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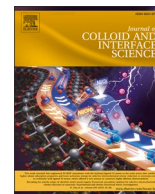
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Determinants of membrane sensitivity to the Mastoparan-1 peptide from *Polybia Paulista*

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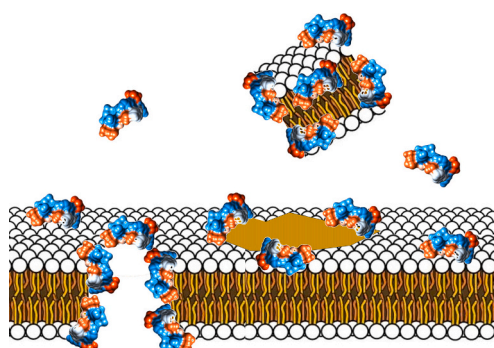
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GRAPHICAL ABSTRACT



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ABSTRACT

The membrane-disrupting peptide Mastoparan-1 (MP-1), derived from the wasp *Polybia paulista* is known to possess antimicrobial properties, and exhibits enhanced activity against a number of cancer cell lines relative to healthy cells. Due to the mechanism of action of MP-1 it is likely that differences in plasma membrane lipid composition arising from cancer associated mutations, such as localisation of phosphatidylserine (PS) lipids to the outer leaflet of the plasma membrane, are involved in driving that enhanced activity. Rapid screening of MP-1 mutants in a combined approach using model membrane and cell-based biological assays, has led to the identification of a number of derivative peptides with enhanced selectivity for cancer-like membrane models and breast cancer cell lines and provided insights into the mechanism of membrane disruption and cell death.

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Notably, the morphology of the membrane perturbations observed by Atomic Force Microscopy (AFM) and activity in cell model systems can change considerably in response to single-point mutations in the MP-1 sequence, indicating a complex structure-activity relationship.

1. Introduction

Mastoparan-1 ('MP-1', [IDWKLLDAKQIL-NH₂]) is an amphiphilic peptide derived from the wasp *Polybia paulista* [1]. MP-1 has been studied for its antimicrobial activity [2], and has exhibited enhanced cytotoxicity against cancer cell lines including prostate and bladder cancer [3,4], without inducing haemolysis [1]. Membrane-disrupting peptides such as MP-1 cause lysis of the cell membrane leading to cell death [5]. The enhanced activity of these peptides against certain cancers is driven, at least in part, by differences in plasma membrane lipid composition in cancer cells resulting from disrupted membrane homeostasis [6,7].

The membrane selectivity of MP-1 has been shown to be enhanced by the presence of cholesterol [8] and phosphatidylserine (PS) lipids, with synergistic enhancement when PS and phosphatidylethanolamine (PE) lipids are both present [9]. Many cancers have increased exposure of PS and PE headgroup lipids on the outer leaflet of the plasma membrane due to reduced activity of the flippase enzymes that ordinarily maintain the location of these lipids on the cytosolic leaflet, as also observed in early apoptosis [7,10,11]. The influence of charged headgroup lipids on MP-1 activity can be rationalised by enhanced electrostatic association between the positively charged MP-1 molecule and a negatively charged membrane. Whereas the distinct behaviour observed in membranes containing multiple phases, or lipids that influence local membrane curvature, suggests that selectivity may also rely on the ability of the peptide to locally influence lipid self-assembly (e.g.: phase behaviour, curvature) [8].

Limited prior studies have investigated a small number of MP-1 mutants, notably with regards to its capacity to induce degranulation in mast cells [12], and membrane disruption in model membrane vesicles [13]. Ye et al. identified key features, such as amidation of the C-terminus and the integrity of the hydrophobic face as critical for its activity in mast cells [12]. While Leite et al. found that replacement of the aspartic acid residue in position D2 with asparagine had a significant effect on the conformation of the peptide in contact with phospholipid membranes and a concurrent diminishing of disruption in vesicle membranes [13]. Both studies point to a more subtle relationship between the amino acid sequence and the membrane activity of MP-1, particularly implicating conformational properties over bulk physicochemical characteristics.

With this in mind, we determined to use a combination of biological and biophysical assays to screen variants of MP-1 and identify those with improved characteristics as candidate anti-cancer agents. By initially producing a subset of single residue substitution variants, we hoped to identify key residues involved in membrane specificity and overall membranolytic potential. Rapid screening of mutants was facilitated by the large unilamellar vesicles (LUV) fluorescent dye leakage assay, which is well-established in this role. Biophysical assays including atomic force microscopy (AFM) of supported lipid bilayers (SLBs) were then undertaken to investigate the mechanism of membrane disruption in model membranes and to identify changes in mechanism. Initially, simplified membrane compositions of DOPC and 80:20 DOPC:DOPS have been employed as model non-cancer and cancer membranes, respectively. Finally, candidate mutants were taken forward into biological assays with 'cancer' and 'non-cancer' model cell lines to assess how improvement in selectivity and potency in model membrane systems relates to behaviour in cell models. While the membrane activity of these peptides is the basis of their activity, model membrane compositions will not precisely match cell plasma membrane or cellular characteristics such as membrane repair processes, which vary by cell type [14]. Further variants of MP-1 can then be produced based on these findings, leading to refined candidate peptides.

2. Results and discussion

2.1. Cell lines demonstrate a wide range of sensitivities to MP-1, but cancer cells are not significantly more sensitive

Our first aim was to determine the sensitivity of a range of mammalian cell lines to MP-1 in an effort to narrow down characteristics that define sensitivity. A range of lines derived from either human cancers or from non-transformed origins (Fig. S1) were treated with different doses of MP-1 and survival was assessed relative to untreated using MTT assays (Fig. 1). MTT assays are a standard assay for quantifying viable cells based on colorimetric detection of mitochondrial dehydrogenase activity that is proportional to viable cell number. IC₅₀ (half maximal inhibitory concentration) values, as shown on Fig. 1, varied almost 6-fold from most sensitive (19 μ M, breast cancer line AU565) to most resistant (111 μ M, non-transformed breast epithelial line MCF-10 A). Cancer cell lines (Fig. 1A) were not significantly more sensitive than non-cancer lines (Fig. 1B) (*t*-Test $p > 0.05$), demonstrating the reported cancer-specificity of MP-1 may be an over-simplification.

Next, we investigated the characteristics of the growth inhibition/cell death induced by MP-1. For further study, we selected 4 representative cell lines, all from the same tissue of origin (breast epithelium) and covering a range of sensitivities (the most sensitive, AU565; the least sensitive, MCF-10 A; two intermediate, MDA-MB-468, BT474 [both cancer lines]). We treated these four lines with their IC₅₀ doses of MP-1, as defined above, and assessed plasma membrane permeability using a pair of DNA-binding fluorescent dyes: propidium iodide, which is not cell-permeable therefore can only access cell nuclei if membrane integrity is lost, and Hoechst 33342, which stains all cell nuclei (Fig. 2A). This assay gives an indirect assessment of plasma membrane integrity, which is especially relevant when working with a membranolytic peptide. All cell lines showed significant increases in proportions of propidium iodide positive cells after treatment, indicating loss of membrane integrity, however the more resistant cells showed smaller increases. Note that these assessments were made using the IC₅₀ doses determined by MTT assays for each cell line, hinting at differences in mechanism of action between relatively sensitive and resistant lines since loss of viability (MTT assays) did not directly correlate with loss of membrane integrity (propidium iodide / Hoechst 33342 assay). In support of this, we demonstrated by patch-clamping that treatment with the same IC₅₀ doses of MP-1 in the relatively sensitive MDA-MB-468 were associated with a loss of plasma membrane electrical potential within 10 min of MP-1 addition, while this was not the case in the resistant MCF-10 A (Fig. 2B). To investigate contributions of apoptotic death to these responses, we performed annexin V/7-AAD staining in these cells, either untreated or treated with IC₇₅ doses of MP-1 (Fig. 2C; Fig. S2 for representative dot plots). This assay allows quantification of apoptosis, which is an active biological response to toxic stimuli that can be distinct from more passive cell lysis mechanisms. The assay quantifies changes in membrane structure that can precede loss of membrane integrity. Higher doses (IC₇₅: AU565 30 μ M, MDA-MB-468 35 μ M, BT474 70 μ M, MCF-10 A 140 μ M) were used for this assay as apoptosis levels were too low for an acceptable signal-to-noise ratio at IC₅₀ doses. The relatively resistant cell lines showed very low levels of MP-1 dependent apoptosis (BT474, MCF-10 A; both <10%), as compared to the more sensitive lines that were up to 70% apoptotic (AU565, MDA-MB-468). None of the cell lines showed MP-1 dependent depolarization of the mitochondrial membrane (Fig. S3). Finally, we assessed the responses to MP-1 of these cell lines in 3D spheroid cultures that may better reflect the cell interactions within tissues, using the fluorescent

dyes as above (Fig. 2D). We found the sensitive cells (AU565, MDA-MB-468) showed substantial cell lysis, while resistant lines demonstrated little lysis in this context.

We concluded that MP-1 acts on different cell types with a range of efficacies, but without cancer-specificity, and that it potentially acts through more than one mechanism. Furthermore, the primary mode of action of MP-1 against sensitive cancer cell lines is consistent with plasma membrane damage, as has previously been reported due to cell uptake of membrane impermeable dyes within minutes of MP-1 addition and visualization of direct damage [4]. Here, we observe loss of membrane potential within 10 min. While apoptosis leads to loss of membrane integrity, this would not be observed until ~12 h [15] and is therefore downstream of the direct membrane damage caused by MP-1.

2.2. Individual residues within MP-1 and membrane constituents define MP-1 activity in membranes

Our next aim was to investigate the functional role of individual residues within MP-1 with respect to membranolytic activity. We employed an *in vitro* assay measuring release of a fluorophore from synthetic membrane vesicles which is suitable for high throughput screening, in order to determine which residues or residue properties are required for MP-1 function and thereby design mutants that might have improved cancer specificity and/or potency.

