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Original

In vitro activity of linezolid in combination with doxycycline, fosfomycin, levofloxacin, rifampicin and vancomycin against methicillin-susceptible *Staphylococcus aureus*

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SUMMARY

The objective of this paper was to investigate the in vitro *effects of linezolid combined with five antistaphylococcal antibiotics – doxycycline, fosfomycin, levofloxacin, rifampicin and vancomycin – upon methicillin-susceptible* Staphylococcus aureus *(MSSA). Five MSSA isolates from clinical specimens of human infections – hf008, hf095, hf295, hf602 and hf946 – were used in this study. The checkerboard method was used to assess synergism between linezolid and the five antibiotics, and time-kill curves were carried out with the most active combinations. Indifference was the most common result achieved by the checkerboard method when linezolid was combined with rifampicin, vancomycin or doxycycline. The combination with levofloxacin yielded antagonism for two of the five isolates. However, four isolates showed synergy for the combination of linezolid plus fosfomycin with a fractional inhibitory concentration index (FICI) ≥0.5. Neither linezolid nor fosfomycin alone inhibited growth at 1/4x minimum inhibitory concentration (MIC); but the combination of both drugs at 1/4 the respective MIC showed a synergistic bacteriostatic effect, a 2–3 log_{10} decrease with respect to the most active antibiotic alone. In summary, the combination of subinhibitory concentrations of linezolid and fosfomycin presented synergism, exerting a bacteriostatic effect.*

Key words: Oxazolidinone – Fosfomycin – Synergism

Actividad in vitro *de linezolid en combinación con doxiciclina, fosfomicina, levofloxacino, rifampicina y vancomicina frente a* Staphylococcus aureus *sensible a meticilina*

RESUMEN

El objetivo de este estudio fue investigar los efectos in vitro *de linezolid en combinación con cinco antimicrobianos antiestafilocócicos (doxiciclina, fosfomicina, levofloxacino, rifampicina y vancomicina) en cepas de* Staphylococcus aureus *sensibles a meticilina (SASM). Se utilizaron cinco cepas de SASM (hf008, hf095, hf295, hf602 y hf946) aisladas de muestras procedentes de infecciones humanas. Se utilizó el método del damero para evaluar la sinergia entre linezolid y los cinco antimicrobianos, y se trazaron curvas de tiempo-muerte con las combinaciones más activas. El resultado más habitual para las combinaciones de linezolid con rifampicina, vancomicina y doxiciclina fue indiferencia. La combinación con levofloxacino produjo antagonismo en dos de las cinco cepas. Sin embargo, frente a cuatro cepas se observó sinergia con la combinación de linezolid y fosfomicina, con un índice de concentración inhibitoria fraccionada (ICIF) ≥0,5. Ni linezolid ni fosfomicina en solitario inhibieron el crecimiento a 1/4x CMI, pero la combinación de ambos fármacos a 1/4 de la CMI respectiva mostró un efecto bacteriostático sinérgico, un descenso de 2-3 log_{10} respecto al antimicrobiano más activo en solitario. En resumen, la combinación de linezolid y fosfomicina a concentraciones subinhibitorias se mostró sinérgica, ejerciendo un efecto bacteriostático.*

Palabras clave: Oxazolidinona – Fosfomicina –Sinergia

INTRODUCTION

Linezolid is a synthetic antibiotic belonging to the oxazolidinone family. It blocks the protein synthesis at the ribosome preventing the formation of the initiation complex. It is active against Gram-positive micro-organisms, including multiresistant strains, being bacteriostatic against *Staphylococcus* spp. (1–6). One of the main features of linezolid is that it has a high oral bioavailability, which makes it easy to administer and adhere to the treatment (7–8). Linezolid has already proven its clinical effectiveness in processes like pneumonia, soft tissue infections and osteomielytis, among others (9–14). Nowadays, it plays a very important role against infections caused by multiresistant Gram-positive pathogens (10, 15–17).

