Lipid membrane composition determines binding, disruption and cytotoxicity of Gomesin peptides

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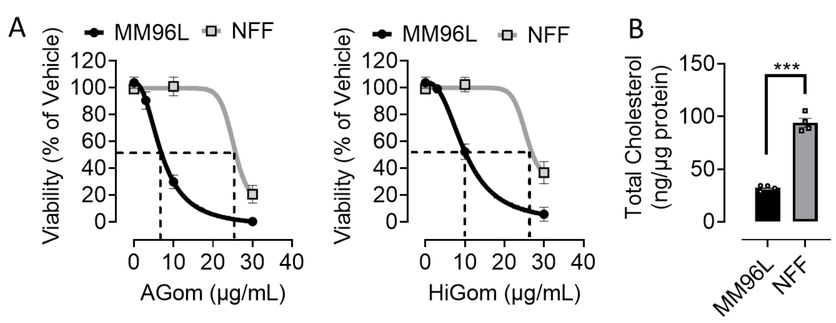
INTRODUCTION:

Gomesin, a cationic peptide initially derived from the haemocytes of the Brazilian tarantula *Acanthoscurria gomesiana*, has demonstrated noteworthy in vitro and in vivo antitumoral properties against various types of cancer[1,3,6]. Numerous mechanisms have been postulated to elucidate the antitumoral activity of spider Gomesins, including its capacity to bind to and disrupt the plasma membrane, as well as its ability to modulate signalling cascades that govern cell death and proliferation[1,4,5,6]. Due to their diverse biological activities and unique mechanisms of action, gomesin peptides have sparked considerable interest in the fields of antimicrobial and anticancer research[6]. Scientists are exploring their therapeutic potential and working on optimizing their structure and bioavailability to enhance their efficacy and minimize potential toxicity.

It is known that Gomesin exhibits an affinity for membranes composed of negatively-charged phospholipids and displays limited affinity for membranes enriched with neutral lipids[6]. However, the role of cholesterol content in these interactions remains incompletely understood. Cholesterol is a vital lipid involved in cellular homeostasis and plays a role in signal transduction by regulating membrane fluidity. Therefore, the purpose of this present study is to delve deeper into the influence of cholesterol on Gomesin's capacity to interact with both artificial membranes and cellular models, ultimately leading to its cytotoxic effects. To achieve that objective, our focus was directed towards examining a natural variant of Gomesin known as AgGom, as well as a closely related homolog called HiGom.By investigating the importance of cholesterol in these interactions, we aim to expand our understanding of the underlying mechanisms through which Gomesin exerts its cytotoxicity. Further investigation into the precise mechanisms underlying their anticancer activities could unlock new possibilities for improving cancer treatment outcomes.

METHODS:

Cell culture: The human BRAFV600E melanoma cells (MM96L) and the non-transformed Neonatal Foreskin Fibroblasts (NFF) were cultured in RPMI-1640 media supplemented with 10% FBS, 1% non-essential amino-acids and 1% penicillin/streptomycin. **Cholesterol content** was analysed with spectrophotometrically at 590 nm with Amplex Red in cell lysates
Cell viability: Cytotoxicity was assessed by MTT assay. Different treatments were incubated for 48 hours and the MTT absorbance was measured at 570 nm in a microplate reader.
RH421 ratiometric fluorescence: dual-wavelength ratiometric fluorescence method (449/524 nm) using the potential-sensitive dye RH421 was used to determine membrane dipole potential as an indication of membrane binding. Reductions in ratiometric fluorescence indicate peptide binding. The data is plotted as a ratio of the RH421 excitation wavelengths in the absence of peptide to the RH421 excitation wavelengths in the presence of peptide vs increasing concentration.
Tethered bilayer lipid membranes (tBLM)/Electrochemical impedance spectroscopy (EIS) for membrane disruption: tBLMs were made using pre-prepared monolayers coated gold slides covered by spacer and tethering molecules. Lipid bilayers were formed using the solvent exchange technique. Experiments were carried out at room temperature. Following addition, each buffer solution was equilibrated on the membrane for ten minutes and afterwards, was exchanged by rinsing with the new solution of different concentration. The conductance was determined by a tethaPad™ swept frequency impedance spectrometer employing real-time modelling of the profiles using tethaQuick™ software.
Statistical analysis: Statistical analysis was performed using the Graphpad Software (Graphpad Prism version 8 for Windows, GraphPad Software, La Jolla California USA). Data are expressed as mean +/- SEM. Statistical significance was considered at *P<0.05, **P<0.01, ***P<0.001.