An initial set of MP-1 mutants were selected with the sequences shown in SI Fig. S4. Of these, twelve comprised an alanine scan to identify key residues, while Lysine or Histidine substitutions were made to examine the role of charge, and a single substitution for a glutamine residue was made to probe the role of peptide-peptide interactions. As MP-1 is known to form an amphipathic helix upon interacting with lipid membranes, the helical wheel structure of the mutant peptide was considered in their design (see SI Fig. S5).

An automated assay was developed to rapidly screen MP-1 mutants based on their ability to disrupt model membranes of various compositions relative to unmodified MP-1. The presence of PS headgroup lipids on the outer leaflet of certain cancers has been implicated as a possible factor in the enhanced activity of MP-1 against these cells [9]. Therefore, parallel leakage assays were performed with vesicles composed of 80:20 DOPC:DOPS and DOPC as simplified models of ‘cancer’ and ‘non-cancer’ membranes (DOPC - 1,2-dioleoyl-sn-glycero-3-phosphocholine, DOPS - 1,2-dioleoyl-sn-glycero-3-phospho-L-serine). Leakage assays followed the well-established Carboxyfluorescein fluorescence dequenching method

as illustrated in Fig. 3A, and the use of a liquid-handling robot enabled medium-throughput screening of peptides.

To aid comparison with cell viability data, peptide-induced leakage was quantified in a manner analogous to calculating IC_{50} values; the concentration of peptide needed to induce a fluorescence value 50% of that obtained in the corresponding positive control experiment (vesicles treated with Triton X-100 detergent) this was termed the ‘50% leakage concentration’, see Fig. 3B. Peptides were assessed against the ‘cancer’ model 80:20 DOPC:DOPS membrane and against the ‘non-cancer’ model DOPC membrane. These data are presented as 50% leakage concentrations in Table S6, and also as potency relative to MP-1 ($Potency = \frac{L_{50} Mutant}{L_{50} MP1}$) in Fig. 3C and D allowing easier visualization of the impact of the mutations. Potency in these two models were also combined to give a measure of relative selectivity for the ‘cancer’ model ($Selectivity_{Peptide} =$

$\left(\frac{Potency \text{ vs. PC:PS}}{Potency \text{ vs. PC}} \right)$, Fig. 3E. These metrics are convenient to assess the key changes in activity resulting from each specific mutation, relative to the MP-1 peptide. While potency against both of the model membrane compositions is correlated (Pearson correlation value: 0.78464, $p \leq 0.0001$), notable ‘selective’ outliers are observed, as shown in Fig. S6, demonstrating that, for these membrane compositions, it is possible to enhance selectivity to PS without sacrificing ‘on-target’ activity.

Some general observations are readily apparent in Figs. 3C–3E, and most clearly in which substitutions cause severe loss of potency. Notably, substitutions that reduce hydrophobicity in the terminal residues (I1, I13, L14), and reduction in hydrophobicity in general, reduce potency against model membranes. This reduction is particularly apparent when a K residue is introduced at these hydrophobic positions. Conversely, replacement of negatively charged D residues with positively charged K residues generally increases potency against both membrane compositions. Interestingly, it might be expected that an increase in positive charge of the peptide enhances selectivity to anionic DOPS-containing membranes, but enhanced potency was also observed against zwitterionic PC-only membranes.

A plot of potency against the 80:20 DOPC:DOPS model vs. selectivity, Fig. 3F, was used to select peptides with improved potency and improved selectivity relative to MP-1 (top right quadrant) as candidates for further study, namely D8K, D2K and L6W. In addition, D2A was selected to take forward into mechanistic studies, due to its comparable potency to MP-1, but reduced selectivity for 80:20 DOPC:DOPS, for possible insights into the mechanism of DOPS selectivity.

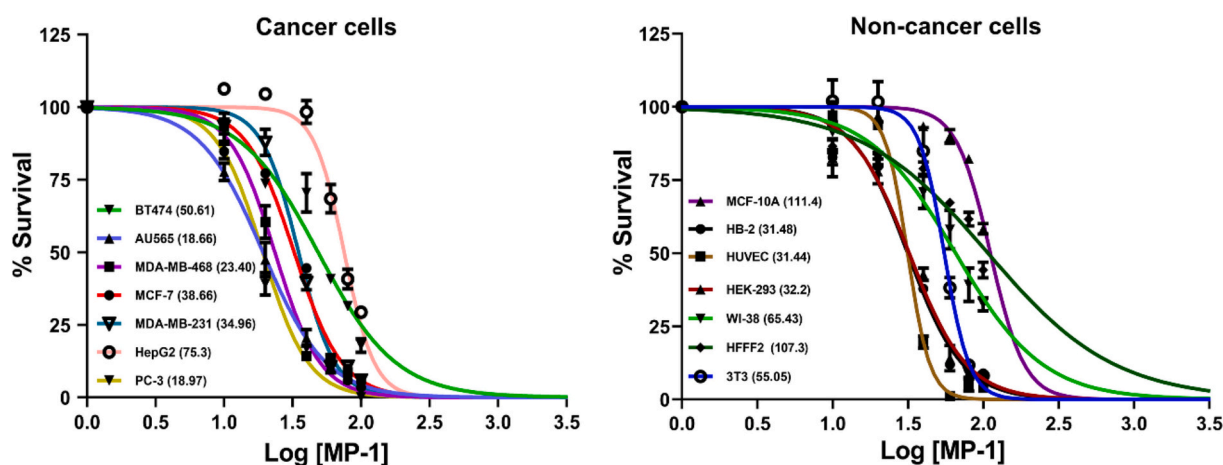


Fig. 1. Cytotoxicity of MP-1 in cancerous and non-cancerous cell lines. MTT assays of cell viability after treatment with 0–200 μ M concentrations of MP-1 in cancer cell lines (A) and non-cancerous cells (B). The IC_{50} values (brackets) are denoted in the figure for respective cell lines in μ M. Data points represent means of three independent biological replicates and error bars represent standard errors. Data points without visible error bars indicate that the datapoint itself obscures the error bars as they are so close to the mean.

2.3. Physicochemical determinants of MP-1 peptide potency and selectivity against model membrane vesicles

Our leakage assay results indicate that bulk physicochemical properties such as charge and hydrophobicity are often not straightforward predictors of peptide activity for MP-1. Similar conclusions have been drawn in previous studies of other membrane-active peptides [16], suggesting that, in general, membrane-disrupting peptides have a complex structure-activity relationship. Nonetheless, we have attempted to find correlations in our data based on categorisation of the physicochemical changes associated with each mutant as shown in

Fig. 4. Being composed of only 14 residues, single mutations result in substantial changes to the overall properties of the peptide. Each plot in Fig. 4 is analogous to Fig. 3F, with axes corresponding to selectivity and potency. Peptides are separated into categories based on qualitative changes to their physicochemical properties, (e.g. the peptide D8A is categorised as a reduction in negative charge, reduction in steric bulk and increase in hydrophobicity). The ‘positive charge’ and ‘negative charge’ categories are assessed independently in these analyses, to reflect the structural differences implied by addition and removal of K and D residues beyond the net physicochemical charge.