Bone and prosthesis infections, where biofilms are likely to be formed, have a high rate of therapeutic failure when an antibiotic is used alone due to problems with antibiotic penetration, inoculum effect or stationary-phase organisms (18). Thus, the search for combinations of antibiotics might yield more effective treatment options, shorter administration periods and fewer major side effects.

The objective of this work was to investigate the *in vitro* effects of linezolid combined with five antistaphylococcal antibiotics – doxycycline, fosfomycin, levofloxacin, rifampicin and vancomycin – upon methicillin-susceptible *Staphylococcus aureus* (MSSA). This was conducted in two steps: First, the checkerboard method was utilized with linezolid plus the other five antibiotics as a screening for synergism; second, the time-kill curve was performed on the most active of them with the aim of strengthening the results by considering data from two different techniques (19).

MATERIALS AND METHODS

Bacterial isolates

The five MSSA isolates of this investigation – hf008, hf095, hf295, hf602 and hf946 – were all isolated from clinical specimens of human infections.

Antibiotics

Doxycycline, fosfomycin, levofloxacin, linezolid, rifampicin and vancomycin were supplied by the Pharmacy Department of our hospital as a stock solution of 20 mg/ml, 250 mg/ml, 5 mg/ml, 2 mg/ml, 60 mg/ml and 5 mg/ml, respectively.

Fosfomycin *E-test*

Fosfomycin *E-test* strips (AB Biodisk N.A. Inc., Solna, Sweden) were used according to manufacturer's guidelines.

Susceptibility tests

Minimum inhibitory concentration (MIC) was determined by microdilution method in Mueller-Hinton broth (20, 21) and defined as the lowest concentration that prevented growth after 20–24 hours of incubation at 35 ºC.

Checkerboard method

A 96 well plate (8 rows × 12 columns) was used. Each well was filled with Mueller-Hinton broth containing linezolid at a concentration ranging from 32 mg/l to 0.0625 mg/l and the other antibiotics from 16× MIC to 1/32× MIC dispensed at two-fold dilutions in a checkerboard manner, plus the bacterial inoculum to a final volume of 200 µl per well. Four wells from the first column were used as a sterility control and were filled only with Mueller-Hinton broth; the other four were used as a growth control and no antibiotic was added. The MIC of both linezolid and the other antibiotic was redetermined every time the checkerboard was conducted. The inoculum was prepared from a 24-hour culture on blood agar plates, and then adjusted to a 0.5 McFarland standard (approximately 1×10^8 CFU/ml) in sterile distilled water, subsequently diluted to a final cell concentration of approximately 5×10^5 CFU/ml, which was confirmed by colony counts in agar plates. The plates were incubated at 35 ºC for 18 ± 2 hours. The interaction was measured by the fractional inhibitory concentration index (FICI) all along the growth–no growth interface by the formula FICI = (MIC of linezolid in combination/MIC of linezolid alone) + (MIC of the antibiotic in combination/MIC of the antibiotic alone). Antagonism was defined as FICI >4, indifference as FICI >0.5 and ≥4, and synergism as FICI ≤0.5 (10, 29).

Time-kill curves

Time-kill curves were performed for the combination of linezolid plus fosfomycin on those isolates that had presented synergy by the checkerboard method (hf095, hf295, hf602 and hf946). Linezolid and fosfomycin were tested alone at 4× and 1/4× MIC of each antibiotic, and both combined at 4× MIC and at 1/4× MIC in a final volume of 5 ml of Mueller-Hinton broth, using an inoculum of approximately 1×10^6 CFU/ml (22, 23). An antibiotic-free flask served as a growth control. Surviving bacteria were counted after 0, 3, 6, 24 and 48 hours of incubation at 35 ºC by

Table 1. MICs by microdilution method and Σ FICIs of the different antibiotics alone and in combination with linezolid by the checkerboard method.

