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The interactions of aurein 1.2 with cancer cell membranes

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Abstract

Here, the interactions of aurein 1.2, a defence peptide, with T98G glioblastoma cell membranes are studied. The peptide induced maximal surface pressure changes of *circa* 9 mN m^{-1} in monolayers of endogenous T98G membrane lipid. Reducing monolayer anionic lipid showed a positive correlation ($R^2 > 0.91$) with decreases in maximal surface pressure changes induced by aurein 1.2 (*circa* 3 mN m^{-1} in the absence of this lipid). Cancer cell membrane invasion by the peptide therefore appears not to be mediated by lipid receptors or specific lipid requirements but rather a general requirement for anionic lipid and/or other negatively charged membrane components.

Keywords: Aurein 1.2; T98G cancer cell membranes; Anionic lipid; Lysine residues; Snorkelling

1. Introduction

Cancer is a problem of global proportions [1,2] yet despite intensive research, the biochemical mechanisms underlying carcinogenesis are not fully understood, limiting the potential to develop efficient anticancer drugs [3,4]. Currently, most anticancer drugs only control tumour growth effectively at concentrations that also affect healthy cells, causing undesirable side effects [5,6]. Moreover, this problem is often exacerbated by multi-drug resistance (MDR) in cancer cells, commonly arising from the over-expression of P-glycoprotein efflux pumps that actively flush conventional anticancer compounds from the cell [7,8].

Some advances have been made in overcoming the problems related to cancer treatment with the development of targeted anticancer agents [9] and MDR protein inhibitors [10]. However, there is still clearly an urgent need for new drugs with novel mechanisms of action [11,12] and one recent focus has been defence peptides [13,14]. Many of these peptides are potent anticancer agents in addition to their established roles as antimicrobial factors and modulators of innate immune systems [15–18]. Generally, these peptides exhibit anticancer activity at low micromolar levels, which are comparable to those of their antimicrobial activity, but which is not accompanied by significant haemolysis or toxicity to other mammalian cells [13]. It is generally accepted that these peptides kill cancer cells *via* membrane invasion although the mechanisms underlying this process are far from fully understood [14]. The majority of these anticancer peptides adopt α-helical structure (α-ACPs) at the interface, thereby facilitating anticancer activity *via* use of the carpet/toroidal pore model, which appears to generally describe membrane invasion by these peptides. In most cases, the resulting disruption of the mitochondrial membrane and/or plasma membrane leads to loss of membrane integrity and cell death *via* a number of mechanisms, typically apoptosis and necrosis [19].

Currently, the best characterised α-ACPs include arthropod cecropins, amphibian magainins and their derivatives [19]. However, amphibians of the *Litoria* and *Uperoleia* genera are proving to be rich sources of α-ACPs with the citropins, dahleins, maculatins, uperins, caerins and aureins recently isolated from these creatures [20–22]. The prototype of this latter peptide family is aurein 1.2 (GLFDIIKKIAESF-NH_2) from *Litoria aurea* and *Litoria raniformis* [23,24], which was recently shown be non-haemolytic and active against 55 human cancers, including: renal, ovarian, prostate, colon, breast, lung

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and bladder cancers, along with melanomas, leukemias and cancers of the central nervous system [22]. However, the ability of the peptide to interact with cancer cell membranes has not been addressed in detail and here, the ability of aurein 1.2 to interact with membranes of cells from the T98G glioblastoma cell line is investigated.

2. Materials and methods

2.1. Reagents

Synthetic aurein 1.2 was supplied by Severn Biotech Ltd., synthesised by solid state synthesis and purified by HPLC to purity greater than 99%, confirmed by Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry. Buffers and solutions for monolayer experiments were prepared from Milli-Q water (specific resistance 18 MΩcm). Dimyristoylphosphatidylserine (DMPS), dimyristoylphosphatidylcholine (DMPC), dimyristoylphosphoethanolamine (DMPE), dimyristoylphosphatidylinistol (DMPI) and dimyristoylphosphatidylglycerol (DMPG) were purchased from Alexis Biochemicals (UK). Sphingomyelin (SP) and all other reagents were purchased from Sigma (UK) unless otherwise stated.