As seen in Fig. 4, increasing hydrophobicity generally results in an

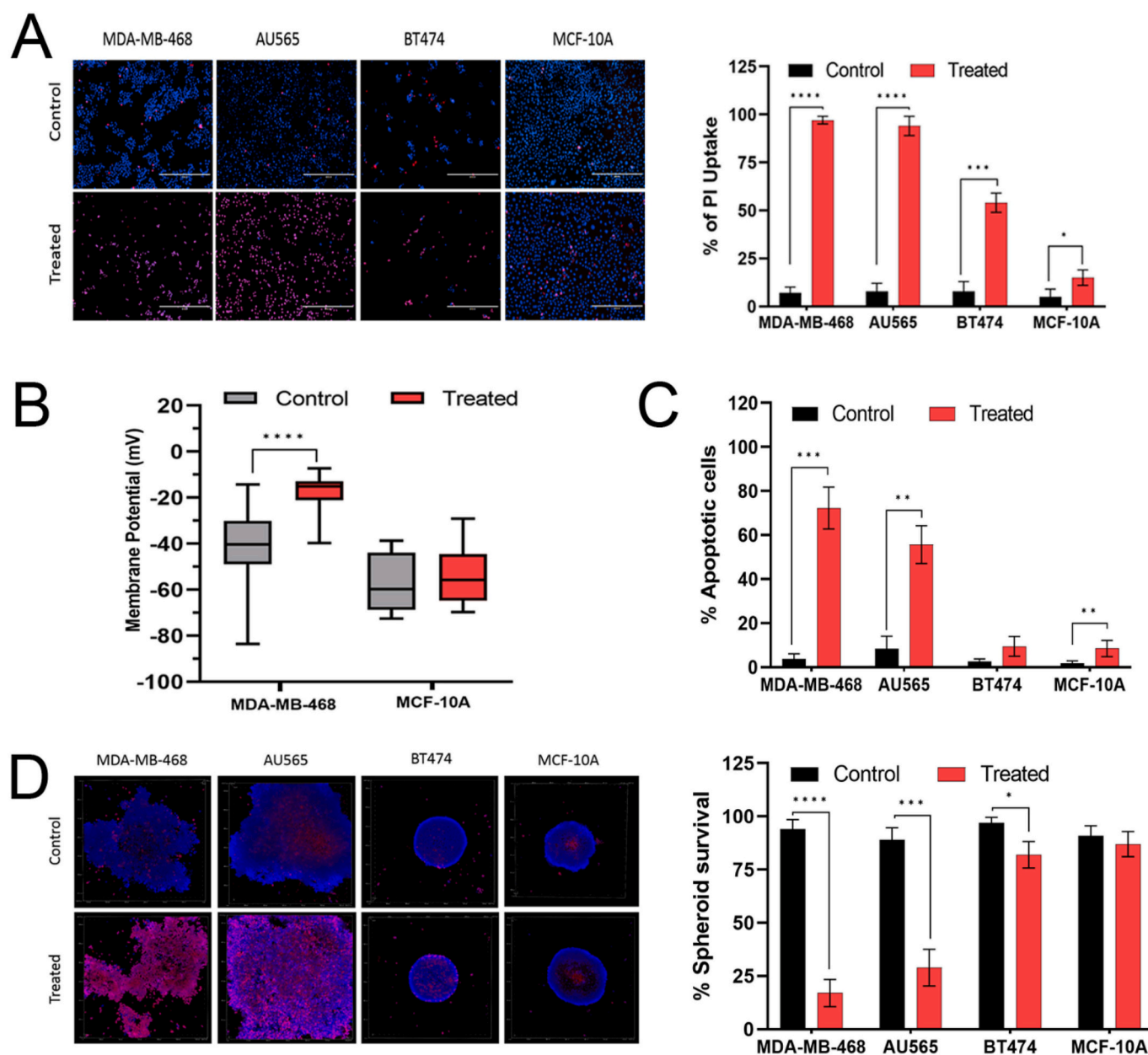


Fig. 2. Actions of MP-1 in breast epithelial cell lines of differing sensitivity. A: Propidium iodide membrane permeability assays, assessed using fluorescence microscopy of cells treated with IC_{50} doses of MP-1 and untreated control cells. (Scale bar 200 μ m). B: Whole cell patch clamp assay, showing membrane potentials for MDA-MB-468 and MCF-10 A cells treated with IC_{50} doses of MP-1 and untreated controls. C: Flow cytometry apoptosis assay. Percentage of apoptotic cells determined by Annexin V-FITC and 7-AAD staining, for cells treated with IC_{75} MP-1 (30 μ M, 35 μ M, 70 μ M and 140 μ M for AU565, MDA-MB-468, BT474 and MCF-10 A respectively) or untreated controls. D: 3D spheroid assay. Spheroids treated with IC_{50} of MP-1 for 24 h followed by staining with Hoechst 33342 and propidium iodide to determine % survival of cells using confocal fluorescence microscopy (Scale bar 200 μ m). For panels A, C and D, data points represent means of three independent biological replicates and error bars represent standard errors. For panel B, the data are readings from a minimum of 10 individual cells, with the boxes showing the interquartile ranges, the whiskers the full ranges, and the bar the medians.

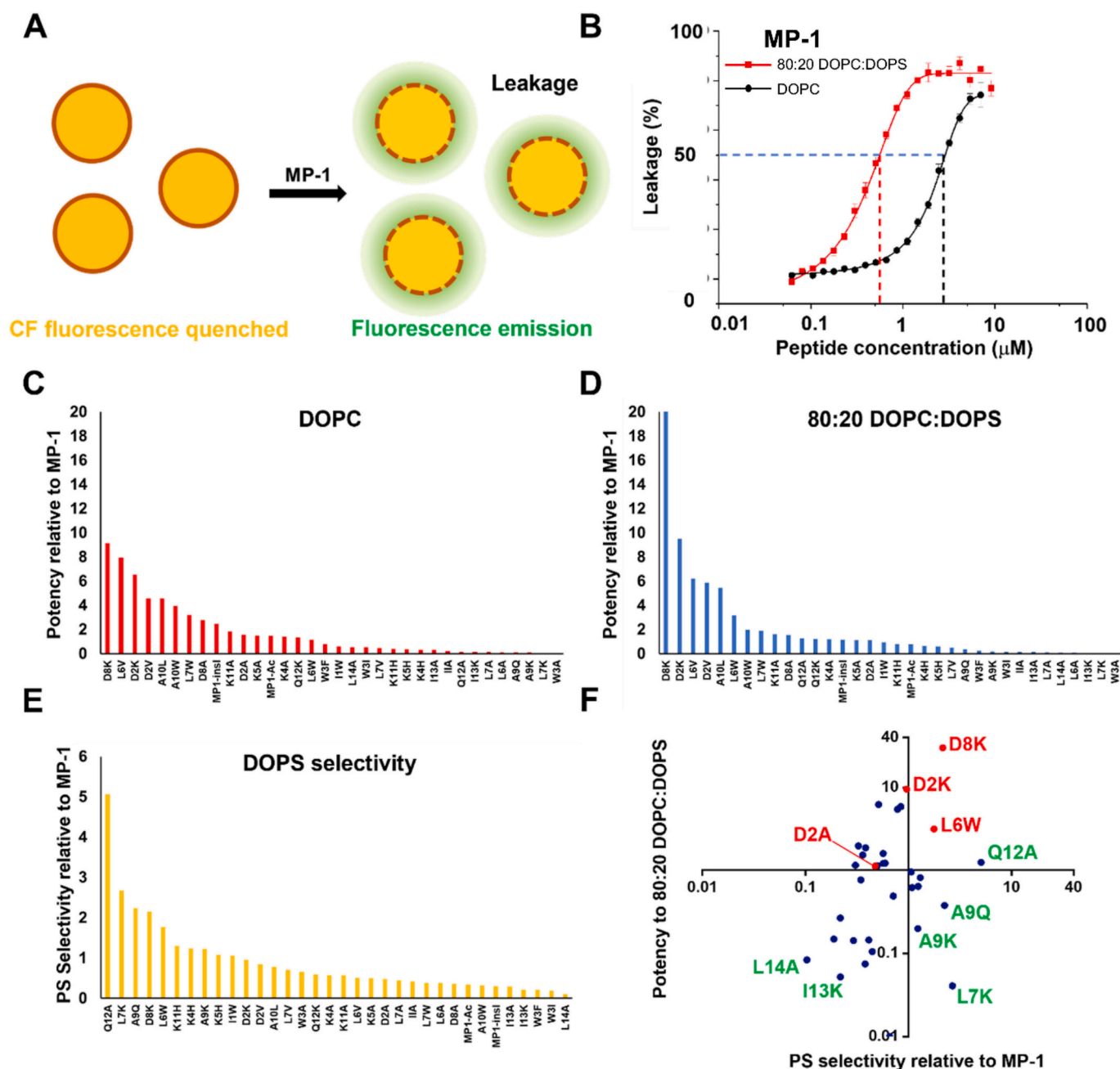


Fig. 3. Dose-response analysis of damage to artificial model membranes induced by MP-1 and derivative peptides. **A** Illustration of the principle of the Carboxyfluorescein (CF) leakage assay. CF is encapsulated in artificial vesicles at a high, self-quenching concentration; peptide-induced leakage of CF causes it to dilute in the surrounding media, leading to an increase in fluorescence emission and, therefore, fluorescence intensity is proportional to the degree of membrane disruption. **B** Example of dose-response leakage data (Black: DOPC LUVs; Red: 80:20 DOPC:DOPS LUVs treated with MP-1): the peptide potency is defined as the concentration of peptide required to induce 50% of the maximal leakage (L_{50}). **C** Potency of mutant peptides vs DOPC LUVs relative to MP-1. **D** Potency of mutant peptides vs 80:20 DOPC:DOPS LUVs relative to MP-1 internal controls, where: $Potency = \frac{L_{50} Mutant}{L_{50} MP1}$. **E** Selectivity for 80:20 PC:PS membranes vs. DOPC membranes for MP-1 mutants, where: $Selectivity_{Peptide} = \left(\frac{Potency_{vs.PC:PS}}{Potency_{vs.PC}} \right)$. **F** Plot of peptide potency vs. 80:20 DOPC:DOPS vesicles against peptide selectivity: candidates for further study are labelled in red; selected additional peptides are labelled in green. MP-1-Ac denotes MP-1 with N-terminal acylation; MP-1-InsI denotes MP-1 with an additional isoleucine amino acid on the N-terminal. For the large screening panels of peptides, dose response curves were collected as a single experiment for most peptides, with a small selection of peptides repeated to ensure reproducibility. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

MP-1 mutants, where: $Selectivity_{Peptide} = \left(\frac{Potency_{vs.PC:PS}}{Potency_{vs.PC}} \right)$. **F** Plot of peptide potency vs. 80:20 DOPC:DOPS vesicles against peptide selectivity: candidates for further study are labelled in red; selected additional peptides are labelled in green. MP-1-Ac denotes MP-1 with N-terminal acylation; MP-1-InsI denotes MP-1 with an additional isoleucine amino acid on the N-terminal. For the large screening panels of peptides, dose response curves were collected as a single experiment for most peptides, with a small selection of peptides repeated to ensure reproducibility. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

increase in potency, and decreased hydrophobicity results in a decrease in potency, however in both instances, most mutants exhibit reduced selectivity for PS membranes.

