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Interaction of Bee Venom Melittin, a Potential Anti-Cancer Drug, with Phosphatidylcholine Membrane Enriched with Phosphatidylserine

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Abstract

Interaction of bee venom melittin, a small basic amphipathic protein, has been studied with dipalmitoyl-phosphatidylcholine (DPPC) liposomes enriched with 10 mol% of dipalmitoyl-phosphatidylserine (DPPS) by methods of erythrosine phosphorescence quenching and differential scanning microcalorimetry. It has been determined that melittin increases fluidity of liposomal membrane and promotes segregation of DPPS to free areas of pure DPPC. It has also been revealed by method of AutoDock simulation that melittin binds to the polar head groups of phosphatidylserine (PS) via intermolecular ionic, ion-polar and hydrogen bonds which involve eight amino acid residues, T11, G12, P14, S18, K21, R22, Q25 and Q26, on molecular surface of melittin. Overall, it has been shown that melittin has a higher binding affinity to PS, a phospholipid commonly found on the outer surface of cancer cells, than to phosphatidylcholine (PC), a phospholipid exposed on the outer surface of healthy cells. In model membrane liposomes made of PC enriched with PS, melittin induces segregation of PS molecules to free and disturb areas of PC molecules. Should the same set of events be triggered by melittin on the surface of cancer cells in vivo, it may attract acidic exogenous PLA2 to hydrolytically destroy cancer cells.

Keywords: *Melittin; Phosphatidylserine; Phosphatidylcholine; Model Membranes; Erythrosine Phosphorescence; Large Unilamellar Liposomes; Differential Scanning Microcalorimetry; Enthalpy Change; AutoDock Simulation*

Abbreviations

PLA_2: Phospholipase A_2; DPPC: Dipalmitoyl-Phosphatidylcholine; DPPS: Dipalmitoyl-Phosphatidylserine; PC: Phosphatidylcholine; PS: Phosphatidylserine; DSMC: Differential Scanning Microcalorimetry; HPLC: High Performance Liquid Chromatography; SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Introduction

Melittin is a 26 amino acid residues cationic amphipathic membrane-active toxin from bee venom [1]. The primary target of melittin is a cell membrane [2]. Melittin disturbs bilayer structure of membranes to affect activities of various membrane-associated enzymes such as phospholipase C [3], phospholipase D [4], G-protein [5], protein kinase C [6] and adenylate cyclase [7].

Modulation of phospholipase A_2 (PLA_2) activity by melittin is an area of active *in vitro* and *in vivo* investigations since alteration of PLA_2 activity has important pharmacological implications [2]. PLA_2 is found in virtually every type of tissues and cells where this enzyme

catalyzes the hydrolysis of the *sn*-2 position of glycerophospholipids to produce lysophospholipids and fatty acids [8]. Lysophospholipids make important material in membrane biosynthesis, while fatty acids serve as substrates in the synthesis of eicosanoids, which are acute mediators of inflammation and play a key role in other pathophysiological processes [9]. Molecular mechanism of PLA_2 activity modulation by melittin is not well understood, however there is a growing evidence suggesting that melittin modulate PLA_2 activity through disturbing the packing order of phospholipids in cell membranes making the packing of phospholipid substrate more conducive for esterase activity of PLA_2 [10,11].

Membrane-active properties of melittin associate with its ability to bind to the membrane surface and to trigger changes in membrane structure leading to formation of membrane pores, fusion of membranes [12,13], changes in membrane potential [14], aggregation of membrane proteins [15], activation or inhibition of membrane associated enzymes [3-7] and stimulation of hormone secretion [16]. Through alteration in membrane structure and through facilitation of processes associated with changes in membrane structure melittin exhibits an array of pharmacological and therapeutical activities including antimicrobial [17], anti-arthritic [18], anti-inflammatory [19], anti-nociceptive [18] and anticancer [20] effects.

