Enhanced active efflux, repression of porin synthesis and development of Mar phenotype by diazepam in two enterobacteria strains

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The aim of this work was to determine whether diazepam could induce the multiple antibiotic resistance (Mar) phenotype in Klebsiella pneumoniae and Escherichia coli strains. The Mar phenotype is characterized by decreased susceptibility to multiple antibiotics due to the loss of porins and/or increased expression of active efflux systems. The effect of subinhibitory concentrations of diazepam on the susceptibility of different antimicrobial agents, outer-membrane protein expression and norfloxacin intracellular accumulation was studied. The results revealed that diazepam concentrations equal or twice adult dosage induced the same Mar phenotype as two well known E. coli marRAB inducers, sodium salicylate and sodium benzoate. Susceptibility to norfloxacin in a K. pneumoniae clinical isolate and E. coli strain Ag100 decreased due to enhanced active efflux and loss of porin expression. A decreased susceptibility to chloramphenicol, tetracycline, nalidixic acid and β-lactam antibiotics was also observed. In conclusion, like sodium salicylate or sodium benzoate, diazepam may induce the Mar phenotype.

Introduction

Several chemicals, such as sodium salicylate (salicylate) and sodium benzoate (benzoate) and chemotherapeutic agents, such as clofibrac and ethacrynic acids (Alekshun & Levy, 1997; Balagué & Vescovi, 2001) induce a multiple antibiotic resistance phenotype (Mar) in Escherichia coli by the activation of the marRAB operon. This activation increases MarA expression (transcriptional activator of mar regulon) resulting in a Mar phenotype that is characterized by decreased susceptibility to multiple antibiotics due to increased active efflux and decreased expression of OmpF (Alekshun & Levy, 1997). RamA is another transcriptional activator, a MarA homologue (Alekshun & Levy, 1997) that has been identified in multidrug-resistant mutants of Klebsiella pneumoniae (George et al., 1995). Thus, we were interested in identifying other chemotherapeutic agents that may also induce Mar phenotype.

Previous works have reported an impaired immune response and decreased survival to bacterial infections as side-effects of treatment with diazepam, both in humans and in mice (Covelli et al., 1991; Galdiero et al., 1995; Laschi et al., 1983). Nevertheless, diazepam has not yet been evaluated as an inducer of bacterial antibiotic resistance. The possibility that diazepam might induce the Mar phenotype in addition to an impaired immune response would increase the risk of refractory infections to antimicrobial treatments. In this study, we compared the behaviour of diazepam with that of salicylate or benzoate as inducers of multiple antibiotic resistance in two strains of both K. pneumoniae and E. coli.

Methods

Bacterial strains and drugs. The effects of salicylate, benzoate and diazepam were studied in a clinical isolate of K. pneumoniae (KP1A02) and in the wild-type E. coli strain Ag100. K. pneumoniae ATCC 10031 (donated by Dr Federico Uruburu, Spanish Type Culture Collection, Valencia, Spain) and strain Ag112 were used as control strains. Strain Ag112 (an OmpF-deficient strain) is a marR mutant derived from strain Ag100 (Oethinger et al., 2000). Strains Ag100 and Ag112 were donated by Dr S. B. Levy, Tufts University School of Medicine, Boston, USA.

Salicylate, benzoate and antimicrobial agents were purchased from...
Sigma. Diazepam was purchased from Roche. An agar dilution method following NCCLS guidelines was used in the susceptibility tests (NCCLS, 2003a).

**Preparation and analysis of outer-membrane proteins (OMPs).**

Bacterial cell pellets were obtained from 1 l of mid-exponential-phase cultures grown in antibiotic medium no. 3 (Difco) with or without diazepam (0.015–0.5 mM) and salicylate or benzoate (0.25–20 mM). Bacterial cells were disrupted by sonication, separating unbroken cells from cell envelopes by centrifugation. A 2 % sodium lauryl sarcosinate solution was used to solubilize cytoplasmic membranes, leaving insoluble outer membranes, which were recovered by centrifugation as a pellet. Each membrane suspension (10 μg) in loading buffer was electrophoresed by SDS-PAGE (Tavío et al., 1999).