The greatest increases in potency against PS membranes were seen for mutants with reduced negative charge (replacement of D residues), but specifically those where D was replaced with K, resulting in an

overall increase in charge of +2, likely resulting in increased electrostatically driven adsorption of the peptide to the vesicle membrane in PS containing vesicles. However, of this category, improved selectivity for 80:20 PC:PS over DOPC was only observed in the case of D8K. D2K, while more potent, has comparable PS selectivity to MP-1. Furthermore, reduced potency was observed for three peptides in the increased

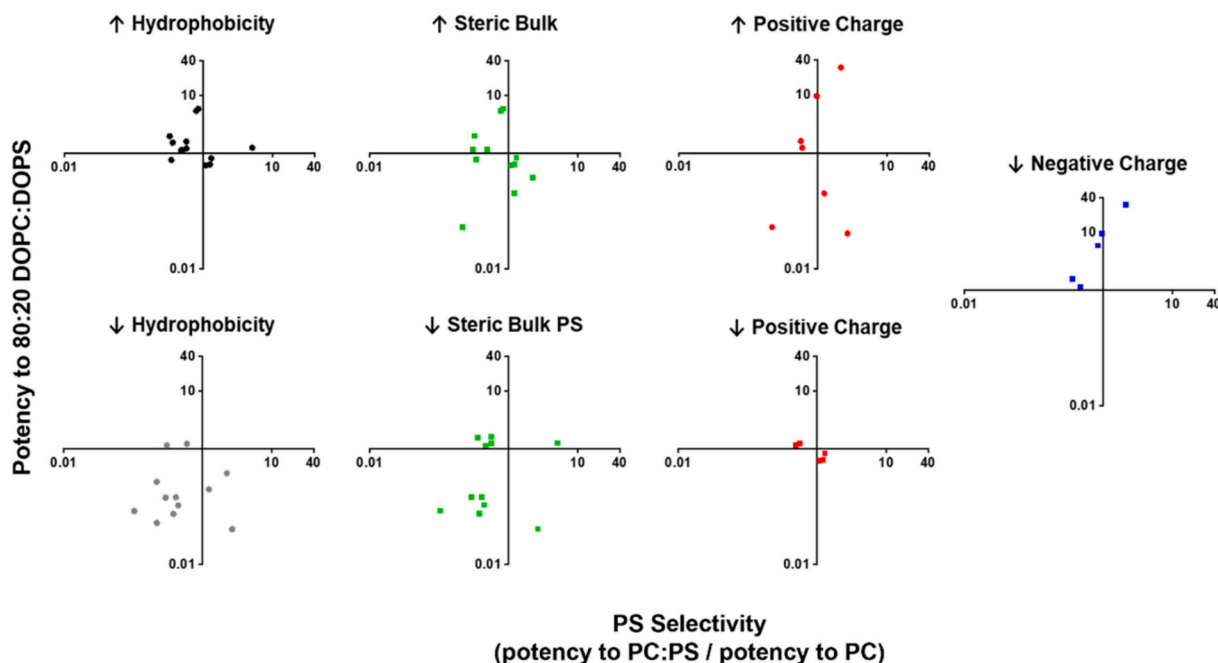


Fig. 4. Analysis of physicochemical characteristics of MP-1-derived peptides that drive the potency and specificity against model membranes. Structural analysis of peptide mutants: plots of potency against 80:20 DOPC:DOPS ($Potency_{PCPS} = \frac{1}{\frac{L_{50PCPS}^{Mutant}}{L_{50PCPS}^{MP1}}}$) vs. selectivity ($Selectivity_{Peptide} = \left(\frac{Potency_{vs.PC:PS}}{Potency_{vs.PC}} \right)$) for mutants characterised by a given category of physicochemical change (increase: ↑, decrease: ↓) relative to MP-1.

positive charge category (I13K, A9K and L7K), with I13K also exhibiting reduced PS selectivity. Particularly in the case of A9K, where the reduction in hydrophobicity is less significant, this suggests that in determining PS-selectivity, hydrophobic effects dominate the effect of increased positive charge. Beyond conferring a negative charge, inclusion of PS lipids in a membrane will alter lipid-packing, peptide insertion and packing in the membrane. Many of these mutations also alter the steric properties of the peptide, both locally and in terms of molecular conformation, with clear implications for insertion and packing.

Clearly, changes to bulk physicochemical properties do not provide a full account of the changes in peptide properties resulting from these substitutions. However, categorising mutants according to whether they have an increased or reduced steric volume relative to MP-1, based on R-group size, also does not result in any clear trend in potency or selectivity, leading us to consider the structural effect of substitutions in each of our candidate mutants on the conformation of the peptide molecule as a whole. Intuitively, such changes at a whole-molecule level could have an effect on insertion/packing that would otherwise seem disproportionate to the steric difference at one substitution site.

To interpret these changes in behaviour, we explored a 3D molecular modelling approach using the PEPFOLD-3 structure prediction tool (<https://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD3/>) to generate predicted conformations of our peptides [17,18]. It should be noted that there are significant caveats in interpreting this data due to the coarse-grain force fields and lack of explicit membrane in the structure prediction tool. PEPFOLD-3 also predicts an α -helical conformation for our peptides in the absence of membrane, while MP-1 is known to have a random coil structure in bulk solution, only undergoing a transition to an α -helix state upon interacting with a lipid bilayer [9]. Despite these limitations, we found the computational analysis to be informative to our thinking and so we include a discussion of this in the supplementary material (Section S7). In the predicted structures, changes in peptide sequence can induce significant alteration in side chain orientations distant from the mutation site. This is suggestive that residue substitutions do not just change the local physicochemical

properties of the peptide but also have global effects on peptide structure that are more difficult to predict a priori. This is consistent with our findings in Fig. 4 that correlations between local changes in amino acid properties and the peptide's membrane perturbation properties are difficult to make. Therefore, this would motivate incorporation of more detailed atomistic simulations in the presence of explicit membrane in the future.

2.4. AFM analysis reveals multiple modes of action in MP-1-like peptides

Taken together, our analysis of membrane permeabilisation data and *in silico* structural analysis of single substitution MP-1 mutants imply a finely balanced, complex relationship between peptide structure, lipid composition and their effect on membrane structural perturbation. AFM studies enable visualization of supported lipid bilayers (SLBs), with nanometre resolution, over time. Due to the relatively low throughput of AFM studies, a subset of 4 peptides plus MP-1 was taken forwards. D2K, D8K, D2A and L6W were selected for the improvements in selectivity and/or potency relative to MP-1 seen in the analysis of vesicle leakage data and, in the case of D2A, due to its selectivity for non-PS membranes.

SLBs of DOPC and 80:20 DOPC:DOPS were prepared and continuously imaged using a FastScan AFM system enabling an effective frame rate of approximately 2 frames/min for a $1.0 \times 1.0 \mu\text{m}^2$ sample area. Representative data for SLBs exposed to each of our selected peptides and MP-1 is presented in Fig. 5.

Peptide solutions were added directly to the supernatant solution to give a final concentration of $1 \mu\text{M}$ initially, although subsequent additions were occasionally made if no membrane perturbation was observed after approximately 10 min. Notably, this concentration of peptide gives comparable peptide-to-lipid ratios to those used in the vesicle leakage assays (see SI section S13). Imaging continued until the membrane perturbations appeared to reach a stable 'end state' or, in some cases, until fouling of the probe tip by non-adherent membrane/peptide material made further imaging impossible. Fig. 5 presents two representative images, from a single run, for each combination of

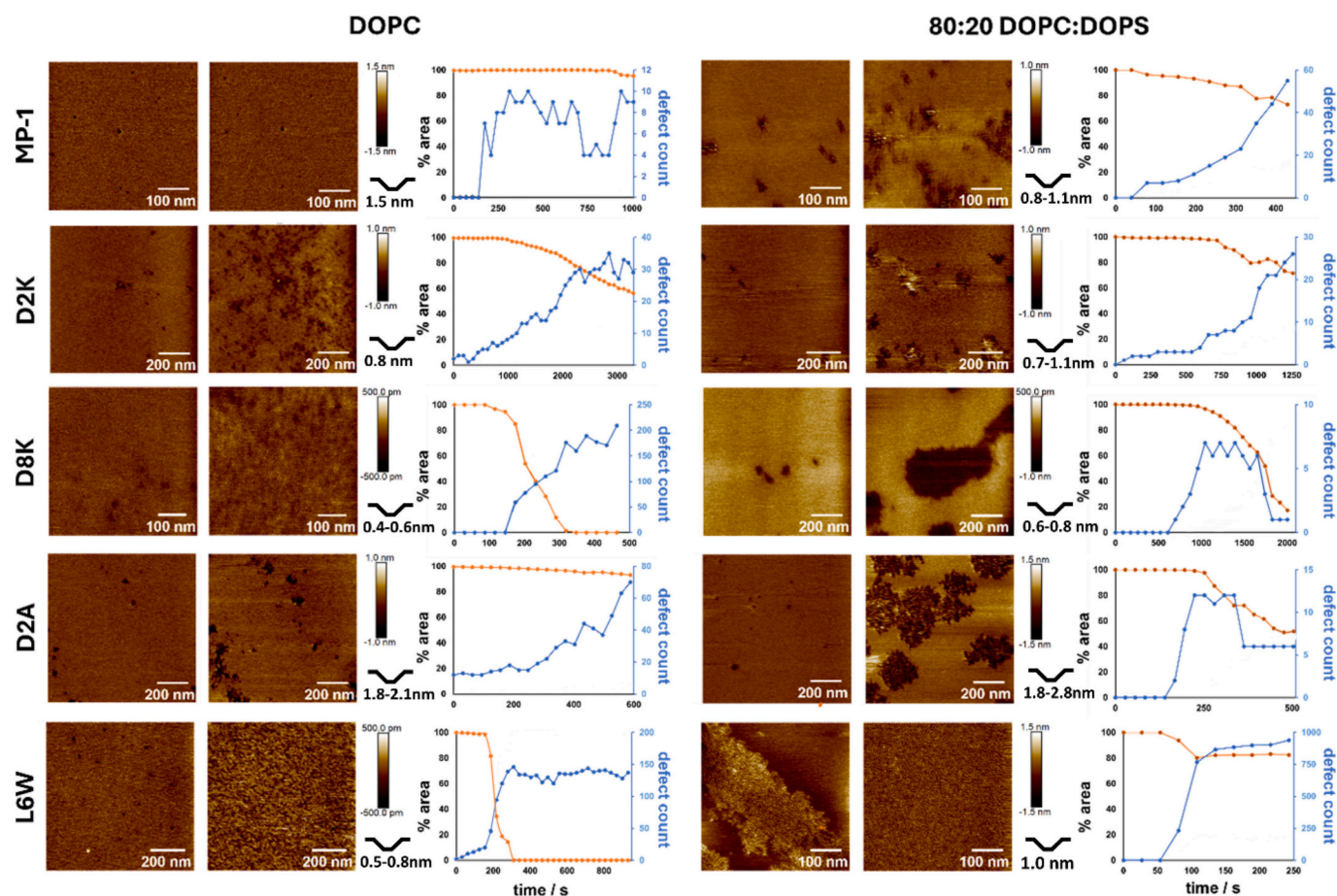


Fig. 5. Nanoscale disruption of model membranes by MP-1-derived peptides. AFM images of DOPC and 80:20 DOPC:DOPS SLBs incubated with 1 μ M peptide introduced at $t = 0$ s. Two images are displayed for each combination of membrane and peptide at different time points to illustrate the progression of peptide-induced membrane disruption. All images in each sequence are from the same area. The corresponding plots record the % area of intact L_d phase membrane (●) and the defect count (●) on the right-hand axis, measured from every image in the sequence. Note that it has not been possible to harmonise all axes across plots due to the time courses varying over an order of magnitude. A minimum of three independent repeat experiments were conducted for each peptide and membrane composition, with similar responses. The plots of defect count/remaining intact membrane area are representative plots from a single time-course experiment (averaging of step/sigma functions tend to blur the time response).