In our recent study we studied molecular mechanisms by which melittin binds to phosphatidylcholine (PC) membrane to trigger disturbance in initial lamellar phospholipid packing which facilitates esterase activity of acidic PLA_2 from Northern black-tailed rattlesnake venom [21]. The results of that study suggested that melittin binds to PC membrane surface with a long molecular axis of melittin parallel to the membrane plane. The five amino acid residues on molecular surface of melittin that bind to the PC polar head groups were identified [21]. In present study by employing methods of erythrosine phosphorescence quenching, differential scanning microcalorimetry and AutoDock simulation we investigate molecular details of melittin binding to PC membrane enriched with phosphatidylserine (PS). PS is a phospholipid which is commonly found on the outer leaflet of cancer cell membranes but not healthy cell membranes [22]. In this study we aim at elucidating whether melittin can specifically target PS on the surface of PC membrane and disturb the packing of phospholipids in PC membranes enriched with PS. We also investigate in this study whether melittin shares same binding sites on its molecular surface for PC and PS. The results of this study may promote engineering of novel melittin based anti-cancer drugs.

Materials and Methods

Reagents

Melittin, dipalmitoyl-phosphatidylcholine (DPPC), dipalmitoyl-phosphatidylserine (DPPS), erythrosine phosphorescent probe and ferrocene quencher were purchased from Sigma Chemical Co. (St. Louis, MO, USA). DPPC and DPPS were purified on silica columns. Melittin was purified from the trace PLA_2 contamination by cation exchange HPLC on a SCX 83-C-13-ET1 Hydropore column (Rainin Instrument, Woburn, MA) as previously described [23]. The purity of melittin was determined by reducing SDS-PAGE and by isoelectric focusing. All other reagents used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Phosphorescence assay

Large unilamellar liposomes of DPPC+10 mol% DPPS at a total lipid concentration of 3 mM were prepared at 46°C by using the ether evaporation method as previously described [24] in a buffer solution containing 10 mM Tris-HCl, pH 7.0, 0.1M NaCl and 0.1 mM $CaCl_2$. Liposomes of DPPC+10 mol% DPPS for phosphorescence assay were incubated with 5×10^{-6} M erythrosine for 20h followed by incubation with 10^{-5} M ferrocene for 2 hours at 4°C. To remove unbound probes, the liposomes were chromatographed on a Sephadex G 50 column (1.8 × 50 cm) according to procedure [25]. In liposomes used for phosphorescence assay, oxygen was enzymatically removed by glucose oxidase treatment. The analyzed liposome samples were contained in a square (10 mm) quartz chamber at 46°C. The erythrosine phosphorescence quenching by ferrocene in the absence or presence of defined melittin concentrations was detected with a filter specific

for a wavelength greater than 700 nm following excitation with a pulsed laser apparatus (Chernogolovka, Russia). The lifetime of the excited state of erythrosine, τ, was estimated as the time dependence of attenuation of the probe glow using semi-logarithmic coordinates. Samples for each data point were made and recorded in triplicate. Each data point for erythrosine exited state lifetime values is a mean of separate experiments with the SD ± 2.1% of the means.

Differential scanning microcalorimeter assay

Large unilamellar liposomes of DPPC or DPPC+10 mol% DPPS at a total lipid concentration of 3 mM for differential scanning microcalorimetry (DSMC) were prepared at 46°C using same protocol described above. Liposome samples were treated with 3×10^{-5} M melittin for 3 minutes at 46°C prior to recording the calorimetric curves. Control liposome samples were incubated for 3 minutes in absence of melittin at 46°C prior to recording the calorimetric curves. Calorimetric curves of liposome samples were monitored at a recording rate of 1°C per minute using a differential scanning microcalorimeter DASM-4 (Saint-Petersburg, Russia) equipped with the software to calculate enthalpy change (ΔH) and the main phase transition peak width at its half-height (T÷2). The instrumental base line calibration mark was obtained by scanning at 50 mW and DT = 4 as previously described [26]. Each liposome sample for the DSMC assay was prepared and tested in triplicate. Each data point for and values is a mean of separate experiments with the SD ± 1.4% of the means.

