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SHORT COMMUNICATION

Inhibitory Effect of Newly-Synthesized Chalcones on Hemolytic Activity of Methicillin-Resistant *Staphylococcus aureus*

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Abstract

Pathogenicity of methicillin-resistant *Staphylococcus aureus* (MRSA) is associated with a broad spectrum of virulence factors, amongst which is α-hemolysin. The aim of this study was to investigate the effect of three newly-synthesized chalcones (1,3- Bis-(2-hydroxy-phenyl)-propenone, 3-(3-Hydroxy-phenyl)-1-(2-hydroxy-phenyl)-propenone and 3-(4-Hydroxy-phenyl)-1-(2-hydroxy-phenyl)-propenone) on α-hemolysin production of clinical isolates of MRSA. Subinhibitory concentrations of the tested compounds reduced hemolytic activity of MRSA strains, with almost complete abolishment of hemolysis at concentrations in the range of 1/2–1/4 x MIC (25–12.5 μg/ml). In conclusion, newly-synthesized chalcones tested in this study showed potent inhibitory activity on α-hemolysin production of multiresistant and genetically diverse MRSA strains.

Key words: α-hemolysin, chalcones, MRSA

Pathogenicity of methicillin-resistant *Staphylococcus aureus* (MRSA) is directly associated with a broad spectrum of virulence factors, amongst which is α-hemolysin (*i.e.* HIa or α-toxin). It is generally considered that α-hemolysin plays a central role in the pathogenesis of staphylococcal infections, especially in pulmonary infections caused by these bacteria (Bubeck Wardenburg *et al.*, 2007; Burlak *et al.*, 2007; Montgomery *et al.*, 2008). Approximately one half of staphylococcal necrotizing pneumonia cases affecting previously healthy adults and children are caused by community-associated MRSA strains (Ragle and Bubeck Wardenburg, 2009). Besides direct lysis of the pulmonary cells, α-hemolysin also activates alveolar macrophages or monocytes, induces massive polymorphonuclear leukocyte influx into lung parenchyma with subsequent degranulation and destruction of microvascular endothelium and adjacent tissues, and induces platelet-neutrophil co-aggregation leading to the host response to toxin that contribute to the severity of lung destruction (Parimon *et al.*, 2013). Such complex pathogenicity and occurrence of multiresistant strains urge the development of antimicrobial compounds that selectively target virulence factors. In past years, several studies investigated the inhibitory activity of various compounds against extracellular virulence factors of *S. aureus* (Escaich, 2008; Cegelski *et al.*, 2008). Previously we have reported antimicrobial activity of three newly-synthesized chalcones against clinical isolates of MRSA, and inhibitory effect on the expression of virulence factors related to the early step of bacterial invasion-adherence (*i.e.* biofilm formation, glycocalyx production and adherence to human fibronectin) (Božić *et al.*, 2014). Therefore, the aim of this study was to investigate the effect of 1,3- Bis-(2-hydroxy-phenyl)-propenone (O-OH), 3-(3-Hydroxy-phenyl)-1-(2-hydroxy-phenyl)-propenone (M-OH) and 3-(4-Hydroxy-phenyl)-1-(2-hydroxy-phenyl)-propenone (P-OH) on α-hemolysin production of clinical isolates of multiresistant and genetically diverse strains of MRSA.

Antibacterial activity of chalcones was tested against 20 clinical isolates of MRSA isolated from blood (4), wound (6), sputum (3), endotracheal tube (2), abdominal drain (1), nose (1), skin (1), urine (1) and external

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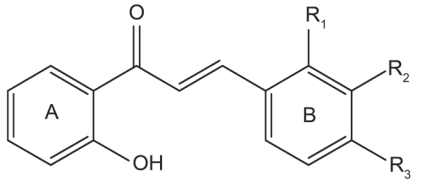

