



Activities of Combinations of Antistaphylococcal Antibiotics with Fusidic Acid against Staphylococcal Biofilms in *In Vitro* Static and Dynamic Models

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ABSTRACT Staphylococcal biofilms are a major cause of therapeutic failure, especially when caused by multiresistant strains. Oral fusidic acid is currently being redeveloped in the United States for skin, skin structure, and orthopedic infections, in which biofilms play a major role. The aim of this study was to examine the activity of fusidic acid alone or combined with other antistaphylococcal drugs against biofilms made by a reference strain and five clinical isolates of *Staphylococcus aureus* or *Staphylococcus epidermidis* in *in vitro* static and dynamic models (microtiter plates and a CDC reactor) exposed to clinically relevant concentrations. In microtiter plates, antibiotics alone were poorly active, with marked differences among strains. At concentrations mimicking the free-drug human maximum concentration of drug in serum (C_{max}), the combination of fusidic acid with linezolid, daptomycin, or vancomycin resulted in increased activity against 4 to 5 strains, while the combination with doxycycline, rifampin, or moxifloxacin increased activity against 1 to 3 strains only. In the CDC reactor, biofilms were grown under constant flow and antibiotic concentrations decreased over time according to human elimination rates. A bactericidal effect was obtained when fusidic acid was combined with daptomycin or linezolid, but not with vancomycin. The higher tolerance of biofilms to antibiotics in the CDC reactor is probably attributable to the more complex architecture they adopt when growing under constant flow. Because biofilms grown in the CDC reactor are considered more similar to those developing *in vivo*, the data support further testing of combinations of fusidic acid with daptomycin or linezolid in models pertinent to chronic skin, skin structure, or orthopedic infections.

KEYWORDS fusidic acid, daptomycin, linezolid, biofilm, CDC biofilm reactor, *Staphylococcus aureus*, *Staphylococcus epidermidis*

Biofilms consist of bacteria encased in a hydrated matrix of polysaccharides, DNA, and proteins, adhering on biotic or abiotic surfaces. Staphylococci are among the bacterial species that are the more prone to cause such biofilm-related infections. As frequent commensals of the skin, they can easily contaminate implanted material during surgery (1), causing biofilm-related infections of drains and catheters (2), implanted cardiac electrophysiological devices and heart valves (3, 4), or orthopedic prostheses (5). Yet, they can also form biofilms on tissues and have been incriminated in chronic rhinosinusitis (6), lung infection in cystic fibrosis patients (7), and wound infections (8, 9).

Bacteria in biofilms are more tolerant to antibiotics than planktonic forms, due to a combination of factors that include not only a restricted access of the drugs to the bacteria embedded in the matrix but also a lower metabolic activity when deep in the structure, which makes them recalcitrant to many antibiotics (10). The problem is made

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even more complex and therapeutically challenging by the fact that staphylococci are often resistant to many currently used antibiotics. In this context, the spread of methicillin-resistant *Staphylococcus aureus* or *Staphylococcus epidermidis* strains that have acquired multiple resistance mechanisms to conventional classes of drugs is alarming (11, 12). While a series of new drugs active against Gram-positive organisms showing enhanced potency against multiresistant strains have been approved over the last years (13), there is also growing interest in reviving old drugs that still show activity against the multiresistant strains, because they have been sparsely used since the time they were brought to the market (14). Fusidic acid is an antibiotic with a long history in Europe and other parts of the world (15) where it has been used since the early 1970s in systemic (intravenous and oral) and topical (ophthalmic and skin preparations) formulations primarily for the treatment of staphylococcal infections, including in orthopedic surgery infections caused by *S. epidermidis* (16). For these indications, it offers the advantages of being available orally and reported as safe (17). Yet, as a consequence to this wide usage (including topical use), resistance has emerged in many European countries. Although still lower than 10% in most surveys (see reference 18 for a review), this resistance incidence has justified a loss of interest for this drug in the affected countries. In contrast, fusidic acid has not been used commercially in the United States as it has never received marketing approval from the Food and Drug Administration (FDA). As a result, resistance to fusidic acid remains rare in North America, including in multiresistant strains (19–21). Fusidic acid is currently being developed in the United States with an optimized dosing regimen for acute bacterial skin and skin structure infections (22) and as salvage therapy for chronic bone and joint infections (18). Although biofilms are preponderant in such infections, only limited data on its activity against biofilms have been published, essentially showing that minimum biofilm eradication concentrations (MBEC) are much higher than MICs for susceptible strains (23–25).

The aim of this study was therefore to evaluate the activity of fusidic acid used alone or in combination with other antistaphylococcal drugs against biofilms. Rodent models with fusidic acid do not enable the mimicking of human pharmacokinetics (plasma concentrations are too low [26, 27]). We therefore used in parallel the static model of biofilms grown in microtiter plates previously developed in our laboratory (28) and a dynamic model of biofilms grown in the CDC reactor (29). This device, where biofilm formation takes place under shear stress, is considered to provide the most conservative estimate of efficacy against biofilms for antiseptics, because it mimics the fluid flow conditions found *in vivo* (30). It can also be used to assess the effects of antimicrobial agents on biofilms (31–33). Here, we also took advantage of the continuous flow in the device to mimic the pharmacokinetic profiles of the drugs in human serum. In a nutshell, we show a strong antibiofilm activity for fusidic acid when combined with daptomycin or linezolid in both the static and dynamic models.

