THE INTERACTION OF AMINOGLYCOSIDES WITH ENDOTHELIAL CELLS INFECTED BY *PSEUDOMONAS AERUGINOSA*

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Abstract. The membrane organization of cultured human endothelial cells (EA.hy926) infected with *Pseudomonas aeruginosa* (PS) was studied in the temperature range 15–37 ^{o}C by using fluorescent depolarization and generalized polarization (*GP*) measurements on the cells suspensions labeled with TMA-DPH and laurdan, respectively. The opportunistic pathogen *Pseudomonas aeruginosa* was chosen since this pathogen is the etiologic agent of resistant and recurrent pulmonary infections in cystic fibrosis (CF) patients, who require sustained treatment with antibiotics. The effect of several aminoglycoside antibiotics – gentamicin, amikacin and kanamycin – on membrane organization of infected cultured cells was recorded. The measurements results showed that the presence of aminoglycosides cations induces a rigidity of the infected cell membrane, gentamicin being the most efficient in this respect. This effect is temperature sensitive, being much more pronounced at temperatures close to physiological range. The above information may be of use in determining the treatment regimen of a CF patient who has recurrent infections and requires antibiotics treatment for a longer time.

Key words: aminoglycosides, *Pseudomonas aeruginosa*, endothelial cells.

INTRODUCTION

Aminoglycosides are polycationic compounds composed by 3–5 hydrophilic amino groups, which are widely used in the treatment of Gram negative and Gram positive bacterial infections [17].

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Although their antibacterial activity is well known, the toxic effects of aminoglycosides are still under research. The prolonged clinical use of aminoglycosides leads to serious complications, mainly due to their nephrotoxicity and ototoxicity.

Nephro and ototoxicity depends on the intracellular accumulation of aminoglycosides [2, 3, 16, 21–23], which leads to minor alterations in the lipid packaging and disruptions of the ion channels and membrane receptors function. Even though it has been proved that aminoglycosides block a variety of ion channels [13, 19, 29], the mechanisms by which these processes occur are still unknown. The most used aminoglycosides are gentamicin, kanamycin and amikacin. Gentamicin (gent) is an oligosaccharide with antibacterial action against Gram positive (*Staphylococcus, Pneumococcus, Enterococcus*) and Gram negative (*Pseudomonas aeruginosa, Escherichia coli, Proteus, Klebsiella, Salmonella, Bacillus pyocyanicus* and *Koch bacillus*) bacteria.

Kanamycin (kana) acts on Gram positive cocci (*Staphylococci, Pneumococci*), the Gram negative bacilli (*E. coli, Salmonella, Haemophilus, Klebsiella, P. aeruginosa)* and the *Koch bacillus,* being indicated for a wide range of infections.

Amikacin (amik) is a semisynthetic derivative of kanamycin A, with a great efficacy against bacteria resistant to other aminoglycosides due to the chemical structure, which is less susceptible to enzymatic inactivation [7, 8, 18, 25–27].

The fact that aminoglycosides are oto and nephrotoxic is related to their ability to interfere with protein synthesis by binding to ribosomal subunits. This ability to intervene in protein synthesis opens a new perspective for using aminoglycosides in the treatment of severe genetic diseases such as Duchenne muscular dystrophy, hemophilia, cystic fibrosis or to limit HIV infection [4, 11, 15, 27, 28]. In order to perform these treatments in the field of genetic disorders, we need a very good knowledge and control of the aminoglycosides mechanisms of action on mammalian cells in different conditions.

