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# Effects of permeabilizers on antimicrobial susceptibility of *Stenotrophomonas maltophilia* and *Acinetobacter* spp.

**Brian Denny[1], Patrick West[2], Debadetta Panigrahi[2]**

*[1]Department of Pharmaceutical Chemistry, Faculty of Pharmacy; and [2]Department of Medical Laboratory Sciences, Faculty of Allied Health Sciences and Nursing, Kuwait University, Kuwait*

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Isolates of the gram-negative bacteria *Stenotrophomonas maltophilia* and *Acinetobacter* spp. are naturally resistant to many classes of antibiotics. Using the disk diffusion technique it was shown that the membrane permeabilizers ethylenediaminetetraacetic acid, sodium citrate, and sodium polyphosphate increased susceptibility to a range of antibiotics including imipenem, ciprofloxacin, tetracycline, and rifampicin. These effects are probably due to the metal chelating properties of the permeabilizers.

**Key words:** *Acinetobacter* spp., antimicrobial susceptibility, permeabilizers, *Stenotrophomonas maltophilia*

*Stenotrophomonas maltophilia* and *Acinetobacter* spp. are gram-negative bacilli. They are widely distributed in nature and increasingly important cause of nosocomial infection [1,2]. At least 14 species of *Acinetobacter* have been described [3]. Many of these nosocomial strains are resistant to multiple antibiotics.

The outer membrane of gram-negative cells is surrounded by an anionic lipopolysaccharide (LPS) surface that is stabilized by $Ca^{2+}$ and $Mg^{2+}$ ions [4]. The outer membrane also contains porins and efflux pumps embedded in the LPS layer [5]. Permeabilizing agents are chemicals that increase the permeability of bacterial membranes to antibiotics and other agents. A variety of substances can act as membrane permeabilizing agents. Generally, these agents are either cationic substances such as peptides or polyamines [4, 6] that bind to the LPS or anionic substances that act as metal chelators. These disrupt the outer membrane by removing $Ca^{2+}$ and $Mg^{2+}$ from the LPS.

The aim of this study was to investigate the effect of the membrane permeabilizers ethylenediaminetetraacetic acid (EDTA), citrate, and sodium polyphosphate (SPP) on the antimicrobial susceptibility of *S. maltophilia* and *Acinetobacter species*. All of these agents are metal chelators. We looked at their effect on antibiotics that are effluxed from the cell (tetracycline, chloramphenicol, ciprofloxacin, and nalidixic acid); enzymatically degraded within the periplasmic space (imipenem); hydrophobic compounds that do not enter gram-negative cells (rifampicin, erythromycin, and vancomycin); and colistin, a cyclic peptide known to disrupt the outer membrane of bacteria.

*Corresponding author: Dr. Brian Denny, PO Box 24923, Safat 13110, Kuwait. E-mail: denny@hsc.kuniv.edu.kw*

## Materials and Methods

Three epidemiologically distinct clinical isolates of *S. maltophilia* plus clinical isolates of *Acinetobacter lwoffii* and *Acinetobacter baumannii* were tested. Species identity was confirmed using the API 20NE method (BioMerieux, Marcy-I' Etoile, France). Samples were incubated in air at 30°C for 24 h and results obtained using the API 20NE software (version 5.1, BioMerieux). Antimicrobial susceptibility tests were performed on Mueller Hinton agar (Acumedia, Baltimore, MD, US) with and without permeabilizers by a disc diffusion method, according to the method of Bauer *et al* [7] as published by National Committee for Clinical Laboratory Standards [8]. Double strength Mueller Hinton medium and permeabilizer solution were prepared in distilled water and autoclaved at 121°C for 15 min. The agar and permeabilizer solutions were cooled to 50°C and equal volumes mixed to produce the media with the appropriate concentrations of permeabilizer. Bacteria suspended in peptone water (Oxoid, Basingstoke, UK) to an optical density of 0.5 McFarland were used to inoculate the media.

Inhibition zone sizes were measured in millimeters after 24 h incubation at 37°C. All experiments were performed at least 3 times. All antibiotic discs were obtained from Oxoid. Ethylenediaminetetraacetic acid, sodium citrate, and SPP were all obtained from Sigma Chemical Co. (St. Louis, MO, US).

