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## **In vitro and in vivo activities of linezolid alone and combined with vancomycin and imipenem against with reduced susceptibility to glycopeptides**

S. Ribes, M. E. Pachón-Ibáñez, M. A. Domínguez, R. Fernández, F. Tubau, J. Ariza, F. Gudiol, C. Cabellos

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Title: In vitro and in vivo activities of linezolid alone and combined with vancomycin and imipenem against *Staphylococcus aureus* with reduced susceptibility to glycopeptides

Article Type: Article

Keywords: GISA; heteroresistance; linezolid; peritonitis; experimental infections

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Abstract: Objective: To evaluate the in vitro and in vivo efficacies of linezolid, vancomycin, imipenem, linezolid+imipenem, linezolid+vancomycin, and vancomycin+imipenem against two clinical *S. aureus* isolates with reduced susceptibility to glycopeptides.

Material and Methods: Time kill curves were performed over 24 hours. Murine peritonitis model: Peritonitis was induced by intraperitoneal inoculation of 10<sup>8</sup> CFU/ml of each bacterial strain. Four hours later (0 h), mice were randomly assigned to a control group or to therapeutic groups receiving treatment for 25 h. Bacterial counts in peritoneal fluid, bacteraemia and mortality rates were determined.

Results: Time-kill curves: Addition of linezolid to imipenem yielded synergistic results after 24 h. Addition of linezolid decreased vancomycin activity. In the animal model, vancomycin and linezolid monotherapies produced comparable bacterial decreases in mice infected with each strain but linezolid achieved higher rates of blood sterilization. Linezolid tested either in monotherapy or in combination showed similar efficacy against both strains in terms of bacterial killing, negative blood cultures and survival. Linezolid and vancomycin were moderately bactericidal and similar in efficacy against glycopeptide intermediate or resistant *S. aureus*.

Conclusions: Linezolid combinations, as effective as linezolid tested alone, could be considered as alternative options for the treatment of GISA infections.

Response to Reviewers: Ms. No. EJCMIID-D-09-00457

In vitro and in vivo activities of linezolid alone and combined with vancomycin and imipenem against *Staphylococcus aureus* with reduced susceptibility to glycopeptides

Dear Dr Jung,

Thank you very much for allowing us to resubmit our manuscript. In accordance with the Reviewers comments, we have modified the manuscript.

We hope that after these modifications, the revised manuscript can be accepted for its publication in European Journal of Clinical Microbiology & Infectious Diseases.

Please do not hesitate to contact me, if there are any further questions.

1. Time kill studies: just two sampling points are insufficient, even if a computer assisted curve fit generates nice graphs.

In the evaluation of bactericidal activity our previous experience as well as some previous reports show that the time kill studies including intermediated points (4 and 8 h) do not provide additional information in comparison to studies that only consider 2 time points (6 and 24 h).

Murillo O, Doménech A, Garcia A, Tubau F, Cabellos C, Gudiol F, Ariza J. Efficacy of High Doses of Levofloxacin in Experimental Foreign-Body Infection by Methicillin-Susceptible *Staphylococcus aureus*. *Antimicrob Agents Chemother*. 2006; 50:4011-7.

Murillo O, Domenech A, Euba G, Verdaguer R, Tubau F, Cabo J, Cabellos C, Gudiol F, Ariza J. Efficacy of linezolid alone and in combination with rifampin in staphylococcal experimental foreign-body infection.

*J. Infection* 2008; 57: 229-35.

Montero A, Ariza J, Corbella X, Domenech A, Cabellos C, Ayats J, Tubau F, Ardanuy C, Gudiol F. Efficacy of colistin versus beta-lactams, aminoglycosides, and rifampin in a model of pneumonia caused by multi-resistant *Acinetobacter baumannii*.

*Antimicrob. Agents Chemother*. 2002; 46: 1946-52.

Mathur T, Singhal S, Khan S, Upadhyay D, Fatma T, Rattan A. Adverse effect of staphylococci slime on in vitro activity of glycopeptides. *Jpn J. Infect. Dis*. 2005; 58: 353-357.

Aeschlimann JR, Hershberger E, Rybak MJ. Analysis of vancomycin population susceptibility profiles, killing activity, and postantibiotic effect against vancomycin-intermediate staphylococcus aureus. *Antimicrob Agents Chemother*. 1999; 43: 1914-1918.

Jacqueline C, Caillon J, Le Mabecque V, Miegeville AF, Donnio PY, Bugnon D, Potel G. In vitro activity of linezolid alone and in combination with gentamicin, vancomycin or rifampicin against methicillin-resistant *Staphylococcus aureus* by time-kill curve methods. *Journal of Antimicrobial Chemotherapy*.

2. Definition of combination effects: the definition is probably based on the end-point (24h) readings; this needs to be clearly defined.

Yes, the definition is based on the 24 h readings. A sentence has been added clarifying this point (page 5).

