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Antibiotic uptake through membrane channels: Role of Providencia stuartii

OmpPst1 porin in carbapenem resistance

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Abbreviations: PBP, Penicillin Binding Protein; OmpPst1, outer membrane protein Pst1; MIC, minimum inhibitory concentration.
ABSTRACT

The role of major porin, OmpPst1 of *Providencia stuartii*, in antibiotic susceptibility for two carbapenems is investigated combining high resolution conductance measurements, liposome swelling and microbiological assays. Reconstitution of single OmpPst1 into planar lipid bilayer and measuring the ion current, in the presence of imipenem, revealed a decrease in the conductance in a concentration dependent manner whereas meropenem produced well resolved short ion current blockages. Liposome swelling assays suggested a small flux of imipenem in contrast to a rapid permeation of meropenem. The lower antibiotic susceptibility of *Providencia stuartii* for imipenem compared to meropenem correlated well with the decreased permeation of the former through OmpPst1 channel.
Gram-negative bacteria have a complex cell envelope comprising an outer and an inner membrane that delimits the periplasm. The outer membrane contains various protein channels, called porins, involved in the influx of hydrophilic compounds including several classes of antibiotics\(^1\)-\(^3\). A major requirement for effective antibacterial activity is the rapid delivery to the target site. The occurrence of bacterial resistance brings an urgent need to understand the molecular mechanisms. Altered porin permeability might be the limiting factor for intracellular target achievement of hydrophilic antibiotics such as β-lactams\(^4\)-\(^8\).

For example, *Providencia stuartii* belonging to the *Proteae* in the *Enterobacteriaceae* family is one of the most pathogenic bacteria in clinics\(^9\),\(^10\). It causes hospital-acquired infections and is usually found in urinary tract of patients undergoing long-term indwelling catheterization. *P. stuartii* strains show high levels of resistance to majority of antibiotic classes but were found to remain susceptible to most of the carbapenems\(^11\). However, carbapenem resistance has occurred in clinical isolates and is frequently related to the alteration of porins, although sometimes in association with an enzymatic mechanism, *e.g.* carbapenemase\(^12\)-\(^15\). Our previous study revealed two major porins in *P. stuartii*: OmpPst1 and OmpPst2. OmpPst1 is known to involve in passive diffusion of β-lactams\(^16\). Ertapenem, a carbapenem molecule, revealed strong antibiotic-channel interaction compared to cephalosporins\(^16\). In the following, focus is on the permeation of two clinically relevant and chemically divergent antibiotics, imipenem and meropenem, through OmpPst1 (Fig. 1A; 2A). An appropriate method is their reconstitution into planar lipid bilayer with subsequent recording of the ion current. As previously shown the penetration of antibiotic molecules into the channel and subsequent interaction of the drug with the channel may interrupt the ion current\(^17\). The analysis of ion current fluctuation allows obtaining permeation rates as previously shown for sugars and ampicillin\(^17\),\(^18\). However, there are limitations of this technique. In particular, due to finite time resolution, the signal of very fast permeation is undistinguishable from no permeation. Thus, a combination of techniques is needed to conclude on antibiotic translocation.
Here, we first determined the antibiotic susceptibility of *Providencia stuartii* for two clinically used antibiotics imipenem and meropenem by measuring their MIC values\(^\text{16}\). We then reconstituted single OmpPst1 into an artificial planar lipid bilayer and characterized time resolved ion current fluctuations in the presence of antibiotics. Single channel analysis of ion currents through a porin in the presence of antibiotics revealed effective binding constants and subsequently the transport parameters at a single molecule level. To further confirm translocation events, we performed liposomes swelling assays\(^\text{19, 20}\), which allowed estimating the flux of the two antibiotics through OmpPst1 channels.

**MATERIALS AND METHODS**

All chemicals used were purchased from Applichem (Darmstadt, Germany), except n-octylpolyoxyethylene (octyl-POE) (Alexis, Läuflingen, Switzerland) and all lipids from Avanti Polar Lipids (Alabaster, AL). Imipenem and meropenem were purchased from Sequoia Research Products (Pangbourne, United Kingdom).

**Antibiotic susceptibility tests**

The MIC values were determined in triplicate by a standard twofold broth dilution method according to the CLSI guidelines as previously reported\(^\text{16}\). Approximately \(10^6\) cells were inoculated into 1mL of MH broth for 18 h at 37°C. The results were scored in µg/mL and the susceptibility was classified according to the Antibiogram Committee of the French Society for Microbiology (http://www.sfm-microbiologie.org/). The bacterial strains were tested against different antibacterial drugs of different classes, among which imipenem (Tienam) and meropenem (Merrem) were obtained from Merck Sharp & Dohme and AztraZeneca (Paris, France) respectively.

