Prevention of rifampicin resistance in *Acinetobacter baumannii* in an experimental pneumonia murine model, using rifampicin associated with imipenem or sulbactam

María E. Pachón-Ibáñez¹, Felipe Fernández-Cuenca², Fernando Docobo-Pérez¹, Jerónimo Pachón¹,³* and Álvaro Pascual²,⁴

¹Service of Infectious Diseases, Hospitales Universitarios Virgen del Rocío, Avda. Manuel Siurot s/n, 41013, Sevilla, Spain; ²Service of Microbiology, Hospital Universitario Virgen Macarena, Avda. Dr Fedriani s/n, 41009, Sevilla, Spain; ³Department of Medicine, University of Sevilla, Avda. Dr Fedriani s/n, 41009, Sevilla, Spain; ⁴Department of Microbiology, University of Sevilla, Avda. Sánchez Pizjuan s/n, 41009, Sevilla, Spain

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**Objectives:** To examine the development of rifampicin resistance in multidrug-resistant *Acinetobacter baumannii* exposed to rifampicin and the prevention of the appearance of rifampicin-resistant mutants when rifampicin is used in association with imipenem or sulbactam.

**Methods:** A clinical strain of multidrug-resistant *A. baumannii* was used to examine the frequency of resistance to rifampicin *in vivo*, in a pneumonia model in immunocompetent C57BL/6 mice. The *in vitro* and *in vivo* prevention of the development of resistance to rifampicin was analysed using rifampicin alone or in association with imipenem or sulbactam, in time–kill studies and in the experimental murine pneumonia, respectively.

**Results:** Rifampicin-resistant mutants were found at 48 and 72 h, both *in vitro* and *in vivo*, when rifampicin was used alone, with the MIC increasing from 4 to ≥128 mg/L. The *in vivo* frequency of rifampicin-resistant mutants was $3 \times 10^{-6}$. On the contrary, no resistant mutants appeared after 72 h, *in vitro* or *in vivo*, when rifampicin was employed in association with imipenem or sulbactam. After six daily passages in rifampicin-free agar plates the resistant mutants maintained the high resistance to rifampicin (≥128 mg/L).

**Conclusions:** These results suggest that rifampicin must not be used alone in the treatment of infections caused by multidrug-resistant *A. baumannii*. In these cases, rifampicin may be used in combination with imipenem or sulbactam, which prevent the development of resistance to rifampicin.

Keywords: resistant mutant, combined treatment, *in vivo* resistance

**Introduction**

*Acinetobacter baumannii* is an important cause of nosocomial infections worldwide. Most nosocomial isolates of *A. baumannii* are resistant to a wide variety of antimicrobials, including imipenem and rifampicin.⁵ Studies on experimental murine pneumonia have reported that rifampicin is active *in vitro* and *in vivo* against multidrug-resistant *A. baumannii*, including those with intermediate resistance to rifampicin.⁶ Consequently, rifampicin has been used successfully, combined with colistin, for the therapy of selected *A. baumannii* infections in humans.⁷ It is well known that, in infections caused by other bacteria, rifampicin must not be used as monotherapy because of the rapid development of resistance *in vitro* and *in vivo*. However, the development of resistance to rifampicin can be avoided with the use of combination treatment. A classic example is the treatment of tuberculosis. Monotherapy with rifampicin induces the development of resistance within 3 months. On the contrary,
the association of isoniazid with rifampicin prevents the development of resistance.

The aim of the present study was to examine the frequency of resistance in multidrug-resistant A. baumannii exposed to rifampicin in vitro and in vivo and the prevention of the development of resistance when rifampicin is used in association with other antimicrobials.

Materials and methods

Antimicrobials

The antimicrobials for the in vitro experiments were obtained as laboratory standard powders: rifampicin (Sigma Chemical Co., Madrid, Spain), imipenem (Merck Sharp & Dohme, Madrid, Spain) and sulbactam (Pfizer, Orsay, France). The anaesthetic for the in vivo experiments was 5% sodium thiopental (B. Braun Medical S.A., Rubi, Barcelona, Spain).