3. Antibiotic assay: Imipenem was analysed microbiologically. It is well documented that imipenem is rather unstable - has this been considered in the course of sampling, storage, and bioassay? - Please specify stability. Detection limits are given for vancomycin (quantitated chemically), but not for imipenem and linezolid - has to be done. How were the standards handled? Throughout the entire course of experimentation in parallel to treatment, sampling etc, etc? How high was the loss in imipenem-activity during this procedure and were some measures taken to adjust for the loss in activity?

Standard curves were constructed with mouse plasma; For imipenem, assay validation indicated linearity, r<sup>2</sup> value of 0.9709 and for linezolid 0.9887.

The detection limit for imipenem was 0.5 mg/ml and for linezolid was 2 mg/ml. Two sentences have been added with these details, page 7, (antibiotic assay).

There were no special measures with imipenem. Samples were immediately centrifugated and frozen.

4. PK: The PK-parameters should be reported, not only the PK/PD surrogates.

Table 1 has been deleted as suggested in point 8.

5. Protein binding is specified for linezolid + vancomycin, but not for imipenem, although free drug levels are reported. Furthermore, protein binding quoted refer to binding to human serum-proteins; binding to animal serum-proteins is quite often different. In this case, the authors are lucky, as the values differ not too much - but there are slight discrepancies which should be corrected and the "correct" references have to be be quoted:

Vanco Knudsen JD et al., AAC 1997; 41: 1910-1915 = 25% in mice

Line: Gentry-Nielsen MJ et al., AAC 2002; 46: 1345-1351 = 26% in rats

Imi: has to searched by the authors

The references about protein binding in rodents have been added. Also the corrected data have been added, including a correction of the original calculation that had a mistake.

A protein binding of imipenem of 13-20% has been reported in human serum.

There is no report concerning the protein binding of imipenem in murine serum but we have found data on rats where the protein binding was considered as "limited" and "negligible":

Lefevre S, Marchand S, Lamarche I, Mimoz O, Couet W. Microdialysis study of imipenem distribution in the intraperitoneal fluid of rats with or without experimental peritonitis. *Antimicrob Agents Chemother.* 2006; 50:34-37.

Lefevre S, Marchand S, Pariat C, Lamarche I, Couet W. Microdialysis study of imipenem distribution in the peritoneal fluid of rats with experimental pancreatitis. *Antimicrob Agents Chemother.* 2008; 52:1516-18

Therefore, free imipenem concentrations were considered to be the same as detected in the bioassay method.

6. Peritonitis model: statistical analysis has to be done on the same basis as for the bacteraemia model, i.e. comparison of the various treatment groups - which may very likely show that there are no differences.

We think that ANOVA is a correct test for comparison of the various treatment groups and we have analyzed by this test the results and published in several journals.

7. Appearance of resistance: this important and clinically relevant aspect is just mentioned in the discussion chapter; however, the authors have had the chance to generate corresponding data themselves. As the authors missed this chance, they should not discuss this aspect.

We have deleted the paragraph.

8. Table 1: PK/PD surrogates are presented - without using them or explaining their use in somewhat greater depth. The authors write that regimens have been selected mimicking human kinetics, i.e. serum conc similar to those found in humans. But these data are not presented, so that the reader has no impression how similar the concentrations were in the end.

On page 11, lines 9-14 the authors refer very briefly and totally speculative to this table. But there is no basis for such a speculation. Others have provided PK/PD rationales for the clinical use of e.g. imi + line

(Ong CT et al., Surgical Infections 2005; 6:419-426 and several others; Gentry-Nielsen quoted above for line). These publications should have been used as a baseline for the interpretation of the results generated in this study - please re-write this para - or delete any reference to PK/PD surrogates.

Table 1 has been deleted.

Human concentrations have been added to mice concentrations. Page 8.

The reviewer strongly recommends to shorten the manuscript by deleting e.g. the resistance- and PK/PD discussion etc, so that the manuscript should be shortened to the format of a concise article.

The paragraphs and Table 1 have been deleted.

Last but not least I'd like to draw the attention of the investigators to a phenomenon which is most frequently not considered at all: chronobiology. PK of almost every agent is strongly dependent from the circadian rhythm of the animals, which is disturbed anyway due to handling of the animals during the "human working hours" - it is a 12h difference + experimental stress. This results in an approx 50% variation of imi-kinetics in the rat, for example, leading to a marked variability of exposure during the day- and night-time. Again, this is not considered when PK data are generated during the lab-working hours only, which has very likely been the case in the course of this study, too. This comment is just food for thought.

Thank you very much for this interesting comment.

Reviewer #3: The paper is well-written and deals with the potential use of the new antimicrobial agent linezolid in combination with either imipenem or vancomycin in an era of increasing antibiotic resistance. It analyzes data and presents concise results from both laboratory and animal studies and concludes that linezolid, either alone or in combination with the other agents, is a promising antimicrobial for the therapy of infections by *S. aureus* strain with reduced glycopeptide susceptibility. In my opinion, there are three major and two minor points the authors should address to:

Major comments

\* The authors should comment on their selection of the peritonitis model, since peritonitis is usually mixed aerobic/anaerobic infection with predominantly Gram negative bacteria. In particular, why is this model a simulation of a Gram positive infection in humans? A reference to a previous experimental study conducted with that model should also be included.