**Expression and purification of *Providencia stuartii* porins**

Expression and extraction of *Providencia* porins were carried out as previously described with minor modifications\(^\text{16, 21}\). Briefly, the expression vector pGOmpPst1 harboring *ompPst1* gene
with signal sequence was electroporated into *E. coli* BL21 (DE3) omp8. Cells were grown in LB broth substituted with 100 µg/mL Ampicillin and 30 µg/mL Kanamycin. At the exponential phase, the cell culture was induced for 6 hours with 0.4 mM IPTG. The cell suspension was harvested using Sorval centrifugation at 10,000 rpm for 30 min at 4°C. The cell pellet was then washed with 20 mM phosphate buffer pH 7.4 and disrupted 2 times by French-press technique using EmulsiFlex-C3 high-pressure homogenizer (Avestin Europe, Mannheim, Germany). The membrane pellet was collected by centrifugation at 22,000 rpm for 1h after 2% SDS stirring treatment at 60°C. The membrane fraction was washed two times with 0.125% octyl-polyoxyethylene (octyl-POE) in 20 mM phosphate buffer followed by ultracentrifugation at 40,000 rpm at 4°C for 1 hour. OmpPst1 porin was extracted with 3% octyl-POE in 20 mM phosphate buffer followed by ultracentrifugation step at 20°C. The extracted porins were concentrated using Amicon® Ultra-15 Centrifugal Filter Units (Millipore) with the molecular cut-off at 30K Daltons. The buffer was exchanged with 1% octyl-POE in final porin dilution for bilayer measurements.

**Conductance measurements**

Planar lipid bilayers were formed according to the monolayer technique of Montal and Mueller. The bilayer is formed by two monolayers juxtaposed and extended across a hole of 50-100 µm in diameter in a 25 µm thick polytetrafluoroethylene (PTFE) film. Prior to bilayer membrane formation, the aperture is pre-painted with 1µL of 1% solution of n-hexadecane in n-hexane to make it lipophilic. After drying for 10 minutes, both chambers are filled with buffer (throughout 1 M KCl, 20 mM MES, pH 6) and a lipid bilayer is prepared by spreading 1 µL from 5 mg/mL solution of 1, 2- diphytanoyl-sn-glycero-3-phosphocholine in a solvent mixture of n-pentane in the aperture. Ag/AgCl electrodes are used to detect the ionic currents. The electrode on the *cis* side of the cell is grounded, whereas the other one in the *trans* side is connected to the headstage of Axopatch 200B amplifier. Purified detergent solubilized porins (1 ng/mL) are added to the *cis* side of the chamber and inserted into bilayer membrane by applying 150-200 mV voltage.
Electrical recordings were made through a pair of Ag/AgCl electrodes (World Precision Instruments, Sarasota, FL), attached to an Axon Instruments 200B amplifier with a capacitive headstage, digitized by an Axon Digidata 1440A digitizer, computer controlled by Clampex 10 software (all by Axon Instruments, Foster City, CA). The data was filtered by an analogue low-pass 4-pole Bessel filter at 10 kHz, and digitally sampled at 50 kHz. Data analysis was carried out on the Clampfit 10.0 software (Axon Instruments, Foster City, CA).

**Liposome swelling assay**

OmpPst1 porin (2 mg/mL) in 1% Octyl-POE is reconstituted into liposomes as described by Nikaido and Rosenberg\(^ \text{20} \). *Escherichia coli* total lipid extract is used to form liposomes, 15% dextran (MW 40,000) is used to entrap in the liposomes and size is checked using a Nano-ZS ZEN3600 zetasizer (Malvern Instruments, Malvern, UK). Control liposomes are prepared in the same manner but without the addition of porin. The isotonic concentration is determined by diluting control and proteoliposomes made in 15% dextran in different concentrations of raffinose measured by Osmomat 30 osmolarimeter (Gonotec, Berlin, Germany). The value obtained for isotonic concentration of raffinose is used as an approximation to facilitate the adjustment of isotonic concentrations for different solutes. 30 µL of liposome or proteoliposome solution is diluted into 630 µL of isotonic test solution in phosphate buffer in a 1 mL cuvette and mixed manually. The change in absorbance at 500 nm is monitored using a Cary-Varian UV-VIS spectrophotometer in the kinetic measurement mode. The swelling rates are taken as averages from at least five different sets of experiments, calculated as previously described\(^ \text{20} \).