2.2. Lipid extracts from T98G glioblastoma cells

Cells of the T98G human Caucasian glioblastoma cell line were purchased from The European Collect ion of Cell Cultures (ECACC at http://www.ecacc.org.uk). The cells were grown in cell culture flasks (25 ml) containing Minimum Essential Medium Eagle (MEM) with Earle's salts, formulated as provided by the maker (Sigma, UK), which was supplemented with: 2 mM glutamine, 1% non essential amino acids (w/v), 1% sodium pyruvate (w/v) and 10% foetal bovine serum (w/v); all at 37 °C with 5% CO_2. The cell culture media was changed every 48–72 h until the cells reached 70–80% confluence and then they were washed three times with Hanks Balanced Salts Solution, treated with 1 ml 0.25% trypsin / EDTA (w/v) and incubated at 37 °C for up to 15 min. On detachment of the T98G cells, 15 ml of fresh growth media was added to neutralise the trypsin and the cells harvested by centrifugation (15000 × g, 20 min). Total lipid extracts of these harvested cells were then obtained using a modified form of the procedure first described by Bligh and Dyer [25]. Essentially, 1 ml of each culture was extracted, washed twice in Tris buffer (10 mM, pH 7.5) and centrifuged (15000 × g, 10 min) to form a pellet. Each pellet was then resuspended in 0.5 ml Tris buffer (10 mM, pH 7.5) and to a 0.1 ml aliquot of this cell suspension, 0.375 ml of a 1:2 (v/v) chloroform–methanol mixture was added. Each of these cell/solvent samples was then vortexed vigorously for 5 min, a further 0.125 ml chloroform added and the whole again vortexed for 5 min. To each sample, 0.125 ml water was added, the whole vortexed for 5 min and then centrifuged at low speed (70 × g, 5 min) to produce two phases. The lower organic layer was transferred to a fresh centrifuge tube, concentrated using a Jouran speed vac (Jouran RC 10.22) and the dried lipid extract stored at −20 °C.

2.3. Lipid monolayer studies on aurein 1.2

2.3.1. Langmuir–Blodgett system

Monolayer investigations were performed using Langmuir–Blodgett equipment supplied by NIMA technology (Coventry UK), which was mounted on a vibration-isolated table. Studies were conducted using a Teflon trough, which possessed surface area dimensions of 5 cm × 16 cm and held a volume of 80 ml, and was fitted with two mechanically coupled Delrin barriers [26]. All experiments were conducted at an operating temperature of 21.0 ± 1 °C and used a subphase of Tris (10 mM, pH 7.5), which had been prepared as described above. Unless indicated otherwise, aurein 1.2 was introduced into the subphase *via* an injection port to give desired final concentrations. The subphase was continuously stirred by a magnetic bar (5 rpm) and surface tension was monitored by the Wilhelmy method using a paper plate (Whatman's Ch1) in conjunction with a microbalance, as described by Brandenburg et al. [27]. To check for surface contamination, the barriers were then set to their maximum distance apart and the surface cleaned by aspiration. The barriers were then compressed at a rate of 100 cm min^{-1} until the smallest possible surface area was achieved. The surface was assumed to be free of contaminants if during this compression the changes in surface pressure observed were less than 0.01 mN m^{-1}. Changes in monolayer surface pressure/area were recorded as graphic output on a PC using NIMA software (version 5.54), which interfaced with the Langmuir–Blodgett microbalance.

2.3.2. Aurein 1.2 surface activity

The barriers of the Langmuir–Blodgett trough were adjusted to their maximum separation (surface area 80 cm^2) and this position maintained. Aurein 1.2 was then introduced into the Tris subphase to give final concentrations ranging between 1.0 and 6.0 μM and at each peptide concentration, changes in

Table 1
Synthetic lipid monolayer compositions

Lipid monolayers	DMPS molar %	DMPG molar %	DMPI molar %	DMPC molar %	DMPE molar %	SP molar %
T98G membrane mix	11.7	–	4.3	35.9	31.2	16.9
T98G membrane mix - anionic lipid absent	–	–	–	42.7	37.1	20.2
PS/PC mix	–	–	–	100		–
PS/PC mix	30	–	–	70		–
PS/PC mix	60	–	–	40		–
PS/PC mix	100	–	–	–		–
PG/PC mix	–	–	–	100		–
PG/PC mix	–	30	–	70		–
PG/PC mix	–	60	–	40		–
PG/PC mix	–	100	–	–		–

Table 1 shows the lipid composition of monolayer mimics of T98G glioblastoma cell membranes, which was derived from Hattari et al. [39], and various PS / PC and PG/PC mixtures. DMPS = dimyristoylphosphatidylserine, DMPG = dimyristoylphosphatidylglycerol, DMPI = dimyristoylphosphatidylinositol, DMPC = dimyristoylphosphatidylcholine, DMPE = dimyristoylphosphatidylethanolamine and SP = sphingomyelin.

