, hederacoside C and primulic acid 1 on the minimal inhibitory concentration (MIC) of linezolid, ampicillin, erythromycin and clindamycin against clinical isolates with vancomycin-resistant *Enterococcus faecium* (VRE). Numbers of isolates showing the respective results out of the total number of isolates are indicated in parentheses. (n/c: no change)

Saponin concentrations (mg/ml)	MIC (mg/l)							
	Linezolid (20 isolates)	<i>n</i>	Ampicillin (20 isolates)	<i>n</i>	Erythromycin (20 isolates)	<i>n</i>	Clindamycin (20 isolates)	<i>n</i>
0	0.5–2		>16– $\geq$ 32		>8		1–>8	
Glycyrrhizic acid								
1.0	$\leq$ 0.25–0.5 (5/18)	33	4 (2/18)	33	4 (3/18)	33	4 (1/18)	33
0.95	n/c (4/4)	9	n/c (4/4)	9	n/c (4/4)	9	n/c (4/4)	9
0.9	n/c (4/4)	9	n/c (4/4)	9	n/c (4/4)	9	n/c (4/4)	9
0.85	n/c (6/6)	11	n/c (6/6)	11	n/c (6/6)	11	$\leq$ 0.125–0.25 (1/6)	11
0.8	1 (1/5)	10	n/c (5/5)	10	n/c (5/5)	10	$\leq$ 0.125 (1/5)	10
0.75	0.5 (1/5)	6	8 (1/5)	6	4 (1/5)	6	n/c (5/5)	6
0.5	n/c (3/3)	4	n/c (3/3)	4	n/c (3/3)	4	n/c (3/3)	4
0.2	n/c (2/2)	2	n/c (2/2)	2	n/c (2/2)	2	n/c (2/2)	2
β-Aescin								
1.0	0.25–0.5 (3/17)	25	n/c (17/17)	25	n/c (17/17)	25	4 (1/17)	25
0.9	n/c (1/1)	1	n/c (1/1)	1	n/c (1/1)	1	n/c (1/1)	1
0.75	n/c (1/1)	4	n/c (1/1)	4	n/c (1/1)	4	n/c (1/1)	4
0.5	0.5 (2/14)	21	n/c (14/14)	21	n/c (14/14)	21	n/c (14/14)	21
α-Hederin								
0.4	0.5 (1/2)	2	n/c (2/2)	2	n/c (2/2)	2	n/c (2/2)	2
0.2	n/c (6/6)	9	n/c (6/6)	9	n/c (6/6)	9	n/c (6/6)	9
Hederacoside C								
1.0	n/c (4/4)	7	n/c (4/4)	7	n/c (4/4)	7	n/c (4/4)	7
0.75	n/c (1/1)	1	n/c (1/1)	1	n/c (1/1)	1	n/c (1/1)	1
0.5	0.5 (1/1)	1	n/c (1/1)	1	n/c (1/1)	1	n/c (1/1)	1
Primulic acid 1								
0.4	n/c (1/1)	2	n/c (1/1)	2	n/c (1/1)	2	n/c (1/1)	2
0.2	n/c (5/5)	8	n/c (5/5)	8	4 (1/5)	8	n/c (5/5)	8

solve the problem, however. In addition to the discovery of new antibiotic drugs, combination of antibiotics or of antibiotics with compounds which do not exhibit an antimicrobial effect *per se* might provide synergistic antimicrobial effects. The presented results provide strong evidence for the therapeutic potential of saponins in combination with defined antibiotic drugs.

In our study, synergetic effects could be observed for the combination of glycyrrhizic acid with gentamicin, teicoplanin and daptomycin. The combination of antibiotic drugs plus glycyrrhizic acid was superior to other saponins under study given that β -aescin, hederacoside C, and primulic acid 1 in the tested concentrations did not result in further antibacterial activities against the clinical isolates. Without antibiotic drug, only glycyrrhizic acid and α -hederin, however, showed at least a weak antibacterial effect on VRE.

Gentamicin high-level resistant isolates changed their MIC of gentamicin in the presence of 0.85 mg/ml or

1 mg/ml glycyrrhizic acid from >1000 mg/l to a MIC between >4 mg/l and $\leq$ 250 mg/l. Thus, it is not clear whether the gentamicin high-level resistance (MIC $\geq$ 128 mg/l) could be overcome. For the gentamicin low-level intrinsic resistant isolates, however, adding of 1 mg/ml glycyrrhizic acid resulted in MIC changes of gentamicin from >8 mg/l to 2 mg/l to $\leq$ 0.125 mg/l and 1 mg/l. Two VRE isolates with a MIC of gentamicin of 4 mg/l were tested with a glycyrrhizic acid concentration of 0.5 mg/ml and showed a reduction of the MIC to 1 mg/l. The pharmacokinetic–pharmacodynamic (PK–PD) breakpoint of the susceptible category of gentamicin is $\leq$ 2 mg/l, applying to intravenous dosage of 3 mg/kg/day to 4.5 mg/kg/day gentamicin [22], to assure a high likelihood of therapeutic success in the treatment of all low-level intrinsic resistant isolates with gentamicin in combination with glycyrrhizic acid.