RESULTS

Activity of fusidic acid in combination with other antistaphylococcal agents in a static biofilm model. In a first step, we measured the activity of antibiotics alone against 24-h-old biofilms grown in microtiter plates. We used in parallel a reference strain and five clinical isolates originating from infection sites where biofilms play a preponderant role (Table 1). Figure 1 summarizes the data obtained when biofilms were exposed to fusidic acid at a fixed concentration corresponding to the predicted human maximum concentration of its free, unbound fraction in serum (fC_{max}) for the therapeutic scheme used in current clinical trials (Table 2). The other antibiotics were also tested at their respective fC_{max} s and used alone or combined with fusidic acid. The reduction in bacterial viability (measured by the conversion of resazurin into resorufin by metabolically active bacteria) for each individual strain is presented in Table 3. When used alone, antibiotics showed a variable degree of activity against the different strains investigated, with only daptomycin, linezolid, and moxifloxacin causing a mean reduction in resorufin fluorescence higher than 40% compared to control values. Interest-

TABLE 1 Origin and antibiotic susceptibility of the strains under study

Strain	Species	Origin	MIC (mg/liter)						
			Fusidic acid	Daptomycin	Linezolid	Vancomycin	Moxifloxacin	Rifampin	Doxycycline
ATCC 25923	<i>S. aureus</i>	Reference	0.25	0.5	1	1	0.032	0.064	0.064
2011S027	<i>S. aureus</i>	Chronic skin infection	2	0.5	2	1	0.125	1	0.125
80224422456	<i>S. aureus</i>	Bone infection	8	0.5	2	1	0.25	0.25	0.25
80124430375	<i>S. aureus</i>	Pacemaker infection	1	1	1	1	1	1	0.125
80124432999	<i>S. epidermidis</i>	Knee joint prosthesis infection	1	0.5	2	2	0.125	0.25	0.125
80124440624	<i>S. aureus</i>	Pacemaker infection	1	1	4	1	0.125	0.25	0.25

ingly, the highest activity was not systematically observed against the same strain for the different antibiotics studied. For fusidic acid alone, a mean reduction in the fluorescence signal of 35% was obtained. When looking at combinations, a significant increase in the mean activity (lower fluorescence signal) compared to those of the drugs alone was observed for fusidic acid with daptomycin or vancomycin (Fig. 1). Yet, when looking at data for individual strains, an increase of $\geq 15\%$ activity versus the most active drug alone in the combination was observed for vancomycin, daptomycin, or linezolid against five, five, or four strains, respectively, but against three strains or less for the other antibiotics (Table 3). To further document these effects, we studied the activity of fusidic acid over a broad range of concentrations when used alone or combined with each of the three most active drugs at their respective minimum concentration of the free, unbound fraction of the drug in serum (fC_{min}) or fC_{max} values against strain ATCC 25923 (Fig. 2) or with each drug at its fC_{max} against one selected clinical isolate (80224422456) (Fig. 3). Against ATCC 25923, all combinations increased the activity of fusidic acid over a broad range of concentrations, including the whole range of clinically achievable ones. Against isolate 80224422456, increased activity was observed only when daptomycin, linezolid, or vancomycin at their fC_{max} was combined with large concentrations of fusidic acid (fC_{max} or higher). A slight improvement of activity was also noticed when doxycycline at its fC_{max} was combined with fusidic acid over the whole range of concentrations. Yet, no gain in activity (or even a loss in activity

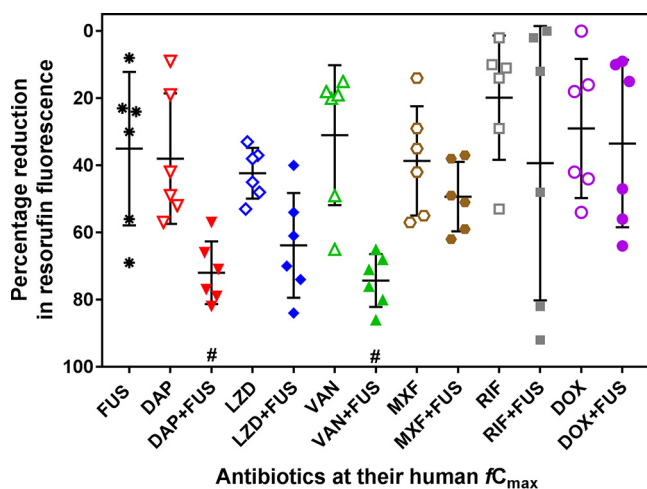


FIG 1 Activity of fusidic acid (FUS) and daptomycin (DAP), linezolid (LZD), vancomycin (VAN), moxifloxacin (MXF), rifampin (RIF), or doxycycline (DOX) alone or in combination with fusidic acid after 48 h of incubation with biofilms from ATCC 25923 and 5 clinical isolates. The graph shows the reduction in bacterial viability evaluated by the percentage reduction in resorufin fluorescence compared to that of the untreated control. Antibiotics are all used at their human fC_{max} values (see Table 2). Each symbol corresponds to a bacterial strain, with the mean reduction and standard deviation appearing as black horizontal lines. Statistical analysis was by one-way analysis of variance (ANOVA) with Tukey's *post hoc* test. #, highlights combinations for which the mean reduction was higher than that observed for the drugs alone. See Table 3 for individual data of each strain.

