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In vitro activity of retapamulin against linezolid and methicillin–resistant *Staphylococcus aureus* isolates

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ABSTRACT

Objectives: To determine the *in vitro* activity of retapamulin and other topical antibiotics (mupirocin, bacitracin, and fusidic acid) usually employed for nasal decolonization, against methicillin-susceptible *Staphylococcus aureus* (MSSA), methicillin-resistant *S. aureus* (MRSA), and linezolid and methicillin–resistant *S. aureus*.

Methods: The minimum inhibitory concentrations (MICs) were determined on Mueller-Hinton agar according to the guidelines of the Clinical and Laboratory Standards Institute and of the European Committee for Antimicrobial Susceptibility Testing. Presence of the *cfr* gene in linezolid and methicillin–resistant *S. aureus* isolates was detected using polymerase chain reaction.

Results: Retapamulin inhibited all the isolates of MSSA and MRSA at 0.125 mg/L, but the 18 linezolid-resistant-MRSA strains proved resistant, with MICs over 32 mg/L. Most MSSA isolates (9/10) were susceptible to mupirocin with MICs under 0.19 mg/L, although this value decreased to half against MRSA, and almost all linezolid-resistant MRSA (17/18) strains were resistant to mupirocin with an MIC range of between 8 mg/L and 28 mg/L. The MIC of fusidic acid increased substantially against linezolid-resistant MRSA, whereas that of bacitracin showed no differences.

Conclusions: Retapamulin demonstrated excellent *in vitro* activity against MSSA and MRSA strains, but not against MRSA isolates harbouring the *cfr* gene. The results of this *in vitro* study support cut-off values for retapamulin of ≤0.5, 1, and ≥2 mg/L for susceptible, intermediate, and resistant strains, respectively.

Key words:

Pleuromutilin, topical antibiotic therapy, cfr gene, nasal carriers

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Actividad *in vitro* de retapamulina frente a cepas de *Staphylococcus aureus* resistente a meticilina y a linezolid

RESUMEN

Objetivos: Determinar la actividad *in vitro* de retapamulina y otros antibióticos tópicos (mupirocina, bacitracina y ácido fusídico) usados habitualmente para la descolonización nasal, contra *Staphylococcus aureus* sensible a meticilina (SASM), *Staphylococcus aureus* resistente a meticilina (SARM) y *Staphylococcus aureus* resistente a meticilina y linezolid (SARM-L).

Material y métodos: Se determinaron las concentraciones mínimas inhibitorias (CMIs) en agar Mueller-Hinton de siguiendo los estándares del Clinical and Laboratory Standards Institute (CLSI) y European Committee for Antimicrobial Susceptibility Testing (EUCAST). La presencia del gen *cfr* en las cepas SARM-L se realizó usando la reacción en cadena de la polimerasa (PCR).

Resultados: Retapamulina inhibió todas las cepas de SASM and SARM alcanzando CMIs sobre 0,125 mg/L, pero las 18 cepas SARM-L se mostraron resistentes, con CMIs en torno a 32 mg/L. La mayoria de los aislados de SASM (9/10) fueron sensibles a mupirocina con CMIs inferiores a 0,19 mg/L, aunque entre las cepas de SARM tan solo fueron sensibles la mitad. La mayoría de las cepas SARM-L (17/18) fueron resistentes a mupirocina con CMIs entre 8 mg/L y 28 mg/L. La CMI de ácido fusídico aumento sustancialmente frente a las cepas SARM-L. Frente a la bacitracina no se observaron diferencias.

Conclusiones: Retapamulina demostró una excelente actividad *in vitro* frente a cepas SASM y SARM, pero no frente a las cepas de SARM portadoras del gen *cfr*. Los resultados *in vitro* de este estudio refrendan los puntos de corte de retapamulina de ≤0,5, 1, y ≥2 mg/L para sensible, intermedio y resistente, respectivamente.