Bacterial strain

A clinical strain of A. baumannii causing nosocomial bacteraemia (HUVR 1327) was used that was collected and identified in a previous study of A. baumannii bacteraemia. This strain was multidrug-resistant, including to imipenem and sulbactam, and was susceptible to rifampicin and colistin.

Determination of MICs and MBCs

MICs of rifampicin, imipenem and sulbactam were determined by the standard microdilution method. Concentrations of antimicrobials from 128 to 0.125 mg/L were tested. MBCs were determined by subculturing 0.1 mL samples from MIC broth cultures on Mueller–Hinton agar plates. Escherichia coli ATCC 25922 was used as a reference strain. Breakpoints for susceptibility and resistance of A. baumannii to rifampicin were those from the French Society for Microbiology.

In vitro selection of rifampicin-resistant mutants

Time–kill curves were used. A. baumannii was incubated with rifampicin, imipenem or sulbactam at concentrations of 1× MIC, 2× MIC and 4× MIC. Furthermore, the 18 possible combinations of rifampicin plus imipenem or sulbactam at these concentrations were tested. Tubes with 20 mL of MHB with an inoculum of 5×10^7 cfu/mL of the strain HUVR 1327 were used. Tubes with the bacterial inoculum and without antimicrobials were used as growth controls. The bacterial growth was counted at 0, 24, 48 and 72 h after incubation. After these passages, we selected a maximum of five colonies to determine the MIC in triplicate.

Stability of rifampicin-resistant mutants

The stability of mutants of A. baumannii resistant to rifampicin was tested by giving them six daily passages in rifampicin-free agar plates. After these passages, we selected a maximum of five colonies to determine the MIC in triplicate.

Laboratory animals

Immunocompetent specific-pathogen-free C57BL/6 young female mice, weighing 16–20 g, were used. They were supplied by Universidad de Sevilla’s facility. Animals were housed in regulation cages and given free access to food and water. The use of mice for these experiments was approved by the Ethics Committee of the University Hospitals Virgen del Rocío (Document 4/2000).

In vivo selection of rifampicin-resistant mutants

An experimental murine pneumonia model was used to evaluate the selection of rifampicin-resistant mutants of A. baumannii. An inoculum of ~10^9 cfu/mL was used. Therapy was started 4 h after the inoculation.

A total of 30 mice were randomly included in the following treatment groups for 72 h: 6 mice for each treatment group: rifampicin 100 mg/kg/day, imipenem 120 mg/kg/day, sulbactam 240 mg/kg/day, rifampicin plus imipenem, and rifampicin plus sulbactam. In each case the total daily dose was divided in three administrations. Rifampicin was administered intraperitoneally and the others intramuscularly. Two mice of each group were sequentially sacrificed every 24 h. The lungs were aseptically removed, weighed and homogenized for 2 min in 2 mL of sterile saline solution (Stomacher 8, Tekmar Co., Cincinnati, OH, USA). Thereafter, they were vortexed for 1 min and centrifuged at 800 rpm for 10 min at 4°C. The supernatant was placed into a new tube and vortexed for 1 min and centrifuged at 4000 rpm for 15 min at the same temperature. The supernatant was taken out and mixed in 600 μL of sterile saline solution. Finally, we plated 100 μL on agar and incubated for 24 h at 37°C; determination of the MIC of rifampicin was carried out in triplicate for a maximum of five colonies from these plates.

Frequency of rifampicin-resistant mutants

Two mice were inoculated with A. baumannii HUVR 1327. One of them was used as control (without treatment). The other was treated with rifampicin 100 mg/kg/day as previously detailed. Both were sacrificed 24 h after the inoculation. The lungs were removed and homogenized for 2 min in 2 mL of sterile saline solution. After 10-fold dilutions, 100 μL was plated on sheep blood agar for 24 h at 37°C and the counts were expressed as log_{10} cfu/g of tissue. To calculate the frequency of appearance of rifampicin-resistant mutants we divided the log_{10} cfu/g of the treated mouse by the same figure from the control mouse. These experiments were carried out twice.