Molecular docking

Docking PS with the molecular surface of melittin was done by using the AutoDockVina Version 4.2 program and using PDB coordinates of PS (crystal structure of Tim-4 bound to PS - PDB ID# 3BIB) and melittin (PDB ID# 2MLT) by applying a similar methodology and parameters as previously published [27], but containing the following minor modifications required for this study. The PS virtual molecule was further edited to remove the alkyl chains using Avogadro as previously published [28], and the overall charges were checked and energy-minimized using AutoDock Vina. A grid box was set up with the following dimensions: center of x = 28.91; center of y = -2.077; center of z = 17.956; length of x = 100 Å; length of y = 72 Å; and length of z = 48 Å. The setting for exhaustiveness was set up as 16, which gave us consistent results in at least three sets of docking for PS polar head and melittin pair. Following each AutoDock run, the best nine docked conformations were analyzed for ionic, ion-polar and hydrogen bond interactions between the PS polar head groups and charged and polar amino acid groups of melittin by using Python Molecular Viewer (MGL Tools, The Scripps Research Institute).

Statistics

The data points in this study are expressed as means ± SD from at least three independent experiments. Data were analyzed by Student's t test (two-tailed) for single comparisons. Multiple group comparisons were done by performing one-way ANOVA followed by Bonferroni-corrected Tukey's test. P values less than 0.05 were considered statistically significant.

Results

Melittin increases fluidity of DPPC+10mol% DPPS liposomes

The phosphorescence probe quenching, a process controlled by diffusion, is a useful method to study the lipid membrane fluidity which is affected by interaction of membrane-active proteins with lipid membrane [25,29]. In this study we employed phosphorescence quenching of erythrosine by ferrocene taking place in membranes of DPPC+10mol% DPPS liposomes to investigate whether melittin interacts with liposomal membranes by monitoring changes in membrane fluidity. We recorded relative lifetime changes of phosphorescence, τ_i/τ_o, where τ_i and τ_o are the lifetimes of phosphorescence in the presence and absence of a defined melittin concentration respectively. A decrease in the lifetime of phosphorescence reflects an increased movement of erythrosine and ferrocene that comes from an increased membrane fluidity, and vice versa. Figure 1 demonstrates that an increase in concentration of melittin in DPPC+10mol% DPPS liposome samples results in increased membrane fluidity as evidenced by the decrease in lifetime of erythrosine phosphorescence. These

results strongly suggest that melittin interacts with DPPC+10mol% DPPS liposomes causing disturbance in membrane structure which makes molecules in membrane to move faster.

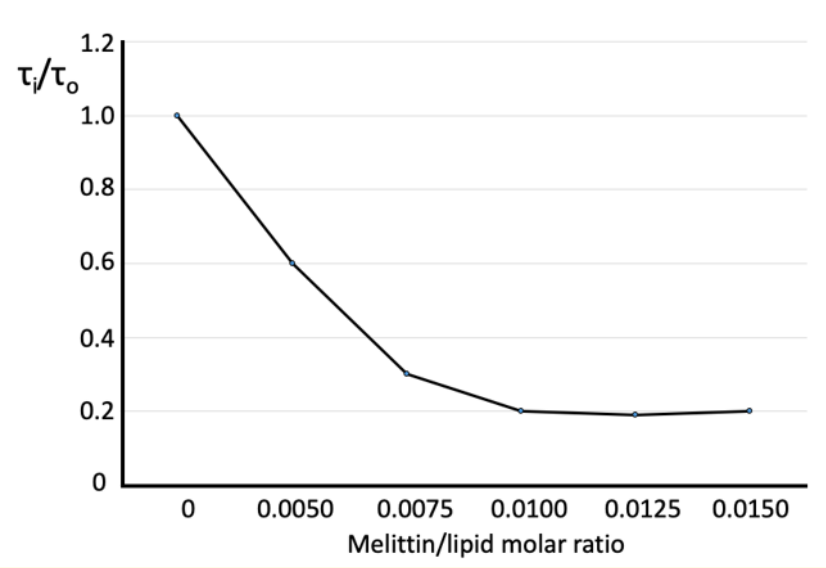

Figure 1: *Dependence of erythrosine phosphorescence quenching by ferrocene on relative concentrations of melittin. τo - Lifetime of erythrosine phosphorescence in large unilamellar liposomes sample of dipalmitoylphosphatidylcholine and 10 mol% dipalmitoylphosphatidylserine (total lipid concentration 3 mM) in absence of melittin. τi - Lifetime of erythrosine phosphorescence with same liposome samples containing defined melittin concentrations. Samples for each data point were made and recorded in triplicate. Each data point for erythrosine exited state lifetime values is a mean of separate experiments with the SD ±2.1% of the means.*