TABLE 2 Pharmacokinetic properties of the antibiotics used to define drug concentrations in experimental models^a

Antibiotic	Daily dose	Protein binding (%)	Total/free C _{min} (mg/liter)	Total/free C _{max} (mg/liter)	Half-life (h)	Reference
Fusidic acid	1,500 mg q12h on day 1 then 600 mg q12h	90–98^b	~75 (day 1) to 100 (steady state) / 7–10	~140/ 14	12–16	22, 61, 67
Daptomycin	6 mg/kg q24h	90	7/0.7	94/ 9.4	8	64, 68
Linezolid	600 mg q12h	30	13–15/ 9–10.5	21–24/15– 17	6	63, 68
Vancomycin	1 g q12h	50	5–10/ 2.5–5	25–40/12.5– 20	6	68
Moxifloxacin	400 mg q24h	40	0.6/ 0.36	3.1/ 1.9	12	69
Rifampin	600 mg q12h	80	1.25/0.03	~10/ 2	2–3	70
Doxycycline	100 mg q12h	90	0.9/0.09	1.3/ 0.13	18–22	71

^aValues in bold are those used in the present study.

^bDepending on the study (all *in vitro* data), the type of sample (albumin solution, serum sample), and the concentration of albumin and of fusidic acid.

with rifampin) was observed for the other combinations compared to the effects obtained for individual drugs in the combinations.

Activity of fusidic acid in combination with other antistaphylococcal agents in a dynamic biofilm model (CDC reactor). On the basis of the results obtained so far, we selected daptomycin, linezolid, and vancomycin for evaluation of their activity in combination with fusidic acid in an *in vitro* dynamic biofilm model (CDC reactor) that enables the mimicking of the pharmacokinetic profiles of the drugs. Figure 4 shows the experimental *in vitro* pharmacokinetic profiles of the drugs compared to the known rate of elimination in humans (Table 2) as programmed in the system by the adjustment of the flow of the peristaltic pumps. Drug concentrations declined over time at a rate close to the projected value. Antibiofilm activity was then evaluated against the reference strain ATCC 25923 and the clinical isolate 80224422456 (Fig. 5). During the conditioning phase (no antibiotic present), bacteria grew over time to reach a density of approximately 10⁸ CFU/cm² for both strains. When used alone, all antibiotics were bacteriostatic against the biofilms in this model, causing a <1-log₁₀ decrease in CFU. Combining fusidic acid with daptomycin or linezolid caused a significant decrease in bacterial counts that follows a one-phase exponential decay, with *k* values 1.7- to 1.8-fold higher against ATCC 25923 (0.18 and 0.12 h⁻¹ for combinations of fusidic acid with linezolid and daptomycin, respectively) than against the clinical isolate (0.10 and 0.06 h⁻¹, respectively, under the same conditions). A maximal reduction of 3 log₁₀ to 3.5 log₁₀ CFU/cm² was obtained at the end of the experiment for these combinations. In contrast, combining vancomycin with fusidic acid did not cause any significant improvement against both strains compared to the activity of each antibiotic alone.

DISCUSSION

This study distinguishes itself by comparing antibiotic activity against staphylococcal biofilms using not only an *in vitro* static model but also a dynamic model that takes into account the human pharmacokinetics of the drugs under study. Its originality also resides in the evaluation of antibiotic combinations in such a setting. The generated

TABLE 3 Activity of fusidic acid alone or combined with antistaphylococcal antibiotics against biofilms of the reference strain ATCC 25923 and 5 clinical isolates grown in microtiter plates

Strain	Percentage reduction in resorufin fluorescence signal in biofilms exposed to: ^a												
	Fusidic acid alone	Daptomycin		Linezolid		Vancomycin		Moxifloxacin		Rifampin		Doxycycline	
		Alone	+FUS	Alone	+FUS	Alone	+FUS	Alone	+FUS	Alone	+FUS	Alone	+FUS
ATCC 25923	23	52	77	45	74	20	86	35	37	10	2	18	10
2011S027	8	42	79	53	54	15	68	57	49	14	12	16	9
80224422456	24	19	57	37	61	19	71	55	62	2	0	0	47
80124430375	30	9	82	33	70	65	80	14	38	11	82	42	15
80124432999	56	49	71	48	40	49	76	29	51	29	48	54	64
80124440624	69	57	66	38	84	18	65	42	59	53	92	44	56

^aAntibiotics used alone were at their respective *f*C_{max} values or combined with fusidic acid (FUS) at its *f*C_{max} (see Table 2 for values). Values in bold highlight combinations that show a reduction in resorufin fluorescence at least 15% higher than that of the most active drug in the combination when used alone.