The election of this particular peritonitis model was based on the previous experience of our working group to study therapeutic options in the management of infections caused by multiresistant Gram positive bacteria. Moreover, it is an interesting model to use as screening model in a small animal.

We have added a reference about the model and a sentence in the text (page 6, reference number 23).

Domenech A, Ribes S, Cabellos C, Domínguez MA, Montero A, Liñares J, Ariza J and Gudiol F. A mouse peritonitis model for the study of glycopeptide efficacy in GISA infections. *Microbial Drug Resistance* 2004;10: 346-53.

Domenech A, Ribes S, Cabellos C, Taberner F, Tubau F, Dominguez MA, Montero A, Liñares F, Ariza J, Gudiol F. Experimental study on the efficacy of the combinations of glycopeptides and B-lactams against *Staphylococcus aureus* with reduced susceptibility to glycopeptides. *J. Antimicrob. Chemother.* 2005; 56: 709-716

\* On page 6 ln 10 of the "mouse peritonitis model", it is stated that "at a given time point, mice were anaesthetised.". That time point is not given, thus its relation to the last administration of antimicrobials is unknown, in order to assess the results and, consecutively, the efficacy of the drugs. Moreover, it is inferred, although not stated, that the total number of doses administered to the sacrificed mice is 5. Is that correct?

"At a given time point, mice were anaesthetized" this time points refers to 25 hours after start of therapy. We have added a sentence clarifying this point page 6, ln 10 of the "mouse peritonitis model".

Yes, we administered a total number of 5 doses: at hours 0, 5, 10, 15 and 20, covering 25 hours of therapy.

· Why were concentrations of linezolid and imipenem not determined using an HPLC method? HPLC is the method of choice for the free (ie active) fraction of an antibiotic in serum and concentrations of the present study should be measured and reported with that methodology.

The HPLC technique is an expensive method that requires specialized equipment, high cost solvents and experience to adjust the most suitable chromatographic conditions. On the other hand, the bioassay method is clearly a less expensive method, ease of handling and requires no specialized equipment.

In a study where linezolid concentrations in human serum and urine were determined, both techniques produced comparable results (Borner K, Borner E, Lode H. Determination of linezolid in human serum and urine by high-performance liquid chromatography. *Int J Antimicrob Agents.* 2001 Sep;18(3):253-8).

· In addition, references 22 and 23 refer to protein binding in humans, who might be different in mice; thus, the murine pharmacodynamics should be recalculated accordingly.

Please see point 5 reviewer 1.

Minor comments

\* The abstract should be structured.

The abstract has been structured.

The volume of the journal is missing from reference #3.

The volume of reference 3 has been added.

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***In vitro* and *in vivo* activities of linezolid alone and combined with vancomycin and imipenem against *Staphylococcus aureus* with reduced susceptibility to glycopeptides**

Sandra Ribes<sup>a,\*</sup>, Maria Eugenia Pachón-Ibáñez<sup>a,\*</sup>, Maria Ángeles Domínguez<sup>b</sup>, Raúl Fernández<sup>b</sup>, Fe Tubau<sup>b</sup>, Javier Ariza<sup>a</sup>, Francesc Gudiol<sup>a</sup>, and Carmen Cabellos<sup>a</sup>.

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## ABSTRACT

**Objective:** To evaluate the *in vitro* and *in vivo* efficacies of linezolid (35 mg/kg/5 h), vancomycin (60 mg/kg/5 h), imipenem (30 mg/kg/5 h), linezolid+imipenem, linezolid+vancomycin, and vancomycin+imipenem against two clinical *S. aureus* isolates with reduced susceptibility to glycopeptides using time kill curves and the murine peritonitis model.

**Material and Methods:** Time kill curves were performed over 24 hours. Murine peritonitis model: Peritonitis was induced by intraperitoneal inoculation of  $10^8$  CFU/ml of each bacterial strain. Four hours later (0 h), mice were randomly assigned to a control group or to therapeutic groups receiving subcutaneous treatment for 25 h. Bacterial counts in peritoneal fluid, bacteraemia and mortality rates were determined.

**Results:** Time-kill curves: Addition of linezolid to imipenem yielded synergistic results after 24 h. Addition of linezolid decreased vancomycin activity. In the animal model, vancomycin and linezolid monotherapies produced comparable bacterial decreases in mice infected with each strain but linezolid achieved higher rates of blood sterilization. Linezolid tested either in monotherapy or in combination showed similar efficacy against both strains in terms of bacterial killing, number of negative blood cultures and survival. Linezolid and vancomycin were moderately bactericidal and similar in efficacy against glycopeptide intermediate or resistant *S. aureus*.

**Conclusions:** Linezolid combinations, as effective as linezolid tested alone, could be considered as alternative options for the treatment of GISA infections.

