Title: The Combination of Daptomycin plus Fosfomycin has Synergistic, Potent and Rapid Bactericidal Activity against Methicillin-Resistant *Staphylococcus aureus* (MRSA) in a Rabbit Model of Experimental Endocarditis (EE).

Running Title: Daptomycin plus fosfomycin or cloxacillin in MRSA EE

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Abstract

This study aims to investigate whether the addition of fosfomycin or cloxacillin to daptomycin provides better outcomes in the treatment of methicillin-resistant *Staphylococcus aureus* (MRSA) experimental aortic endocarditis in rabbits. Five MRSA strains were used to perform *in vitro* time-kill studies at standard (10^6) and high (10^8) inocula. Combined therapy was compared with daptomycin monotherapy treatment in the MRSA experimental endocarditis model. A human-like pharmacokinetics model was applied and the equivalents of cloxacillin 2 g/4h iv, fosfomycin 2 g/6h iv and daptomycin 6-10 mg/kg/d iv were administered. The combination of daptomycin and fosfomycin or cloxacillin was synergistic in the five strains tested at both inocula. A bactericidal effect was detected in four out of five strains tested with both combinations. The MRSA-277 strain (vancomycin MIC, 2 mcg/mL) was used for the experimental endocarditis model. Daptomycin plus fosfomycin significantly improved the efficacy of daptomycin monotherapy at 6 mg/kg/d in terms of both the proportion of sterile vegetations (100% vs. 72%, *P*=.046) and the decrease in the density of bacteria within the vegetations (*P*=.025). Daptomycin plus fosfomycin was as effective as daptomycin monotherapy at 10 mg/kg/d (100% vs. 93%, *P*=1.00) and had better activity than daptomycin plus cloxacillin when daptomycin was administered at 6 mg/kg/d (100% vs. 88%, *P*=0.48), but the differences were not statistically significant. Daptomycin non-susceptibility was not detected in any of the isolates recovered from vegetations. In conclusion, for the treatment of MRSA experimental endocarditis, the combination of daptomycin plus fosfomycin showed synergistic, potent, and rapid bactericidal activity.

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Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) endocarditis is a difficult-to-treat infection, which is frequently associated with undesirable outcomes (1-3).

Daptomycin is recommended for the treatment of MRSA native valve endocarditis (4, 5). It is a concentration-dependent lipopeptide antibiotic that has shown bactericidal activity against the stationary and logarithmic phases of growth in Gram-positive bacteria (6, 7) and a great ability to penetrate cardiac vegetations (8, 9). However, clinical failures have been frequently described in MRSA bacteremia and endocarditis (10). In a randomized clinical trial, only one (11%) of nine patients with left-side infective endocarditis treated with intravenous daptomycin at 6 mg/kg daily was cured, while the emergence of resistance was observed in six of 19 patients with microbiological failure (2). Different strategies to improve daptomycin efficacy against MRSA infections have been evaluated in recent years. The use of higher doses of daptomycin showed greater *in vitro* activity compared with standard doses (11). Moreover, daptomycin toxicity did not increase when used at such doses (12), making 8-10 mg/kg the current recommended dose of daptomycin in MRSA endocarditis (4, 5).

Combined antibiotic therapies have been evaluated, aiming to improve daptomycin efficacy. Although scarce experience has been reported to date concerning the treatment of left-sided endocarditis with daptomycin combinations, associations with β-lactams have shown *in vitro* synergism and great clinical efficacy in MRSA bacteremia. For example, Dhand et al. reported microbiologic and clinical cure in seven episodes of MRSA complicated bacteremia treated with daptomycin and nafcillin, and described synergistic activity between the antibiotics (13). In another study, ceftaroline combined

with daptomycin rapidly cleared the blood cultures of patients with refractory staphylococcal bacteremia (14). Other β-lactams have also shown *in vitro* synergism (15), and today these combinations are being evaluated in a randomized clinical trial so as to clarify their real benefits with respect to monotherapy (ClinicalTrials.gov Id: NCT02365493) (16).