Statistical analysis

χ^2 and Student t-tests were performed. A value of P < 0.05 was considered significant. The statistical package SPSS 13.0 (SPSS Inc., Chicago, IL, USA) was used.

Results

MICs and MBCs

The MICs/MBCs of imipenem, sulbactam and rifampicin for the HUVR 1327 strain were 32/32, 32/32 and 4/8 mg/L, respectively.

In vitro selection of rifampicin-resistant mutants

The bactericidal activity of the drugs at different concentrations is shown in Figure 1. The susceptibility to rifampicin did not change in the tubes containing imipenem or sulbactam alone, at 24, 48 and 72 h of incubation. In the tubes with rifampicin alone the MIC of rifampicin remained 4 mg/L at 24 h and changed to ≥128 mg/L after 48 and 72 h of incubation.
In the same way, at 24 h with the combinations of rifampicin plus imipenem or sulbactam, the MIC of rifampicin did not change from 4 mg/L. No bacteria were recovered from the tubes in the experiments made with the different combinations of rifampicin plus imipenem or sulbactam at 48 and 72 h of incubation.

Stability of rifampicin-resistant mutants

The rifampicin-resistant mutants from HUVR 1327 A. baumannii maintained their resistance (MIC ≥ 128 mg/L) over six daily passages in fresh rifampicin-free agar plates.

In vivo selection of rifampicin-resistant mutants

Susceptibilities of the strains of A. baumannii recovered from the lungs of the infected mice treated with the different monotherapies or combinations are shown in Table 1.

<table>
<thead>
<tr>
<th>Basal</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rifampicin (RIF)</td>
<td>4</td>
<td>4</td>
<td>≥128</td>
</tr>
<tr>
<td>Imipenem (IPM)</td>
<td>4</td>
<td>4</td>
<td>ND</td>
</tr>
<tr>
<td>Sulbactam (SUL)</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>RIF + IPM</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>RIF + SUL</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

ND, not done, because no survivor mice at these time-points.

Frequency of rifampicin-resistant mutants

The number of colonies was $2 \times 10^8$ cfu/g of lung in the untreated mice and $0.66 \times 10^2$ cfu/g in the mice treated with rifampicin. The MIC of rifampicin for the isolates from treated mice increased to $\geq 128$ mg/L. Thus, the frequency of appearance of rifampicin-resistant mutants was $3 \times 10^{-6}$.

Discussion

These results show that A. baumannii develops resistance to rifampicin when this drug is used alone, both in vitro and in vivo. The association of imipenem or sulbactam with rifampicin prevents the development of resistance to rifampicin. We used a strain susceptible to rifampicin, because in A. baumannii experimental pneumonia rifampicin was efficacious as monotherapy using strains showing MICs of rifampicin between 4 and 8 mg/L.9

The appearance of rifampicin resistance in staphylococcal infections can be avoided with the use of rifampicin in association with other antimicrobials,10 like happened in our experiments when we used rifampicin in combination with imipenem or sulbactam. The association of ciprofloxacin or vancomycin with rifampicin in the treatment of methicillin-resistant S. aureus experimental osteomyelitis in rats prevented the emergence of resistance to rifampicin, whereas rifampicin-resistant mutants appeared in the animals treated with rifampicin alone.10

In our experiments, the in vivo mutation rate in A. baumannii was $10^{-6}$, which is higher than that found in other bacteria, which ranges from $10^{-7}$ to $10^{-8}$.11 We also found that the rifampicin-resistant mutants, both in the in vitro and in vivo experiments, were stable and maintained their resistance through six daily transfers in rifampicin-free agar.

In summary, as occurred in the treatment of infections by other bacteria, our results suggest that rifampicin must not be used alone in the treatment of infections caused by A. baumannii. In these cases, rifampicin may be used in association with imipenem or sulbactam, which prevents the development of resistance to rifampicin.

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Transparency declarations
None to declare.

References

β-lactams, 