**Results**

**Antibiotic susceptibility assays**

The ability of β-lactams to traverse the outer membrane barrier via OmpPst1 channel was initially approached using microbiological assays (MIC)\(^ {16} \) that determines the lowest concentration of a particular antibiotic needed to inhibit the growth of bacteria. The MIC results is a biological assay corresponding to the complete mechanism of antibiotic action: this includes, (i) the diffusion
through the porin channel, (ii) the affinity constant for the binding site of the periplasmic target (PBP) and (iii) the inhibitory constant on the PBP. These data confirmed the involvement of OmpPst1 porin in β-lactam susceptibility\textsuperscript{16}. We further measured the activity of carbapenems by exposing \textit{P. stuartii ATCC 29914} bacteria cells. MIC test of the type strain \textit{P. stuartii ATCC 29914} shows higher MIC to imipenem while significantly lower MIC value for meropenem given that the two molecules belong to the same carbapenem class in β-lactam family. The test indicated MIC value of 2 µg/mL with imipenem compared to ≤ 0.06 µg/mL with meropenem. It has been reported that carbapenems, as many other hydrophilic antibiotics use porin channels as the intracellular influx pathway. Our previous study has confirmed that the \textit{P. stuartii ATCC 29914} does not produce any extended-beta-lactamases or metallo-beta-lactamases that are capable of hydrolyzing carbapenems\textsuperscript{16}. To further confirm the role of OmpPst1 in antibiotic permeation porin-deficient \textit{E. coli} BL21 (DE3) omp8 strain is used to express OmpPst1 porin and the MIC value is determined for carbapenems (Table S1). The data suggested that the lower MIC value of \textit{P. stuartii} to meropenem, as compared to imipenem, may be due to a faster rate of influx of meropenem across the membrane channels, thereby accelerating the intra periplasmic concentration of the drug and the access to the target.

\textbf{Conductance measurements}

Single OmpPst1 channel was reconstituted into planar lipid membrane and showed a single trimer channel conductance of 2.7 ± 0.3 nS in 1 M KCl as shown previously\textsuperscript{16}. In the absence of antibiotics, the ion current through the channel was stable without any modification in the flow of ions. Addition of imipenem to one or both sides of the lipid membrane caused a drop in ionic conductance that is strongly concentration dependent. Fig. 1B shows that addition of 5 mM imipenem to both sides of the chamber reduces the single channel conductance from 2.5 nS to 2.2 nS. Further increasing the imipenem concentration to 15 mM reduces the conductance to 2 nS at 100 mV in 1 M KCl. Corresponding amplitude histogram is shown in Fig. 1C. It must be noted that the direct effect of the antibiotic in solution on the bulk conductance is negligible in the
applied concentration range, i.e. up to 15 mM (Table S2). The resolution limit of our technique indeed restricts the detection of events occurring below 100 µs. Previous studies have shown that by lowering the temperature the kinetics of translocation slows down and thus allows to resolve the translocation events. In the case of imipenem, lowering the temperature did not allow us to resolve individual translocation events even at a temperature as low as 5°C (Fig. S1). Thus, we hypothesize that imipenem binds to the channel surface, resulting in the reduction of ion current. In contrast, addition of meropenem to the system caused transient blockage of the ionic current (Fig. 2B). At low drug concentration, meropenem interacts with OmpPst1 channel resulting in monomer blocking. Increasing antibiotic concentration increases the number of events. The dwell time, \( \tau_c \) does not depend on the concentration of the antibiotic used and was calculated to be around 150 µs at 100 mV. Kinetic analysis of the antibiotic binding at different voltages and ionic strengths of the solution demonstrated that the interactions are of electrostatic origin. Previous studies on OmpF have revealed a charge reversion of the negatively charged aspartic acid in presence of multivalent cations\(^{23}\). To elucidate a similar contribution we tested the effect of divalent and trivalent cations. The addition of 10 mM MgCl\(_2\) in presence of imipenem resulted in partial closure of the channel (Fig. S2), while addition of 10 mM LaCl\(_3\) in presence of imipenem caused modulation of the ion current and highly resolvable blocking events (Fig. 3). This is in contrast with meropenem, for which binding kinetics were not significantly affected in the presence of trivalent cations (Fig. S3).