Melittin causes DPPS segregation in DPPC+10mol% DPPS liposomes and disturbs the packing of phospholipids

Differential scanning microcalorimetry (DSMC) is a powerful method to study the lipids packing order in membrane composed of pure saturated lipids. A calorimetric curve of a phase transition from solid crystalline to liquid gel phase of saturated tightly packed highly purified lipids has a shape with a narrow main transition peak (width at peak's half-height between 1 to 2°C) and a preceding small pretransition peak [26]. When packing order of saturated lipids in solid crystalline state is disturbed by an impurity or by the action of a membrane-active protein, the width of a main transition peak broadens and a pretransition peak disappears. Also, the phase transition enthalpy change decreases because it takes less energy to drive a phase transition when lipids in membrane are more disturbed and less orderly packed [26].

The calorimetric curve of large unilamellar liposomes made of DPPC has a narrow main transition peak at about 40.5°C and a small preceding pretransition peak at about 36°C (Figure 2A) which indicates that DPPC molecule in liposomal membranes are tightly packed in a high order. The calorimetric curve of large unilamellar liposomes made of DPPC+10 mol% DPPS does not have a pretransition peak and has a main transition peak at about 37°C (Figure 2B). Also, the change in the phase transition enthalpy in DPPC+10 mol% DPPS liposomes is somewhat less than that in DPPC liposomes (Figure 2B) which points to a somewhat disturbed packing of phospholipids in membranes of DPPC+10 mol% DPPS liposomes. DPPS has a higher phase transition temperature and a different polar head than those of DPPC. Thus, it is reasonable to conclude that the shift of a main transition peak towards higher temperatures and a disturbed packing of phospholipids are caused by addition of DPPS into liposomal membrane of DPPC.

Addition of melittin to DPPC+10 mol% DPPS liposomes caused significant decrease in the phase transition enthalpy change and increase in width at peak's half-height (Figure 2C). Plus, the main transition peak of calorimetric curve is returned back to 40.5°C (Figure 2C). The return of the main transition peak to the temperature of the main transition of DPPC strongly suggest that acidic DPPS molecules are segregated by the force of electrostatic attraction to basic melittin. The high temperature shoulder on the main transition peak (Figure 2C) is likely caused by a small amount of DPPS molecules which are not segregated due to small concentration of melittin which was not enough to segregate all DPPS molecules. Overall, the shape of calorimetric curve in figure 2C and values for transition enthalpy change and width at peak's half-height strongly suggest that melittin interacting with DPPC+10 mol% DPPS liposomes causes not only segregation of acidic DPPS but also significant disturbance in packing of phospholipids.