Our group recently reported the experience of three patients diagnosed with left-sided staphylococcal endocarditis and cured with daptomycin combined with fosfomycin, demonstrating the existence of *in vitro* synergy between the two antibiotics (17). Fosfomycin is a cell-wall synthesis inhibitor and is FDA approved for the treatment of uncomplicated urinary tract infections. However, it has also shown good antimicrobial activity against a broad spectrum of pathogens including MRSA (18), as well as synergism with daptomycin against *S. aureus* (19). There have been reports of successful therapy of MRSA invasive infections with fosfomycin since the 1980s (20, 21). Currently our group is participating in a clinical trial to evaluate the efficacy of daptomycin and fosfomycin versus daptomycin in MRSA bacteremia (Trial registration number: NCT01898338) (22).

Since daptomycin-based combinations with β-lactams or fosfomycin have not been compared *in vivo*, it is not known which show greater activity in left-sided MRSA endocarditis. The aim of this study then was to evaluate the activity of daptomycin plus fosfomycin in comparison with daptomycin plus cloxacillin in the treatment of the experimental endocarditis caused by MRSA.

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Results

Susceptibility testing

The MICs/MBCs for cloxacillin, fosfomycin and daptomycin of the five strains are summarized in Table 1. All strains were susceptible to daptomycin, fosfomycin and vancomycin according to the CLSI standard MIC breakpoints (23).

In vitro time-kill studies

In Table 2 are displayed the results of the time-kill synergy studies for the daptomycin plus fosfomycin or cloxacillin combinations. Two different initial inocula were tested: a standard inoculum (ISI) of 10^5-10^6 colony-forming units (CFU)/ml and a higher inoculum (IHI), to mimic the density of cfu in mature infected vegetation, equal to 10^8 cfu/ml.

After 24h of incubation with daptomycin plus fosfomycin (Table 2A) with ISI, synergistic activity was observed in the five strains, and bactericidal effect was observed in 4/5 the strains. When IHI was used, the five strains retained the synergy and 4/5 presented bactericidal effect. The daptomycin plus cloxacillin combination (table 2B) showed synergistic activity for all five strains and bactericidal effect against 4/5 of the studied strains with both ISI and IHI. Thus, daptomycin plus cloxacillin showed similar activity to daptomycin plus fosfomycin.

Human PK simulation studies

The values of the pharmacokinetic parameters for daptomycin and fosfomycin antibiotics have been previously described (8, 21). The results of the human-like approach of cloxacillin simulating the human dose of 2 g/4h (24) are shown in Figure 1

and Table 3 (PK parameters, mean maximum (C_{max}) and trough (C_{min}) concentrations achieved were 150 mcg/ml and 1 mcg/ml, respectively, for a 2 g/4h simulated dose).

Treatment of established endocarditis

The relative effectiveness of drugs in monotherapy and in combined therapy is shown in Table 4. All control rabbits had infected aortic valve vegetations, with a median bacterial titer equal to 10 $\log_{10}$ cfu/g veg. For this strain, the daptomycin plus fosfomycin arm (16/16, 100% sterilization) was significantly more active than daptomycin at 6 mg/kg both in the proportion of sterile vegetations (13/18, 72%; *P*= 0.046) and in the reduction of the density of bacteria within the valve vegetations (*P*=0.025). It also showed a slightly better activity (with no statistical significance) than daptomycin monotherapy at 10 mg/kg/qd (14/15, 93%; *P*=1). Daptomycin plus cloxacillin also showed good activity but did not significantly improve the activity over daptomycin monotherapies. When comparing the two, combined therapy arms, daptomycin plus fosfomycin showed a trend towards being more active than daptomycin plus cloxacillin but without reaching the statistical significance: *P*=0.48 for the sterile vegetations rate and *P*=0.15 for the reduction of bacterial density in the vegetations. In any case, daptomycin-resistant strains were recovered from vegetations.