**Keywords:** GISA; heteroresistance; linezolid; peritonitis; experimental infections



## INTRODUCTION

1  
2 Methicillin-resistant *Staphylococcus aureus* (MRSA) is the most important  
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4 cause of antibiotic-resistant healthcare-associated infections [1]. In addition,  
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6 clinical isolates of *S. aureus* with heterogeneous resistance to vancomycin  
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8 (hGISA) and, more rarely, glycopeptide-intermediate resistant strains (GISA)  
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10 (hGISA) and, more rarely, glycopeptide-intermediate resistant strains (GISA)  
11  
12 have emerged worldwide over the past years [2-4].

13  
14 Reduced vancomycin and teicoplanin activities against hGISA and GISA  
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16 isolates have been reported in experimental studies [5-7], while in the clinical  
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18 setting vancomycin has appeared to be sub-optimal in deep-seated and difficult-  
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20 to-treat infections caused by these strains [7, 8]. Furthermore, the antagonistic  
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22 effect or false synergy showed by *in vitro* studies with the combination of  
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24 glycopeptides and  $\beta$ -lactams refuses its use as a potentially promising  
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26 alternative to glycopeptide monotherapy [9,10]. The oxazolidinone linezolid, one  
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28 of the new treatment options for multidrug-resistant Gram-positive bacteria [11-  
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30 13], shows high *in vitro* activity against resistant staphylococcal strains [13, 14].  
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32 In patients with MRSA infections, linezolid has shown comparable efficacy to  
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34 vancomycin [15, 16]. Moreover, it shows excellent oral bioavailability and does  
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36 not require dose adjustment for renal insufficiency [14]. Its unique mechanism of  
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38 action by inhibiting ribosomal protein synthesis at an early stage of bacterial  
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40 replication leads to the absence of cross resistance with other antimicrobials  
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42 [15]. Although linezolid-nonsusceptible strains are unusual [17], long courses of  
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44 oxazolidinone therapy could select resistant mutants [18], hence the use of a  
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46 combined strategy might be considered in clinical practice. To date, the efficacy  
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48 of linezolid as part of a combination has been studied against MRSA strains but  
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50 very few data has been reported against hGISA or GISA strains [19, 20].  
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Linezolid plus  $\beta$ -lactams exhibited bactericidal and synergistic activity against MRSA and hGISA strains in experimental models of endocarditis and meningitis [19]. Linezolid plus rifampicin was an effective prophylactic regimen for preventing staphylococcal prosthetic vascular graft infection, although the combination did not show higher efficacy than linezolid monotherapy [20].

We aimed to evaluate and compare the efficacies of linezolid alone and in combination with either vancomycin or imipenem against two *S. aureus* strains with reduced susceptibility to glycopeptides.

## **MATERIALS AND METHODS**

### **Bacterial strains**

Two clinical isolates of *S. aureus* with different degrees of resistance to glycopeptides were included: a hGISA strain isolated in our hospital and belonging to the Iberian clone, growing on 4 mg/l vancomycin Mueller–Hinton plates with a sub-population frequency of  $3.6 \times 10^{-6}$  CFU/ml (this strain was equivalent to the Mu3 heteroresistant strain) [8]; and a GISA strain (Mu50, ATCC 700699) reported as the first GISA strain [3]. MICs (mg/l), determined using the Etest and the macrodilution method [21], were for the hGISA strain: cloxacillin, 1024; cefotaxime, 1024; teicoplanin 8; vancomycin (VAN), 2; linezolid (LZD), 1; and imipenem (IMP), 32. MICs for the GISA strain were: cloxacillin, 1024; cefotaxime, 2048; teicoplanin, 16; VAN, 8; LZD, 2; and IMP, 64.

### ***In vitro* time-kill studies**

The bactericidal activities of the drugs were tested in glass tubes containing Mueller–Hinton broth and a final inoculum of  $1 \times 10^5$  to  $1 \times 10^6$  CFU/ml [21].

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Linezolid (Pfizer, Madrid, Spain), imipenem-cilastatin (Merck, Sharp & Dohme, Madrid, Spain) and vancomycin (Normon, Madrid, Spain) were provided by the manufacturers. Antibiotics were tested for a range of concentrations according to their MICs and their achievable levels in human serum: linezolid concentrations ranging from 1/4 x to 8 x MIC, vancomycin concentrations ranging from 1/4 x to 1 x MIC, imipenem levels from 1/8 x to 1 x MIC, as well as concentrations of 1/4 x, 1/2 x and 1x MIC of each drug in combination. In all experiments, growth control was assessed using an extra tube without antibiotic. At 0, 6 and 24 h of incubation, aliquots of 100 µl were taken from each tube to perform direct and 10-fold dilutions, and were cultured onto 5% sheep blood agar plates (SBA) at 37°C for 24 h. Experiments were performed in duplicate. **The following effects were studied in combinations after 24 h of incubation:** a bactericidal effect was defined as a decrease in the initial inoculum of  $\geq 3 \log_{10}$ CFU/ml. Synergy of a combination was defined as a  $> 2 \log_{10}$ CFU/ml reduction over the most active agent alone, with one of the drugs at subinhibitory concentration. An indifferent effect was defined as  $< 1 \log$  (increase or decrease) in killing.