The antibiotics and the concentrations used (μg/disk) were as follows: chloramphenicol (30),

**Table 1.** Effect of the permeabilizer EDTA on the growth inhibition of *S. maltophilia* by antibiotics

| EDTA concentration (molar) | Inhibition zone of antibiotics in diameter (mm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | C | CIP | CL | IPM | NA | NOR | RA | Te |
| Strain 55[a] | | | | | | | | |
| 0 | 13 | 9 | 15 | 6 | 9 | 6 | 9 | 12 |
| 0.002 | 30 | 6 | 34 | 22 | 6 | 14 | 11 | 29 |
| Strain 85[a] | | | | | | | | |
| 0 | 9 | 21 | 10 | 6 | 13 | 6 | 10 | 12 |
| 0.002 | 11 | 15 | 28 | 42 | 6 | 6 | 23 | 36 |
| Strain 86 | | | | | | | | |
| 0 | 26 | 13 | 9 | 6 | 16 | 6 | 7 | 13 |
| 0.002 | 26 | 12 | 19 | 42 | 16 | 6 | 9 | 23 |
| 0.004 | 28 | 16 | 29 | 48 | 14 | 6 | 17 | 32 |

Abbreviations: EDTA = ethylenediaminetetraacetic acid; C = chloramphenicol; CIP = ciprofloxacin; CL = colistin; IPM = imipenem; NA = nalidixic acid; NOR = norfloxacin; RA = rifampicin; Te = tetracycline

[a]There was no growth with 0.004 M EDTA.

ciprofloxacin (5), colistin (10), imipenem (10), nalidixic acid (30), norfloxacin (10), rifampicin (5), and tetracycline (30).

## Results and Discussion

The results for 3 strains of *S. maltophilia* tested against different antibiotics in the presence of permeabilizers are shown in Tables 1, 2, and 3. Susceptibility to tetracycline was increased for all strains and all 3 permeabilizers. This effect is probably due to chelation of $Ca^{2+}$ and $Mg^{2+}$ by the permeabilizers since the non-chelated form of tetracycline can enter cells more easily [9].

Susceptibility to quinolones (nalidixic acid, norfloxacin, ciprofloxacin) was increased to a limited extent by all 3 permeabilizers. The mechanism in this case may be related to the permeabilizers limiting the availability of divalent cations such as $Ca^{2+}$ and $Mg^{2+}$. These have been shown to inhibit quinolones [10].

Two of the strains (no. 85 and no. 55) were resistant to chloramphenicol and the other strain (no. 86) was susceptible. One of the resistant strains (no. 55) showed increased susceptibility with EDTA and SPP (but not citrate). The other resistant strain (no. 85) showed no enhanced effect in the presence of the permeabilizers. This suggests that the 2 strains have different mechanisms of chloramphenicol resistance.

The marked effect of EDTA (compared with citrate and SPP) on the activity of imipenem could be related to zinc chelation [11]. Imipenem hydrolysis in *S.*

**Table 2.** Effect of the permeabilizer sodium citrate on the growth inhibition of *S. maltophilia* by antibiotics

| Citrate concentration (% w/v) | Inhibition zone of antibiotics in diameter (mm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | C | CIP | CL | IPM | NA | NOR | RA | Te |
| Strain 55 | | | | | | | | |
| 0 | 14 | 12 | 15 | 6 | 14 | 6 | 9 | 11 |
| 1.0 | 15 | 14 | 18 | 6 | 11 | 6 | 12 | 21 |
| 2.5 | 16 | 19 | 18 | 6 | 12 | 6 | 16 | 21 |
| 5.0 | 16 | 18 | 20 | 6 | 12 | 6 | 18 | 21 |
| Strain 85 | | | | | | | | |
| 0 | 10 | 18 | 16 | 6 | 19 | 11 | 11 | 14 |
| 1.0 | 11 | 25 | 21 | 6 | 15 | 12 | 13 | 22 |
| 2.5 | 12 | 28 | 25 | 10 | 20 | 16 | 15 | 29 |
| 5.0 | 13 | 32 | 29 | 13 | 15 | 17 | 15 | 26 |
| Strain 86 | | | | | | | | |
| 0 | 26 | 13 | 9 | 6 | 16 | 6 | 7 | 13 |
| 1.0 | 25 | 19 | 11 | 8 | 17 | 9 | 8 | 21 |
| 2.5 | 26 | 23 | 12 | 11 | 22 | 13 | 13 | 22 |
| 5.0 | 24 | 29 | 15 | 14 | 23 | 16 | 14 | 25 |

Abbreviations: C = chloramphenicol; CIP = ciprofloxacin; CL = colistin; IPM = imipenem; NA = nalidixic acid; NOR = norfloxacin; RA = rifampicin; Te = tetracycline