Discussion

Our study is the first to compare the *in vivo* activity of daptomycin combined with cloxacillin or fosfomycin in an MRSA experimental endocarditis model. Both combinations had previously shown *in vitro* synergism and clinical efficacy in a few case reports (13, 17); however, they had never been compared *in vivo*. Our results provide some evidence of the comparable bactericidal activity of both antibiotic regimens. It is worth noting that both combinations showed superior efficacy to daptomycin monotherapy at 6 mg/kg in terms of the proportion of sterile vegetations and the reduction of the density of bacteria within the valve vegetations. Daptomycin plus fosfomycin had slightly better activity than daptomycin plus cloxacillin and daptomycin administered at higher doses – equivalent to 10 mg/kg – although these differences were not statistically significant. Therefore, the combination appears to be a promising option in clinical practice for patients with methicillin-resistant *Staphylococcus aureus* infective endocarditis, regardless of any allergy to β-lactams.

Despite β-lactams lack of direct *in vitro* activity against MRSA, it is worth noting that two different mechanisms have been proposed explaining their synergistic activity with daptomycin. On the one hand, they increase daptomycin activity through cell-wall charge reduction mediated by the β-lactams, in general, and by nafcillin, in particular, driving an increase in daptomycin binding (13). In addition, PBP1-selective beta-lactam inhibition enhances the antimicrobial efficiency of daptomycin (25,26), resulting in an increased frequency of septation and cell-wall abnormalities, although in this scenario, nafcillin is not a selective PBP inhibitor. On the other hand, they attenuate MRSA virulence and boost innate immunity (27,28). Beta-lactams improve MRSA

opsonisation and phagocytosis by leukocytes (28,29) and the activity of cationic antimicrobial peptides (CAP) (28,29). Meanwhile, in our study, daptomycin and fosfomycin were both active against MRSA strains and, when combined, had synergistic and bactericidal activity. This synergistic activity may be explained by fosfomycin PBP-1 inhibition (21,25,26) and by fosfomycin's ability to modify cell-wall protein composition (30). It is not known if some of the other indirect effects on MRSA strains described above for nafcillin may also be observed in MRSA fosfomycin-resistant strains.

This study has some limitations. First, combined therapies using high doses of daptomycin were not evaluated, although the synergistic effect would probably be maintained. Second, while other studies have shown that the combination of daptomycin plus nafcillin is synergistic against daptomycin non-susceptible strains (13,31), it would have been of interest to know if daptomycin plus fosfomycin was synergistic against these strains. And third, although the efficacy of daptomycin plus either cloxacillin or fosfomycin was evaluated in only one strain in the animal model, the synergism and bactericidal activity was demonstrated *in vitro* in five strains with both the standard and the high inocula.

In conclusion, both the regimens of either cloxacillin or fosfomycin combined with daptomycin had bactericidal activity against MRSA in a rabbit model of experimental endocarditis, with fosfomycin plus daptomycin being the more potent and rapid of the two. This combination is currently being studied in a clinical trial (EUDRACT# 2013-000586-37) to assess its efficacy and safety when compared with daptomycin monotherapy at high doses for MRSA bacteremia and IE (22).

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MATERIALS AND METHODS

Bacterial isolates

For the *in vitro* studies, five SARM isolates were selected: SARM-196, SARM-277, SARM-513, SARM-726, and SARM-835, isolated from blood cultures in patients diagnosed with IE at our center. From among these five strains, MRSA-277 was selected for the *in vivo* study. The isolates were stored at -80º C in skim milk.

Antimicrobial agents

Daptomycin powder was supplied by Cubist Pharmaceuticals, (Lexington, MA, USA); fosfomycin and cloxacillin were purchased from Sigma (St Louis, MO). The drugs were prepared according to the manufacturer's recommendations.

Susceptibility Testing

Minimal inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs) were determined using the broth microdilution method according to standard recommendations (23). Fosfomycin susceptibility testing was done in Mueller-Hinton broth supplemented with D-glucose 6-phosphate (Sigma, St Louis, MO, USA) to a final concentration of 25 mg/L. For daptomycin, the broth was supplemented with Ca^{2+} to 50 mg/L according to the manufacturer's recommendations. *S. aureus* ATCC 29213 was used as the test control strain. All the results were double-checked.