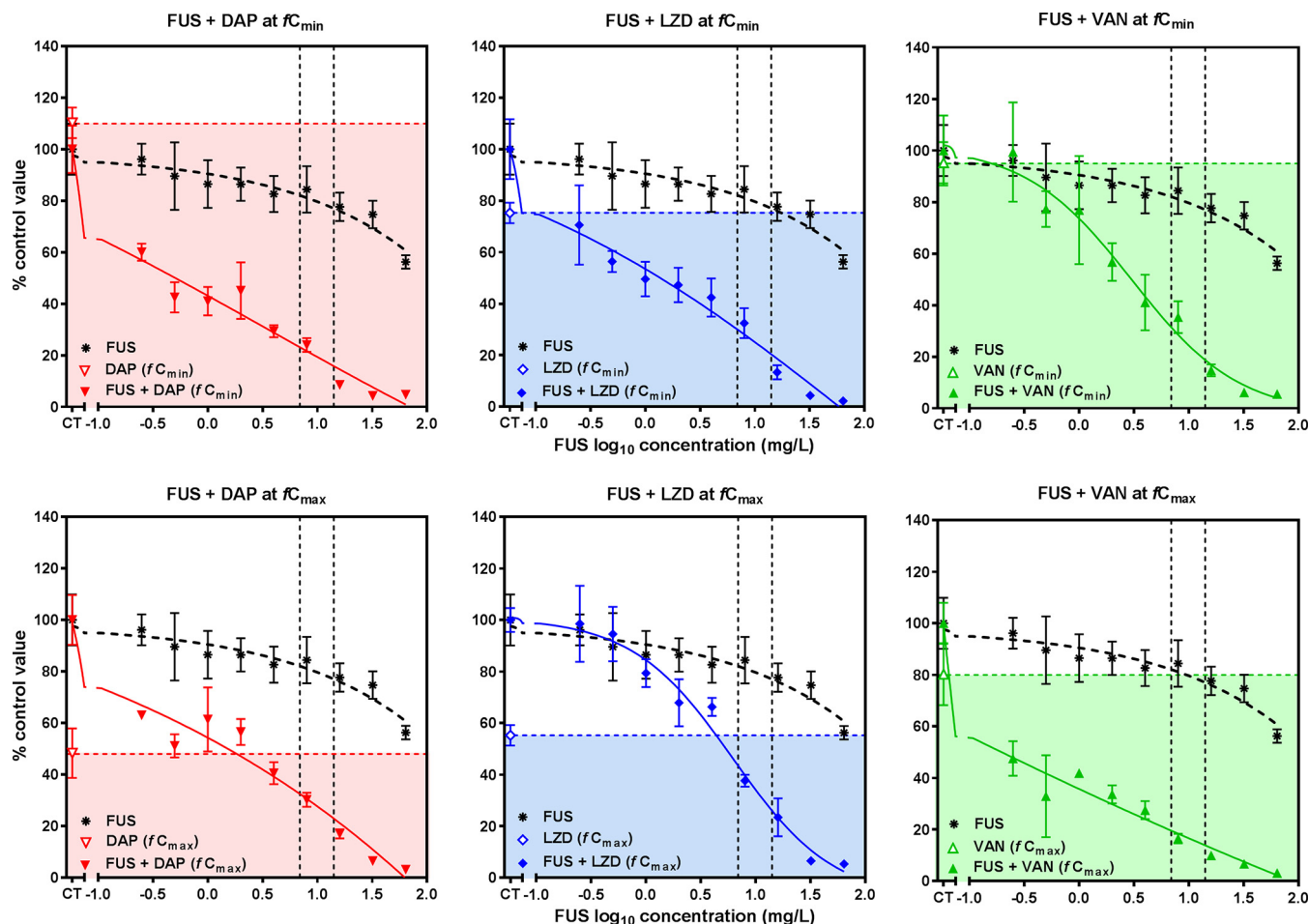


FIG 2 Activity of fusidic acid (FUS) alone or combined with selected antibiotics (daptomycin [DAP], linezolid [LZD], and vancomycin [VAN]) after 48 h of incubation with biofilms from ATCC 25923. The graphs show the residual viability evaluated by the reduction in resorufin fluorescence compared to that of the untreated control (CT) as a function of the concentration of fusidic acid. Combined antibiotics were added at a fixed concentration corresponding to their human fC_{min} (top) or fC_{max} (bottom). The horizontal, colored dotted line in each panel shows the effect of the combined antibiotic used alone at the selected concentration; therefore, all data points located in the colored area correspond to activities higher than that measured for this combined antibiotic alone. The black vertical dotted lines show the fC_{min} and fC_{max} of fusidic acid. All data are the means \pm SDs from quadruplicates.

data highlight the possible interest of combining fusidic acid with daptomycin or linezolid in the context of biofilm-related infections.

Considering first the activity of antibiotics alone in the static biofilm model, we show here that it is globally rather poor, with a <40% reduction in the viability signal being observed in most of the cases with drugs tested at clinically relevant concentrations. This is consistent with previous data obtained in the same model (28, 34). Since the intrinsic potency of the drugs against planktonic cultures (MIC values) are essentially of the same order of magnitude against all strains, the huge variation in activity of each individual drug against the same strains when studied in biofilm points to a major impact of biofilm thickness and/or matrix composition on the expression of their activity in this setting (34, 35). Conversely, it is not surprising that the activity of all antibiotics is not affected to the same extent against a specific strain, since drug interactions with matrix constituents (via hydrogen, hydrophobic, electrostatic, or van der Waals bonds [36]) are largely dependent on their chemical structure. Altogether, these observations underline the difficulty of drawing general conclusions from unique or sparse data and thus the necessity of testing a panel of clinically relevant isolates when evaluating antibiotic activity against biofilms. Taking fusidic acid as an example, a previous study on a single isolate concluded it is inactive against biofilms (37), which is clearly not the case here, at least for some of the isolates investigated. Moreover,