**Table 3.** Effect of the permeabilizer sodium polyphosphate on the growth inhibition of *S. maltophilia* by antibiotics

| SPP concertration (% w/v) | Inhibition zone of antibiotics in diameter (mm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | C | CIP | CL | IPM | NA | NOR | RA | Te |
| Strain 55 | | | | | | | | |
| 0 | 13 | 9 | 15 | 6 | 9 | 6 | 6 | 12 |
| 0.25 | 16 | 13 | 13 | 6 | 15 | 6 | 10 | 24 |
| 0.5 | 16 | 11 | 12 | 6 | 16 | 6 | 10 | 26 |
| 1.0 | 20 | 9 | 12 | 6 | 16 | 6 | 10 | 30 |
| Strain 85 | | | | | | | | |
| 0 | 9 | 21 | 10 | 6 | 13 | ·11 | 10 | 12 |
| 0.25 | 14 | 22 | 11 | 12 | 13 | 12 | 15 | 34 |
| 0.5 | 13 | 23 | 10 | 13 | 26 | 16 | 21 | 36 |
| 1.0 | 13 | 20 | 13 | 20 | 21 | 17 | 21 | 36 |
| Strain 86 | | | | | | | | |
| 0 | 26 | 13 | 9 | 6 | 16 | 6 | 7 | 13 |
| 0.25 | 34 | 17 | 11 | 11 | 15 | 9 | 9 | 21 |
| 0.5 | 26 | 16 | 14 | 15 | 17 | 13 | 10 | 23 |
| 1.0 | 29 | 16 | 13 | 19 | 22 | 16 | 13 | 30 |

Abbreviations: SPP = sodium polyphosphate, C = chloramphenicol; CIP= ciprofloxacin; CL = colistin; IPM = imipenem; NA = nalidixic acid; NOR = norfloxacin; RA = rifampicin; Te = tetracycline

*maltophilia* is mediated by the presence of a zinc-β-lactamase [12]. The effect of the citrate and SPP could be explained by leakage of the enzymes from the periplasmic space. One of the strains (no. 55) tested was far less susceptible to imipenem in the presence of permeabilizers.

The hydrophobic antibiotic rifampicin showed an increase in susceptibility similar to that reported for *Pseudomonas aeruginosa* [13]. This effect could also be related to limiting the availability of $Ca^{2+}$ and $Mg^{2+}$. These ions have been shown to block the action of rifampicin against *Escherichia coli* [14]. Rifampicin functions by inhibiting RNA polymerase. This enzyme contains $Mg^{2+}$ at its active site [15].

No increases in susceptibility were observed for vancomycin and erythromycin. These antibiotics were reported to have increased effects on a strain of *P. aeruginosa* in the presence of the same permeabilizers [11].

Colistin disrupts both outer and inner bacterial membranes by complexing with membrane phospholipids [4]. Its effect was potentiated by citrate and EDTA but not by SPP.

The results for *Acinetobacter* tested against different antibiotics in the presence of permeabilizers are shown in Tables 4, 5, and 6. *A. lwoffii* was much more susceptible to antibiotics than *A. baumannii*. Lower concentrations of the permeabilizers EDTA and SPP were needed to see an effect with the *Acinetobacter* strains compared to *S. maltophilia*. This effect was not observed for citrate. These results may be explained by differences in the LPS for the 2 strains of *Acinetobacter* and *S. maltophilia*.

Susceptibility to tetracycline was increased for both *Acinetobacter* strains and for all 3 permeabilizers. The probable explanation is the same as with *S. maltophilia*, that is, the non-chelated form of tetracycline can enter

**Table 4.** Effect of the permeabilizer EDTA on the growth inhibition of *Acinetobacter* spp. by antibiotics

| EDTA concentration (molar) | Inhibition zone of antibiotics in diameter (mm) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | C | CIP | CL | IPM | NA | RA | Te | E | Va |
| *A. baumannii* | | | | | | | | | |
| 0 | 6 | 6 | 14 | 19 | 6 | 10 | 6 | 9 | 6 |
| 0.0005 | 8 | 6 | 15 | 22 | 6 | 15 | 8 | 8 | 6 |
| 0.001 | 10 | 6 | 18 | 28 | 6 | 30 | 11 | 10 | 15 |
| *A. lwoffii* | | | | | | | | | |
| 0 | 6 | 26 | 13 | 31 | 20 | 11 | 22 | 15 | 6 |
| 0.0005 | 11 | 28 | 13 | 34 | 25 | 15 | 30 | 24 | 6 |
| 0.001 | 34 | 34 | 16 | 41 | 27 | 24 | 35 | 37 | 10 |

Abbreviations: EDTA = ethylenediaminetetraacetic acid; C = chloramphenicol; CIP = ciprofloxacin; CL = colistin; IPM = imipenem; NA = nalidixic acid; RA = rifampicin; Te = tetracycline; E = erythromycin; Va = vancomycin