Palabras clave:

Pleuromutilinas, tratamiento antibiótico tópico nasal, gen cfr, portadores nasales

INTRODUCTION

The number of infections due to methicillin-resistant *Staphylococcus aureus* (MRSA) is increasing, especially in surgical patients and patients in long-term care units. This rise is also evident in the number of MRSA isolates in patients receiving regular care in hospital day units (e.g., haemodialysis, oncologic patients). Nasal carriage of *S. aureus* is a factor that can increase the risk of nosocomial infection by this pathogen up to three-fold[1]; therefore, control of colonization becomes necessary. During the last two decades, different topical anti-staphylococcal antibiotics have been tested in order to reduce the impact of infection. The traditional antibiotics of choice have been topical chlorhexidine, bacitracin, and fusidic acid and, more recently, mupirocin, although, in some cases, patients have been treated with systemic antibiotics to control colonization[2].

Although only a few prospective studies establish the efficacy of intranasal mupirocin in preventing nosocomial infection, this agent has been widely used in nasal MRSA carriers and its incidence has been reduced. However, failure of decolonization and increasing resistance rates are becoming increasingly common, mainly due the widespread use of mupirocin[3]. Some types of resistance can be horizontally transmitted through PSK-41, a conjugative plasmid that carries multiple resistance determinants including the *mupA* gene, which is responsible for high-level resistance with no substantial fitness cost[4]. Retapamulin is a novel, semi-synthetic member of the pleuromutilins. It is active against the main skin pathogens, in particular *Streptococcus pyogenes* and *S. aureus*, and its use is approved for some skin infections (impetigo, infected traumatic or surgical injuries) (http://www.emea.europa.eu/humandocs/PDFs/EPAR/altargo/H-757-PI-en.pdf). Retapamulin has a complex mode of interaction with the ribosome, and acts by inhibiting 50S ribosomal subunit formation and protein synthesis[5,6]. This dual inhibitory effect differentiates retapamulin from other bacterial protein synthesis inhibitors, such as macrolides and ketolides, and makes it fully active against clinical strains that are resistant to protein synthesis inhibitors and also to strains that are resistant to antibiotics with different mechanisms of action[5].

We recently reported the first outbreak of *cfr*-mediated linezolid- and methicillin–resistant *S. aureus* (LR-MRSA) in intensive care patients[7,8], and we considered that it would be of interest to test the *in vitro* activity of retapamulin against the strains involved in the LR-MRSA outbreak. As comparators, we tested other topical antibiotics (fusidic acid, mupirocin, and bacitracin) that are habitually administered for nasal decolonization.

MATERIALS AND METHODS

Bacterial isolates and susceptibility testing

We collected 18 clinical LR-MRSA isolates: 12 were from patients in the intensive care unit during the outbreak, 3 from patients in other hospital wards during the same period, and 3 from patients admitted 5 to 6 months before the outbreak. For comparison, we included 10 methicillin-sensitive *S. aureus* (MSSA) isolates and 10 MRSA isolates from our collection.

The agents tested were retapamulin, fusidic acid, mupirocin, and bacitracin. MICs were determined on Mueller-Hinton agar using Etest strips (AB BIODISK, Solna, Sweden) and according to CLSI guidelines. The European Committee for Antimicrobial Susceptibility Testing (EUCAST) criteria were applied for fusidic acid, with a minimum inhibitory concentration (MIC) breakpoint of $\leq$1 mg/L for susceptibility. Susceptibility cut off values recommended by EUCAST (http://www.srga.org/eucastwt/MICTAB/index.html) for mupirocina was $\leq$0.5 mg/L, and for retapamulin we established values of $\leq$0.5, 1, and $\geq$2 mg/L for susceptible, intermediate, and resistant proposed by Traczewski et al.[9]

Molecular typing

LR-MRSA isolates were genotyped by PFGE. LR-MRSA suspensions were adjusted to a density equivalent of 5 x 10^8 cells/mL, mixed with an equal volume of 2% low melting agarose gel, and poured into the block molds. The agarose plugs obtained were treated with proteinase K/lysostaphin and digested with 20 U of *Sma*I restriction enzyme. The plugs were loaded into 1% agarose gels and electrophoresed using a CHEF-DRIII apparatus (Bio-Rad Laboratories) in 0.5 TBE buffer. Run time was 20 h with an initial switch time of 5 s and a final switch time of 40 s. The ramping factor was linear. Temperature was set at 14C, voltage at 6 V/cm, and the included angle at 120. The gels were stained with ethidium bromide, visualized under ultraviolet light, and documented using the Molecular Imager ChemiDoc XRS (Bio-Rad Laboratories). Digital images were stored as tiff files and analyzed visually or with use of FPQuest, version 4.5 (Bio-Rad Laboratories).