Biological cell membrane is the first structure that comes in contact with antibiotics and the present study aims to identify various changes induced *in vitro* by gentamicin, kanamycin and amikacin in the membrane fluidity and *laurdan* generalized polarization in cultured endothelial cells infected with *Pseudomonas.*

Pseudomonas aeruginosa is a common bacterium that can cause disease in animals and humans. It is found in soil, water, skin flora, and most man-made environments throughout the world. *Pseudomonas aeruginosa* is a versatile opportunistic pathogen that causes infections which are resistant and difficult to treat, especially within the respiratory airways and lungs. It is a Gram-negative bacterium, negatively charged, which enters the cell by receptor-mediated endocytosis [5]. Other authors argue that *P. aeruginosa* internalization occurs by an actin-dependent Toxin B-inhibited pathway which becomes down regulated as cells become polarized, suggesting that one or more of the Rho family GTPases is involved in bacterial internalization [12].

Cell-surface polysaccharides play diverse roles in the bacterial "lifestyle". They serve as a barrier between the cell wall and the environment, mediate host-pathogen interactions, and form structural components of bacterial biofilms [20, 24].

Since in real life aminoglycosides are used against bacterial pathogens, it is of interest to know how the presence of the pathogen modulates the changes induced in the cultured cells by these antibiotics.

Endothelial cells were chosen because they are found in the blood vessels of the lung alveoli, working as selective barriers between blood vessels and the surrounding tissue. Vascular endothelial cells line the entire circulatory system, from the heart to the smallest capillaries.

Patients with cystic fibrosis who have recurrent lung infections with *Pseudomonas aeruginosa* require prolonged treatment with aminoglycosides, especially gentamicin (it is known that *P. aeruginosa* is more sensitive to gentamicin). Since endothelial cells are found in the lungs, we considered them a good model for studying the effects of aminoglycosides on lung located cells, infected with *P. aeruginosa*.

MATERIALS AND METHODS

CELL CULTURES

Human endothelial cells (EA.hy926 – human umbilical vein cell line) (EA) were purchased from American Type Culture Collection (ATCC). The basic medium for this cell line is Dulbecco's Modified Eagle's Medium with 2 mM L-glutamine, glucose 4.5 g/L (PAA, Austria) supplemented with 10% fetal bovine serum (Gibco, Scotland, United Kingdom) at 37 °C, 5% CO_2. Sub-confluent cells were harvested by trypsinization (0.05% w/vTrypsin- + EDTA 4Na, Biochrom AG, Germany).We used 500 μg/mL (final concentration) concentrations of gentamicin, amikacin and kanamycin; these were established in order to be comparable to those used *in vivo* to treat various infections with gram-negative bacteria.

BACTERIAL CULTURES

Pseudomonas aeruginosa ATCC 27853 strain was purchased from American Type Culture Collection (ATCC). One overnight *P. aeruginosa* colony was stab inoculated in 5 mL Luria Broth (Oxoid) and incubated for 24 h at 37 ^{o}C. The bacterial inoculum was obtained by diluting washed *P. aeruginosa* cell sediment in sterile PBS to an optical density of 0.5 McFarland corresponding to a microbial suspension of ~ 10^8 units forming colonies (colony forming units)/mL (~10^8 CFU/mL). This inoculum was used for all further experiments.

We used 500 μL of a bacterial suspension per 75 cm^2 flasks and the bacteria were allowed to invade the host cells for 2 h (previous studies of cell adhesion and invasiveness of *Pseudomonas* showed this time to be necessary to invade the host [9, 10]).

FLUORESCENCE ANISOTROPY MEASUREMENTS PERFORMED ON EA CELLS

Fluorescence anisotropy of lipophilic probes dissolved in a membrane is directly related to the microfluidity of the lipid environment. The more fluid is the environment, the smaller is the value of the fluorescence anisotropy. Fluorescence anisotropy (r) (equations 1, 2) is defined as:

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \tag{1}$$

with

$$G = \frac{I_{HV}}{I_{HH}} \tag{2}$$

where the fluorescence intensities (I) are recorded in polarized light at λ_{ex} = 385 nm and λ_{em} = 434 nm, with polarizers oriented as specified by the indices (V = vertical, H = horizontal); G is a correction factor related to the specific sensitivity for polarized light of the fluorometer. The fluorescence anisotropy may vary between 0 (for a "perfect fluid" environment) and 1 (for a "perfect rigid" environment). However, some considerations related to the statistics of the excitation and emission processes limit the anisotropy to lower values ($r_{max} \leq 0.4$).