### 41 **Pharmacokinetics**

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Pharmacokinetic studies were performed to select dose regimens that result in serum concentrations similar to those found in humans [6, 22]. Groups of 21 healthy mice were used for each pharmacokinetic study. A single weight-adjusted antibiotic dose was administered subcutaneously (sc) to each animal. At different time points, sets of three animals were anaesthetised intraperitoneally (ip), and blood samples (0.5 ml) were obtained by an intracardiac puncture. Blood was centrifuged and serum stored at -80°C.

1 Pharmacokinetic and pharmacodynamic parameters were obtained by a  
2 computer-assisted method (PK Functions for Microsoft Excel. Usansky, Desai  
3 and Tang-Liu, Pharmacokinetics and Drug Metabolism Dept, Allergan, Irvine,  
4 CA 92606) after determination of antibiotic concentrations over the time. Based  
5 on the obtained parameters the final selected doses were: vancomycin 60  
6 mg/kg every 5 h (300 mg/kg/day), linezolid 35 mg/kg every 5 h (175 mg/kg/day)  
7 and imipenem 30 mg/kg every 5 h (150 mg/kg/day).  
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### 10 **Mouse peritonitis model**

11 **This mouse peritonitis model has been previously characterized in our**  
12 **laboratory [6,23].** Inbred, female C57BL/6 mice (6 weeks; 14–16 g) were used  
13 (Harlan Int. Ibérica, S.A., Barcelona, Spain). Inoculation was performed via a  
14 26-gauge syringe by ip injection of 0.5 ml of the inoculum consisting of a  $5 \times 10^8$   
15 CFU/ml staphylococcal suspension with 5% (w/v) mucin in sterile saline. A  
16 group of control mice ( $n \geq 18$ ) were killed 4 h after inoculation (hour 0) and  
17 antibiotic sc therapy was initiated. The rest of the mice were randomized to the  
18 control group receiving saline ( $n \geq 25$ ) or to one of the following therapeutic  
19 schedules ( $n \geq 10$  per therapy): Linezolid, vancomycin, imipenem,  
20 linezolid+vancomycin, linezolid+imipenem and vancomycin+imipenem receiving  
21 sc treatment over 25 h. **At 25 h of therapy (5 h after the last antibiotic dose),**  
22 mice were anaesthetised ip with ketamine/xylazine and peritoneal washes were  
23 performed by injecting 2 ml of sterile saline ip followed by a massage of the  
24 abdomen. Immediately, 0.1 ml of blood was withdrawn by cardiac puncture and  
25 animals were then sacrificed by cervical dislocation. Next, the abdomen was  
26 opened and 0.2 ml of peritoneal fluid (PF) was recovered from peritoneum.  
27 Undiluted and ten-fold diluted PF samples (0.1 ml) were plated on SBA plates to  
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1 perform bacterial determinations. Mortality was recorded after 25 h of therapy.  
2 Blood samples were grown in TSB at 37°C for 24 h and then 0.1 ml of broth  
3 was cultured on SBA plates to assess *S. aureus* bacteraemia.  
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### 6 **Antibiotic assays**

7 Vancomycin serum concentrations were determined by fluorescent polarization  
8 immunoassay using a TDx analyzer (ABBOTT CIENTIFICA, S.A., Diagnostics  
9 Division, Costa Brava 13, 28034 Madrid, Spain) with a detection limit of 2.0  
10 µg/ml. Serum concentrations of linezolid and imipenem were measured using  
11 the agar disc diffusion method and *Bacillus subtilis* ATCC 12432 and  
12 *Escherichia coli* ATCC 25922, respectively, as assay organisms. Standard  
13 curves were constructed using mouse plasma. Assay validation indicated  
14 linearity ( $r^2$  value) of 0.9709 for imipenem and 0.9887 for linezolid. The  
15 detection limit was 0.5 µg/ml and 2 µg/ml for imipenem and linezolid,  
16 respectively.  
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### 33 **Statistics**

34 Statistical analysis was performed with SPSS 12.0. ANOVA with Dunnett's post-  
35 hoc tests was used to analyse multiple bacterial count comparisons between  
36 therapeutic and control groups. Two-tailed Fisher's exact test was used for  
37 analyse survival and bacteraemia data. A *P* value of < 0.05 was considered  
38 statistically significant.  
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## 51 **RESULTS**

### 52 ***In vitro* time–kill studies**

53 Linezolid achieved a bacterial decrease up to 2 log CFU/ml when tested at 4-16  
54 mg/l against both strains. Vancomycin achieved a bacterial decrease of 2 log  
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CFU/ml when studied at 2 and 8 mg/l against the hGISA and the GISA strain, respectively. Imipenem failed to inhibit bacterial growth at any tested concentration (8-64 mg/l).