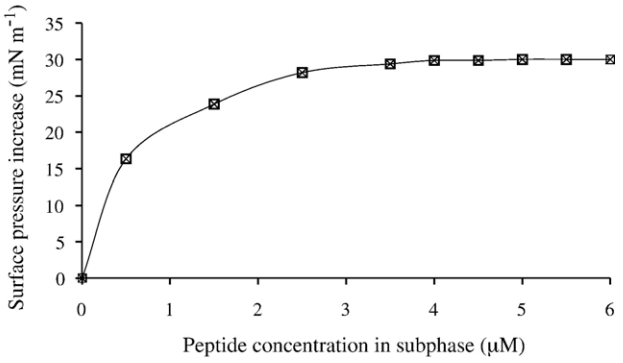

Fig. 1. Shows aurein 1.2 surface pressure as a function of peptide concentration. Increasing levels of aurein 1.2 were injected into a Tris buffer subphase (10 mM, pH 7.5) of a Langmuir–Blodgett system. At each aurein 1.2 concentration, the peptide was allowed to equilibrate, the surface pressure determined and plotted, all as described above.

surface pressure at the air/water interface were monitored for 1 h. The maximal values of these surface pressure changes were then plotted as a function of the peptide's final subphase concentration.

2.3.3. Aurein 1.2 interactions with lipid monolayers

The ability of aurein 1.2 to penetrate lipid monolayers was studied. Monolayers were formed by spreading onto a Tris buffer subphase, chloroform solutions of either total lipid extract from cells of the T98G glioma cell line, derived as described above, or various synthetic lipid mixes, prepared according to Table 1. After spreading, the solvent was allowed to evaporate off the subphase surface over 30 minutes and then the lipid monolayer compressed at a velocity of 5 cm^2 min^{-1} to give a surface pressure of 30 mN m^{-1}, mimetic of naturally occurring membranes [28]. To study the interactions of aurein 1.2 with lipid monolayers at constant surface area, the barriers were maintained in this position and aurein 1.2 was introduced into the Tris subphase to achieve a peptide concentration of 4 μM, which was determined as optimal for these experimental conditions (Fig. 1). Interactions of the peptide with lipid monolayers were monitored as changes in monolayer surface pressure versus time (Fig. 2A). To study the interactions of aurein 1.2 with lipid monolayers at constant surface pressure (Fig. 2B), lipids were spread onto the Tris subphase as described above except that once a surface pressure of 30 mN m^{-1} had been attained, this pressure was maintained. The lipid monolayer was allowed to equilibrate for 10 minutes and aurein 1.2 was introduced into the Tris subphase to give a final peptide concentration of 4 μM. Interactions of aurein 1.2 with lipid monolayers were monitored as changes in monolayer surface area per lipid molecule versus time. The interactions of aurein 1.2 with DMPS/DMPC and DMPG/DMPC monolayers (Table 1) were studied at constant surface area and maximal surface pressure changes induced by the peptide were plotted as a function of their DMPS (Fig. 3A) and DMPG (Fig. 3B) concentrations respectively. Regression analysis of these data (Table 2) was performed using Minitab version 14.

3. Results and discussion

Aurein 1.2 is a potent α-ACP [22–24] and several recent studies have suggested generic models for membrane invasion by the peptide [22,29–31]. However, this appears to be the first detailed study on the peptide's ability to invade cancer cell membranes.

Aurein 1.2 was found to be strongly surface active with a surface pressure of 30 mN m^{-1} (Fig. 1), which is highly comparable to that of other α-ACPs from amphibian sources [19,29]. To study the interaction of the peptide with cancer cell membranes, monolayer mimics of these membranes were constructed and studied at constant area with an initial surface pressure of pressure of 30 mN m^{-1}. With monolayers formed from endogenous lipid of T98G glioblastoma cell membranes (Fig. 2A), AP1 was found to rapidly partition into these monolayers over a period of approximately 10 s, inducing large maximal surface pressure changes of *circa* 9.5 mN m^{-1} (Fig. 2A, curve A). These levels of interaction are consistent with disruption of the monolayer acyl chain region by aurein 1.2 and are comparable to those reported for other strongly membrane invasive peptides [32,33]. In combination, these