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CARC Domain	CARC domain 1																	
	K/R	1-5x	1-5x	1-5x	Y/F	1-5x	1-5x	1-5x	1-5x	LV								
CRAC DOMAIN					CRAC domain 1						Crac domain 2							
					L/V	1-5x	Y	1-5x	x	K/R		L/V	1-5x	Y	1-5x	K/R		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	
AgGom	Z	C	R	R	L	C	Y	K	Q	R	C	V	T	Y	C	R	G	R	NH2
HiGom	Z	C	R	R	L	C	Y	R	N	R	C	V	T	Y	C	R	G	R	NH2

Figure 1. Identification of cholesterol recognition motifs in Gomesins

A) Cytotoxicity of Gomesins is 3 times higher in melanoma cells than in fibroblasts. Concentration response curve for the effects of AgGom and HiGom in the viability of MM96L and NFF at 48h (n=3)

B) Total cholesterol is 3 times lower in melanoma cells. Bar-chart illustrates total cholesterol content analysed spectrophotometrically (n=4)

C) Identification of the Cholesterol Recognition Aminoacid Consensus sequence (CRAC) or its reverse form (CARC) in AgGom and HiGom

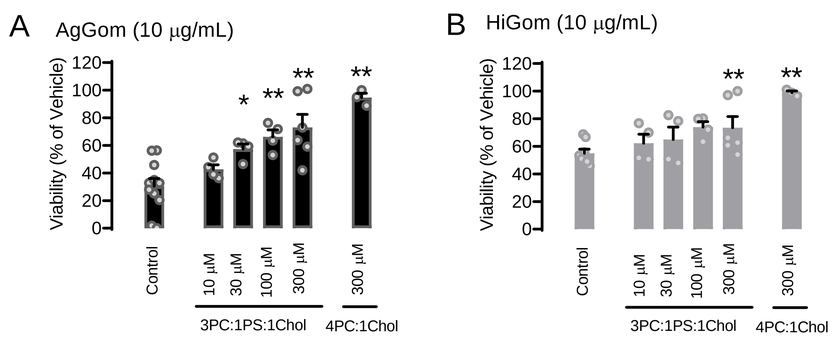

Figure 3. Cholesterol supplementation reduces Gomesin's cytotoxicity in melanoma cells.

Effects of a 48h treatment with **(A)** AgGom or **(B)** HiGom in the viability of MM96L cells under control conditions or supplemented with increasing concentrations of PC:PS: Cholesterol at a ratio 3:1:1 or PC: Cholesterol at a ratio 4:1 (n=3-8)