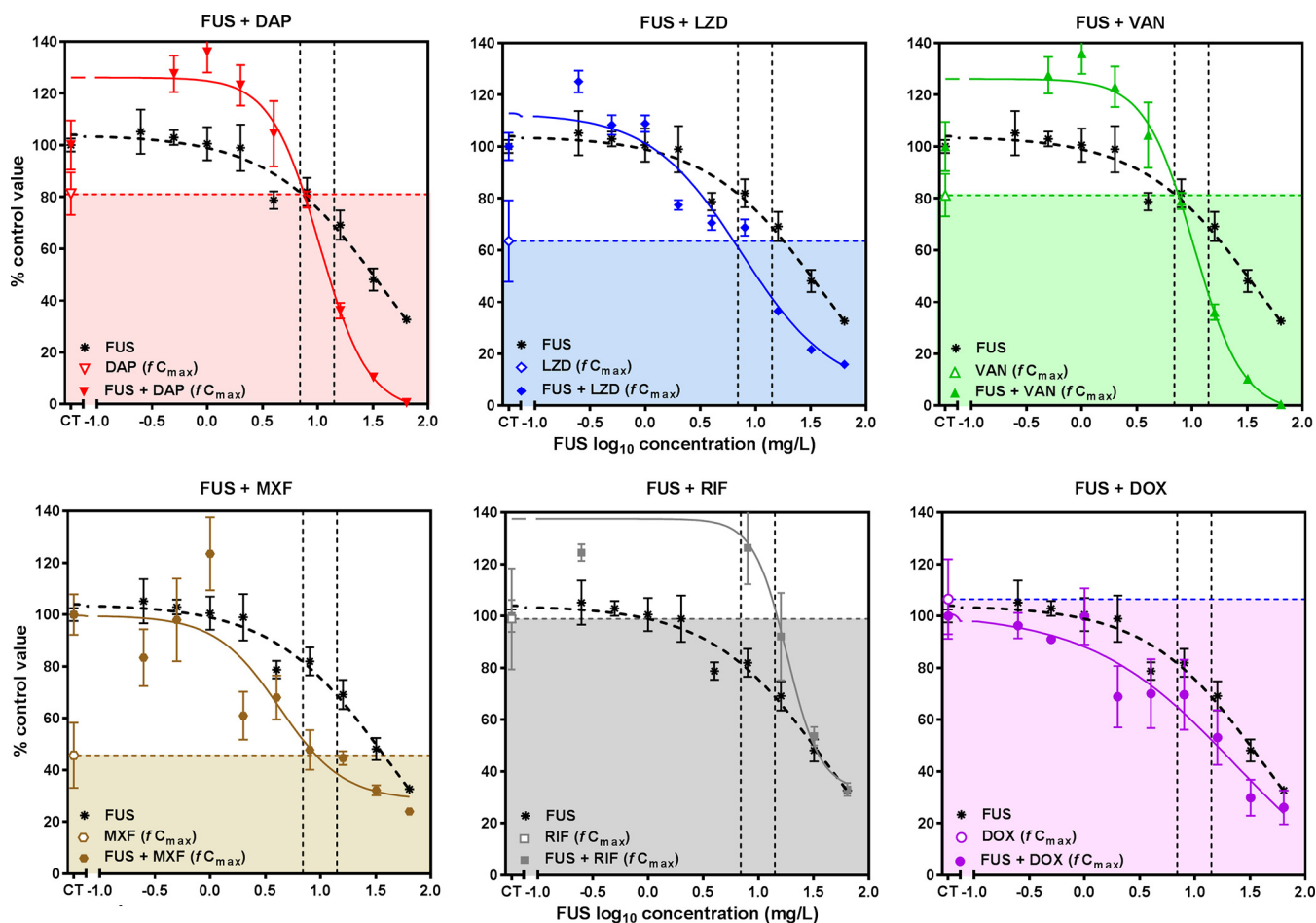


FIG 3 Activity of fusidic acid (FUS) alone or combined with selected antibiotics (daptomycin [DAP], linezolid [LZD], vancomycin [VAN], rifampin [RIF], moxifloxacin [MXF], and doxycycline [DOX]) after 48 h of incubation with biofilms from the clinical isolate 80224422456. The graphs show the residual viability evaluated by the reduction in resorufin fluorescence compared to that of the untreated control (CT) as a function of the concentration of fusidic acid. Combined antibiotics were added at a fixed concentration corresponding to their human fC_{max} . The horizontal, colored dotted line in each panel shows the effect of the combined antibiotic alone at the selected concentration; therefore, all data points located in the colored area correspond to activities higher than that of the combined antibiotic alone. The black vertical dotted lines show the fC_{min} and fC_{max} of fusidic acid. All data are the means \pm SDs from quadruplicates.

most published studies (including those with fusidic acid [23–25]) only focus on MBEC determinations, which does not provide any information about the concentration-response relationship we explored in the present work.

Considering, then, combinations of fusidic acid with other antibiotics in the static model, we see that these can enable marked increases in activity, but again, not in a systematic fashion and depending not only on the associated antibiotic but also on the strain. The most effective and constant effects of combinations in this model were obtained when fusidic acid was associated with daptomycin, linezolid, and vancomycin. Notably, no synergy was observed between fusidic acid and linezolid against isolate 80124432999, which is the only *S. epidermidis* in our collection. Thus, generalization of this observation would require using other *S. epidermidis* strains. Previous works with planktonic cultures have shown synergy between fusidic acid and daptomycin or linezolid (38, 39) and indifference with vancomycin (40), but no mechanistic explanation has been provided. The latter study also reports indifference when combined with ciprofloxacin and synergy with rifampin, which is less clear in our biofilm model. Other studies on static staphylococcal biofilm models demonstrated synergy between fusidic acid and minocycline (23), vancomycin or gentamicin (25), or rifampin (23, 41). However, the interest of the latter combination for therapeutics is limited, because rifampin induces fusidic acid metabolism *in vivo*, generating subtherapeutic serum levels (42, 43).