**Measurements of norfloxacin accumulation.** Norfloxacin intracellular accumulation was measured as previously described (Tavío et al., 1999). The norfloxacin uptake measurements were simultaneously performed in strains KPA102 and Ag100 grown with and without subinhibitory concentrations of diazepam (0.03–0.5 mM) and salicylate or benzoate (5–20 mM). Salicylate and benzoate MICs were 20 mM in strain Ag100 and 40 mM in strain KPA102. Diazepam MICs were 0.5 mM in strain Ag100 and 1 mM in strain KPA102. Likewise, all the above were assayed with and without the presence of 50 and 100 μM carbonyl cyanide m-chlorophenylhydrazone (CCCP), an inhibitor of proton motive force (Oethinger et al., 2000; Alekshun & Levy, 1997). Norfloxacin concentration in each cellular extract was measured at least six times by bioassay using *K. pneumoniae* ATCC 10031, as previously described (Tavío et al., 1999). *K. pneumoniae* ATCC 10031 is recommended by Spanish Type Culture Collection for susceptibility tests. Norfloxacin intracellular concentration in extracts was determined by a disk-diffusion method (NCCLS, 2003b). The inhibition zones produced by 20 μl aliquots of each extract were compared with those produced by 20 μl aliquots of different known norfloxacin concentrations using nonlinear regression. The accepted standard deviation for all the norfloxacin uptake results was always <5 % with respect to each mean value of the three measurements that were taken at 5, 10, 15 and 20 min, with and without CCCP.

The norfloxacin uptake basal level was defined as intracellular norfloxacin concentration in the strains grown without salicylate, benzoate, diazepam or CCCP.

**Results and Discussion**

Several drugs have been identified as inducers of the Mar phenotype in *E. coli* by the activation of the marRAB operon (Alekshun & Levy, 1997; Balagué & Vescovi, 2001). Diazepam is not among the chemotherapeutic agents analysed as such (Alekshun & Levy, 1997; Balague & Vescovi, 2001). Diaze-

![Fig. 1. (a) SDS-11 % PAGE of OMPs prepared from the following strains: 1, KPA102; 2, KPA102 in 20 mM sodium benzoate (SB); 3, KPA102 in 10 mM SB; 4, KPA102 in 5 mM SB; 5, KPA102 in 0.5 mM diazepam (DZ); 6, KPA102 in 0.25 mM DZ; 7, KPA102 in 0.12 mM DZ; 8, KPA102 in 20 mM sodium salicylate (SL); 9, KPA102 in 10 mM SL; 10, KPA102 in 5 mM SL; 11, *K. pneumoniae* ATCC 10031; 12, Molecular mass standards corresponding to ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa). The 42 kDa OMP is marked by →, the 34 kDa OMP by •. (b) 6 M Urea SDS-10 % PAGE of OMPs prepared from the following strains: 1, *E. coli* Ag100 in 10 mM SB; 2, *E. coli* Ag100 in 5 mM SB; 3, *E. coli* Ag100 in 0.5 mM SB; 4, *E. coli* Ag100 in 0.25 mM SB; 5, *E. coli* Ag112; 6, *E. coli* Ag100. (c) 6 M Urea SDS-10 % PAGE of OMPs prepared from the following strains: 1, *E. coli* Ag100; 2, *E. coli* Ag112; 3, *E. coli* Ag100 in 0.25 mM DZ; 4, *E. coli* Ag100 in 0.12 mM DZ; 5, *E. coli* Ag100 in 0.03 mM DZ (▼); 6, *E. coli* Ag100 in 0.015 DZ. C, OmpC; F, OmpF; A, OmpA.

-0.03–0.25 mM diazepam repressed OmpF expression in strain Ag100, leading to the same OMP profile as *E. coli* strain Ag112 (Fig. 1c), matching the effect of benzoate (Fig. 1b) and coinciding with previous descriptions (Alekshun & Levy, 1997). Diazepam concentrations ≤0.015 mM were not assayed more extensively since they did not induce OMP changes in either of these two strains.