Detection of the *cfr* gene

The presence of *cfr* was assessed by PCR using oligonucleotide primers[10]. PCR conditions were as follows: denaturation for 2 min at 94ºC, 30 cycles of denaturation for 10 s at 94ºC, annealing for 30 s at 55ºC, extension for 30 s at 72ºC, and a final extension of 7 min at 72ºC.

RESULTS AND DISCUSSION

Several explanations have been put forward to explain the reduced susceptibility to pleuromutilins in *Staphylococcus* species isolates. One is the expression of the methyltransferase encoded by the c*fr* gene, which also confers cross-resistance to phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin A antibiotics ($PhLOPS_A$ resistance phenotype)[11]. The new pleuromutilin, retapamulin, has been tested *in vitro* and has demonstrated excellent activity against *S. aureus* isolates resistant to mupirocin and fusidic acid, probably as a result of its dual mechanism of action. For this reason we considered interesting to test retapamulin against LR-MRSA strains.

Table 1 **Susceptibility of the *Staphylococcus aureus* isolates studied**

Organism (n)		Minimum Inhibitory Concentrations (mg/L)			
		Retapamulin	Mupirocin	Fusidic acid	Bacitracin
MSSA[a] (10)	MIC_{50}	0.094	0.125	0.125	64
	MIC_{90}	0.094	0.25	0.19	64
	Range	0.094-0.125	0.125-256	0.094-0.19	8-128
MRSA[b] (10)	MIC_{50}	0.125	0.25	0.19	128
	MIC_{90}	0.125	1024	0.5	256
	Range	0.094-0.125	0.125-≥1024	0.094-2	32-≥256
LR-MRSA[c] (18)	MIC_{50}	≥32	16	1.5	24
	MIC_{90}	≥32	24	2	64
	Range	≥32	0.19-24	0.125-2	16-64

[a]MSSA, methicillin susceptible *S. aureus*.

[b]MRSA, methicillin resistant *S. aureus*.

[c]LR-MRSA, methicillin and linezolid resistant *S. aureus*.

Table 2 **MIC distributions of retapamulin, mupirocin, fusidic acid, and bacitracin in *Staphylococcus aureus* isolates**

Antibiotic	Organism (n)	Minimum Inhibitory Concentrations (mg/L)																	
		≤0.094	0.125	0.19	0.38	0.5	0.75	1	1.5	2	4	8	12	16	24	32	64	128	≥256
Retapamulin	MSSA[a] (10)	9	1																
	MRSA[b] (10)	4	6																
	LR-MRSA[c] (18)															18			
Mupirocin	MSSA[a] (10)		6	3															1
	MRSA[b] (10)		1	5				1							1				2
	LR-MRSA[c] (18)			1								1	6	6	4				
Fusidic acid	MSSA[a] (10)	2	5	3															
	MRSA[b] (10)	3	1	1	3	1				1									
	LR-MRSA[c] (18)		1		1		1	1	10	4									
Bacitracin	MSSA[a] (10)											2					6	2	
	MRSA[b] (10)															1	1	3	5
	LR-MRSA[c] (18)													5	5	5	3		

[a]MSSA, methicillin susceptible *S. aureus*.

[b]MRSA, methicillin resistant *S. aureus*.

[c]LR-MRSA, methicillin and linezolid resistant *S. aureus*

PCR was performed to establish the presence of the *cfr* gene in all 18 LR-MRSA strains of this study, and we found in all of them the presence of a band of 746 bp, which was compatible with the *cfr* fragment (data not shown). The amplification fragment sequences of selected strains—one of each PFGE type—were identical to the *cfr* sequence in the National Center for Biotechnology Information gene database.