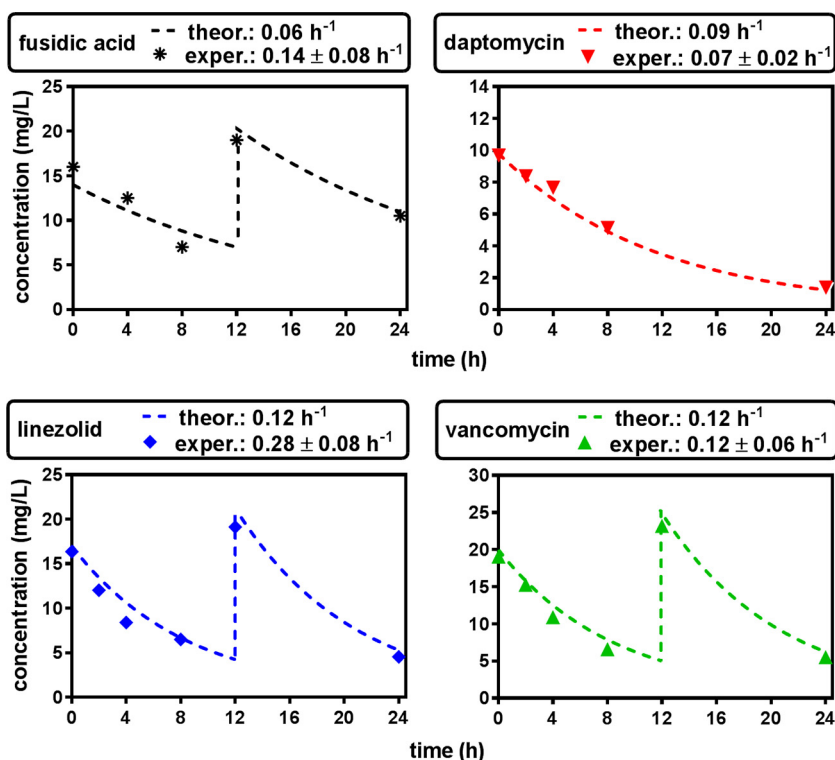


FIG 4 Experimental (exper.) versus theoretical (theor.) concentrations of antibiotics in the CDC reactor. The values at the top of each graph are the elimination constants (k). Fusidic acid (FUS), linezolid (LZD), and vancomycin (VAN) were injected twice (0 h and 12 h), and daptomycin (DAP) once (0 h), at the following concentrations (to mimic fC_{max}): FUS, 14 mg/liter; DAP, 9.8 mg/liter; LZD, 17 mg/liter; VAN, 20 mg/liter. Peristaltic pumps were set up to mimic a half-life ($t_{1/2}$) of 12 h for FUS, 8 h for DAP, and 6 h for LZD and VAN.

Moving, then, to the dynamic model, we noticed an almost complete loss of activity for all antibiotics when tested alone and a remarkable synergy between fusidic acid and daptomycin or linezolid, but not with vancomycin. Biofilms grown in the CDC reactor are considered a valid surrogate for *in vivo* biofilms, which usually form more complex and robust tridimensional structures than in microplates (30, 44, 45). Moreover, biofilms grown under turbulent flow conditions display higher antimicrobial tolerance than those grown under laminar flow or in the absence of flow (46). Of interest, previous work using another type of shear-exposed biofilm showed that vancomycin was less effective than ciprofloxacin (47). We may therefore suggest that vancomycin, previously shown to penetrate to lower levels than daptomycin or the fluoroquinolone delafloxacin in biofilms grown in microtiter plates (34), could be affected more than the other drugs by the more robust and tighter architecture of the biofilm grown under shear stress in the CDC reactor. *In vivo*, vancomycin is also less active than daptomycin in a model of foreign body infection (48). Also noteworthy, a recent study using the CDC reactor and mimicking antibiotic pharmacokinetics (as we did here) demonstrated the low activity of linezolid when used alone (as observed here) and the benefit when using it in combinations (49).

Our work has at least four major limitations. First, we did not determine the concentrations of the drugs within the biofilms nor did we examine the composition of the matrix, both of which play critical roles in the global activity of the antibiotics in biofilms and for which variations may have contributed to explain the differences observed between strains or models. This would need the development of adequate methodologies, which was beyond the scope of the current pharmacologically oriented study. Second, while the CDC reactor enables variations in antibiotic concentrations to be mimicked over time (pharmacokinetics), our experimental setting, using bacterial