In killing curves with the hGISA strain, linezolid combined with vancomycin showed lower activity than vancomycin alone. Vancomycin activity was decreased between 1-1.5 log<sub>10</sub>CFU/ml at 24 h. The same combination improved the activities of antibiotics tested alone against the GISA strain (Figure 1).

The combination of linezolid at concentrations above the MIC and imipenem did not improve upon the activity of linezolid tested alone against either strain. The addition of sub-MIC concentrations of linezolid to imipenem produced a synergistic effect against both strains (Figure 2).

The combination of vancomycin with imipenem was bactericidal and synergistic against the hGISA strain. Vancomycin tested at 2 mg/l in combination with imipenem (8-64 mg/l) was also bactericidal and improved upon the activity of vancomycin alone against the GISA strain.

### **Pharmacokinetics.**

Linezolid and vancomycin free maximum concentrations in serum were 18.16 and 37.73 mg/l, respectively (with a protein binding of 26 % for linezolid and 25 % for vancomycin [24, 25]) Imipenem free maximum concentration found in serum was 38.26 mg/l. Drug serum concentrations in humans are 12-15 mg/l for linezolid (dose 600 mg/12 h), 30-40 mg/l for vancomycin (dose 1 g/12 h) [26] and 32.1 mg/L (dose 500 mg/6 h) for imipenem [27]

**Mortality and bacteraemia rates.** In control mice, mortality rates were 90% and 69% after 25 h of infection with the hGISA and GISA strains, respectively. At the same time point, mortality in mice infected with the hGISA strain was 0%

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in all therapeutic groups except for the imipenem (20%, 2/10) and the  
vancomycin plus imipenem (7%, 1/14) groups. In mice infected with the GISA  
strain, mortality was 0% in all treated animals except for those receiving  
imipenem monotherapy (45.5%, 5/11). Data from GISA-infected animals treated  
with imipenem monotherapy were not considered for any statistical analysis  
because of the low number of animals that survived after 25 h of therapy (n=6).  
Bacteremia in control animals at 0 h, expressed as percentage of positive blood  
cultures, was 100% for each strain. Bacteraemia rates in control and  
therapeutic groups after 25 h of therapy are shown in Table 1. Imipenem alone  
and in combination with vancomycin failed in blood bacterial clearance.  
Linezolid alone and its combinations significantly reduced the bacteraemia  
rates achieved by the control group in hGISA-infected mice ( $P < 0.04$ ). Mice  
treated with linezolid combinations also showed lower number of positive blood  
cultures than imipenem-treated group ( $P < 0.04$ ). In GISA-infected mice,  
linezolid alone and in combination with imipenem significantly reduced the  
bacteraemia rates reached by the control ( $P \leq 0.02$ ) and the vancomycin plus  
imipenem ( $P < 0.05$ ) groups.

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**Murine peritonitis model. Therapeutic efficacy.** Bacterial counts in PF (mean  
 $\log_{10}$ CFU/ml  $\pm$  SD) of control animals at hour 0 were  $8.17 \pm 0.81$  for the hGISA  
strain (n = 25) and  $7.82 \pm 0.57$  for the GISA strain (n = 18). Bacterial counts in  
PF of control and treated mice after 25 h are shown in Table 2. Efficacy of an  
antibiotic therapy was defined as the decrease in the number of CFU ( $\Delta\log$   
CFU/ml) in PF between 0 and 25 h. All regimens were statistically more  
effective than the control group for both strains ( $P < 0.001$ ). Linezolid  
monotherapy produced similar bacterial decreases against both isolates.

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Linezolid was as effective as vancomycin against the hGISA strain but slightly improved vancomycin activity against the GISA strain.

Linezolid combinations showed comparable efficacies to linezolid monotherapy against both strains. The association of linezolid with vancomycin was more active reducing bacterial counts than vancomycin alone in mice infected with the GISA strain. The addition of linezolid to imipenem showed enhanced activity upon imipenem alone against both strains. The association of linezolid with either vancomycin or imipenem showed higher activity than vancomycin plus imipenem against both strains ( $P = 0.048$ , linezolid plus vancomycin vs vancomycin plus imipenem against the GISA strain).

## DISCUSSION

The increasing incidence of nosocomial infections due to *S. aureus* antibiotic-resistant strains and the report of therapeutic failures associated with standard glycopeptide therapy highlight the importance of identifying new synergistic drug combinations [1, 7, 8]. Linezolid has demonstrated good activity against most staphylococci, including methicillin-resistant strains [12, 13].