Subconfluent endothelial cells (EA) cultured were washed with PBS, trypsinized and centrifuged at 250 × g for 5 min; the resulting pellet was resuspended in PBS at a cell density equivalent to an optical density of 0.18 at 450 nm.2 mL of the cell suspension was then incubated with 1 μM TMA-DPH (final concentration) for 5 min and subsequently, with 500 μg/mL (final concentration) of antibiotics for 5 min.

I_{VV} and I_{VH} were measured simultaneously for each sample in the 15–37°C temperature range.

LAURDAN FLUORESCENCE SPECTROSCOPY OF EA CELLS

Subconfluent EA.hy926 cultured cells were washed with PBS, trypsinized and centrifuged at 250 × g for 10 min; the resulting pellet was resuspended in PBS at a cell density equivalent to an optical density of 0.18 at 450 nm. 2 mL of cell suspension was then incubated with 1 μM (final concentration) laurdan for 5 min and, subsequently, with 500 μg/mL (final concentration) gentamicin, amikacin or kanamycin for 5 min. The cell suspension was placed into a fluorometric cuvette

with 10 mm optical path length. A Horiba Jobin-Yvon Fluorolog 3 spectrofluorometer with Peltier Quantum thermostated sample holder (TLC50, Quantum Northwest Inc, USA) was used for fluorescence measurements. All measurements were done under magnetic stirring.

Laurdan emission spectra were recorded in the temperature range of 15–37°C using λ_{ex} = 350 nm and λ_{em} = 400–550 nm.

Laurdan is a fluorophore sensitive to the polarity of the environment [14]. In nonpolar media, its emission spectrum is characterized by an emission peak at around 430 – 440 nm (called "emission in blue", I_B, while in polar media, the emission spectrum presents a maximum at 480–500 nm ("emission in red", I_R). In intermediate situations, the overall recorded spectra will be a mixture of the two components. The so-called "generalized polarization" (*GP*) (Equation 3) is defined as:

$$GP = \frac{I_B - I_R}{I_B + I_R} \tag{3}$$

where I_B and I_R are the fluorescence intensities emitted by laurdan at the specific wavelengths for the gel phase (less polar) and liquid crystalline phase of the membrane respectively; the liquid crystalline phase is more polar due mainly to water penetration in the polar part of the lipid bilayer. According to this definition, a higher *GP* value corresponds to the prevalence of the gel phase, while a lower *GP* corresponds to the predominantly liquid crystalline phase. *GP* may vary between +1 and –1.

STATISTICS

Five independent measurements were performed for each temperature in case of each antibiotic. The data at each temperature are presented as averages of these data ± SD (standard deviation calculated with Excel software). The statistical significance of the antibiotics effect was evaluated using two tailed unpaired t-Student test (Tables 1 and 2 in Results section). The effect was considered significant for p values ≤ 0.05.

RESULTS

FLUORESCENCE ANISOTROPY ON ENDOTHELIAL CELLS

The fluorescence anisotropy was tested in a large temperature interval (15–37^{o}C) in order to get a broader picture of membrane organization changes in presence of the antibiotic polycations. It is known that, similar to artificial lipid

bilayers, the living membrane also displays a temperature interval of phase transition (from gel to liquid crystalline states), which is essential for many vital membrane functions.