Antibiotic	Isolate	MIC (mg/ml)			Σ FICI (interpretation)
		AB	LNZ	AB/LNZ	
Doxycycline	hf 008	0.0625	1	0.03/0.125	0.6 (I)
	hf 295	0.0625	1	0.03/0.125	0.6 (I)
	hf 946	0.03	2	0.007/0.5	0.5 (S)
	hf 095	0.0625	2	0.003/1	0.55 (I)
	hf 602	0.0625	2	0.015/1	0.75 (I)
Fosfomycin	hf 008	0.5	1	0.25/0.25	0.75 (I)
	hf 295	4	1	1/0.25	0.5 (S)
	hf 946	0.5	2	0.06/0.5	0.375 (S)
	hf 095	0.5	2	0.125/0.25	0.375 (S)
	hf 602	4	2	1/0.5	0.5 (S)
Levofloxacin	hf 008	0.0625	2	0.125/1	2.5 (I)
	hf 295	0.03	2	0.125/1	4.5 (A)
	hf 946	0.0625	1	0.06/0.5	1.5 (I)
	hf 095	0.03	1	0.125/0.5	4.5 (A)
	hf 602	0.0625	2	0.125/1	2.5 (I)
Rifampicin	hf 008	0.064	2	0.032/0.125	0.56 (I)
	hf 295	0.032	2	0.016/1	1 (I)
	hf 946	0.032	1	0.016/0.5	1 (I)
	hf 095	0.064	2	0.016/0.5	0.5 (S)
	hf 602	0.032	2	0.016/0.5	0.75 (I)
Vancomycin	hf 008	0.5	1	0.5/0.125	1.125 (I)
	hf 295	0.5	1	0.5/0.125	1.125 (I)
	hf 946	0.5	1	0.5/0.125	1.125 (I)
	hf 095	0.5	2	0.25/1	1 (I)
	hf 602	0.5	2	0.5/0.125	1.06 (I)

AB: current antibiotic; LNZ: linezolid; I: indifference; S: synergism; A: antagonism.

subculturing 100 µl serial dilution of every flask on sheep blood agar plates. The aliquots were deposited as a spot onto the agar plates and allowed to soak. After the plate had dried, spreading was performed; no antibiotic carry-over was detected. The lower limit of accurate and reproducible detectable colony count was 100 CFU/ml.

Synergism and indifference were defined as a decrease ≥ 2 $\log_{10}$ and <2 $\log_{10}$ CFU/ml with respect to the most active drug, antagonism as an increase ≥ 2 $\log_{10}$.

Bacteriostatic effect was defined as a decrease <3 $\log_{10}$ CFU/ml compared to the initial inoculum and bactericidal as a decrease ≥ 3 $\log_{10}$ CFU/ml compared to the initial inoculum.

RESULTS

Susceptibility

MICs of the antibiotics are displayed in Table 1. Small variations of the MIC (±1 doubling dilution) occurred occasionally when the MIC was determined at the time the checkerboard method was performed.

Checkerboard

Indifference was the most common result achieved by the checkerboard method for doxycycline, rifampicin and vancomycin, although isolate 095 showed synergism with rifampicin and linezolid at concentrations of 0.016/0.5 mg/ml respectively, and isolate hf946 showed synergism for the combination of doxycycline and linezolid at concentrations of 0.007/0.5 mg/ml. Isolates hf095 and hf295 presented antagonism for the combination of levofloxacin plus linezolid at 0.125/1 and 0.125/0.5, respectively. The other three isolates resulted in indifference (Table 1).

Four isolates showed synergy for the combination of linezolid plus fosfomycin by the checkerboard method with a FICI ≥ 0.5 (Table 1).