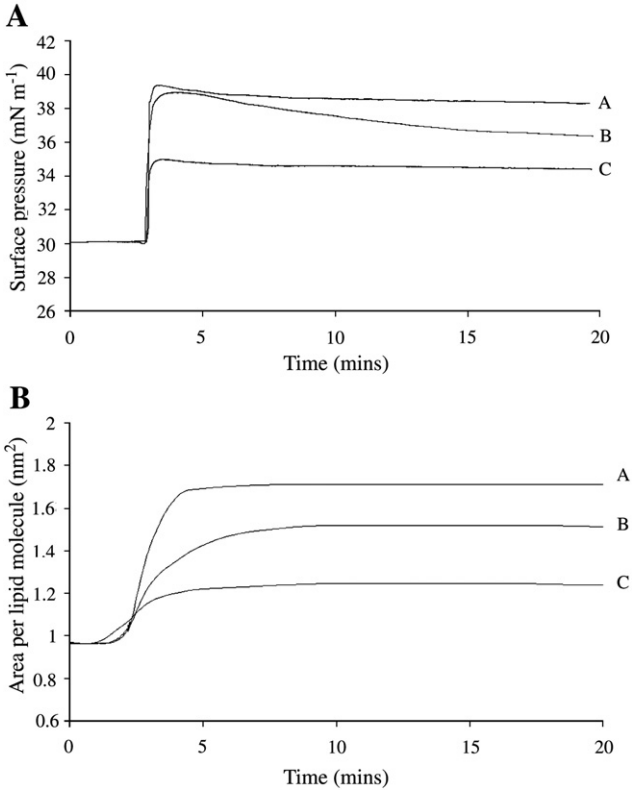

Fig. 2. Shows the time course of interactions between aurein 1.2 and lipid monolayers at constant surface area and an initial surface pressure of 30 mN m^{-1} (A), and at a constant surface pressure, maintained at 30 mN m^{-1} (B). In both A and B, curve (A) represents interactions of the peptide with monolayer mimics of T98G glioblastoma cell membranes, which were formed from the endogenous lipid of these cells. Curve (B) represents interactions of the peptide with monolayers, which were formed from an artificial lipid mixture of composition equal to that of T98G glioma cell membrane (Table 1). Curve (C) represents interactions of the peptide with monolayers of the same composition as in curve (B) but excluding the anionic lipid component (Table 1).

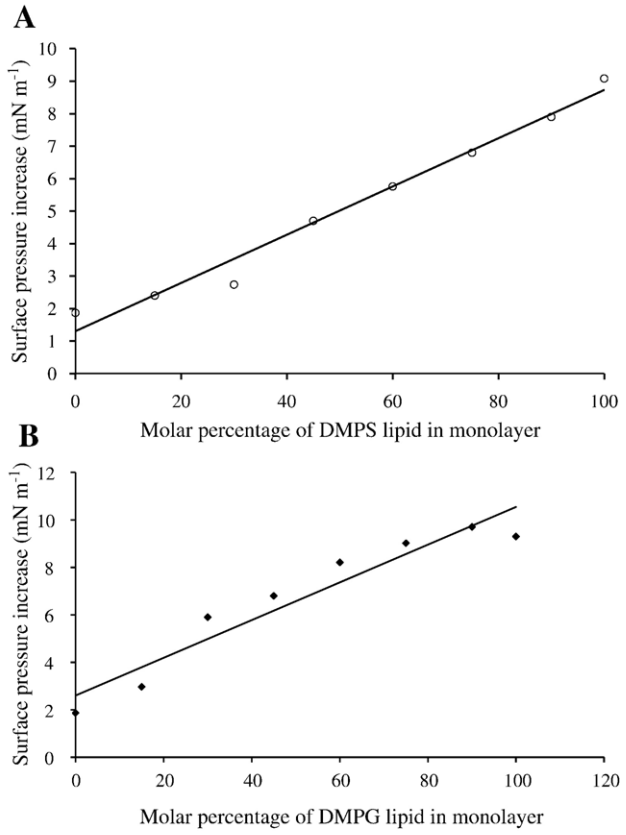

Fig. 3. Shows the effect of varying anionic lipid levels in monolayers on the levels of interaction shown by aurein 1.2 with these monolayers. Fig. 3A shows that PS (○) levels exhibit a strong positive linear correlation (Table 2) with the surface pressure changes induced by the peptide in PS/PC monolayers. Fig. 3B shows that a similar strong positive linear correlation (Table 2) exists when the experiments represented in Fig. 3A were conducted with PG (♦) substituted for PS.

data clearly show that aurein 1.2 has a high affinity for the membrane lipid of T98G cells and suggest that the peptide has a strong ability to invade the membranes of these cancer cells.