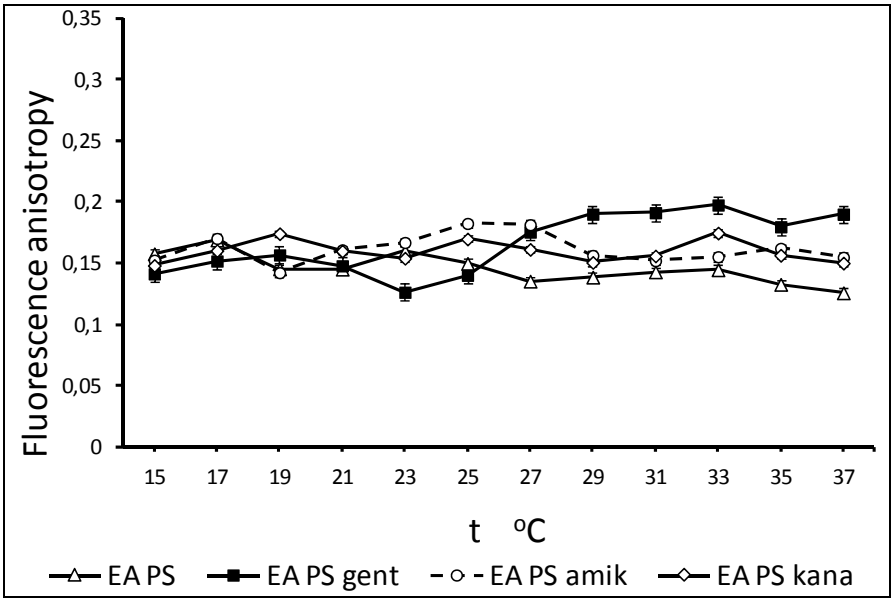

Fig. 1. The effect of gentamicin (gent), amikacin (amik) and kanamycin (kana) on fluorescence anisotropy of endothelial cells (EA.hy926) infected with *Pseudomonas aeruginosa* (PS), in the temperature range 15–37 °C.

It may be observed that, unlike the control *P. aeruginosa* infected cells, which show a slight increase in membrane fluidity (decrease in fluorescence anisotropy) with the increasing temperature, the infected cells treated with antibiotics, all display a more rigid membrane (higher *r* values) comparatively to controls, especially at temperatures close to physiological 37 °C.

Gentamicin exceeds by far the rigidity effect induced by amikacin and kanamycin.

Table 1

T-test values for fluorescence anisotropy measurements

Temperature	Gentamicin	Amikacin	Kanamycin
˚C	*p*	*p*	*p*
15	0.0458	0.7196	0.6564
17	0.2517	0.9605	0.6943
19	0.3354	0.9163	0.3866

Table 1 (continued)

21	0.4712	0.4901	0.5437
23	0.0973	0.6226	0.6626
25	0.3736	0.2426	0.4166
27	0.0507	0.0488	0.1377
29	0.0253	0.1801	0.3394
31	0.0249	0.6302	0.5034
33	0.0327	0.6248	0.1844
35	0.0099	0.0351	0.0224
37	0.0013	0.0164	0.0128

LAURDAN EMISSION SPECTROSCOPY OF ENDOTHELIAL CELLS

Fluorescence emission spectra of laurdan incubated EA.hy926 cells suspensions were recorded at temperatures between 15 °C and 37 °C allowing the computation of generalized polarization which reflects the amount of polar species within the membrane and, indirectly, its integrity.