The problems of oral treatment with glycyrrhizic acid are the low bioavailability caused by the poor absorption and the hydrolysis in the gastrointestinal tract [23, 24]. In

Table 5. Influence of glycyrrhizic acid, β -aescin, α -hederin, hederacoside C and primulic acid 1 on the minimal inhibitory concentration (MIC) of flucloxacillin, rifampicin, penicillin, moxifloxacin against clinical isolates with vancomycin-resistant *Enterococcus faecium* (VRE). Numbers of isolates showing the respective results out of the total number of isolates are indicated in parentheses. (n/c: no change)

Saponin concentrations (mg/ml)	MIC (mg/l)							
	Flucloxacillin (20 isolates)	<i>n</i>	Rifampicin (20 isolates)	<i>n</i>	Penicillin (20 isolates)	<i>n</i>	Moxifloxacin (20 isolates)	<i>n</i>
0	8–>8		>4		>0.5		≥ 8	
Glycyrrhizic acid								
1.0	0.5 (1/18)	33	1 (1/18)	33	n/c (18/18)	33	n/c (18/18)	33
0.95	n/c (4/4)	9	n/c (4/4)	9	n/c (4/4)	9	n/c (4/4)	9
0.9	n/c (4/4)	9	n/c (4/4)	9	n/c (4/4)	9	n/c (4/4)	9
0.85	n/c (6/6)	11	n/c (6/6)	11	n/c (6/6)	11	n/c (6/6)	11
0.8	n/c (5/5)	10	n/c (5/5)	10	n/c (5/5)	10	n/c (5/5)	10
0.75	n/c (5/5)	6	n/c (5/5)	6	n/c (5/5)	6	n/c (5/5)	6
0.5	n/c (3/3)	4	n/c (3/3)	4	n/c (3/3)	4	n/c (3/3)	4
0.2	n/c (2/2)	2	n/c (2/2)	2	n/c (2/2)	2	n/c (2/2)	2
β -Aescin								
1.0	n/c (17/17)	25	n/c (17/17)	25	n/c (17/17)	25	n/c (17/17)	25
0.9	n/c (1/1)	1	n/c (1/1)	1	n/c (1/1)	1	n/c (1/1)	1
0.75	n/c (1/1)	4	n/c (1/1)	4	n/c (1/1)	4	n/c (1/1)	4
0.5	n/c (14/14)	21	2 (1/14)	21	n/c (14/14)	21	n/c (14/14)	21
α -Hederin								
0.4	n/c (2/2)	2	n/c (2/2)	2	n/c (2/2)	2	n/c (2/2)	2
0.2	n/c (6/6)	9	n/c (6/6)	9	n/c (6/6)	9	n/c (6/6)	9
Hederacoside C								
1.0	n/c (4/4)	7	n/c (4/4)	7	n/c (4/4)	7	n/c (4/4)	7
0.75	n/c (1/1)	1	n/c (1/1)	1	n/c (1/1)	1	n/c (1/1)	1
0.5	n/c (1/1)	1	n/c (1/1)	1	n/c (1/1)	1	n/c (1/1)	1
Primulic acid 1								
0.4	n/c (1/1)	2	n/c (1/1)	2	n/c (1/1)	2	n/c (1/1)	2
0.2	n/c (5/5)	8	n/c (5/5)	8	n/c (5/5)	8	n/c (5/5)	8

contrast to other saponins, glycyrrhizic acid, which is used in Japan for the intravenous treatment of chronic hepatitis [25], exerts a very low haemolytic activity (haemolysis index <2000) [14]. The LD₅₀ values of monoammonium glycyrrhizinate were 325 and 478 mg/kg body weight in mice and rats, respectively [26]. Hence, the required saponin concentrations are much too high for the possibility of parenteral saponin application to this end. An alternative mode of treatment is the topical application of preparations with glycyrrhizic acid. The *in vitro* glycyrrhizic acid concentration of 1 mg/ml corresponds to a topical preparation with 0.1% glycyrrhizic acid. Preparations with 0.1% glycyrrhizic acid were well-tolerated and showed no irritation on the intact skin [27].