It is generally accepted that α-ACPs do not use protein-based receptor mechanisms to invade cancer membranes [13,14,19]. However, the use of lipid-based receptors to enhance the lipid affinity of membrane invasive peptides is well established in the case of some peptides [34,35], suggesting the possibility that use of such receptors may underlie the high affinity and interactivity shown by aurein 1.2 for monolayers derived from the membrane lipid of T98G cells (Fig. 2A, curve A). To gain insight into this possibility, the putative role of anionic lipid in the peptide's interactions with T98G cell membranes was investigated. Using synthetic lipids, monolayer mimics of T98G membranes were constructed with precisely known composition. Aurein 1.2 showed reaction kinetics and maximal surface pressure changes (*circa* 9.0 mN m^{-1}) with these monolayers (Fig. 2A, curve B) that closely paralleled those observed for interaction of the peptide with monolayers formed from endogenous T98G lipid (Fig. 2A, curve A) over 5 min. These observations established that the former monolayers provided good models of the latter and when the level of anionic lipid in these synthetic monolayer mimics was reduced to zero, maximal surface pressure changes induced by aurein 1.2 decreased by approximately two third to *circa* 3.5 mN m^{-1} (Fig. 2A, curve C). These data strongly suggested that anionic lipid may play a major role in the ability of aurein 1.2 to target and invade the membranes of cancer cells.

The monolayer data of Fig. 2A was strongly supported by experiments using the same lipid/peptide systems, which were conducted at constant pressure with the surface pressure of the monolayer held at 30 mN m^{-1} (Fig. 2B). Fig. 2B shows the time course for variations in the area per lipid molecule induced by the interaction of aurein 1.2 with monolayers possessing lipid compositions that corresponded to those of the monolayers analysed in curves 'A', 'B' and 'C' of Fig. 2A. It can be seen from Fig. 2A and B that these monolayers follow the same rank order in terms of the peptide's induction of maximal increases in area per lipid molecule of a monolayer and maximal changes in surface pressure of a monolayer.

It is well established that PS on the outer membrane surface of cancer cells makes a major contribution to the negative charge carried by these cells [36,37]. To study the putative role played by this lipid in the membrane interactions of aurein 1.2, monolayers with varying PS levels were constructed, as described in Table 1. It was found that the maximal surface pressure changes induced by aurein 1.2 in these monolayers showed a correlation with their PS levels (Fig. 3A), which regression analysis indicated was a strong positive linear relationship (R^2=0.98). However, a similar positive linear relationship (R^2=0.92) was observed when corresponding experiments were performed with monolayers where PS was substituted by PG (Fig. 3B), an anionic lipid not found in eukaryotic membranes. Analysis of variance showed that statistically, there were no significant differences between the curves shown in Fig. 3A and 3B ($F_{1,23}$=1.323; p=0.262). These data clearly show that under the experimental conditions used here, PS and PG have similar capacities to promote the interaction of aurein 1.2 with membranes but that this capacity is greatly reduced in the absence of these lipids. In combination, these results strongly suggest that the invasion of cancer cell membranes by aurein 1.2 is not based on mechanisms mediated by specific lipid requirements such as a lipid-receptor or a particular anionic lipid(s) but rather a general requirement for such lipid and possibly other negatively charged membrane components. Indeed, it has been recently suggested that in addition to anionic lipid, the affinity of α-ACPs for cancer cell membranes may be related to the increased levels of negatively charged sialic acids associated with these membranes [19].

Table 2
Regression analysis of monolayer data in Fig. 3

Monolayer	Anionic Lipid	R^2	F value	Significance of F-value
DMPS/DMPC	DMPS	0.98	272.6074	3.15×10^{-6}
DMPG/DMPC	DMPG	0.92	68.85425	1.66×10^{-4}

Table 2 shows summary statistics for regression analysis of curves depicting the relationship between surface pressure changes induced by aurein 1.2 in lipid monolayers and the levels of anionic lipid in these monolayers. Curves analysed were for PS/PC monolayers (Fig. 3A) and PG/PC monolayers (Fig. 3B).