Compound	R1	R2	R3
O-OH	– OH	– H	– H
M-OH	– H	– OH	– H
P-OH	– H	– H	– OH

Fig. 1. The chemical structures of the tested chalcones:
1,3- Bis-(2-hydroxy-phenyl)-propenone (O-OH)
3-(3-hydroxy-phenyl)-1-(2-hydroxy-phenyl)-propenone (M-OH)
3-(4-hydroxy-phenyl)-1-(2-hydroxy-phenyl)-propenone (P-OH)

auditory canal (1) and one laboratory control strain of methicillin-resistant *S. aureus* ATCC 43300 (KWIK-STIK™, Microbiologics, USA). Identification of the isolates and methicillin resistance were determined by VITEK 2 test cards GP and AST-P580 (bioMérieux, France) and confirmed by PCR for *nuc* (Brakstad *et al.*, 1992) and *mec*A (Bignardi *et al.*, 1996) genes. Genotyping (SCC*mec*, *agr*, *pvl* and *spa* typing) of MRSA was performed according to previously described protocols (Boye *et al.*, 2007; Lina *et al.*, 2003; Harmsen *et al.*, 2003). The *spa* types were clustered into *spa* clonal complexes (CCs) using the algorithm based upon repeat pattern (BURP) with Ridom Staph-Type 1.4 software (http://www.ridom.de). The multiresistance profile of MRSA strains was determined by VITEK 2 test card AST-P580 and further supplemented with disc diffusion test according to CLSI guidelines (CLSI, 2007). Chalcones tested in this study (Fig. 1) were obtained from the Department of Pharmaceutical Chemistry, University of Belgrade-Faculty of Pharmacy, Belgrade, Serbia. Compounds were prepared and characterized as previously described (Božić *et al.*, 2014) and their antimicrobial activity was determined by broth microdilution test according to CLSI guidelines (CLSI 2007). Hemolytic activities of MRSA culture supernatants were determined according to the method of Rowe and Welch (1994). The data obtained in this study were analyzed in SPSS statistical program (PASW statistics for Windows, Version 18.0, Chicago: SPSS Inc. USA) using methods of descriptive statistics, Chi square test and Mann-Whitney U test.

Clinical isolates of MRSA were genetically heterogeneous and expressed multiresistance phenotype (Table I). MRSA strains were classified into SCC*mec* type I (55.5%), II (5.0%), III (20.0%), IV (10.0%) and V (10.0%) and *agr* type I (35.0%), II (60.0%) and III (5.0%). Strains belonged to 10 *spa* types and were clustered into 5 *spa* CCs, with most frequent CC5 (55.0%) and CC8 (20.0%).

Tested chalcones exerted inhibitory activity against MRSA, with the order of potency of chalcones (average MIC ± SD) as following: O-OH (MIC = 37.5 ± 13.2 μg/ml) > M-OH (MIC = 97.5 ± 27.5 μg/ml) > P-OH (MIC = 110.8 ± 21.1 μg/ml). The most significant dose-dependent inhibition of hemolysis was observed in MRSA supernatants cultivated with O-OH chalcone (Fig. 2). A 91.6–99.7% reduction of hemolysis was detected in all MRSA strains cultivated with ½ × MIC of O-OH. Hemolytic activity of MRSA strains cultivated with 1/2 × MIC (25.0 μg/ml) and 1/4 × MIC (12.5 μg/ml) of O-OH was in the range of 3.8–8.2% of the positive control ($p < 0.001$). Mild increase of the hemolytic activity was detected after cultivation with 1/8 × MIC (6.2 μg/ml) up to 19% ($p < 0.01$), and 1/16 × MIC (3.1 μg/ml) up to 34.6% ($p < 0.01$). The effect of M-OH

Table I
Genotyping and resistance profiles of MRSA strains.