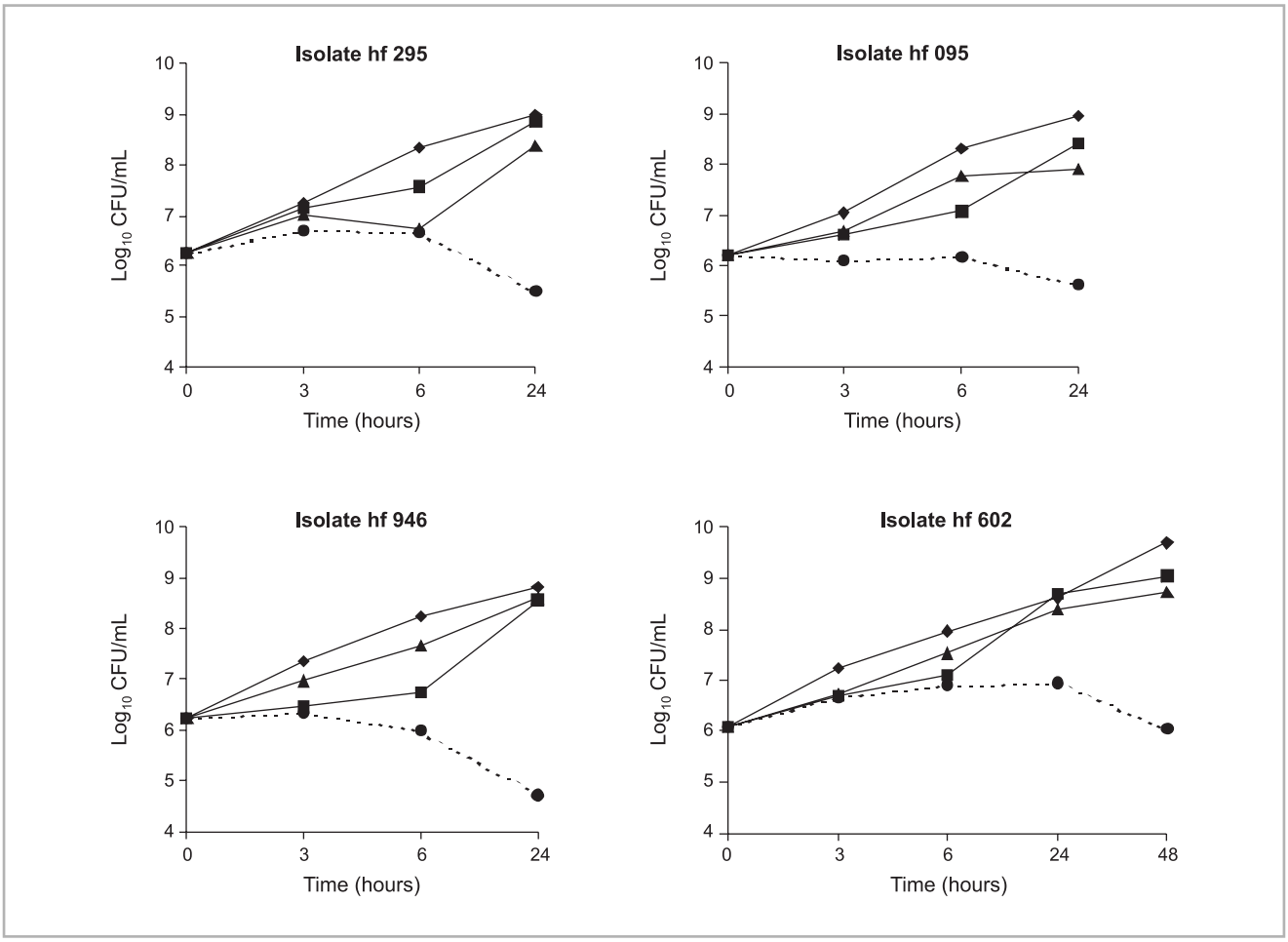

Figure 1. Time-kill curve plots of linezolid and fosfomycin alone and in combination at 1/4x MIC. (◆, filled diamond) growth control, (■, filled square) 1/4x MIC linezolid, (▲, filled triangle) 1/4x MIC fosfomycin, (●, filled circle) 1/4x MIC linezolid plus 1/4x MIC fosfomycin.