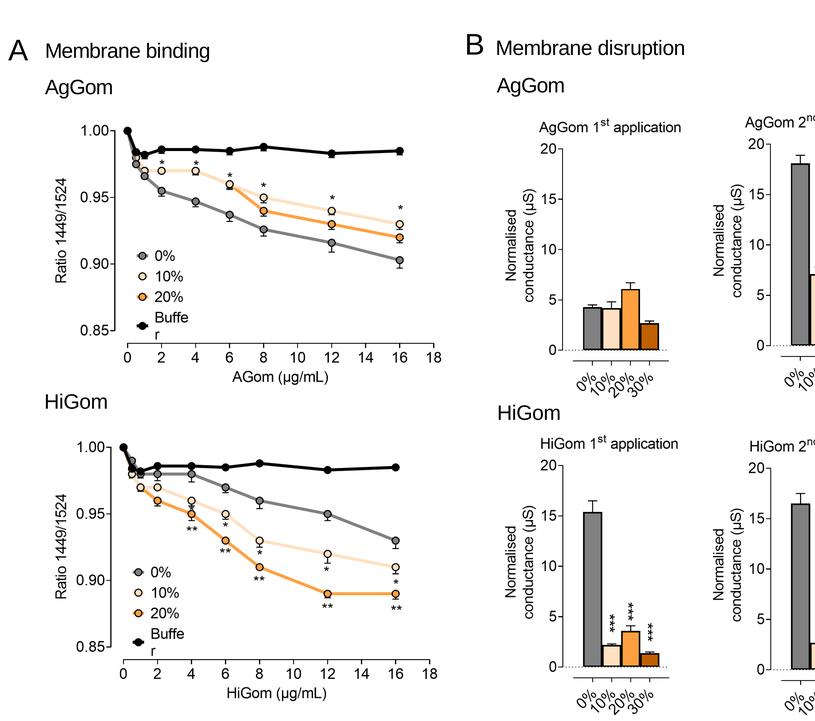

Figure 2. Cholesterol modulation of binding and membrane disruption in artificial membranes

A) Ratio of the RH421 fluorescence intensity for increasing concentrations of AgGom (top) or HiGom (bottom) in artificial membranes (4 POPC: 1 POPS) with increasing % of cholesterol. Cholesterol content inversely affects AgGom binding to artificial membranes. HiGom binding to membrane is favoured by increasing % of cholesterol (n=5-9)

B) Electrochemical impedance spectroscopy determines membrane disruption induced by AgGom (top) or HiGom (bottom) and the effects of acute (1st application) or cumulative (2nd application). AgGom shows cumulative membrane disruption. Increasing % of cholesterol reduced membrane disruption by AgGom and HiGom (n=5-9)

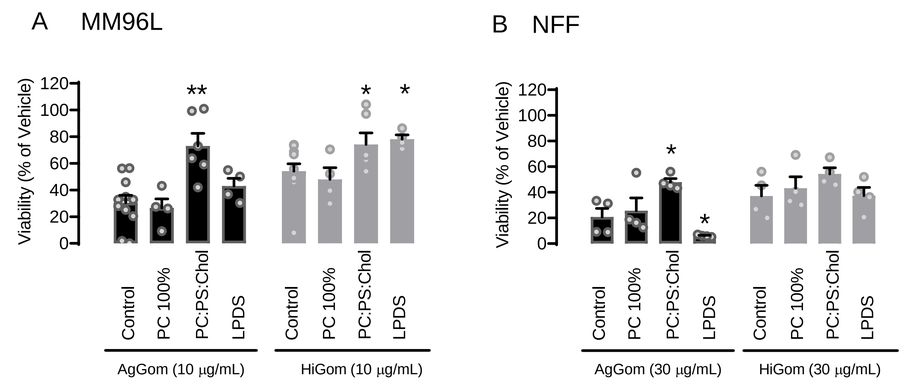

Figure 4. Cholesterol differently modulates cytotoxicity in melanoma cells and fibroblasts.

Effects of a 48h treatment with AgGom or HiGom in the viability of **(A)** MM96L and **(B)** NFF cells under control conditions or supplemented with 300 µM of the indicated lipids (PC, 3PC:1PS:1Chol) or 10% of lipoprotein-deficient serum (LPDS for cholesterol restriction). Increases in cholesterol blunted the cytotoxicity of AgGom in MM96L and NFF cells, but only reduced the cytotoxicity of HiGom in MM96L cells. Interestingly, cholesterol restriction (LPDS) potentiates the effects of AgGom only in NFF cells. (n=3-8)

CONCLUSIONS:

1. **Gomesins contain a consensus sequence likely susceptible to cholesterol binding that could explain its preferential cytotoxicity towards melanoma cells**
2. **Cholesterol impairs membrane disruption induced by Gomesins, while differently regulating binding of AgGom and HiGom to artificial membranes**
3. **Cytotoxicity of Gomesins was blunted by increasing concentrations of cholesterol in melanoma cells but potentiated by cholesterol depletion in healthy fibroblasts.**
4. **Cholesterol plays a key role in the cytotoxicity of Gomesins by modulating binding and disruption of lipid membranes**

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