The susceptibilities of the isolates to retapamulin, mupirocin, fusidic acid, and bacitracin are summarized in table 1, and the MIC distributions are shown in table 2. All MSSA and MRSA isolates were susceptible to retapamulin, with an MIC_{90} of 0.094 mg/L and 0.125 mg/L respectively, but retapamulin MICs for the 18 LR-MRSA strains were over 32 mg/L in all cases. Similarly, almost all mupirocin MICs for MSSA isolates (9/10) were under 0.25 mg/L. This susceptibility rate decreased to 6 out of 10 against MRSA, and almost all linezolid-resistant MRSA strains (17/18) were resistant to mupirocin with an MIC range of between 8 mg/L and 24 mg/L. The MICs of fusidic acid increased substantially against LR-MRSA, with 16 isolates over 0.5 mg/L, whereas all MSSA and MRSA isolates were susceptible. We did not observe differences between linezolid-resistant or linezolid-susceptible isolates when bacitracin was used.

Strains carrying *cfr* are resistant not only to linezolid, but also to phenicols, lincosamides, pleuromutilins, and streptogramin A class antibiotics[11]. The type of resistance expressed by these LR-MRSA strains could get implications in epidemiologic control, because of a selective pressure due to the uncontrolled use of any these drug classes may lead to the spread of this resistance determinant.

In conclusion, retapamulin demonstrated excellent *in vitro* activity against MSSA and MRSA strains, but not in those strains harbouring the *cfr* gene. *In vitro* results of this study support the cut-off values of EUCAST and Traczewski et al.[9] for retapamulin (≤0.5 mg/L, susceptible; 1 mg/L, intermediate, and ≥2 mg/L resistant). The results of this study are relevant, as the clinical utility of retapamulin could be reduced against strains carrying the *cfr* gene and should be taken in account if trials of retapamulin are conducted.

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The authors declare no conflicts of interest.

REFERENCES

1. Wertheim, HFL, Melles, DC, Vos, MC et al. The role of nasal carriage in *Staphylococcus aureus* infections. Lancet Infect Dis 2005; 5: 751-62.
2. McConeghy, KW, Mikolich, DJ, LaPlante, KL. Agents for the decolonization of methicillin-resistant *Staphylococcus aureus*. Pharmacotherapy 2009; 29: 263-80.
3. Patel, JB, Gorwitz, RJ, Jernigan, JA. Antimicrobial resistance: Mupirocin resistance. Clin Infect Dis 2009; 49: 935-41.
4. Mongkolrattanothai, K, Pumfrey, L, Mankin, P et al. Acquisition of high-level mupirocin resistance and its fitness cost among methicillin-resistant *Staphylococcus aureus* strains with low-level mupirocin resistance. J Clin Microbiol 2009; 47: 4158-60.
5. Yan, K, Madden, L, Choudhry, AE et al. Biochemical characterization of the interactions of the novel pleuromutilin derivative retapamulin with bacterial ribosomes. Antimicrob Agents Chemother 2006; 50: 3875-81.
6. Champney, WS, Rodgers, WK. Retapamulin inhibition of translation and 50S ribosomal subunit formation in *Staphylococcus aureus* cells. Antimicrob Agents Chemother 2007; 51: 3385-7.
7. Morales, G, Picazo, J, Baos, E et al. Resistance to linezolid is mediated by the *cfr* gene in the first reported outbreak of linezolid-resistant *Staphylococcus aureus*. Clin Infect Dis 2010; 50: 821-5.
8. Sánchez García M, De la Torre MA, Morales G, Peláez B, et al. Clinical outbreak of linezolid-resistant *Staphylococcus aureus* in an intensive care unit. JAMA 2010; 303: 2260-4.
9. Traczewski, MM, Brown, SD. Proposed MIC and disk diffusion microbiological cutoffs and spectrum of activity of retapamulin, a novel topical antimicrobial agent. Antimicrob Agents Chemother 2008; 52: 3863-7.
10. Kehrenberg, C, Schwarz, S. Distribution of florfenicol resistance genes *fexA* and *cfr* among *chloramphenicol-resistant Staphylococcus isolates*. Antimicrob Agents Chemother 2006; 50: 1156-63.
11. Long, KS, Poehlsgaard, J, Kehrenberg, C et al. The Cfr rRNA methyltransferase confers resistance to phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin A antibiotics. Antimicrob Agents Chemother 2006; 50: 2500-5.