For the susceptibility tests, all the assayed diazepam concentrations increased MICs to β-lactams, quinolones, tetracycline and chloramphenicol in both the KPA102 and Ag100 strains, similar to the effect of salicylate or benzoate (Table 1). In line with these results, the Mar phenotype in *E. coli* (or its equivalent in *K. pneumoniae*) is characterized by increased MICs of antibiotics that cross the outer membrane preferentially through porins and/or which are efflux pump substrates, such as β-lactams, nalidixic acid, norfloxacin, tetracycline and chloramphenicol (Alekshun & Levy, 1997; Doménech-Sánchez et al., 2003). Mar phenotypes induced by diazepam, salicylate and benzoate did not always result in MICs ≥ the breakpoints for assayed antibiotics. Thus, norfloxacin MICs were ≤2 μg ml⁻¹, as in strains that show decreased permeability in the outer membrane and enhanced active efflux to fluoroquinolones (Martínez-Martínez et al., 2002; Kern et al., 2000). In this way, MICs of the antimicro-
bial agents assayed in strain Ag100 grown with diazepam, salicylate or benzoate matched in many cases those found in strain Ag112 (a Mar phenotype strain) (Oethinger et al., 2000) grown without the presence of any of these chemicals (Table 1).

Thus, changes in OMP expression and development of the Mar phenotype were induced by some of the standard clinical doses of diazepam, bearing in mind that the usual maximum adult dosage for diazepam is 40 mg day\(^{-1}\) in tablets and 160 mg intramuscular or intravenous (Roche dosage recommendations). This means that the normal adult dosage per day could be 0.03 mM or 0.12 mM, although therapeutic doses may sometimes reach 400 mg day\(^{-1}\) (0.3 mM).

Changes in norfloxacin uptake were also induced by diazepam, salicylate or benzoate. Norfloxacin uptake levels induced by 20 mM salicylate or benzoate and 0.5 mM diazepam (Table 2) in strain KP1A02 (3.2–4.1-fold less than the basal level) were characteristic of \(K.\ pneumoniae\) strains that do not express OmpK36 and show enhanced active efflux (Martínez-Martínez et al., 1998). The concomitant 16-fold increase in norfloxacin MICs in strain KP1A02 (Table 1) might be explained by the participation of the above two mechanisms of resistance, as previously described in other strains (Doménech-Sánchez et al., 2003; Schneider et al., 2003). Likewise, 5 or 10 mM salicylate or benzoate and 0.12 or 0.25 mM diazepam decreased norfloxacin uptake 2–3.2-fold in strain KP1A02 (Table 2), concomitant with a four to eightfold increase in norfloxacin MIC, probably due to both the decreased expression of 36 kDa OMP (not total loss) and enhanced active efflux, as previously reported (Martínez-Martínez et al., 1998; Doménech-Sánchez et al., 2003). Moreover, diazepam, salicylate or benzoate decreased norfloxacin uptake 3–10.3-fold in strain Ag100 (Table 2), an effect similar to that which occurs when the \(mar\) regulon is activated (Kern et al., 2000; Oethinger et al., 2000) (Table 2). Reduced norfloxacin uptake was concomitant with a two to eightfold increase in norfloxacin MIC, as previously associated with the development of the Mar phenotype in \(E. coli\) strains (Oethinger et al., 2000).

### Table 1. Effect of salicylate, diazepam and benzoate on antimicrobial agents MICs (µg ml\(^{-1}\))

FOX, cefoxitin; CP, cephalothin; TE, tetracycline; CL, chloramphenicol; NA, nalidixic acid; NOR, norfloxacin; ND, not done.