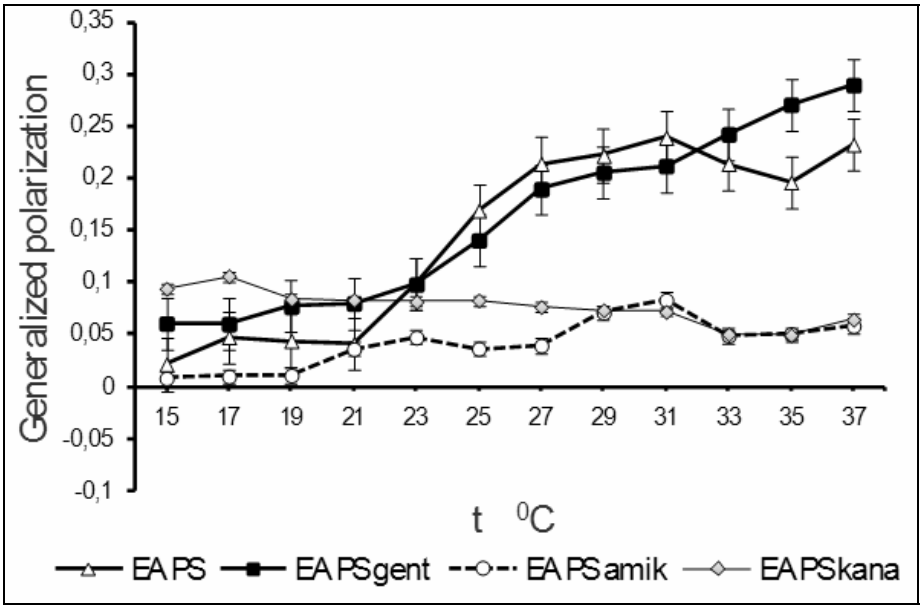

Fig. 2. The effect of gentamicin (gent), amikacin (amik) and kanamycin (kana) on membrane generalized polarization of endothelial cells (EA.hy926) infected with *Pseudomonas aeruginosa* (PS), in the temperature range 15–37 °C.

Table 2

T-test values for laurdan generalized polarization measurements

Temperature	Gentamicin	Amikacin	Kanamycin
˚C	*p*	*p*	*p*
15	0.4556	0.7212	0.1208
17	0.8408	0.4370	0.2183
19	0.5019	0.3791	0.3731
21	0.4680	0.9246	0.4069
23	0.9840	0.2754	0.6791
25	0.5988	0.0110	0.0918
27	0.6577	0.0023	0.0134
29	0.7970	0.0312	0.0183
31	0.5730	0.0043	0.0004
33	0.6808	0.0084	0.0170
35	0.3226	0.0086	0.0161
37	0.4561	0.0066	0.0092

Except for kanamycin, in all situations, the cell membrane *GP* of infected cultured cells displays a sort of sygmoidal evolution with the inflection point around 21–23 ^{o}C, increasing dramatically at temperatures over 23 ^{o}C (especially in controls and gentamicin treated cells).

DISCUSSION AND CONCLUSIONS

Analyzing the obtained fluorescence anisotropy and general polarization data, we may conclude that the presence of the aminoglycoside polycation molecules induces a membrane rigidity in the infected endothelial cells, gentamicin being the most effective in this respect.

This tendency becomes obvious above the temperature of 23 ^{o}C and is more pronounced when approaching the physiological temperature of 37 ^{o}C.

We assume that the fluorescence marker is incorporated only in endothelial cells, due to the complexity of the bacterial wall which would not allow the marker penetration to the internal PS lipid bilayer.

The external bacterial membrane is lipopolysaccharidic, being negatively charged and fixed by electrostatic bridges made by Ca^{2+} and Mg^{2+} ions bound to negative lipopolysaccharides (LPS).

This network assures the stability of the external PS membrane. It is however susceptible to cationic antibiotics such as aminoglycosides, which compete with Ca^{2+} and Mg^{2+} and avidly bind to LPS , disorganizing the external membrane with lethal effects to the bacterium [1, 6, 30].

In our study we are interested however in the membrane organization changes induced by aminoglycosides in the infected cells and not in the bacteria. Our observation that the cell membrane becomes more rigid in infected cells treated with aminoglycoside cations and this effect is temperature sensitive, increasing as temperature approaches the physiological range, is surprising. It suggests that at least at physiological temperatures, the cell membrane of the infected cells is somehow "protected" and the access of polar species (water, e.g.) into the lipid bilayer is denied. This tendency is maintained in the presence of antibiotics, especially gentamicin (Fig. 1). Laurdan measurements confirm this effect of gentamicin, contrary to that of amikacin and kanamycin which indicates a membrane destabilization and penetration of polar species (*GP* decrease) at temperatures close to physiological 37 °C (Fig. 2).

It might be useful to know this in determining the treatment regimen of CF patients who have recurrent infections and require antibiotics treatment for a longer time.

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