peptide and membrane, with time points selected to best illustrate the progression of the particular mode of membrane disruption observed, the first showing the initial visible signs of membrane changes, and the second from a later time point showing the progression. Corresponding image analysis data for each run is presented with percentage area of intact membrane and the number of distinct ‘defects’ at each time point, with the peptide injection occurring at $t = 0$.

2.5. PS-containing membranes develop larger defects

The most general observation in these studies was the formation of larger defects in PS-containing membranes. This is observed quite consistently, with the exception of peptide L6W, which exhibits very distinct membrane disruption behaviour (discussed below). The generality of this observation suggests that one of the drivers of PS selectivity seen in MP-1 may, in part, be more severe membrane disruption. This is possibly due to enhanced electrostatic binding of the peptides to the membrane, where 20 mol% PS lipids have previously been shown to enhance membrane partitioning of MP-1 peptides by a factor of 7–8 compared to comparable zwitterionic lipid membranes [9].

2.6. Distinct modes of action are characterised by the morphology and kinetics of pore nucleation and growth

In Fig. 6 we present diagrammatical representations for the common modes of progression of membrane disruption. Many of the membrane disrupting effects we observe using AFM in this study have been described previously and interpreted [19]. DOPC membranes treated with MP-1 exhibit a unique poration not observed for other peptide-membrane combinations in this study. Discrete uniform pores of a highly regular diameter of approximately 13 nm are observed to form, with many appearing to be transient, persisting for approximately 2 frames of imaging time (~ 40 s), although a smaller number of more stable pores were also observed. The ‘pores’ are around 1.5 nm deep, so about a monolayer depth (although their small diameter could mean the AFM probe cannot penetrate far enough to image the full depth) and thus cannot be defined as trans-membrane pores. The defects observed in this system did not undergo any growth or morphological change. We classify this mode of action as ‘small transient defects’ (Fig. 5A).

Contrasting this with MP-1 vs. 80:20 PC:PS membranes, we see ‘progressive poration’ (Fig. 5B), where defects of varied diameter and morphology nucleate and undergo growth, with continuing nucleation eventually leading to a membrane with a high percentage of the observed area being composed of ‘disrupted phase’, that being the

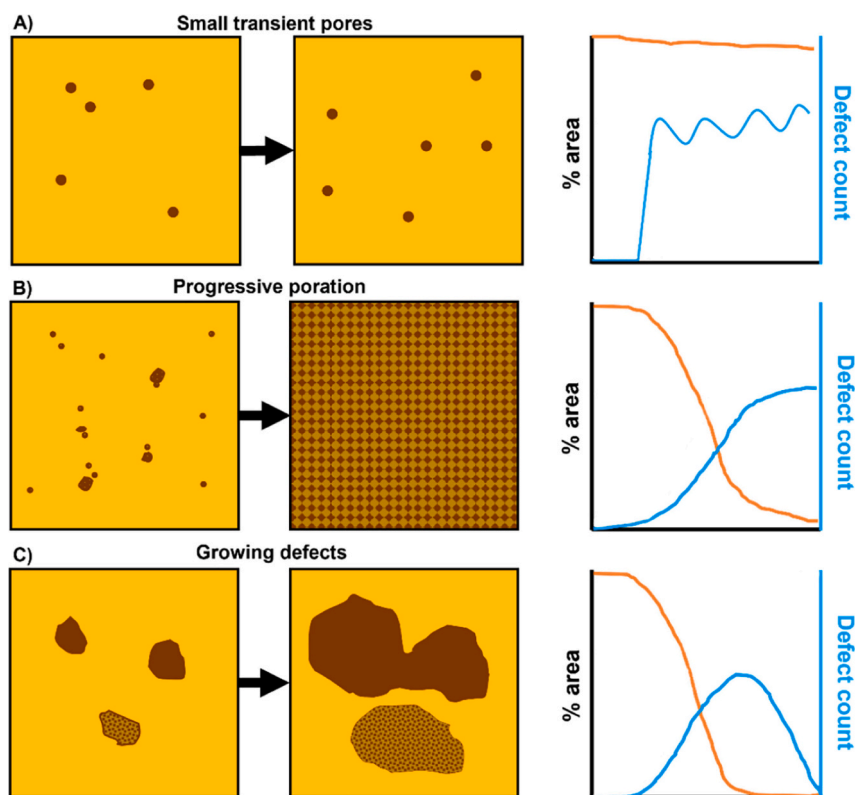


Fig. 6. Diagrammatic representations of mechanisms of peptide action on SLBs. **A)** Small transient pores of uniform size (≈ 13 nm). **B)** Progressive formation of small stable pores leading to widely disrupted surface. **C)** Widespread disruption resulting from the growth and merger of large defects.

membrane type which comprises densely-packed small defects. Again, the shallow depth of these larger defects indicates these are not trans-membrane pores, but rather regions of membrane thinning or collapse. This patchy disruption is observed on SLBs more often than complete poration, but it is not understood why [19]. The leakage assay data on free vesicles indicates trans-membrane pores *are* forming, so this is likely to be a factor of supported lipid bilayers. It could be explained by the directly-supported leaflet having more order, and being resistant to disruption, both by its stabilising influence on membrane structure and resistance to perturbation, but also restricting the ability of the peptide to achieve its preferred orientation, perhaps with the peptide protruding from the membrane surface to some degree.

This second type of 'progressive poration' is the one most commonly observed, where small defects gradually accumulate in number, leading to a membrane increasingly disrupted as time progresses. This applies to D2K, D8K, and L6W vs. DOPC membranes. The third general mode of action was observed with MP-1, D2K, D8K and D2A vs PS containing membranes, as well as D2A on DOPC. These appear as 'growing defects' (Fig. 5C), characterised by fewer pore nucleation events but with rapid growth, particularly in the case of D8K. This process is most likely to reach an end point when the membrane in the imaging area is fully disrupted. The defect count in this scenario thus tends to reduce over time as previously separate defects merge.

The defect boundary of these larger features appears rough, and upon closer examination have a distinctive fractal-like pattern, although at the scale presented this is difficult to see. A detail of D2K on PS lipid highlights the flower-like growth pattern indicative of a fractal growth pattern (Fig. S10B). This growth mechanism has been studied previously [20], and explained by peptides binding to the membrane surface then diffusing, gathering until they reach a critical local concentration and inserting into the membrane (note that we cannot resolve individual peptides as they are too mobile at the time resolution of a single AFM image). A defect is nucleated at this point, but growth of the defect is

governed by the 2-dimensional diffusion rate of peptides to the exposed membrane edge. At this boundary, lipids are micellised when the peptide concentration is high enough and the membrane is exfoliated by a detergent-like action. The balance between fractal growth vs a smooth and circular growth front is governed by the rate of 2D peptide diffusion, the balance between peptide adsorption at a defect boundary versus the intact membrane, and the concentration required to micellise the membrane [20]. All of our membranes exhibiting this exfoliation and micellisation mechanism appear visually to have a similar fractal dimension (although this has not been quantified), indicating that the partitioning and diffusion rates of our MP-1 variants are similar. Micellisation is also indicated by occasional vesicles observed momentarily associated with the defect edge and tip-clogging. Our illustration in Fig. 6C also depicts two different types of defect area / disrupted phase. Some disrupted phases possess a relatively smooth, uniform surface, such as in D8K against 80:20 DOPC:DOPS, while others, such as D2K possess height irregularities. Imaging of the latter type was often subject to fouling of the AFM probe tip, suggesting that lipid or lipid-peptide aggregate material is lost from the surface. This is indicative of a carpet mechanism, where the peptide adsorbs to the membrane surface.

The action of L6W against DOPC membranes follows the most commonly observed progressive poration (Fig. 6B) process. This continues until the whole membrane is peppered with small defects approximately 13 nm in diameter, with a depth 0.5–0.8 nm (Fig. S10A). However, in some instances, a filamentous structure was observed moving across the surface. Although the structure cannot be definitively identified from these images, its appearance informs our interpretation of anomalous behaviour of L6W against PS-containing membranes. In these experiments, no membrane disruption or poration was observed over a long period of imaging. Due to this lack of activity, a subsequent aliquot of L6W was added to the sample to give a peptide concentration of 10 μ M. After several minutes, the appearance of plaque-like height

anomalies was observed. These features evolve rapidly into an ordered network which rapidly fills the observed area. This structure can somewhat resemble the end state of the progressive poration mode of action due to the dense pattern of roughly circular depressions with a regular diameter of ~ 6 nm. However, the progression over three frames (Fig. S10C-E) indicates that this is a surface carpet of adsorbed peptide which is forming an interlocking network on the membrane surface. It sits as a layer 1.3 nm deep on the membrane, and the regular periodicity of the “cells” is 7.5 nm (slightly larger than the individual pore diameter, Fig. S10F-G). Taken together with the observed filamentous structures induced by L6W on DOPC membranes, we interpret this structure as a network of peptide or peptide/lipid filamentous structures rather than an expanding area of dense poration. Alternatively, they could be locally-curved membrane; induction of membrane curvature being a key part of the action of MP-1 and other membrane disrupting peptides.