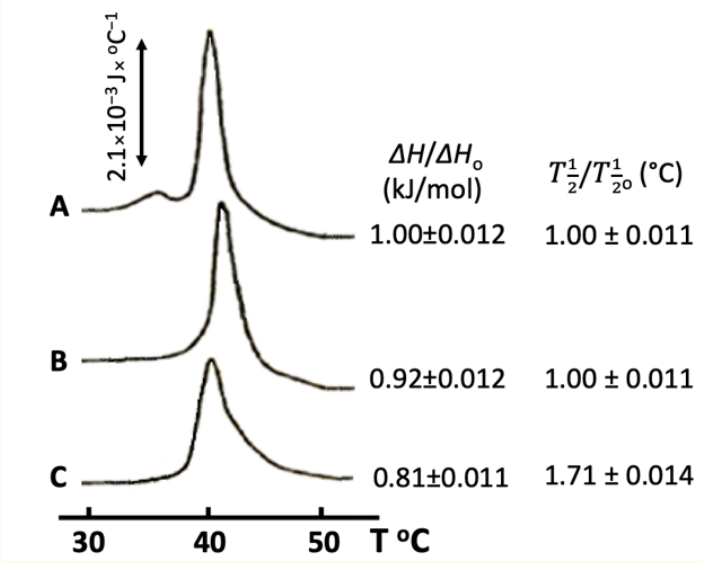

Figure 2: *Calorimetric curves of large unilamellar dipalmitoylphosphatidylcholine (DPPC) liposomes (A), DPPC + 10 mol% dipalmitoylphosphatidylserine (DPPS) liposomes in absence of melittin (B) and DPPC + 10 mol% DPPS liposomes treated with melittin (C). Total lipid concentration was 3 mM and melittin concentration was 3×10-5M. ΔHo and T(1/2)o are respectively the values for the enthalpy change and the width of main transition peak at its half-height in control DPPC liposomes (A). ΔH and T(1/2) are respectively the values for the enthalpy change and the width of main transition peak at its half-height recorded in samples of liposomes in (B) or (C).*

Phosphatidylserine binding sites on molecular surface of melittin identified by AutoDock simulation

In our recent study on interaction of melittin with phosphatidylcholine (PC) membrane we employed AutoDock Vina Version 4.2 program to identify putative ionic, ion-polar and hydrogen intermolecular bonds between melittin and PC polar head groups [21]. We identified five amino acid residues, T10, S18, R22, Q25 and Q26, on molecular surface of melittin which bind to polar head groups of PC. In this study we also employed the same program for docking simulation of melittin with phosphatidylserine (PS), a molecule commonly found on outer leaflet of cancer cell membranes. We used only PS polar head groups without hydrophobic alkyl chains as alkyl chains are not likely get involved in initial interaction with amphipathic proteins on the membrane surface [30]. The AutoDock Vina program identified nine best binding sites with the best affinity energies for PS polar head groups on molecular surface of melittin and we analyzed the types of intermolecular bonds between amino acid residues of melittin and polar head groups of PS in these binding sites. One can see in table 1 that in the nice binding sites there are three types of intermolecular bonds, ionic, ion-polar and hydrogen, identified by AutoDock

simulation. With exception of binding site 9, all have at least two bonds of different types in each binding site (Table 1). In binding sites 1, 5, 6, 7 and 8 ionic bonds involve R22 of melittin and carboxyl or phosphate groups of PS polar head, while binding sites 2 and 3 involve K21 of melittin and carboxyl or phosphate groups of PS polar head to form ionic bonds (Table 1). Interestingly, choline group, $-N^{+}H_3$, of PS polar head did not make ionic bonds and made only ion-polar bonds in binding sites 2, 3 and 4 (Table 1) which is an expected outcome as melittin does not have acidic amino acid residues. There are no ionic bonds identified in binding sites 4 and 9, and there is only ion-polar bond identified in binding site 9 (Table 1). As an example of ion-polar bond, figure 3A shows intermolecular bond in yellow broken line between $-COO^{-}$ group of PS polar head (sticks representation) and $-OH^{\delta+}$ of T11 of melittin (lines representation) which is also listed in table 1. Overall, there are eight amino acid residues, T11, G12, P14, S18, K21, R22, Q25 and Q26, identified by AutoDock simulation on molecular surface of melittin which are predominantly located on hydrophilic side of melittin (Figure 3B) suggesting that melittin binds to PS containing membrane with helical rod parallel to the membrane plane. Although, melittin shares four amino acid residues, S18, R22, Q25 and Q26, in binding to PC and PS polar head groups, AutoDock simulation revealed the higher affinity (more negative energy values) binding to PS polar head groups suggesting that melittin has higher tendency for binding to PS than for binding to PC.