<table>
<thead>
<tr>
<th>Agent</th>
<th>Strain</th>
<th>Salicylate (mM)</th>
<th>Diazepam (mM)</th>
<th>Benzoate (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td><strong>KP1A02</strong></td>
<td>FOX</td>
<td>4</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>CP</td>
<td>4</td>
<td>32</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>TE</td>
<td>2</td>
<td>32</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>CL</td>
<td>2</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>NA</td>
<td>8</td>
<td>16</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>NOR</td>
<td>0.03</td>
<td>0.12</td>
<td>0.25</td>
</tr>
<tr>
<td><strong>Ag100/Ag112</strong></td>
<td>FOX</td>
<td>4/32</td>
<td>32</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>CP</td>
<td>2/16</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>TE</td>
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</tr>
<tr>
<td></td>
<td>NOR</td>
<td>0.06/0.25</td>
<td>0.25</td>
<td>0.25</td>
</tr>
</tbody>
</table>

### Table 2. Effect of salicylate, diazepam and benzoate on norfloxacin uptake by bacterial cells [ng norfloxacin (mg dry cell)\(^{-1}\)]

ND, Not done.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Salicylate (mM)</th>
<th>Diazepam (mM)</th>
<th>Benzoate (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td><strong>KP1A02</strong></td>
<td>123</td>
<td>53</td>
<td>42</td>
</tr>
<tr>
<td>+50 µM CCCP</td>
<td>196</td>
<td>248</td>
<td>175</td>
</tr>
<tr>
<td>+100 µM CCCP</td>
<td>196</td>
<td>248</td>
<td>186</td>
</tr>
<tr>
<td><strong>Ag100</strong></td>
<td>127</td>
<td>15</td>
<td>12.3</td>
</tr>
<tr>
<td>+50 µM CCCP</td>
<td>206</td>
<td>40</td>
<td>30.8</td>
</tr>
<tr>
<td>+100 µM CCCP</td>
<td>206</td>
<td>45</td>
<td>49.2</td>
</tr>
</tbody>
</table>

http://jmm.sgmjournals.org
The use of CCCP for evaluating active efflux showed that incubation of strains KP1A02 and Ag100 in 50 or 100 μM CCCP increased the intracellular norfloxacin basal level 1.6-fold (Table 2). This increase is within the range of response to CCCP in susceptible strains (Kern et al., 2000; Oethinger et al., 2000). Earlier studies have confirmed that fluoroquinolone-susceptible E. coli cells use energy to reduce the intracellular level of norfloxacin. In these cases, when proton motive force is dissipated by CCCP, the increase in fluoroquinolone accumulation is twofold the basal level (Oethinger et al., 2000). By contrast, a 2.5- to 1.0-fold increase in norfloxacin uptake in strain KP1A02, grown with diazepam, salicylate or benzoate, was induced by 50–100 μM CCCP (Table 2). Thus, incubation with CCCP restored or exceeded the intracellular norfloxacin basal level in strain KP1A02. These results agree with previous studies on K. pneumoniae that attribute a main role to active efflux in norfloxacin uptake (Martinez-Martinez et al., 2002; Domenech-Sanchez et al., 2003). Nevertheless, despite a 2- to 3-fold increase in norfloxacin uptake through the effect of 50 or 100 μM CCCP in strain Ag100 grown in salicylate, benzoate and diazepam (Table 2), norfloxacin uptake was always less than the basal level in strain Ag100, concurrent with the previously described role of OmpF in outer membrane permeability to norfloxacin in E. coli strains (Kern et al., 2000; Mortimer & Piddock, 1993).

In conclusion, diazepam effects on outer-membrane protein expression, active efflux and antimicrobial agent MICs in strains KP1A02 and Ag100 matched to a notable extent those induced by two recognized marRAB inducers (salicylate and benzoate) (Alekshun & Levy, 1997). The above results suggest that diazepam concentrations equal or twice adult dosage might induce the expression of MarA in E. coli strains or RamA in K. pneumoniae strains (George et al., 1995; Schneiders et al., 2003). Thus, diazepam might play a role not only as a cause of impaired immunity to bacterial infections as previously described by Laschi et al. (1983), but also as an inducer of the bacterial Mar phenotype. The conjunction of both mechanisms induced by diazepam might lead to a refractory infection. Therefore, the potential development of the bacterial Mar phenotype in a concomitant infection with a diazepam treatment in humans should not be ruled out.

Acknowledgements

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References