Time-kill curves were performed with daptomycin and fosfomycin or cloxacillin at concentrations of ¼xMIC and 1xMIC, using two different initial inocula: a standard inoculum of 10^5-10^6 colony-forming units (CFU)/ml (ISI), according to previously described criteria (21), and a higher inoculum to mimic the density of cfu in a mature

infected vegetation equal to 10^8 cfu/ml (IHI). Before inoculation, each tube of fresh cation-adjusted Mueller-Hinton broth was supplemented with Ca^{2+} to 50 mg/L and D-glucose 6-phosphate to test fosfomycin, as described previously (21). All experiments were performed in duplicate, as recommended (32). Bactericidal activity was defined as a ≥3-log10 decrease in cfu/ml of the initial inoculum at 48h. At 24h, the results of the combination were compared with those of the most active single drug; synergy, indifference, and antagonism were then defined as a ≥2-log increase in killing, a <2-log change (increase or decrease) in killing, and a ≥2-log decrease in killing, respectively.

Study animals

Female, New Zealand white rabbits (body weight, 2.5 Kg) provided by San Bernardo farm (Pamplona, Spain) were housed in the animal facilities of the University of Barcelona's School of Medicine, which is equipped with high-efficiency particulate air filter in an automatic air exchange system, as well as a circadian light cycle. They were nourished *ad libitum.* The Committee of Animals Ethics of the University of Barcelona approved all animal experimentation in this study.

Human pharmacokinetics (PK) simulation studies

Following the recommendations of the AHA (5) and IDSA (33) guidelines, the following antibiotic regimens were chosen: Cloxacillin (2 g/4h iv), daptomycin (6 mg/kg or 10 mg/kg iv once daily) and fosfomycin (2 g/6h iv).

Antibiotics were administered using a computer-controlled infusion pump system designed to reproduce human serum pharmacokinetics in rabbits. The *in vivo* experimental pharmacokinetics of daptomycin and fosfomycin have already been described (8,21). To determine the cloxacillin animal pharmacokinetic parameters (24),

a single dose of cloxacillin 50 mg/kg iv was administered to five healthy rabbits. At different times (0, 0.025, 0.5, 0.75, 1, 1.25, 1.5 and 2 hours), a milliliter of blood was collected through a catheter placed in the carotid artery. Samples were centrifuged at 13,000 rpm for 20 min, plasma was removed and cloxacillin concentration was measured by high-performance liquid chromatography assay. The method was validated and applied for drug quantification in rabbit plasma at the Pharmacy Research Laboratory. Plasma samples were analyzed according to the methodology described by Giang do T et al. (34). Cloxacillin was extracted from the rabbit plasma by protein precipitation with acetonitrile. The chromatographic column was a NovaPak C_{18} (150*3.9 mm) (Waters Corporation, Milford, MA, USA). The mobile phase composition was 0.01 M KH_2PO_4 – methanol (45:55, v/v). Isocratic flow rate was set at 0.7 ml/min and UV-absorbance detection at 225 nm. Under these chromatographic conditions, the retention time was found to be 5.1 min. The method showed a good linearity: 0.5-64 mcg/ml (r^2 = 0.99). Inter-inaccuracy was 3.6-5.6%. Intra- and inter-imprecision were 1.5-3.8% and 4.5-5.03%, respectively. Mean recovery was 105.7%. The limit of detection and lower limit of quantitation were 0.3 and 0.5 mcg/ml, respectively.

Endocarditis model

The experimental aortic valve IE model was induced according to the method described by Garrison and Freedman (35). Briefly, a catheter was inserted through the right carotid artery into the left ventricle of anaesthetized rabbits; the catheter used for antibiotic administration was placed into the inferior vena cava through the jugular vein (8). The infusion pump delivered 2 ml/h of 0.9% saline solution until the beginning of antimicrobial administration. Twenty-four hours later, each animal was inoculated via

the marginal ear vein with MRSA-277 strain (1 ml of 5.5 x 10^5 colony forming units [cfu/ml). Before the initiation of antimicrobial therapy, one milliliter of blood was obtained to confirm bacteremia. Antibiotic treatments were started and animals were treated for 48h using a computer-controlled pump. After completion of the treatment, an additional six half-lives were allowed to elapse before the animals were sacrificed (anesthetized and euthanized using an intravenous bolus of pentobarbital). Aortic valve vegetations were obtained, weighed, homogenized in 2 ml of saline solution, and quantitative and qualitative cultures were performed.