The addition of a second Tryptophan residue in L6W, is predicted in our molecular modelling (Section S7) to result in a dominant conformation where the aromatic moieties of the W6 and W3 Tryptophan side chains are arranged in a stacked conformation that implies a π -stacking interaction. Such interactions, along with Π -cation interactions, are known to play a role in intermolecular interactions of peptides [21,22], and W residues have been shown to be of critical functional importance for a number of membrane-disrupting peptides [23,24].

2.7. Relating mode of action to leakage assay data

There are a few general observations in our AFM studies, for which it is tempting to infer a relationship between the apparent mode of action and the performance of these peptides in our leakage assays.

The general observation of larger defects in PS-containing membranes is consistent with the increased potency of MP-1 (and most of its mutant derivatives) in leakage assays against this membrane composition.

In the DOPC experiments, the highly potent mutants D8K and D2K are notable for the very high density of defects they are able to induce compared to MP-1. A similar end-state is observed in DOPC vs. L6W, although this must be interpreted more cautiously. This contrasts with D2A vs. DOPC, where nucleation of defects progressed much more slowly, and, uniquely for peptides against DOPC membranes, appears to possess a mechanistic character combining ‘progressive poration’ and ‘growing defects’ (Fig. 6B/C), with continuous nucleation of new defects at a slow rate, but those defects show significant growth over time. Its end state more closely resembles those observed for MP-1 and D8K against PS-containing membranes. However, D2A is unique in the peptides taken into AFM studies in that it has a reduced PS-selectivity relative to MP-1 (due to enhanced activity against DOPC). This may therefore reflect the convergence in membrane disruption processes we observe in our AFM studies and the ‘growing defects’ mode of action observed against SLBs may represent a more disruptive mechanism in a vesicle membrane.

D8K, which is the most potent peptide in our leakage studies, exhibits exceptional potency against PS-containing membranes, where it causes rapid growth of defects. In the supported lipid bilayer, this process progresses to an end state where the majority of the observed membrane is of the ‘disrupted phase’. A high proportion of the membrane has been exfoliated and thinned, with lipids lost to micellization, leaving behind a roughened surface, the most extreme membrane disruption we observed. The activity of D2K and D2A follows a similar but less extreme pattern on PS-containing membrane, with fewer growing pores rather than an increasing density of small pores. D2K's action against PS-containing membranes is comparable to MP-1 (growing defects) but defects were significantly smaller and slower growing than those of D8K and D2A. All PS-containing membranes exhibit the same fractal growth pattern at the domain boundary, revealing a process of diffusion limited peptide attack at the exposed defect boundary and removal of lipid in a detergent-like mechanism. The exception to this is D2A, where we also observe growing defects.

The anomalous activity of L6W was not anticipated based on leakage assay data. It was selected for further study purely based on its enhanced potency and PS-selectivity relative to MP-1. That L6W performs well against DOPC membranes is not surprising given the rapid ‘progressive poration’ process. The structures observed for L6W in our PS-membrane experiments is harder to interpret as it is not clear if they contain defects comparable to those in our other experiments. The high potency of L6W in the leakage assays might be used to inform our interpretation of these structures as representative of a membrane perturbation sufficient to induce leakage in an unsupported vesicle membrane. We observed mobile linear structures on the DOPC membrane, and a dense 1.3 nm-deep carpet of peptide forming a fine mesh structure with 7.5 nm periodicity at higher L6W concentration on PS-containing membrane. It is possible that the supported lipid membrane is stable enough to resist these overlying structures which presumably have inserted into the membrane. In contrast, the effect of these structure on an unsupported vesicle membrane could be extreme, tearing or inducing strong curvature.

2.8. Cytotoxicity of MP-1-derivatives mostly correlates with model membrane studies but with nuance that indicates broader cell-specific properties are also important

In Fig. 1, we selected a panel of cell lines to represent relatively ‘resistant’ cells (BT474, MCF-10 A) and relatively ‘sensitive’ cells (MDA-MB-468, AU565). One goal of selecting cell lines with different extremes of MP-1 responsiveness was to assess whether these differential responses would be maintained with key mutant peptides, and whether cells would reproduce the trends seen with synthetic membranes (Figs. 3 and 5). Selected mutants identified in leakage assay screening (Fig. 3) were tested against these cell lines to assess their activity in the more complex cellular context. IC_{50} values for D2K, D8K, D2A and L6W were determined in cell viability assays in all four cell lines and are presented alongside the values for MP-1 in Fig. 7A.

D2A has greatly reduced IC_{50} values for the ‘resistant’ cell lines (blue and grey bars in Fig. 7A) and much smaller reductions in IC_{50} values in ‘sensitive’ cell lines (blue and red bars). In our prior leakage assays (Fig. 3), D2A was selected for further study because of its reduced PS-selectivity, driven by enhanced potency against DOPC membranes and negligible improvement in potency against DOPC:DOPS 80:20. This appears to be reflected directly in the increased potency (reduction in IC_{50} values) in resistant cell lines seen, with similarly negligible improvement in the sensitive lines (Fig. 7A), although this differential in cell lines is even more striking. The AFM mechanistic studies detailed in Fig. 5, did indicate a divergence in mechanistic behaviour for D2A where behaviour against DOPC membranes more closely resembled activity against PS-membranes by other peptides.

The observed IC_{50} values for D2K and D8K are correlated with what we might anticipate based on our leakage data, an overall reduction or similar IC_{50} values to MP-1 for most cell lines. D8K is the most potent peptide against MDA-MB-468 cell lines but its increase in potency against the MP-1-resistant MCF-10 A cell lines is more significant, making this peptide less selective than MP-1. This reduction in IC_{50} for ‘resistant’ cell lines is somewhat greater than we might have expected based on leakage data, however due to the complexity of cell models, both in terms of membrane composition and other non-membrane factors influencing survivability, a very close correlation should not be expected. Importantly, however, for D2K we observe both a decrease in IC_{50} against the sensitive, cancer cell line MDA-MB-468 and increase against the resistant, non-cancer cell line MCF-10 A. Overall, this makes D2K more selective than MP-1 for these two cell lines, which makes it an interesting candidate peptide to progress alongside MP-1 in future work. It should be noted that this increased selectivity is nuanced, as D2K is less potent (and therefore less selective) than MP-1 against the other two cancer cell models: AU-565 and BT474. Therefore, as could be expected, this improved potency and selectivity is dependent on phenotypic properties of specific cancer cell lines.

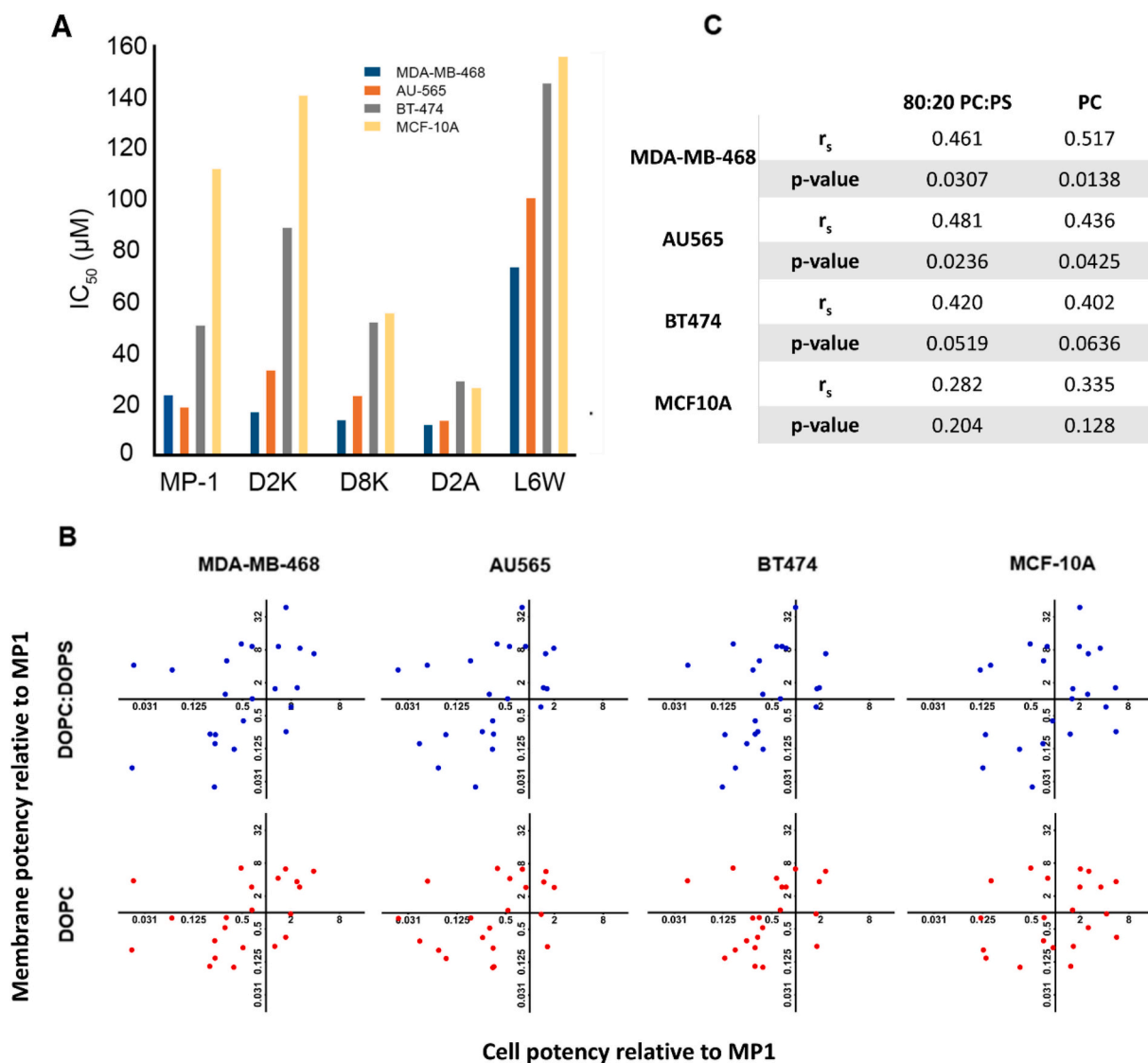


Fig. 7. Correlation between cell viability and model membrane leakage assays. A: IC₅₀ values for selected peptide mutants and cell lines. B: Scatter plots of potency values ($Potency = \frac{IC_{50}(MP1)}{IC_{50}(Mutant)}$), for all studied peptide mutants versus each model membrane (y-axes, DOPC: ●, 80:20 DOPC:DOPS: ●) and against the different cell lines ($IC_{50}(MP1)/IC_{50}(mutant)$) (x-axis, Log₂ scale). C: Spearman analysis of data in 7B. r_s – Spearman correlation coefficient.