Time-kill curves

Linezolid alone at 4× MIC exerted a bacteriostatic effect. However, fosfomycin at 4× MIC had a killing effect the first six hours; after that, a regrowth occurred. When both antibiotics were combined at 4× MIC, the time-kill curve followed that of the linezolid alone.

Neither linezolid nor fosfomycin alone inhibited growth at 1/4× MIC; but the combination of both drugs at 1/4 the respective MIC showed a synergistic effect, a 2–3 $\log_{10}$ decrease with respect to the most active antibiotic alone, although this decrease was less than 3 $\log_{10}$ with respect to the initial inoculum, thus being bacteriostatic (Fig. 1).

In order to determine whether the regrowth observed when fosfomycin was used alone was due to selection of spontaneous mutants, aliquots of 100 µl were withdrawn from the flasks onto sheep blood agar plates with a fosfomycin *E-test* strip at the beginning of the experiment and after 24 hours of incubation. No colonies were observed within the inhibition zone at the beginning of the experiment. However, colonies emerged inside the inhibition zone of the cultures from the fosfomycin flasks after 24 hours of exposure, but not when it was combined with linezolid. The MIC of the cultures from the 4× MIC fosfomycin flasks increased more than five doubling dilutions, shifting from 4 mg/l to 96 mg/l in two isolates, whereas it did not change in the cultures from the flasks where linezolid was used in combination.

DISCUSSION

Linezolid is an antibiotic with a unique mechanism of action – the inhibition of the initiation process of the protein transcription, bacteriostatic against *Staphylococcus aureus*. It has proven to be a good option for the treatment of a great variety of infections, such as those of bone or soft tis-

sue, where Gram-positive micro-organisms play an important role. At the present time, combined therapies are being considered as a way to enhance antibiotic activity, reduce the duration of the process as well as the duration of the administration of drugs, and to avoid the emergence of resistance.

In our study, we have tested the interaction of linezolid with different antibiotics by the checkerboard and time-kill curve methods. With the checkerboard method we have observed that indifference was the most frequent effect when combined with rifampicin, vancomycin and doxycycline, although two isolates presented synergism, one with rifampicin and another with doxycycline. The combination with levofloxacin yielded antagonism for two of the five isolates. Other authors have also reported indifference using the time-kill curve method for rifampicin and vancomycin (4, 25), and antagonism for the combination with levofloxacin (17, 26). In contrast, the combination of fosfomycin and linezolid exhibited synergism in four of the five isolates tested, similar to the findings of Grif *et al.* (26), who detected synergism against MSSA and *Staphylococcus epidermidis* isolates.

With the time-kill curve method, the combination of both linezolid and fosfomycin at concentrations above the MIC did not show any enhancement in their killing activity, linezolid being the leading drug. Still, the combination of linezolid with fosfomycin prevented the emergence of resistance observed when fosfomycin was used alone; other authors found that linezolid in combination with rifampin or fusidic acid avoided the appearance of resistance to the partner drug (4, 25, 27). Notably, when combined at subinhibitory concentrations, they experience synergism, despite the fact that both drugs when tested alone were not able to inhibit growth, the combination accomplished a bacteriostatic effect. This is noteworthy due to the fact that both antibiotics are widely distributed into human tissues and biofilms (7, 8, 28, 29); consequently, common doses of both drugs could reach faster and maintain therapeutic levels of antibiotics at the site of infection in circumstances where it is difficult to reach drug concentrations similar to those in serum.

In summary, we can presume that linezolid combined with fosfomycin could perform an important role as an alternative treatment of diseases that are complex to heal and that have a tendency to become chronic, such as osteoarticular or prosthesis infections (10–12, 28), where biofilms are likely to be produced by micro-organisms, since the association of linezolid with fosfomycin is active at lower concentrations than the single agents. This combination could also prevent the emergence of resistance to current antistaphylococcal drugs.

Nevertheless, *in vivo* studies, as well as analyses on biofilms, need to be carried out in order to consider this association of antibiotics as an option for the treatment of staphylococcal infections.

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