Treatment group

The infected rabbits were separated into different treatment arms simulating human pharmacokinetics. Monotherapy: daptomycin high dose (HD) of 10 mg/kg/d; daptomycin low dose (LD) of 6 mg/kg/d. Combined therapy: daptomycin 6 mg/kg/d plus cloxacillin 2 g/4h or fosfomycin 2 g/6h. Each group included from 15 to 18 animals.

Analysis of endocardial vegetations

The cfu counts recovered from vegetations were expressed as the number of $\log_{10}$ cfu per gram of vegetation ($\log_{10}$ cfu/g veg). The result was assigned a value of 2 $\log_{10}$ cfu per gram of vegetation if there was no growth on the quantitative plates but there was growth in the qualitative culture and from the homogenate cultures for a week. The result was assigned a value of zero, and the vegetation was considered sterile if there was no growth from the initial quantitative and qualitative culture or from the homogenates cultured for a week.

All the isolates recovered from vegetations were stored, and their MICs re-tested to detect *in vivo* emerging resistance to daptomycin.

Statistical analysis

The results were expressed as the median and the interquartile range (IQR) of the number of $\log_{10}$ cfu/g veg. The Mann Whitney non-parametric test was used to compare the $\log_{10}$ cfu tissue values among the different treatment groups. The Fisher exact test was used to compare the rate of sterilized vegetations and to analyze whether there were differences between treatment groups.

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528 **Table 1. *S. aureus* strains tested and MICs/MBCs.**

529

MIC/MBC (mg/liter)				
STRAINS	**Daptomycin**	**Cloxacillin**	**Fosfomycin**	**Vancomycin**
MRSA-196	0.25/0.5	16/64	4/8	0.5/8
MRSA-277*	0.5/0.5	512/512	4/4	2/2
MRSA-513	0.5/0.5	512/512	8/64	1/128
MRSA-726	0.5/0.5	16/64	4/32	0.5/0.5
MRSA-835	0.5/0.5	128/256	8/16	1/16

530

531 **In vivo* study strain

Table 2. *In vitro* time-kill synergy study.

2A. Methicillin-resistant *S. aureus* (MRSA) Daptomycin (DAP) + Fosfomycin (FOM) Time-Kill Curves (antibiotic concentrations tested at 1 x MIC with two different inocula).

Strains tested		CONTROL Δ Change (x hours) in log_{10} cfu/ml		DAP Δ Change (x hours) in log_{10} cfu/ml		FOM Δ Change (x hours) in log_{10} cfu/ml		DAP+ FOM Δ Change (x hours) in log_{10} cfu/ml	
Baseline (0 hours) Log_{10} CFU/mL		4h	24h	4h	24h	4h	24h	4h	24h
Standard inoculum (10^6 cfu/ml)									
MRSA-196	6.3	+1	+1.8	-1	+2.6	-0.6	+1.8	-2.4	-3.8
MRSA-277*	6	+2	+3.1	-3.4	+0.5	-1.4	+0.5	-4	-4
MRSA-513	6	+2	+3	-1.3	+2.9	-0.4	+1.1	-2.5	-2.6
MRSA-726	6.1	+2	+2.6	-1.2	+2.2	-0.7	+0.7	-2.6	-4.1
MRSA-835	6	+2	+2.9	-0.4	+1.6	-1	+2.9	-2	-4
High inoculum (10^8 cfu/ml)									
MRSA-196	8	+0.3	+0.6	-2.1	-1.7	-0.1	-2.4	-3	-4.9
MRSA-277*	8	+0.7	+1	-3.3	+0.8	-0.4	-2	-4	-4.2
MRSA-513	8	+0.9	+1	-2.9	-0.8	-0.2	-1.5	-3.2	-6
MRSA-726	8	+1	+1	-2.1	+1	-0.2	-1	-3.5	-1.9
MRSA-835	8.1	+0.9	+0.9	-2	-4	-0.2	-1.5	-2.8	-5.3

In vivo study strain

2B. Methicillin-resistant *S. aureus* (MRSA) Daptomycin (DAP) + Cloxacillin (CLO) Time-Kill Curves (antibiotic concentrations tested at 1 x MIC with two different inocula).