The combination of gentamicin and saponins has not been examined in other studies yet. So far, the combination of ginsenoside and kanamycin, another aminoglycoside, was tested on *B. subtilis* and MRSA [17, 18]. Synergy was demonstrated for the combination of kanamycin

with ginsenoside in concentrations of 0.3 mg/ml and 0.6 mg/ml on *B. subtilis*. Both concentrations led to a MIC reduction of kanamycin from 0.32 mg/l to 0.16 mg/l [18]. The combination of kanamycin and ginsenoside on three clinical MRSA isolates resulted in synergistic or additive antimicrobial effects. Adding ginsenoside in concentrations of 0.025 mg/ml or 0.05 mg/ml resulted in four and eight times lower kanamycin MICs, respectively, as compared to the antibiotic alone (from 400 mg/l to 100 mg/l and from 100 mg/l to 12.5 mg/l, respectively) [17]. Enhancement of the sensitivity by ginsenoside could also be found for ampicillin, oxytetracycline, and chloramphenicol against *B. subtilis* and for ampicillin, cephalixin, and cefotaxime against *S. aureus* [17, 18]. Compared to glycyrrhizic acid, the concentrations of ginsenoside resulting in MIC reduction are lower, whereas the exerted antibacterial effects of ginsenoside are clearly more distinct.

Two glycopeptide antibiotics under study, vancomycin and teicoplanin, displayed changes in their MIC upon

Table 6. Clinical breakpoints of the antibiotics under study on *Enterococcus* spp. [22]

Antibiotics	Clinical breakpoints (MIC in mg/l)		
	Resistant	Intermediate	Susceptible
Ampicillin	>8		≤4
Clindamycin		No breakpoints	
Daptomycin	Insufficient evidence of the effectiveness of the therapy		
Erythromycin		No breakpoints	
Flucloxacillin		No breakpoints	
Fosfomycin		No breakpoints	
Gentamicin	Isolates with gentamicin MIC ≤ 128 mg/l are wild type and low-level intrinsic resistant, isolates with gentamicin MIC > 128 mg/l are high-level resistant		
Linezolid	>4		≤4
Moxifloxacin		No breakpoints	
Mupirocin		No breakpoints	
Rifampicin		No breakpoints	
Teicoplanin	>2		≤2
Tigecyclin	>0.5		≤0.25
Trimethoprim-sulfamethoxazole	>1	>0.03–≤1	≤0.03
Vancomycin	>4		≤4

combination with glycyrrhizic acid. Remarkably, at the highest glycyrrhizic acid concentration of 1 mg/ml, the MIC for teicoplanin changed in three of seven teicoplanin resistant isolates to the susceptible range, whereas five isolates remained resistant, but with a lower MIC. The antimicrobial effects for teicoplanin were significantly stronger and more uniform as compared to vancomycin, despite the same antibacterial mechanism. The combination of vancomycin and glycyrrhizic acid led to a change in the MIC only in some isolates. From 18 vancomycin resistant isolates only two exerted a reduced MIC to the susceptible range.

Glycyrrhizic acid in combination with daptomycin resulted in a reduction of the MIC in all studied isolates. Synergy between saponins and glycopeptides or lipopeptides was also described in a study with the synthetic saponin diosgenyl 2-amino-2-deoxy-β-D-glucopyranoside hydrochloride (HSM-1) [20]. The combination of HSM-1 with vancomycin or daptomycin against *E. faecalis* and *S. aureus* was shown to exert synergistic antimicrobial effects *in vitro*. The synergistic effect was also described in an *in-vivo* wound model with *E. faecalis* and *S. aureus* for the combination of intraperitoneal vancomycin or daptomycin and local saponin treatment [20]. The antibacterial properties of HSM-1 against *E. faecalis* (MIC of 0.002 mg/ml to 0.032 mg/ml) were much higher than the antibacterial properties of the saponins under study against *E. faecium* [20].

The results for the enhanced sensitivities of VRE towards fosfomycin and trimethoprim–sulfamethoxazole in the presence of glycyrrhizic acid were inconsistent compared with the antibiotics above (Table 3).

When speculating about the synergistic mechanisms exerted by saponins, a higher uptake of the antibiotic drugs

into the bacterial cell might have been induced by the interaction of the saponins with the bacterial membrane and, hence, have been responsible for the observed beneficial effects. The reduction of the MIC of gentamicin, teicoplanin, and daptomycin in combination with glycyrrhizic acid points towards a potential therapeutic option of saponins in the coapplication with antibiotics for the treatment of wound infections. The higher antimicrobial activity of the antibiotic compound by the combination with saponins is associated with a higher likelihood of therapeutic success. In conclusion, our study points towards a therapeutic potential of saponins in the coapplication with antibiotics for bacterial infections. Future studies, however, are needed to further investigate the combinatory effects between antibiotic compounds and saponins *in vivo*.

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