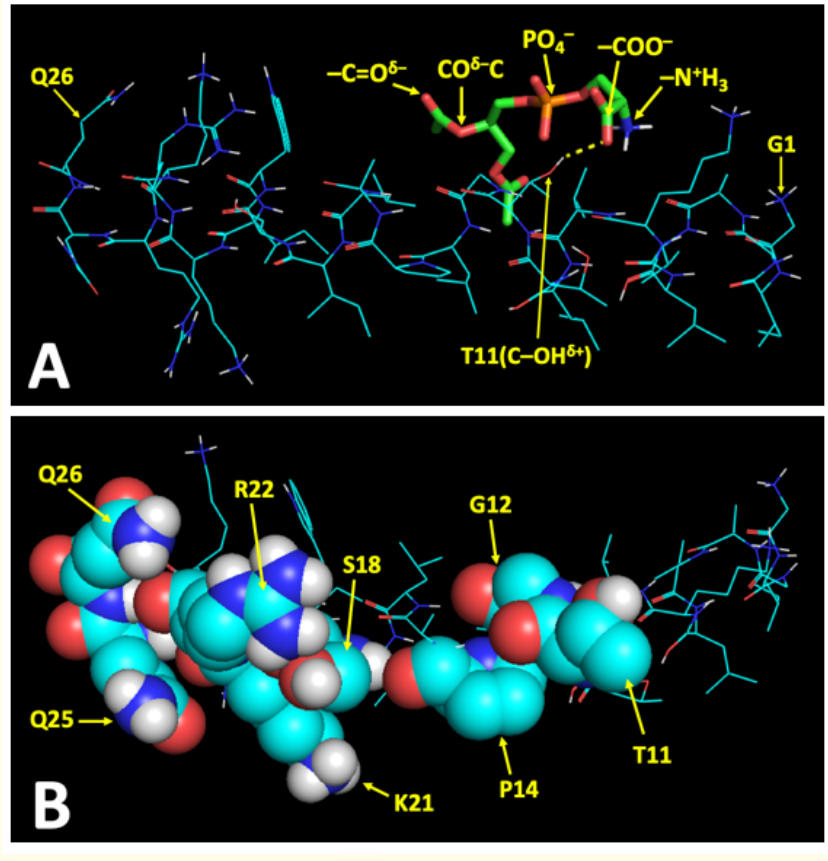

Figure 3: *Pymol diagram (A) shows the interaction of melittin with the polar head of PS represented in lines for melittin and in sticks for PS polar head at the binding site 9 with the affinity energy (-3.2 kcal/mol) determined by AutoDock simulation. The intermolecular bond between -COO- group of PS and -OHδ+ of T11 of melittin is shown in yellow broken line in diagram (A) which is also listed in table 1. Orientation of melittin in space given in diagram (A) does not suggest the melittin binding orientation on PC membrane surface and was only chosen for better visualization of intermolecular bond. Pymol diagram (B) shows melittin in cartoon and lines representations. Amino acid residues T11, G12, P14, S18, K11, R22, G25 and G26 shown in spheres representation. These residues are involved in binding to PS polar head at the top nine binding sites via ionic, ion-polar and hydrogen bonds as suggested by Autodock simulation. The type of bonds and affinity energies for each of the nine binding sites are given in table 1.*

Binding site # and affinity energy values	PS polar head groups	Melittin a. a. residues' charged and polar groups	Bond type
Binding site 1 Affinity: -3.60 kcal/mol	$-COO^-$ $-COO^-$	R22($C=N^+H_2$) R22($C-NH_2^{\delta+}$)	Ionic Ion-Polar
Binding site 2 Affinity: -3.50 kcal/mol	$-COO^-$ $-N^+H_3$	K21($-N^+H_3$) P14($C=O^{\delta-}_{pb}$)	Ionic Ion-Polar
Binding site 3 Affinity: -3.40 kcal/mol	PO_4^- $-N^+H_3$ $-C=O^{\delta-}$	K21($-N^+H_3$) P14($C=O^{\delta-}_{pb}$) S18($C-OH^{\delta+}$)	Ionic Ion-Polar Hydrogen
Binding site 4 Affinity: -3.40 kcal/mol	PO_4^- $-N^+H_3$ $CO^{\delta-}C$ $-C=O^{\delta-}$	Q25($C-NH_2^{\delta+}$) Q25($C=O^{\delta-}$) Q25($C-NH_2^{\delta+}$) Q26($C-NH_2^{\delta+}$)	Ion-Polar Ion-Polar Hydrogen Hydrogen
Binding site 5 Affinity: -3.40 kcal/mol	PO_4^- $-COO^-$ $-C=O^{\delta-}$ $CO^{\delta-}C$	R22($C=N^+H_2$) Q25($C-NH_2^{\delta+}$) R22($C-NH_2^{\delta+}$) S18($C-OH^{\delta+}$)	Ionic Ion-Polar Hydrogen Hydrogen
Binding site 6 Affinity: -3.30 kcal/mol	$-COO^-$ $C=O^{\delta-}$	R22($C=N^+H_2$) G12($-NH^{\delta+}_{pb}$)	Ionic Hydrogen
Binding site 7 Affinity: -3.30 kcal/mol	$-COO^-$ $-COO^-$	R22($C=N^+H_2$) R22($C-NH_2^{\delta+}$)	Ionic Ion-Polar
Binding site 8 Affinity: -3.30 kcal/mol	$-COO^-$ $-COO^-$	R22($C=N^+H_2$) R22($C-NH_2^{\delta+}$)	Ionic Ion-Polar
Binding site 9 Affinity: -3.20 kcal/mol	$-COO^-$	T11($C-OH^{\delta+}$)	Ion-Polar