Strains tested		CONTROL Δ Change (x hours) in log_{10} cfu/ml		DAP Δ Change (x hours) in log_{10} cfu/ml		CLO Δ Change (x hours) in log_{10} cfu/ml		DAP+ CLO Δ Change (x hours) in log_{10} cfu/ml	
Baseline (0 hours) Log_{10} CFU/mL		4h	24h	4h	24h	4h	24h	4h	24h
Standard inoculum (10^6 cfu/ml)									
MRSA-196	6.1	+1.8	+3.2	-0.6	+3.1	-0.3	+1.7	-1.6	-1
MRSA-277*	5.8	+2.2	+3.3	-2.5	+2.3	-0.2	+2.8	-3.8	-3.8
MRSA-513	6	+2.1	+3	-1.4	+2.9	-0.1	+2.6	-2.2	-2.7
MRSA-726	6	+2	+3	-0.8	+2.8	+1	+2	-3	-4
MRSA-835	6	+2.3	+3.5	-1.5	+3.5	-0.9	+3.2	-2.8	-4
High inoculum (10^8 cfu/ml)									
MRSA-196	8.1	+0.3	+0.8	-3.2	-2.1	-0.3	-0.2	-3.7	-4.9
MRSA-277*	7.9	+0.7	+1.2	-3.3	+1.1	-1.9	+0.8	-3.8	-3.6
MRSA-513	7.9	+0.9	+1.1	-2.3	-0.6	-1	+0.7	-1.9	-5.1
MRSA-726	8.1	+0.9	+0.9	-2.1	+1	-0.1	+0.4	-2.9	-1.7
MRSA-835	7.9	+1	+1.1	-1.5	-1.4	-0.1	+0.7	-2.3	-5

* *In vivo* study strain

547 **Table 3. Cloxacillin pharmacokinetic parameters.**

pK parameters[a]	**Previously Reported Human Values (Single Dose)**[24]	**Animal Values (n = 5)**	**Human-like Values in Animals (n = 5)**
Dose	2 g iv	-	-
C_{max}/C_{min} (μg/ml)	150/0.6	-	160/0.96
k_{el} (h^{-1}) [mean ± SD]	1.39	2.42 ± 0.44	1.43 ± 0.12
$t_{½}$ β (h) [mean ± SD]	0.5	0.29 ± 0.06	0.49 ± 0.04
AUC_{0-4} (μg·h/ml) [mean ±SD]	108.2	63.41 ± 12.55	105.1 ± 8.95

548

549 [a]C_{max}/C_{min}, maximum/minimum concentration of drug in serum; k_{el}, elimination rate

550 constant; SD, standard deviation; $t_{½}$β, terminal half-life; AUC, area under the

551 concentration-time curve.

552

Table 4. Treatment of experimental endocarditis caused by MRSA-277

Treatment group	No. of rabbits with sterile vegetations / total no. of rabbits (%)	Log_{10} cfu/g vegetation [median (IQR)]
Control	0/15 (0)	10 (9.8-10)
Daptomycin (simulating 6 mg/kg/qd)	13/18 (72)[a,b,c]	0 (0-1.5)[f,g]
Daptomycin (simulating 10 mg/kg/qd)	14/15 (93)[a,d]	0 (0-0)[h]
Daptomycin + Fosfomycin (simulating 6 mg/kg/qd + 2g/6h)	16/16 (100)[b,d,e]	0 (0-0)[f,h,i]
Daptomycin + Cloxacillin (simulating 6 mg/kg/qd + 2g/4h)	14/16 (88)[c,e]	0 (0-0)[g,i]

[*]The control animals were sacrificed 24h after the infection was started; [a] *P*=.19; [b]*P*=.046; [c]*P*=.40; [d]*P*=1; [e]*P*=.48; [f]*P*=.002; [g]*P*=.46; [h]*P*=.35; [i]*P*=.15. cfu, colony-forming unit; IQR, interquartile range.

Figure legend.

Figure 1. Serum levels of cloxacillin. “Human-profile” represents the antibiotic serum values obtained in a human being (24). “Human-like profile” represents the antibiotic serum values obtained in rabbits when the fitting model was used.

Figure 1. Serum levels of cloxacillin.