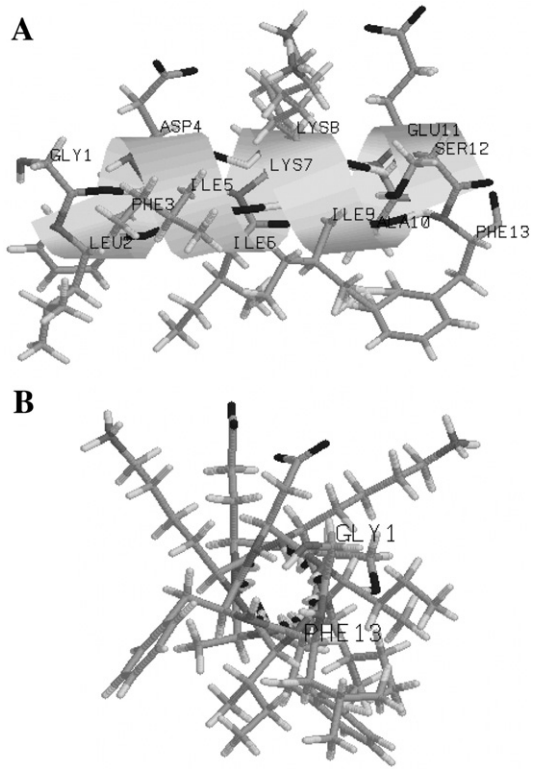

Fig. 4. Shows aurein 1.2 modelled in an-helical conformation. A front projection of this α-helix (A) shows a strong centrally placed cationic region formed by the peptide's adjacent lysine residues. This projection also shows that the negatively charged glutamic acid and aspartic acid residues of the peptide are distal from its cationic region, thus forming a residue distribution that facilitates the interaction of aurein 1.2 with negatively charged membranes by allowing the peptide to effectively function as a positively charged molecule. This front projection also demonstrates that the lysine residues of the aurein 1.2 α-helix define the boundary between its narrow polar and wide apolar faces, a residue arrangement that is strongly associated with the class A α-helices and the snorkelling mechanism of Segrest et al. [38]. According to this mechanism, the long side-chains of these lysine residues can snorkel or extend from the membrane surface as illustrated in the side projection of the aurein 1.2 α-helix (B). This snorkelling would allow the bulk of the aurein 1.2 α-helix to penetrate more deeply into the membrane hydrophobic core, whilst permitting the cationic moieties of its lysine residues to maintain electrostatic interactions with the membrane lipid headgroup region.

The ability of α-ACPs to interact with negatively charged moieties on cancer cell membranes usually derives from the fact that these peptides carry a net positive charge. To examine the charged residue distribution of aurein 1.2, the three-dimensional structure of the peptide was modelled using PyMOL version 0.98 and when a front projection of this α-helix was viewed, a strong centrally placed cationic region formed by adjacent lysine residues was revealed (Fig. 4A). This projection also showed that the negatively charged glutamic acid and aspartic acid residues of the peptide are distal from this cationic region. It seems likely that these positively charged residues would stabilise the membrane interactions of aurein 1.2 through association of these residues with negative charge arising from moieties in membrane lipid headgroups. Moreover, it can be seen from this front projection of the α-helix that its lysine residues define the boundary between the narrow polar and wide apolar faces of the α-helix, a residue arrangement that is strongly associated with the class A α-helices and the snorkelling mechanism of Segrest et al. [38]. According to this mechanism, the long side-chains of these lysine residues can snorkel or extend from the membrane surface as illustrated in the side projection of the aurein 1.2 α-helix (Fig. 4B). This snorkelling would allow the bulk of the aurein 1.2 α-helix to penetrate more deeply into the membrane hydrophobic core, whilst permitting the cationic moieties of its lysine residues to maintain electrostatic interactions with the membrane lipid headgroup region. Indeed, the use of this mechanism of membrane interaction could contribute to the high levels of monolayer penetration exhibited by aurein 1.2, leaving the more negatively charged residues of the peptide to interact with positively charged moieties within the polar headgroup region of the membrane.

In summary, aurein 1.2 interacts strongly with lipid mimics of glioma cell membranes, suggesting that the anticancer activity of the peptide involves membrane invasion. These interactions appear to be promoted by a general requirement for a negatively charged membrane rather than by a lipid-receptor or specific lipid requirement. Aurein 1.2 appears to interact with cancer cell membranes *via* a localised cationic region, which we speculate uses the snorkelling mechanisms to promote deeper levels of membrane penetration by the peptide.

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