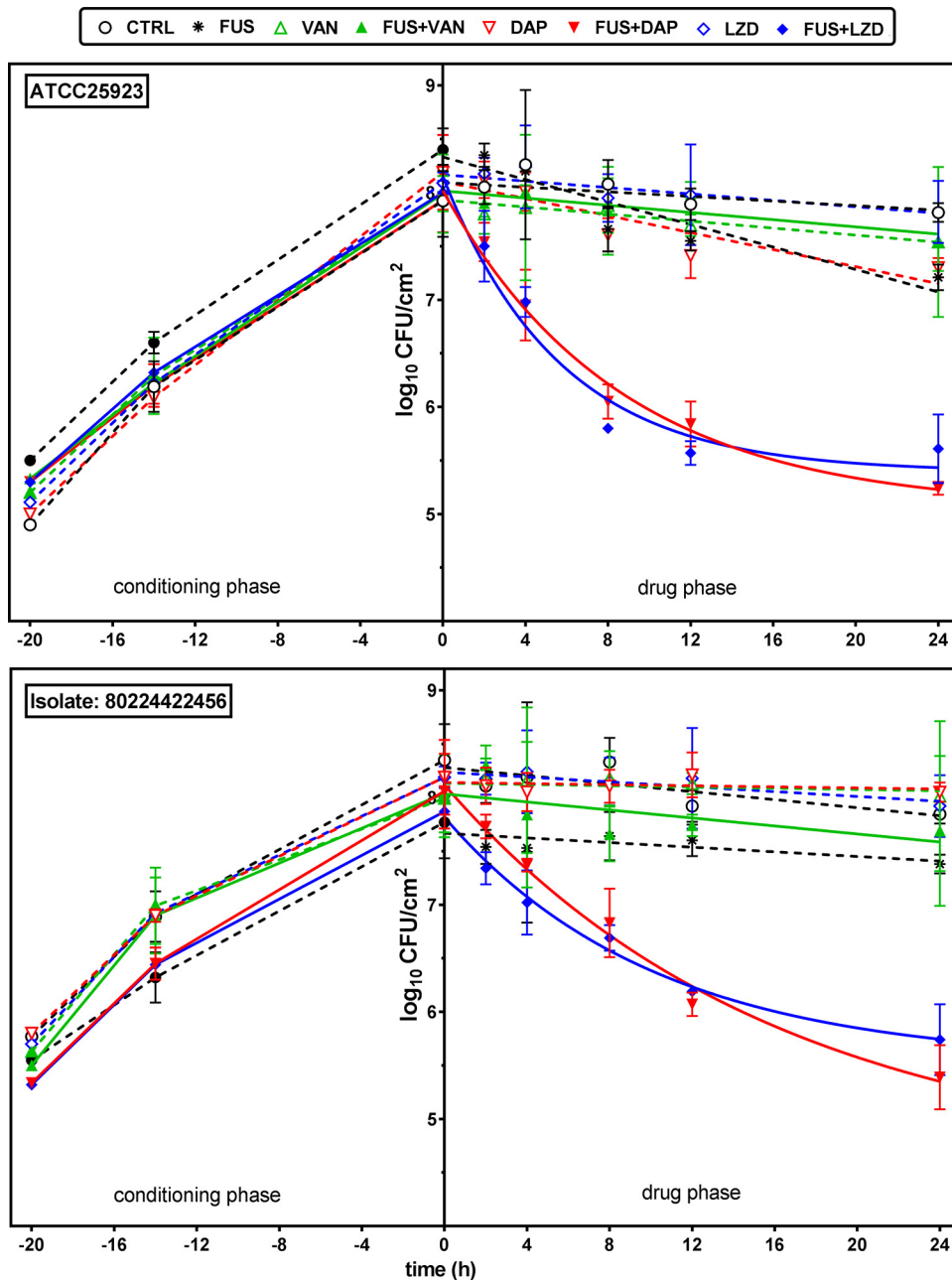


FIG 5 Activity of fusidic acid (FUS) alone or combined with daptomycin (DAP), linezolid (LZD), or vancomycin (VAN) against biofilms from the reference strain ATCC 25923 (top) or the clinical isolate 80224422456 (bottom) in the CDC bioreactor. FUS, LZD, and VAN were injected twice (0 h and 12 h), and DAP once (0 h), at the following concentrations (to mimic $f_{C_{max}}$): FUS, 14 mg/liter; DAP, 9.8 mg/liter; LZD, 17 mg/liter; VAN, 20 mg/liter. Peristaltic pumps were then started to infuse antibiotic-free medium at rate set up to mimic a $t_{1/2}$ of 12 h for FUS, 8 h for DAP, and 6 h for LZD and VAN.

broth as the culture medium, did not optimally take into account the influence of the environment on bacterial responsiveness to drugs or on the expression of antibiotic activity (pharmacodynamics). Third, the treatment duration in the dynamic model was limited to 24 h, which is shorter than conventional antibiotic regimens. Preliminary experiments, however, showed that longer incubation times are associated with a spontaneous disassembly of the biofilm, preventing us from following drug activity. Fourth, and directly related to the short duration of drug exposure, we could not assess the possible emergence of resistance in our models, which should, however, be

minimized by the use of antibiotic combinations. Taking these limitations into account, our work nonetheless demonstrates that combining fusidic acid with linezolid or daptomycin could be a useful therapeutic option against staphylococcal biofilm-related infections in areas and/or situations where resistance to these drugs is low (essentially, in North America [19–21], but not in Europe [50, 51] or other regions of the world [52, 53]). Linezolid and daptomycin, although recommended for short-term use, sometimes need to be used for long periods of time (>3 weeks [54–57]) in difficult situations, such as those involving biofilms, which may expose the patients to higher risks of adverse events. Synergic combinations, as those evidenced here with fusidic acid, may offer a real opportunity in this context. The present study, therefore, supports the development of appropriate *in vivo* studies using *ad hoc* animal models to confirm the efficacy of these combinations and to better define their conditions of use (dose and treatment duration).