CC type	*spa* type	N° of strains	SCC*mec* type	*agr* type	PVL	Resistance profile (> 50% of strains)
CC5	*t041*	9	1	2	–	AM, GEN, KAN, TOB, STR, LIN, CLIN, ER, CLA, AZ, SPIRA, TET, DOX, CHL
	t041	1	1	1	–	AM, GEN, KAN, TOB, STR, LIN, CLIN, ER, CLA, AZ, SPIRA, CHL
	t003	1	2	2	–	KAN, TOB, LIN, CLIN, ER, CLA, AZ, SPIRA
CC8	*t030*	2	3	1	–	AM, GEN, KAN, TOB, STR, ER, CLA, AZ, TET, DOX, MIN, CHL
	t030	1	3	2	–	AM, GEN, KAN, TOB, STR, LIN, CLIN, ER, CLA, AZ, SPIRA, TET, DOX, MIN
	t969	1	3	1	–	AM, GEN, KAN, TOB, STR, ER, CLA, AZ, TET, DOX
CC45	*t015*	1	1	2	–	AM, GEN, KAN, TOB, STR, LIN, CLIN, ER, CLA, AZ, SPIRA, CHL
	t583	1	4	1	–	AM, GEN, KAN, TOB, STR, ER, CLA, AZ
CC80	*t044*	1	4	3	+	KAN, STR, TET
CC152	*t595*	1	5	1	+	GEN, KAN, TOB, LIN, TET
	t6371	1	5	1	–	GEN, KAN, TOB, ER, CLA, AZ

AM – amikacin, GEN – gentamicin, KAN – kanamycin, TOB – tobramycin, STR – streptomycin, LIN – lincomycin, CLIN – clindamycin, ER – erythromycin, CLA – clarithromycin, AZ – azithromycin, SPIRA – spiramycin, TET – tetracycline, DOX – doxycycline, MIN – minocycline, CHL – chloramphenicol.

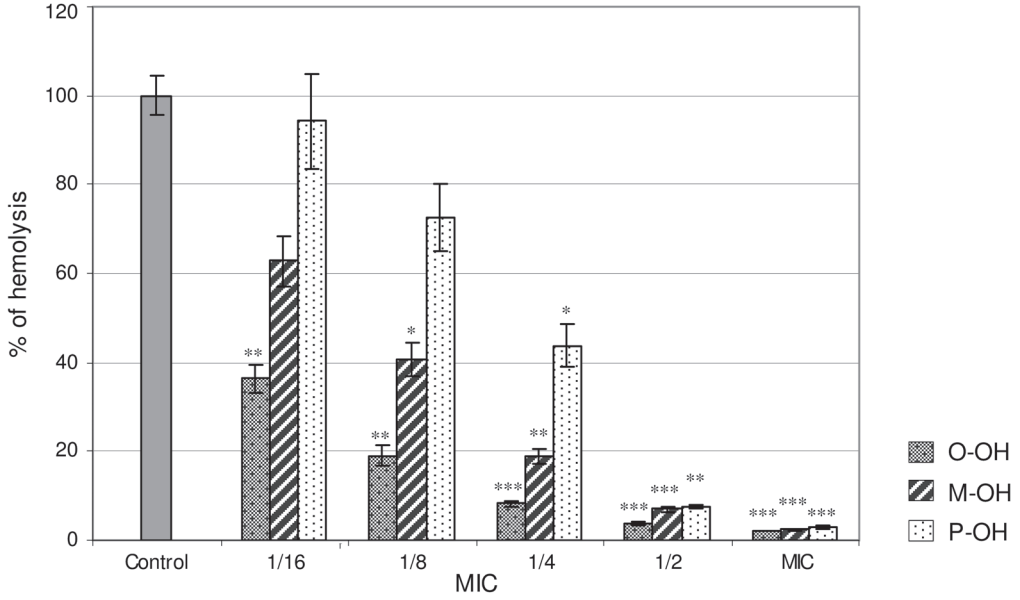

Fig. 2. The inhibitory effect of the O-OH, M-OH and P-OH chalcone on α-hemolysin production of MRSA strains. Results are present as mean percent of hemolysis ± SD of 20 clinical isolates of MRSA and one control strain (MRSA ATCC 43300), compared to the positive control (*i.e.* hemolytic activity of supernatants of untreated MRSA strains presented as 100% hemolysis); * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

and P-OH was tested in the two-fold higher concentrations than O-OH chalcone (50.0–6.2 μg/ml), corresponding the same range of MIC's (1/2 × MIC – 1/16 × MIC). Hemolytic activity of MRSA strains cultivated with 1/2 × MIC and 1/4 × MIC of M-OH was in the range of 7.1–18.8% of the positive control ($p < 0.01$; $p < 0.001$), and cultivation with 1/8 × MIC lead to the increase of the hemolysis up to 40.7% of the positive control ($p < 0.05$). The lowest applied concentration of M-OH (1/16 × MIC, 6.2 μg/ml) did not reduce the hemolytic activity of MRSA ($p > 0.05$). Statistically significant inhibition of hemolytic activity of MRSA strains cultivated with P-OH chalcone occurred only in concentrations of 1/2 × MIC (7.7%; $p < 0.01$) and 1/4 × MIC (43.8%; $p < 0.05$) (Fig. 2). Chalcones themselves did not perform any hemolytic activity or induced hemolysis of rabbit erythrocytes in the same range of MIC's (1/2 × MIC – 1/16 × MIC; $p > 0.05$).

