Spectral analysis of Laurdan fluorescence reveals changes induced by AMPs in lipid membranes

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Introduction

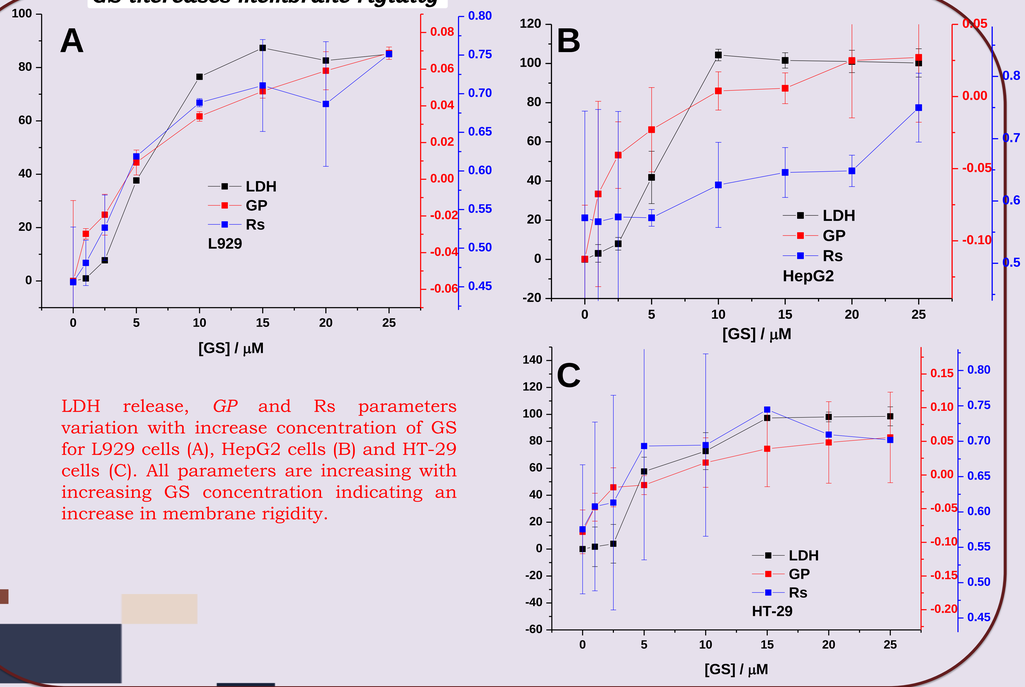
Antimicrobial peptides (AMPs) are a class of molecules synthetized by various organism with the aim of protecting themselves against pathogens. In order for the peptides to enter the cells they have to pass or interact with the plasma membrane. The effect of various peptides at the lipid membrane level were investigated using Laurdan fluorescence. Laurdan, a lipid membrane probe, is sensitive to polarity changes in the environment. The fluorescence spectra were interpreted using the classic generalized polarization (GP) parameter, as well as using the previously described log-normal deconvolution. The later method allowed us to infer other parameters: the difference between the relative areas of elementary peak (ΔSr), and the ratio of elementary peaks areas (Rs). The parameters analyzed allowed us to highlight different changes induced the peptides investigated.

Considering that plasma membrane has a protective role, for both eukaryotic and bacteria cells, but it also represents the first gateway for peptides to cells, fully understanding the way peptides interact and affect them is essential to help researchers develop more efficient structures in the future. In this study, we investigate the effect of Gramicidin S (GS) with a circular structure, on cell viability and membrane fluidity.