Table 1: *Summary of the charged and polar groups of amino acid residues in the melittin binding sites that interact with polar head groups of truncated PS. Hypothetical binding sites in melittin that bind to the charged and polar groups of PS polar head were determined by AutoDock modeling. Designation of $-COO^-$, $-N^+H_3$, PO_4^-, $CO^{\delta-}C$ and $C=O^{\delta-}$ in PS polar head is shown in figure 3A. Pb in $NH^{\delta+}_{pb}$ and $C=O^{\delta-}_{pb}$ denotes a peptide bond.*

Discussion

Melittin, a small basic amphipathic membrane-active protein from bee venom, has been an object of extensive investigation for several decades [1,2,12,13,31-34]. Melittin exhibits an array of potential pharmacological properties which are thought to come from the melittin's direct action on cell membranes to facilitate or inhibit activities of various membrane-associated enzymatic proteins [2-7]. A range of physiological reactions modulated via interaction of melittin with cell membranes presents novel avenues for possible therapeutic application of melittin in treatment of a number of diseases [4,14,17,18,20]. One of the most promising therapeutic applications of melittin is in treating cancer [4,18,20], however a lot of work has to be done to elucidate molecular mechanism(s) of melittin action in killing cancer cells and suppressing oncological processes.

Mechanism of melittin anti-cancer activity is not well understood. Cardiotoxins from cobra venom [23] and *Pyrularia* thionin from *Pyrularia pubera* [35], which are basic amphipathic membrane-active proteins like melittin is, kill cancer cells by targeting PS, acidic phospholipid exposed on outer leaflets of cancer cell membranes [22]. *Pyrularia* thionin which exhibits a particular high specificity for binding to PS also exhibits a very high specificity in killing only cancer cells *in vitro* [35]. Cardiotoxins and *Pyrularia* thionin do not bind to PC

membranes and as a result do not kill healthy cells outer membrane leaflet of which is made of PC [23,35]. In opposition to all known basic amphipathic protein toxins from natural sources, melittin has high binding affinity to the PC polar head groups [33]. In our recent study we have revealed five amino acid residues, T10, S18, R22, Q25 and Q26, of melittin which bind to PC polar head groups [21]. Amino acid residue R22 seems to be a key force driving interaction of melittin with PC polar head. The charged, $C=N^{+}H_2$, and polar, $C\text{-}NH_2^{\delta+}$, groups of R22 on surface of melittin establish intermolecular ionic and ion-polar bonds with phosphate group, PO_4^{-}, of PC polar head [21] which appears to play a major role in attraction and binding of melittin to PC membrane.

Melittin has a 3D structure of alpha-helical rod with the hydrophobic and hydrophilic amino acid residues segregated on opposite sides of the long axis of the helix. The five amino acid residues of melittin involved in interaction with PC polar head groups identified in our recent study [21] were found on hydrophilic side of the helix. This suggests that melittin binds to PC membrane with the long axis of the helix parallel to the membrane plane which agrees well with findings of other researchers [32]. We think that after embedding into polar area of PC membrane melittin twists along its long axis to orient hydrophobic side chains of the helix toward nonpolar area of alkyl chains of lipids in PC membrane [21]. Thus, melittin stays in the interface between lipid polar head region and nonpolar area of alkyl chains [32,34] and does not enter the inner leaflet of bilayer. Accumulation of melittin at only outer leaflet of the bilayer increases the surface area of outer monolayer over the inner monolayer. We have previously published a mathematical model that predicts formation of convexities on the outer surface of membrane to release stress caused by asymmetric enlargement of monolayer surfaces of the bilayer [35]. We believe that convexities on the outer membrane surface exposing phospholipids to solution may facilitate esterase activity of exogenous body phospholipids [21]. At a lipid to melittin molar ratio exceeding 100 to 1, a stress caused by asymmetric enlargement of membrane's two monolayer surfaces is released via formation of pores [34] which helps to balance surface areas of two monolayers. When this happens, melittin helices start changing molecular orientation in membrane from parallel to perpendicular to promote formation of stable pores [34]. Obviously, during these processes orderly bilayer packing of phospholipids is disturbed on both sides of membrane interface which may provide more opportunities for the increased esterase activity of exogenous and endogenous body PLA_2.