The behaviour of L6W is perhaps the most poorly aligned with our leakage assay results, being a potent and PS-selective peptide in those experiments and displaying an unusual combination of changes to IC₅₀ values, being less potent against both ‘sensitive’ cell lines and BT474 cells, while being more potent against MCF-10 A cells. AFM studies with L6W indicate that it has a highly divergent mechanism contrasting with all other peptides studied, therefore we might conclude that this mechanism of activity is less effective in cell membranes. We postulated that the structures, indicating peptide self-assembly on the SLB, could be highly damaging to a free-floating vesicle in the leakage assay. However, a real cell membrane could behave more like a SLB, being supported by the cytoskeleton, and thereby resistant to the particular mode of action of L6W.

With the partial exception of L6W, the overall potency of the selected peptides is consistently reflected in the model membrane leakage and cell viability assays. Further refinement of the membrane models, with better compositional and structural alignment to cell plasma membranes should enable us to extract more detail relating peptide activity and membrane composition, to better understand the membrane properties that are driving selectivity in sensitive and resistant cells. The potential

complicating factor of other cellular structures and processes, such as membrane repair activity, might be investigated with a bioinformatics approach to further enrich the findings.

2.9. Closer biomimetic membrane models are required to strengthen the correlation between model membrane disruption and cell-line cytotoxicity

In Fig. 7B, scatter plots were generated for all peptide mutants for which cell viability assays were performed (see also values given in Fig. S11). The relative potency of peptide mutants in model membranes is compared with their relative potency against each cell line to look for those combinations of model membrane and cell lines where the activity of the peptides is best correlated. A Spearman's correlation test is presented in Fig. 7C. The r_s (Spearman correlation coefficient) values obtained generally indicate moderate correlations for the two sensitive cell lines (MDA-MB-468 and AU-565) and also the BT474 cell line, which is intermediate between the sensitive cells and the highly resistant MCF-10 A cells. In comparison, there is only a weak correlation for the resistant MCF-10 A cells. Furthermore, the correlations for the two sensitive cell lines are statistically significant ($p < 0.05$), with the

intermediate BT474 cells being close to this threshold of significance, but the correlation for the highly resistant MCF-10 A cells is not found to be significant. Taken together, this indicates that our model membranes are more reliable for determining the effect of these peptides against cell lines sensitive to MP-1 but do not capture the resistance mechanisms of cells that exhibit low sensitivity to the MP-1 peptide. Therefore, the model membrane screening approach is more attuned to identifying novel peptides of increased potency, but less reliable for predicting peptides that might have improved selectivity for sensitive over resistant cells.

The stronger correlation between cell and leakage studies for sensitive cell lines may indicate a better correlation in the membrane composition or physical properties between those categories, where the model membranes in this study contain unsaturated lipids in the L_{α} phase, which are loosely-packed in the membrane compared to vesicles containing saturated phospholipids and/or cholesterol. Indeed, tightly packed regions of membrane may be more important for modelling the mechanisms of cell resistance to MP-1 and its derivatives, where much poorer correlations are observed for resistant cell lines. However, other native membrane structural features such as membrane asymmetry or electrochemical membrane potentials may also play an important role. Furthermore, active cellular protection/repair mechanisms are likely less prevalent in sensitive cell lines, making their behaviour easier to model with passive model membranes. Model membranes with improved composition or physical properties, more similar to native membranes, could provide more accurate screening, within the limitations of a model lacking cellular processes such as membrane repair or structures such as the glycocalyx. However, this identifies an opportunity to add further complementary biological assays downstream of model membrane screening and add value to the complete data set. Unfortunately, essentially no data are available regarding the relative plasma membrane compositions of the representative cell lines used in our studies, therefore we are not able to test associations between MP-1 sensitivities and specific membrane lipids. Overall, these analyses support the conclusion that the model membrane screening approach is a valid methodology for identifying candidate peptides, particularly for improved potency against sensitive cell lines, but requires refinement in terms of model membrane composition and to control for other cellular properties, which may have a stronger influence in the outcomes for cells that have a high resistance to the activity of MP-1.

3. Conclusions

Development of cancer therapeutics derived from membrane disrupting peptides is currently held back by insufficient understanding of their mechanism of action. We have therefore developed a hybrid approach to rapidly screen candidate peptides based on MP-1. Our hypothesis was that, by screening a comparatively small panel of mutant peptides with rational design of candidates based on the importance of amphiphilicity and cationic charge in the membrane perturbation interaction, we could identify a more selective and potent variant of MP-1. We find substantial variation in the membrane activity of MP-1 mutants, allowing us to take a selection forward to biological assays of activity and biophysical studies to develop our understanding of the processes of membrane disruption. These findings can then be fed back to refine our assays through a design-test-learn cycle, e.g. through better biomimetic model membrane compositions.

MP-1 is capable of disrupting cellular membranes leading to cell lysis, with the extent of activity varying significantly across the range of cell lines studied. There was not a simple selectivity for cancer-derived cell lines, but sensitive and resistant categories were identified as a basis for our combined biological and biophysical screening approach. Differences in membrane permeabilization between these categories suggests a different mechanism of MP-1 activity or cellular response.

Rapid screening of mutants in our LUV leakage assays and subsequent cell viability assays indicate that the membrane disrupting

activity of MP-1 is finely balanced, with single residue mutations able to greatly enhance or disrupt its overall potency and selectivity for particular model membrane compositions or cell types. Notable findings include the essential nature of the I1, I13 and L14 terminal hydrophobic residues and D to K replacement boosting activity against both membrane compositions. A significant correlation between peptide hydrophobicity and potency against model membranes was also observed, but no strong indication as to the structural basis of PS-selectivity was apparent in these results. This led us to investigate the role of molecular conformation by computational modelling. Using the PEPFOLD-3 structure prediction software, we observed how single point mutations could radically alter the predicted conformation of the molecule and the amino acid side chains in particular, at positions remote from the mutated site due to changes in the global energy balance and interactions within the peptide (Section S7). Mutants that preserved a MP-1-like geometry on their hydrophobic face were found to be more potent. We hypothesise that these differences in the most favourable conformations might drive membrane selectivity through preferential insertion and packing in membranes of particular lipid compositions. However, these computational observations must be treated tentatively due to the coarse-grained force field and lack of explicit membrane in the PEPFOLD-3 structural analysis. Future work would benefit from atomistic modelling with explicit membrane to increase the confidence in computational structure predictions. Computational predictions could be further verified experimentally by techniques, such as NMR and EPR, that are sensitive to conformational changes of the peptides in the membrane and the initial perturbations to membrane structure upon peptide binding.

The mode of action for disruption in model membranes was also found to vary considerably with these single point mutations, as observed directly by AFM. Transient and highly uniform pores were observed in DOPC SLBs exposed to MP-1, while D8K induced rapidly expanding regions of disruption in membranes of the same composition. Generating more, or larger size, defects correlated well with potency observed in leakage assays. This suggests mechanistic differences can drive changes in activity. Leakage assay data on our selected mutants were found to correlate with corresponding cell viability assays, validating the methodology. This correlation is moderate and statistically significant for cell lines that are sensitive to the action of MP-1, but only moderate-to-weak correlation for resistant cell lines with no or weak evidence to reject the null hypothesis of there being no correlation. This implies our current model membrane screening approach is valid for identifying peptides with improved potency, but is unable to reliably predict whether a peptide may have improved selectivity for sensitive cells compared to resistant cell lines, as these assays are poor predictors for the peptide's effect on the latter. This reinforces the need for refinement of the model membrane systems and supplementation with other methods to investigate the interaction of these peptides with other cellular components and processes (e.g. glycocalyx, lipid rafts, membrane potential).

We have found that MP-1 derivatives are able to induce disruption in model membranes and cell lysis in a manner that can be highly selective for particular cell types or membrane compositions and is highly sensitive to single mutations in its amino acid sequence. Such mutations are capable of radically altering potency, selectivity and mechanism of membrane disruption, but the effect of a given mutation cannot be easily predicted, with changes to molecular conformation likely playing a role.

The findings presented here provide a basis for further refinement of our approach and indicate that rational mutation of MP-1 to enhance characteristics desirable in a targeted cancer therapeutic is achievable. For example, the D2K mutant peptide showed enhanced potency and selectivity compared to MP-1 when comparing activity against the sensitive MDA-MB-468 (a triple negative breast cancer cell line derived from a metastatic tumor) and the resistant MCF-10 A (a non-malignant breast epithelial cell line), and is therefore a good candidate for further investigation alongside MP-1.