MATERIALS AND METHODS

Antibiotics. The following antibiotics were obtained as microbiological standards: sodium fusidate (also known as CEM-102; potency, 98.5%) from Cempra, Inc. (Chapel Hill, NC), moxifloxacin HCl (potency, 90.9%) from Bayer HealthCare (Leverkusen, Germany), and doxycycline (potency, 90.2%) from Sigma-Aldrich (St. Louis, MO). The other antibiotics were obtained as the corresponding branded products registered for human parenteral use in Belgium or France and in compliance with the provisions of the European Pharmacopoeia (vancomycin as Vancomycine Mylan [Mylan, Inc., Canonsburg, PA], linezolid as Zyvoxid [Pfizer Inc., New York, NY], daptomycin as Cubicin [Novartis, Horsham, UK], rifampin as Rifadine [Merrell Dow Pharmaceuticals Inc., Strasbourg, France]).

Bacterial strains. *S. aureus* ATCC 25923 was used as a reference. Five clinical isolates from patients suffering from chronic tissue infections or infected medical devices were selected from the collection of the microbiology department of the Cliniques Universitaires Saint-Luc (Université catholique de Louvain, Brussels, Belgium) (Table 1). MICs were determined by broth microdilution according to the recommendations of the Clinical and Laboratory Standards Institute (58).

***In vitro* static biofilm model.** Biofilms were grown for 24 h in 96-well microtiter plates (European cat. number 734-2327; VWR [Radnor, PA]) in Trypticase soy broth supplemented with 2% NaCl and 1% glucose (TGN), with a starting inoculum adjusted to an optical density at 620 nm (OD_{620}) of 0.005 in a volume of 200 μ l, as previously described (34). Biofilms were then exposed for 48 h to either fusidic acid alone at concentrations spanning from 0.25 to 64 mg/liter (to obtain full concentration-response curves) or to fusidic acid at the same concentrations but combined with another antibiotic (linezolid, daptomycin, or vancomycin) adjusted to a concentration corresponding to its human free trough level (fC_{min}) or free peak level (fC_{max}). The biofilms were then washed twice with phosphate-buffered saline (PBS). Bacterial viability in biofilms was quantitated using the blue-colored phenoxazin dye resazurin, which is reduced by viable bacteria to the pink fluorescent compound resorufin (59). As previously described (28), the biofilms were incubated with 10 mg/liter resazurin (Sigma-Aldrich) in H₂O for 30 min at room temperature in the dark, after which resorufin fluorescence was measured (excitation λ [λ_{exc}], 560 nm; emission λ [λ_{em}], 590 nm) using a SPECTRAMax M3 spectrometer (Molecular Devices LLC, Sunnyvale, CA, USA).

***In vitro* dynamic biofilm model mimicking antibiotic human pharmacokinetics.** Biofilms were grown on polycarbonate coupons inserted into rods (3 coupons per rod) mounted in a CDC biofilm reactor (BioSurface Technologies, Bozeman, MT) according to the recommendations of the U.S. Environmental Protection Agency (60). Briefly, a 20-h conditioning phase was performed to obtain biofilms, consisting of a 6-h incubation at 37°C of 10⁵ CFU/ml in TGN, followed by 14 h of continuous flow of TGN at a rate of 11.6 ml/min, adjusted using peristaltic pumps (Masterflex L/S precision modular drive; Metrohm, Belgium). At the end of this conditioning phase, antibiotics were injected into the reactor to reach a concentration corresponding to their fC_{max} observed in patients receiving conventional doses. Fresh, antibiotic-free medium was then infused at a rate set to simulate the half-lives of the antibiotics in human serum (fusidic acid, 12 h [shorter value reported] [61]; vancomycin [62] and linezolid [63], 6 h; daptomycin, 8 h [64]). When antibiotics were used in combination, the infusion rate of fresh medium was adapted to the antibiotic with the shorter half-life, but a second peristaltic pump was connected to the system to inject the antibiotic with the longer half-life at a rate compensating for its excessive loss. For fusidic acid, vancomycin, and linezolid, the whole cycle (injection of antibiotics followed by infusion of antibiotic-free medium) was repeated at 12 h to mimic their twice-daily mode of administration. Only one injection followed by a 24-h decay was used for daptomycin. Three coupons were aseptically removed at 0, 2, 4, 8, and 24 h. Bacteria were liberated from biofilms by three alternating 1-min cycles of vortexing and sonication. The samples were then serially diluted and plated onto tryptic soy agar (TSA), and bacterial colonies were counted after an overnight incubation.

Determination of antibiotic concentrations in the CDC reactor. Antibiotic concentrations in the reactor were measured at regular intervals to verify that they declined at the expected rate. Fusidic acid was measured by a microbiological bioassay (agar diffusion) using ATCC 25923 as test organism (65). Daptomycin was assayed by fluorimetry, according to a previously described procedure (66). Linezolid was assayed by high-performance liquid chromatography (HPLC) using a Waters Alliance 2695 separation module combined with a Waters 2998 photodiode array detector (Waters Corp., Milford, MA), an

XTerraRP18 column as stationary phase, and a mixture of A (water plus 0.5% acetic acid) and B (methanol plus 0.5% acetic acid) as mobile phase. Tedizolid (Merck & Co., Kenilworth, NJ) was used as an internal standard. A volume of 50 μ l of sample (diluted in B) was injected, and separation was achieved at room temperature using a flow rate of 1 ml/min, a linear gradient from 80:20 to 20:80 (A:B) in 15 min. Linezolid was detected at 253.5 nm and tedizolid at 300 nm. Vancomycin was measured by fluorescence polarization immunoassay on a TDx analyzer (Abbott Diagnostics, Louvain-la-Neuve, Belgium).

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