In this study we showed by the method of erythrosine phosphorescence quenching that increase in DPPC+10 mol% DPPS membrane fluidity caused by melittin reaches plateau at lipid to melittin molar ratio at 100 to 1, which is the same molar ratio at which PC membrane starts forming stable pores with melittin helixes starting to orient perpendicular to the membrane plane. This suggests that changes in erythrosine phosphorescence quenching most likely reflect the melittin caused disturbance in lipid packing from bilayer to pore formation at which membrane fluidity increases. It appears that at a stage of pore formation, membrane fluidity remains constant even when more pores are being formed. It is reasonable to assume that melittin induced membrane perturbation that triggers transformation in lipid packing modulates hydrolytic activity of both exogenous and endogenous body PLA_2 enzymes and may bring to total collapse of membrane integrity and subsequent cell death.

Another important finding in this study revealed by DSMC method is that melittin causes phase segregation of DPPS molecules in DPPC+10 mol% DPPS membrane which is likely caused by electrostatic attraction of basic melittin to acidic DPPS molecules. This suggestion is supported by our AutoDock simulation of melittin and PS interaction in this study that identified eight amino acid residues, T11, G12, P14, S18, K21, R22, Q25 and Q26, on molecular surface of melittin. All eight amino acid residues were found on hydrophilic side of melittin (Figure 3B) to suggest that melittin binds to PS containing membrane in a similar fashion at which melittin interacts with PC membrane, that is with melittin helixes binding in parallel orientation to the membrane plane. Eight amino acid residues of melittin involved in binding to PS as opposed to five residues involved in binding to PC, plus two basic residues, K21 and R22, binding to PS as opposed to one basic residue binding to PC, explains the higher affinity of melittin binding to PS than for PC. This higher attraction of basic melittin to acidic PS than to zwitterionic PC seems to be a driving force of the melittin induced segregation of PS molecules in membrane containing both PS and PC. Segregation of PS by melittin frees pure PC areas on membrane surface which increases esterase activity of acidic exogenous PLA_2 enzymes which are otherwise repelled by acidic PS. A phase segregation of acidic phospholipids by basic cobra venom cytotoxins in PC liposomes enriched with acidic phospholipids with subsequent activation of acidic PLA_2 has been shown by us

previously [11,36]. Secreted exogeneous PLA_2 are acidic enzymes [37], and accumulation of acidic PS on the outer surface of cancer cells which repel exogenous PLA_2 may explain immortality of cancer cells. Thus, the mechanism of anticancer activity of melittin may be based on its ability to segregate PS and disturb freed neutral PC areas on the outer surface of cancer cells to promote hydrolytic lysis of cancer cells by acidic exogenous PLA_2.

Conclusion

In conclusion, our present study revealed that melittin has a higher binding affinity to PS, a phospholipid commonly found on the outer surface of cancer cells, than to PC, a phospholipid exposed on the outer surface of healthy cells. In model membrane liposomes made of PC enriched with PS, melittin induces segregation of PS molecules to free and disturb areas of PC molecules. Should the same set of events be triggered by melittin on the surface of cancer cells *in vivo*, it may attract acidic exogenous PLA_2 to hydrolytically destroy cancer cells.

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Conflict of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study, in the collection, analysis, or interpretation of data, in the writing of the manuscript or in the decision to publish the results.

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Volume 8 Issue 12 December 2020