Antistaphylococcal activity of various chalcones is strongly correlated with their chemical characteristics. It is generally considered that lipophilicity of the ring A (Nowakowska, 2007) and presence of free hydroxyl group/s at various positions of the ring B are necessary for antistaphylococcal activity of these compounds (Sato *et al*., 1996; Alcaraz *et al*., 2000; Kromann *et al*., 2004; Talia *et al*., 2011). Three compounds investigated in our study carrying free hydroxyl group at various positions of the B ring exerted strong anti-MRSA activity, with 1,3- Bis-(2-hydroxy-phenyl)-propenone (O-OH) bearing the hydroxyl group at the position 2 of ring B as the most active one. These results are in accordance with the findings of other investigators (Alcaraz *et al*., 2000; Kromann *et al*., 2004). Subinhibitory concentrations of the tested compounds also inhibited hemolytic activity of MRSA strains in a dose dependent manner, with almost complete abolishment of hemolysis at concentrations in the range of 1/2–1/4 MIC (25–12.5 μg/ml). Inhibition of hemolytic activity of α-hemolysin was dose dependent. Chalcone with free hydroxyl group at the position 2 of ring B was the most active one, since the lowest applied concentration of this compound (1/16 × MIC, 3.1 μg/ml) exerted statistically significant inhibition of hemolysis. Higher concentrations of other tested compounds exerted similar inhibitory effect, with partial or complete recovery of hemolytic activity of α-hemolysin when applied in the lowest concentrations (1/16 × MIC, 6.2 μg/ml). Previously it has been reported that subinhibitory concentrations of chalcones with similar chemical structure isolated from natural sources, like licochalcone A and E, inhibit α-hemolysin production (Qiu *et al*., 2010a; Zhou *et al*., 2012). Licochalcone A possess anti-MRSA activity in the range of MIC's from 2.0–8.0 μg/ml (Fukai *et al*., 2002; Qiu *et al*., 2010a), and inhibitory effect on α-hemolysin production in the range of 1/2–1/8 × MIC (Qiu *et al*., 2010a). Licochalcone E was found to be the most active licochalcone against *S. aureus*, since the inhibitory effect was detected in the range of MIC's from 1.0–4.0 μg/ml, and inhibition of α-hemolysin production in the range of 1/4–1/16 × MIC (Zhou *et al*., 2012).

Precise mechanism of antimicrobial activity of chalcones and inhibition of α-hemolysin synthesis has not been clarified yet. Several different mechanisms could be involved, like direct enzyme inhibition, inhibition

of DNA or RNA synthesis (Mori *et al*., 1987; Ohemeng *et al*., 1993) or interference with energy metabolism by inhibition of NADH-cytochrome *c* reductase, reducing the energy required for active uptake of various metabolites and biosynthesis of macromolecules (Haraguchi *et al*., 1998; Cushnie and Lamb, 2011). Licochalcone A (Qiu *et al*., 2010a) or thymol (Qiu *et al*., 2010b) perform inhibitory effect on α-hemolysin synthesis through inhibition of *agr*A transcription, one of the components of the *agr* global regulatory system of *S. aureus*. Besides influence on *agr* global regulatory system, licochalcone E also directly inhibits expression of *hla*-gene encoding α-hemolysin synthesis (Zhou *et al*., 2012). Accordingly, it is possible that chalcones also inhibit production of α-hemolysin through mechanisms proposed by Qui *et al*. and Zhou *et al*. Further studies are necessary to elucidate the precise mechanisms of inhibitory effect of tested chalcones on hemolytic activity of α-hemolysin.

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