Linezolid was tested *in vitro* at achievable concentrations in human serum after oral administration of 500 and 600 mg regimens [22]. At 4-16 mg/l linezolid was effective against both hGISA and GISA strains. Its association with different drugs exerted distinct effects. Linezolid combined to imipenem was synergistic against both strains. The synergistic interaction between low concentrations of linezolid and imipenem has been previously reported against MRSA strains [28]. An indifferent effect was the most common result achieved with the interaction between linezolid and vancomycin accordingly to previous studies



1 involving MRSA and hGISA strains [29, 30]. Of particular interest was our  
2 finding of a synergistic killing with sub-MIC concentrations of both antibiotics in  
3 combination against the GISA strain. This enhanced effect has been reported  
4 on another GISA strain in an *in vitro* pharmacodynamic model [31].  
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9 In the murine peritonitis model caused by the hGISA strain no differences were  
10 found between linezolid and vancomycin monotherapies in terms of bacterial  
11 counts in peritoneal fluid and survival. In contrast, in GISA-infected mice  
12 linezolid showed a slightly higher activity than vancomycin, although this did not  
13 reach statistical significance. Moreover, linezolid achieved higher percentages  
14 of blood culture sterilization in comparison to vancomycin against both isolates.  
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16 The use of combined regimens would be a good approach to improve the  
17 effectiveness of linezolid in the management of drug-resistant infections. In our  
18 experimental setting, the addition of linezolid to vancomycin showed similar  
19 efficacy but decreased the bacteraemia rates in comparison to vancomycin and  
20 linezolid monotherapies against the hGISA strain. The same combination  
21 enhanced vancomycin activity against the GISA strain but did not improve the  
22 rates of blood sterilisation achieved with monotherapy regimens.  
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27 Even though  $\beta$ -lactam antibiotics do not show any activity against MRSA and  
28 hGISA strains, their use in combination with linezolid has been shown to be  
29 highly effective against MRSA strains *in vitro* and in experimental endocarditis  
30 [28]. In our study, linezolid combined to imipenem was an effective therapy in  
31 mice infected with hGISA/GISA strains in terms of bacterial and bacteraemia  
32 reduction.  
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37 Our study found some discrepancies between *in vitro* and *in vivo* results. It  
38 should be emphasized that *in vitro* interaction may not translate into clinical  
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1 efficacy, mainly because of the diversity of mechanisms involved in *in vivo*  
2 antibiotic interactions which can not be analyze by the use of *in vitro* techniques  
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4 [33]. In deed, experts recommend to use the *in vivo* efficacy more than the *in*  
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6 *vitro* data when selecting an antistaphylococcal drug as a therapeutic option  
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9 [34].  
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11 To date, few studies have addressed the role of linezolid combinations against  
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13 glycopeptide-resistant *S. aureus*. The present study confirms the  
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15 antistaphylococcal activity of linezolid in association with vancomycin or  
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17 imipenem indicating that linezolid combinations preserves the activity of  
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19 linezolid alone and might be considered as therapeutic options in the  
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21 management of infections caused by *S. aureus* strains with reduced  
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23 susceptibility to glycopeptides.  
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## 31 **ACKNOWLEDGEMENTS**

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**Table 1.** Bacteraemia rates of control and therapeutic groups of infected mice after 25 h of subcutaneous therapy. Data are expressed as percentages of positive blood cultures ( $n \geq 10$  mice/group except for imipenem against the GISA strain where  $n = 6$ ).

LZD, linezolid; VAN, vancomycin; IMP, imipenem

Therapy (25h)	% Positive blood cultures	
	hGISA strain	GISA strain
Control	100	100
LZD 35 mg/kg/5 h	73 <sup>a</sup>	67 <sup>a, c</sup>
VAN 60 mg/kg/5 h	93	81
IMP 30 mg/kg/5 h	100	81*
LZD + VAN	64 <sup>a, b</sup>	79
LZD + IMP	64 <sup>a, b</sup>	67 <sup>a, c</sup>
VAN + IMP	93	100

<sup>a</sup>  $P < 0.04$  vs. Control group

<sup>b</sup>  $P < 0.04$  vs. IMP group

<sup>c</sup>  $P < 0.05$  vs. VAN + IMP group

\* Small "n"; this group was excluded from statistical studies.

**Table 2.** Bacterial counts in peritoneal fluid (PF) for therapeutic and control groups after 25 h of subcutaneous therapy.

Therapy (25h)	PF bacterial counts $\pm$ SD (log CFU/ml) [n]	
	hGISA strain	GISA strain
Control	8.19 $\pm$ 0.57 [29]	8.29 $\pm$ 0.9 [29]
LZD 35 mg/kg/5 h	5.88 $\pm$ 0.61 [15] <sup>a</sup>	5.60 $\pm$ 0.61 [18] <sup>a, b</sup>
VAN 60 mg/kg/5 h	5.90 $\pm$ 0.31 [14] <sup>a</sup>	6.02 $\pm$ 0.56 [16] <sup>a</sup>
IMP 30 mg/kg/5 h	6.40 $\pm$ 0.80 [10] <sup>a</sup>	7.38 $\pm$ 1.38 [6] <sup>*</sup>
LZD + VAN	5.94 $\pm$ 0.30 [14] <sup>a</sup>	5.61 $\pm$ 0.56 [14] <sup>a, b</sup>
LZD + IMP	5.81 $\pm$ 0.41 [14] <sup>a</sup>	5.74 $\pm$ 0.52 [15] <sup>a</sup>
VAN + IMP	6.15 $\pm$ 0.68 [14] <sup>a</sup>	6.28 $\pm$ 0.49 [14] <sup>a</sup>

<sup>a</sup>  $P < 0.001$  vs. Control group

<sup>b</sup>  $P < 0.05$  vs. VAN + IMP group

\* Small "n"; this group was excluded from statistical analysis.