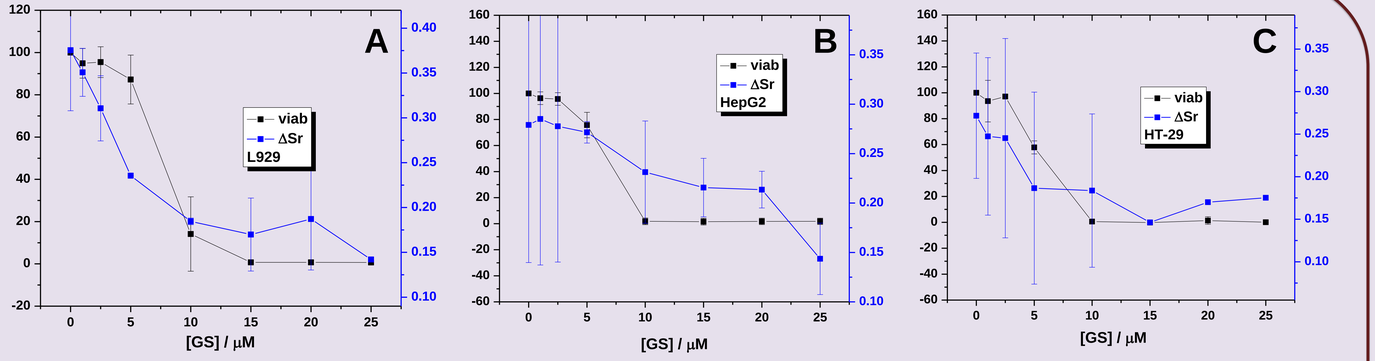
Material and Methods

Cell line: Human colorectal adenocarcinoma cell line HT-29 (ATCC) and human hepatocytes carcinoma cell line HepG2 (ATCC) were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), L-glutamine and penicillin/streptomycin at 37°C in a humidified atmosphere with 5% CO_2. **MTT assay:** The MTT solution Promega (Madison, WI, USA) at a final concentration of 5 mg/ml was prepared in PBS, filtered through a 0.22 µm filter and stored in the dark at -20 °C until use. The cytotoxic effect of the AMPs was determined using MTT from according to the following protocol. Briefly, cells were seeded into 96- well plates (8000 cells/well) for 24 h prior the experiment. Cells were treated with GA or GS at various concentrations. After 24 h 20 µl of MTS solution was added into each well and viability was evaluated after additional 4 h incubation at 37 °C in a humidified atmosphere with 5% CO_2. Thereafter, the MTT solution was carefully removed and formazan crystals were dissolved in dimethyl sulfoxide (DMSO). Finally, the absorbance was measured at 570 nm using a microplate reader Mithras 940 (Berthold, Germany). The data were corrected for the background and the percentage of viable cells was calculated according to the equation: [(A570 of treated cells)/(A570 untreated cells)]*100%. **LDH Assay:** Membrane integrity after peptides treatment was assessed based on LDH release using CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega) according the manufacturer's instructions. Cells treated with different peptide concentrations were harvested, centrifuged at 250 g for 10 min at 25°C, and the supernatant was used for the activity assay. Maximal LDH release was obtained by full cell lysis induced by the use 1 % of Triton X-100. The absorption resulting from LDH activity was measured in a microplate reader at 490 nm and the % LDH was calculated as: [(corrected absorbance of the LDH released after treatment) / (corrected absorbance of the total LDH released)] *100 %.
Laurdan Measurements and Spectra Processing: Steady-state fluorescence measurements were performed using a FluoroMax 3 spectrofluorimeter (Horiba Jobin Yvon, Edison, NJ, USA) equipped with a Peltier thermostated cell holder. The spectra were recorded in the range 400–600 nm, with the excitation set at 378 nm. The slits of excitation and emission monochromators were 3 nm. The recorded spectra were corrected for the spectral sensitivity of the emission channel of the spectrofluorimeter and for Raman and scattering artifacts. Laurdan was added at 1 µM in cell suspension and left for 20 min at 37 °C to allow full insertion into lipid membranes. Fluorescence spectra were recorded for every concentration, by adding small aliquots of melittin over liposome solution, leaving them for 10 min, prior spectra measurement, for reaching equilibrium in solution. Data obtained were processed using the OriginPro 2016 software package (OriginLab Corporation, Northampton, MA, USA) to calculate the GP (generalized polarization) parameter as follow: GP=(I440−I490)/(I440+I490), where I440 and I490 are Laurdan emission intensities at 440 nm and 490 nm. We used the deconvolution method previously reported [Bacalum et al. 2013] to fit the emission spectra of Laurdan with two log-normal (LN) functions: one characterizing the emission of Laurdan molecules found in a more rigid environment (the blue part of the spectra) and the other one the emission of molecules found in the fluid phase (the green part of the spectra). Few parameters have been calculated using the data resulted from spectrum deconvolution: (i) elementary peak positions, (ii) relative areas of the elementary peaks against the total area of the spectrum, (iii) difference between relative areas of elementary peaks (ΔSr) and (iv) ratio of elementary peaks areas (RS).

GS increases membrane rigidity



LDH release, *GP* and Rs parameters variation with increase concentration of GS for L929 cells (A), HepG2 cells (B) and HT-29 cells (C). All parameters are increasing with increasing GS concentration indicating an increase in membrane rigidity.



Cell viability and ΔSr parameters variation with increase concentration of GS for L929 cells (A), HepG2 cells (B) and HT-29 cells (C). Cell viability is decreasing with increasing GS concentration which is correlated with ΔSr

Conclusions

- Laurdan parameters (GP, ΔSr and RS) reported in the study are able to sense membrane changes after GS addition.
- Changes are correlated indicating an increase in membrane rigidity of the cells treated.

Acknowledgments

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