The number of events and residence time obtained from ion current blockages can be readily inserted into a simplified enzymatic model, where it is considered that the channel catalyzes antibiotic translocation. The association rate constant \( k_{on} \) gives the permeation of antibiotic molecule from cis- or trans-side to the affinity site in the channel calculated from the number of antibiotic binding events per second. The dissociation rate constant \( k_{off} \) gives the rate at which antibiotic molecules release from the channel affinity site to cis or trans aqueous phase calculated from the average residence time of antibiotic blockage\(^{17,23-25}\) (Table 1).
Permeation assays through liposomes

To support the conclusion on transport from the ion current fluctuation analysis, we performed liposome swelling assays. Diffusion rate of antibiotics through OmpPst1 is calculated by reconstituting channels in liposomes and by measuring the change in optical density in the presence of isotonic concentration of antibiotics. A requirement to carry out liposome swelling assay is that the molecule of interest is zwitterionic, making imipenem and meropenem suitable candidates for such measurement. To scale the flux we first use raffinose a high molecular weight sugar too large to diffuse through the porins and second arabinose, a small sugar which permeates through the channel. Permeation rates for different sugars are obtained together within the same batch allowing us to normalize the antibiotic diffusion values with respect to arabinose. The value obtained for arabinose, which is set to 100%, is 20% higher than that obtained for galactose and 80% higher than maltopentaose confirming that swelling rate decreases as the size of the solute increases (Fig. 4). However, in presence of imipenem, there is no significant change observed in the absorbance of proteo-liposomes. Swelling rate obtained for imipenem is around 10%, i.e. comparable to that of big sugar molecules like raffinose and maltopentaose which are known to display poor or no permeation through porin channels. From the above results, we can conclude that imipenem translocates very slowly through the OmpPst1 channel. In contrast, meropenem showed a very high diffusion rate of around 90% indicating it is able to translocate at a fast rate through OmpPst1. The results from liposome swelling assays thus complement the microbiological assays and bilayer measurements in suggesting a poor permeation of imipenem through OmpPst1, which contrasts with meropenem that translocates efficiently through the channel.
DISCUSSION

Currently carbapenems are the most recent available β-lactams against Gram-negative bacteria. Due to their broad activity, they have become widely used in clinics, e.g. imipenem is the most used carbapenem in hospital wards. However, carbapenem efficacy is being threatened by the dissemination of bacterial resistance. During the treatment of infected patients, a correlation between the level of antibiotic resistance and the absence of porins was observed\(^\text{11}\). In *Providencia stuartii*, the major porin OmpPst1 provides main pathway for antibiotic penetration through the outer membrane\(^\text{16}\). Our focus here is on the mechanism of antibiotic uptake through porins and its role in antibiotic resistance. In particular we investigated the relation between pore properties, the structure of the antibiotic and the correlation with the uptake of these molecules. Our results indicate that both imipenem and meropenem interacts with OmpPst1, albeit with different binding kinetics. From the observation that imipenem reduces the channel conductance without resolvable single blocking events we hypothesize that either imipenem translocates very fast through the channel where the time resolution of instrument limits visualizing of well defined events or imipenem binds to the channel, inhibiting flow of ions hence reducing the channel conductance. To further conclude on the above observation we performed temperature measurements as shown previously\(^\text{26}\) in order to catch the fast events but these measurements were inconclusive. To differentiate binding from translocation liposome swelling assay was performed which suggested low flux of imipenem in contrast to rapid permeation of meropenem. Combining single channel measurements and liposome swelling assays we could conclude that imipenem binds to the channel, where it may interact with side chains of amino acids present in the channel surface. Thus, imipenem can be envisaged as a plug that reduces the passage of other ions.

Similar studies have shown that enrofloxacin, a fluoroquinolone antibiotic, blocks the OmpF channel\(^\text{23}\). The interactions between the enrofloxacin and the OmpF channel wall are strong enough to close the pore for about 3 ms revealing a strong affinity of antibiotic to the channel.
without efficient translocation \(^{23}\). Interestingly, in presence of magnesium chloride, the affinity of
enrofloxacin for OmpF channel was altered, as well the orientation of the antibiotic during
translocation \(^{23},^{27}\). Similarly in the case of OmpPst1, the presence of trivalent cations caused a
dramatic change in the imipenem binding kinetics (Table 1). The number of imipenem blocking
events increased with the increase in the antibiotic concentration and the average residence time
was calculated to be around 150 µs. It is important to note that trivalent cations have no effect on
binding and translocation kinetics of meropenem interaction with OmpPst1 (Fig. S3). Kinetic
constants of on and off rates of imipenem and meropenem binding to OmpPst1 in the presence of
trivalent cations are presented in Table 1.