**Figure 1.** Time-kill curves of the combinations of linezolid plus vancomycin that improved the activities of both monotherapies against the GISA strain.

LZD, linezolid; VAN, vancomycin.

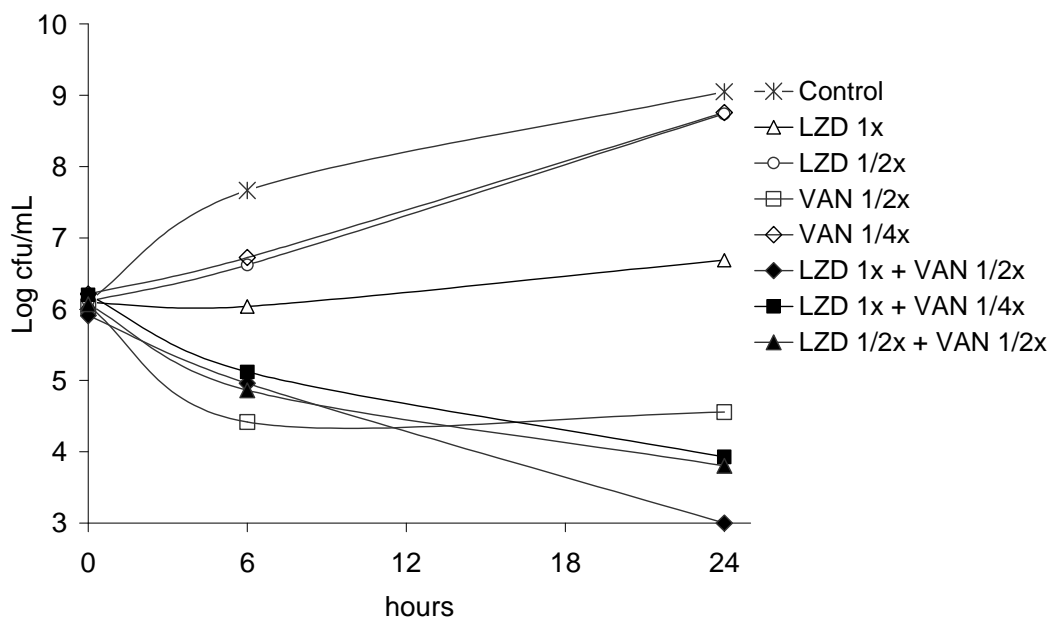
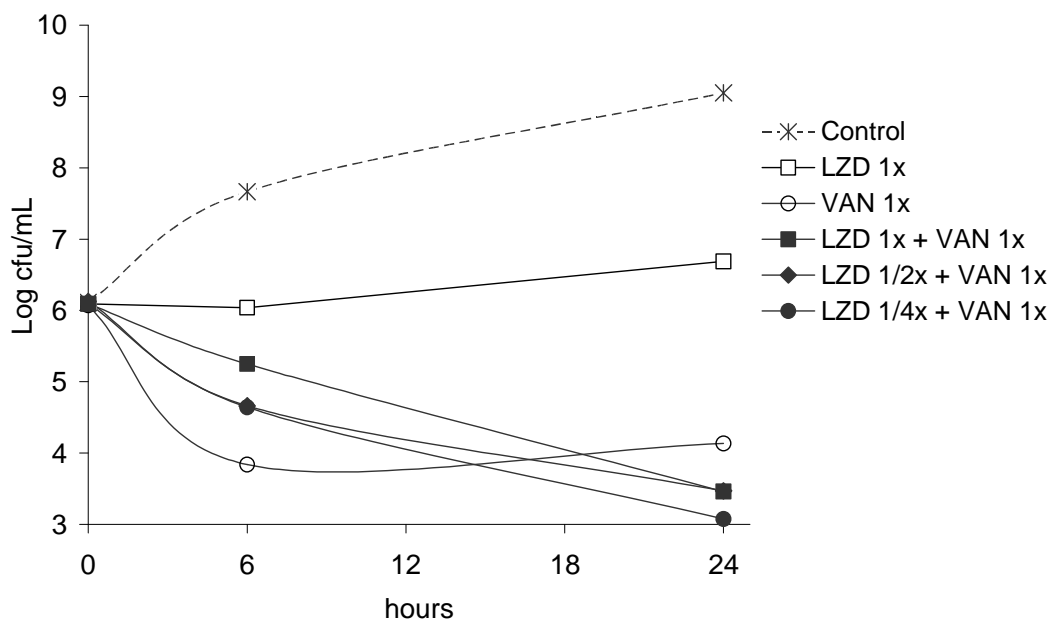


Fig.2

**Figure 2.** Time-kill curves with synergistic activity for linezolid in combination with imipenem against hGISA and GISA strains.

LZD, linezolid; IMP, imipenem