**Table 5.** Effect of the permeabilizer sodium citrate on the growth inhibition of *Acinetobacter* spp. by antibiotics

| Citrate concentration (% w/v) | Inhibition zone of antibiotics in diameter (mm) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | C | CIP | CL | IPM | NA | RA | Te | E | Va |
| *A. baumanii* | | | | | | | | | |
| 0 | 6 | 6 | 13 | 19 | 6 | 6 | 6 | 9 | 6 |
| 1.0 | 17 | 6 | 17 | 25 | 14 | 19 | 13 | 21 | 8 |
| 2.5 | 19 | 6 | 13 | 38 | 19 | 25 | 13 | 27 | 8 |
| *A. lwoffii* | | | | | | | | | |
| 0 | 6 | 27 | 14 | 30 | 22 | 9 | 17 | 12 | 6 |
| 1.0 | 11 | 32 | 15 | 33 | 23 | 16 | 29 | 29 | 6 |
| 2.5 | 20 | 37 | 25 | 55 | 27 | 30 | 40 | 36 | 8 |

Abbreviations: C = chloramphenicol; CIP = ciprofloxacin; CL = colistin; IPM = imipenem; NA = nalidixic acid; RA = rifampicin; Te = tetracycline; E = erythromycin; Va = vancomycin

cells more easily [9]. The 2 *Acinetobacter* strains showed completely different behavior towards the quinolones nalidixic acid and ciprofloxacin. *A. baumannii* was resistant to the quinolones but *A. lwoffii* was susceptible. The 3 permeabilizers showed no significant enhancement of susceptibility for *A. lwoffii* but *A. baumannii* showed increased susceptibility in the presence of citrate and SPP (but not EDTA). Both *Acinetobacters* were resistant to chloramphenicol, but in each case the permeabilizers made them susceptible.

The results for the quinolones and chloramphenicol could be explained by differences in the efflux mechanisms for the 2 strains [16]. Susceptibility to imipenem was increased for both citrate and EDTA but not SPP. The effect in this case cannot be related to a metallo β-lactamase since the strains tested do not possess such enzymes.

The hydrophobic antibiotics erythromycin and rifampicin showed increased effects in the presence of the permeabilizers. Vancomycin had a smaller effect in each case. Ayres *et al* [13] obtained similar results with *P. aeruginosa*. Colistin has been shown to be highly effective at treating multidrug-resistant strains of *Acinetobacter* [17]. Ethylenediaminetetraacetic acid and SPP had little additional effect on the susceptibility of the organisms to colistin. Citrate increased the effect of colistin against *A. lwoffii* but not against *A. baumannii*.

At the concentrations used in this study the permeabilizers did not show any antibacterial effects. For both *Stenotrophomonas* and *Acinetobacter*, the results are complicated by the fact that variations were observed between different strains. Thus caution should be taken in extrapolating results from a single strain to an entire species. Consistent results were obtained on retesting the same strains.

Many antibiotics are losing their clinical usefulness because of resistance. For example imipenem resistance in *S. maltophilia* is mediated by the production of a metallo β-lactamase enzyme [12]; increased resistance to ciprofloxacin in *Acinetobacter* is mediated by changes in the permeability to the drug [3]. The permeabilizers used in this study would be highly toxic to patients. Nevertheless, an understanding of membrane permeability effects on antibiotic sensitivity may allow for the development of agents that increase the effectiveness of currently available antibiotics.

**Table 6.** Effect of the permeabilizer SPP on the growth inhibition of *Acinetobacter* spp. by antibiotics

| SPP concentration (% w/v) | Inhibition zone of antibiotics in diameter (mm) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | C | CIP | CL | IPM | NA | RA | Te | E | Va |
| *A. baumannii* | | | | | | | | | |
| 0 | 6 | 6 | 13 | 19 | 6 | 10 | 6 | 9 | 6 |
| 005 | 15 | 6 | 13 | 21 | 8 | 13 | 9 | 13 | 7 |
| 0.1 | 16 | 6 | 13 | 23 | 13 | 16 | 11 | 15 | 11 |
| *A. lwoffii* | | | | | | | | | |
| 0 | 8 | 27 | 13 | 30 | 18 | 11 | 21 | 16 | 6 |
| 0.05 | 10 | 32 | 12 | 32 | 21 | 13 | 27 | 26 | 6 |
| 0.1 | 12 | 30 | 15 | 35 | 24 | 19 | 30 | 30 | 10 |

Abbreviations: SPP = sodium polyphosphate; C = chloramphenicol; CIP= ciprofloxacin; CL = colistin; IPM = imipenem; NA = nalidixic acid; RA = rifampicin; Te = tetracycline; E = erythromycin; Va = vancomycin

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