Previously we used homology modeling to predict the structure of the OmpPst1 channel, using as
a starting model to the homologous porin OmpF\(^{16}\). Important differences in amino acid residues
were predicted for OmpPst1, with regard to OmpF. For example, M38 which in OmpF forms an
important hydrophobic pocket above the constriction region is substituted by an aspartic acid in
OmpPst1. We conjecture that this specific residue modification is correlated to the reduced uptake
of imipenem through OmpPst1 porin. The side chain of D38 could indeed act as a sensor that
recognizes and binds the exposed NH\(^{2+}\) group in the iminomethyl-amino-ethyl strongly polar side
chain of imipenem, thereby stopping its progression in the channel and conferring partial
insusceptibility to \(P. stuartii\). In this context, our interpretation of the effect of trivalent cations is
that they reverse the charge of D38, and thereby allow imipenem uptake by OmpPst1. This
proposition is supported by the observation that meropenem, whose side chain a more bulky
dimethycarbomyl-pyrrolidin group, translocates through OmpPst1 and shows efficiency against
\(P. stuartii\).

Thus, our results highlight the importance of efficient influx through porins for \(\beta\)-lactams to
reach their target site and provide useful information for rational drug design exhibiting enhanced
bacterial penetration. We show an example where porins screen antibiotic molecules entering the
channel surface and those attractive forces facilitate translocation through the channel. In
addition, our study provides clues that could explain some paradoxical susceptibilities to carbapenems in other clinical isolates (Lavigne et al, unpublished results). Thus, not only the affinity of each carbapenem for its PBP target, but also, the efficiency of its translocation across the outer membrane participate in the regulation of the bacterial susceptibility to this class of antibiotics.

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Supporting Information:
1. MIC values of carbapenems against porin deficient E. coli BL21 (DE3) omp8 strain (Table S1).

2. Bulk conductivity of 1M KCl, pH- 6 in absence and in presence of imipenem (Table S2).

3. Ion current trace of OmpPst1 in presence of imipenem at two different temperatures (Fig. S1).

4. Ion current trace of single OmpPst1 channel in presence of divalent cation (Fig. S2)

5. Single OmpPst1 channel recording in presence of meropenem and La^{3+} (Fig. S3).

6. Dwell time histogram of 10 mM meropenem in presence of LaCl3 (Fig. S4).

This material is available free of charge via the Internet at http://pubs.acs.org.
References:


### Table 1: Rate constants of entry and exit of carbapenems through OmpPst1.

<table>
<thead>
<tr>
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<th>10mM Imipenem in the presence of LaCl$_3$ at 100 mV</th>
<th>10mM Meropenem in the presence of LaCl$_3$ at 100 mV</th>
</tr>
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<tbody>
<tr>
<td>$k_{on}$ ($10^3$ M$^{-1}$ s$^{-1}$)</td>
<td>9 ± 3</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>$k_{off}$ (10$^3$ s$^{-1}$)</td>
<td>10 ± 3</td>
<td>8 ± 1.7</td>
</tr>
</tbody>
</table>

$k_{on} = (\text{number of events s}^{-1})/(3[c]$ where $[c]$ is antibiotic concentration), $k_{off} = 1/(\text{average residence time})$. 
FIGURE LEGENDS

Fig. 1: (A) Chemical structure of imipenem,
(B) Ion current trace through single OmpPst1 channel reconstituted into planar lipid membranes
in the presence of imipenem, and (C) corresponding amplitude histogram of OmpPst1 channel in
the absence and in the presence of imipenem.

Fig. 2: (A) Chemical structure of meropenem,
(B) Ion current recordings through single OmpPst1 channel in the absence of meropenem and in
the presence of 10mM meropenem added to both sides of the chamber. Conditions: 1M KCl,
20mM MES pH-6 and applied voltage 100mV.

Fig. 3: Ion current trace of single OmpPst1 channel reconstituted into bilayer in the presence of
10 mM imipenem (A) without La$^{+3}$,(B) with La$^{+3}$ and (C) Dwell time histogram in presence of 10
mM imipenem and LaCl$_3$.

Fig. 4: Relative rate of diffusion of imipenem compared to various sugars and meropenem
through proteo-liposomes. The swelling rates, which were averaged over at least three different
sets of experiments, were calculated as described$^{12}$, normalized by setting the rate of arabinose
diffusion to 100%.
Figure 1

A.

B.

C.
Figure 2

A. 

B. 

No Meropenem

10mM Meropenem

Dwell Time Histogram

Count (N)

0 0.2 0.4 0.6 0.8 1

Dwell Time (ms)
Figure 3

A. No LaCl₃

B. 10mM LaCl₃

C. No of events vs Dwell time (ms)
Figure 4

![Bar graph showing relative diffusion rates for different sugars](image-url)

- Arabinose: 100 ± 3
- Maltose: 92 ± 10
- Galactose: 85 ± 7
- Maltopentase: 22 ± 5
- Imipenem: 10 ± 4
- Raffinose: 3 ± 2
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![Image of antibiotic uptake through membrane channels]