4. Materials and methods

4.1. Materials

Peptides were synthesised by Bio-Synthesis Inc., Lewisville, TX, USA to >95% purity with counterion exchange to HCl. Peptides were lyophilised and shipped on dry ice. Quality control was conducted by HPLC and mass spectrometry by Bio-Synthesis Inc., and we checked selected peptides for purity by analytical HPLC (see SI Fig. S12). All lipids: DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) and DOPS (1,2-dioleoyl-sn-glycero-3-phospho-L-serine (sodium salt)) were obtained from Avanti Polar Lipids Inc. (Alabaster, AL, USA). NaCl, HEPES buffer, were all obtained from Sigma-Aldrich Company Ltd. (UK).

5. Methods

5.1. Production of large unilamellar vesicles (LUVs)

A lipid solution (1 ml, 15 mM) of the desired composition was dried under a stream of nitrogen, to a thin film, further dried under vacuum and rehydrated in 5(6)-Carboxyfluorescein (CF) (Sigma-Aldrich, UK) solution (120 mM in 10 mM HEPES pH 7.4) followed by 5 freeze-thaw cycles in liquid N₂. The suspension was then extruded through a 400 nm polycarbonate membrane using an Avestin LF-1 extruders (biopharma process systems, UK) and unencapsulated CF was removed by size exclusion chromatography using PD-10 gel filtration columns (GE Healthcare Bio-Sciences AB, Uppsala, SE). Final lipid concentration was determined by colourimetric total phosphorus assay [25,26].

5.2. CF leakage assays

A custom program was written for a Hamilton Microlab Star M liquid handling robot (Hamilton robotics Ltd.) wherein a serial dilution of peptide solution into HEPES (pH 7.4 or 6.5, 10 mM) buffer was performed, followed by a fixed concentration of CF-loaded vesicles. Negative and positive controls were established by addition of vesicles to peptide-free buffer and buffer containing 0.6 mM Triton X-100 (Sigma-Aldrich, UK). The final assay plate (384-well black OptiPlate, PerkinElmer LAS (UK) Ltd) was transferred to a Perkin Elmer Envision plate reader where the CF fluorescence intensity (λ_{ex} : 495 nm / λ_{em} : 517 nm). Peptide stock solutions were prepared fresh from lyophilised powder dissolved in the desired buffer and concentration was determined by tryptophan absorbance using a Nanodrop spectrophotometer, measuring the characteristic absorbance at 280 nm.

Serial dilution of the peptide was used to achieve solutions with final peptide concentrations of between 0.01 and 10 μM , to which were added a fixed aliquot of vesicles, to a final concentration of 20 μM , plus a control where no peptide is added to the vesicles and a '100% release' control, where an aliquot of Triton-X100 is added instead of peptide to a final concentration of 0.06 M. CF fluorescence was quantified after incubation for 5 min. Each experiment was carried out in triplicate. Release was calculated as a percentage of the 100% release value after subtraction of the background fluorescence of the no peptide control. 50% leakage values were calculated by fitting the log plot of leakage % against peptide concentration using a sigmoidal dose-response fit.

5.3. Molecular modelling

Peptide 3D structures were generated using the PEP-FOLD3 program hosted at <https://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD3/>¹⁷. This program performs de novo structure prediction of short chain peptides. This prediction was then used to generate hydrophobicity map space-filling models to assess the geometry of MP-1 and its derivatives in the USCF Chimera software [18].

5.4. Preparation of Supported Lipid Bilayers (SLBs)

Dried lipid films of the desired composition were rehydrated in MilliQ water to give 1 ml of solution at a lipid concentration of 0.5 mg/ml, suspension was achieved by thorough vortex mixing. The resulting suspension was subjected to 5 min of sonication using a probe-type sonicator, or until the solution appeared less turbid. The resulting suspension (75 ml) was applied to freshly cleaved mica substrates and incubated at 40 °C in a petri dish containing a small piece of wet tissue to prevent drying out. After 1 h, 25 ml of a 20 mM MgCl₂ (Sigma-Aldrich, UK) solution was added to each substrate and incubated for a further 30 min at room temperature. Immediately prior to use, MilliQ water (30 ml) was added to the substrate, followed by 8 washings of the surface by sequential addition and removal of further 30 ml aliquots of MilliQ water using a pipette. The final volume of supernatant solution during imaging was approximately 200 ml.

5.5. Atomic force microscopy (AFM) of supported lipid bilayers (SLBs)

AFM experiments were conducted using a Bruker dimension FastScan instrument and FastScan-D probes operating in tapping mode. Typically, a 1 $\mu\text{m} \times 1 \mu\text{m}$ area was imaged continuously throughout the experiment. After imaging the membrane for some time to observe that it has a stable morphology, an aliquot of peptide solution of approximately 10 ml volume is added directly to the supernatant solution during imaging, to give a final peptide concentration in the solution of 1 μM . Imaging was continued until the development of peptide-induced changes was complete. Occasionally, imaging was disrupted by lipid material coming away from the surface due to peptide-induced disruption, although this generally only occurred once disruption was well advanced. To maintain good condition, probes were rinsed thoroughly with dilute detergent solution, milliQ water, and finally isopropanol, followed by drying under a stream of nitrogen, failure to do so led to rapid deterioration due to contamination with lipid / peptide material.

5.6. Cell culture

Cells were originally obtained from ATCC and were subjected to mycoplasma testing and STR typing (Source Bioscience, UK) before use. HepG2, PC-3, BT474, AU565, MDA-MB-468, MDA-MB-231, MCF-7, HB-2, HEK-293, WI-38, HFFF2 and 3 T3 were all grown in DMEM medium (Thermo Scientific, Waltham, USA) supplemented with 10% fetal calf serum (FCS; Thermo Scientific, Waltham, USA) and 100 units/ml of penicillin/streptomycin (ThermoScientific, Waltham, MA, USA). Whereas MCF-10 A cells were grown in DMEM/F12 (Thermo Scientific, Waltham, USA), containing 10% horse serum (Thermo Scientific, Waltham, USA), 20 ng/ml epidermal growth factor (PeproTech, UK), 500 $\mu\text{g}/\text{ml}$ Hydrocortisone (Sigma-Aldrich, UK), 500 ng/ml Cholera Toxin (Sigma-Aldrich, UK), 10 $\mu\text{g}/\text{ml}$ Insulin (Sigma-Aldrich, UK) and 100 units/ml of penicillin/streptomycin (ThermoScientific, Waltham, MA, USA). Cells were cultured and maintained in a humidified incubator with 5% CO₂ at 37 °C. All experiments were conducted at cell densities that allowed exponential growth or mentioned otherwise.

5.7. MTT assays

Bioactivity of peptides was evaluated on cell lines using MTT assays [27]. AU565, MDA-MB-468, BT474 and MCF-10 A cells were seeded in 96 well plates with respective growth medium containing 10% FCS at 1×10^4 cells per well and incubated for 18 h. Cells were then treated with various concentrations (0–100 μM) of MP-1 or mutant peptides for 24 h in growth medium without FCS. Cells were then incubated with 0.5 mg/ml MTT (Thermo Scientific, Waltham, USA) dissolved in growth medium without FCS for 3 h. Formazan crystals were then dissolved in isopropanol and absorbance was measured at 570 nm using a spectrophotometer microplate reader (Shimadzu, UK).

5.8. Membrane permeability assay

Cells were seeded in 24 well plates at 2.5×10^4 cells/well for 18 h. Then cells were treated with MP-1 peptide at the respective IC_{50} doses for 24 h. Cells were stained with 5 μ g/ml Hoechst 33342 (Sigma-Aldrich, UK) in phosphate buffer saline (ThermoScientific, Waltham, MA, USA) for 30 min, and further with 1 μ g/ml propidium iodide (Sigma-Aldrich, UK) for 5 min before observing under the fluorescent microscope (EVOS FL auto, Thermo fisher Scientific, UK).

5.9. Whole cell patch clamp recording

MCF-10 A and MDA-MB-468 cells were grown on glass coverslips for 24 h prior to whole cell patch clamp recording to measure the plasma membrane potential [28]. Current clamp recordings were made using an Axopatch 200B amplifier (Molecular Devices) at room temperature. Data were digitized using a Digidata 1440 A interface (Molecular Devices), low-pass filtered at 10kHz and analysed using pCLAMP 10.4 software (Molecular Devices). The standard extracellular physiological saline solution (PSS) contained the following components (in mM): 144 NaCl, 5.4 KCl, 1 $MgCl_2$, 2.5 $CaCl_2$, 5 HEPES, and 5.6 glucose adjusted to pH 7.2 with NaOH. For the Na^+ -free PSS, NaCl was replaced with 144mM *N*-methyl-D-glucamine (NMDG) or choline chloride (ChoCl) adjusted to pH 7.2 with HCl. The standard intracellular (pipette) solution contained (in mM) 5 NaCl, 145 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, and 11 EGTA adjusted to pH 7.4 with KOH. In the mode ' $I = 0$ ', a steady V_m was recorded within 5 s of achieving the whole cell configuration. Cells were held at the whole cell configuration for up to 10 min with constant flow of fresh PSS and the V_m was recorded. MP-1-containing PSS was directly perfused onto the cells using a ValveLink 4-channel gravity perfusion controller (AutoMate Scientific) at a rate of ~ 1.5 ml/min. The solution was allowed to equilibrate for ~ 4 min following switching prior to measurement at steady state. Thus, the mean V_m over the last 5 s in control vs. MP-1-containing PSS was used to compare the V_m data. The V_m signals were sampled at 200 Hz. Liquid junction potentials were corrected using the integrated tool in Clampex 10.4.

5.10. Mitochondrial membrane potential

JC-1 is a membrane permeant dye used to monitor the mitochondria membrane potential (MMP). Cells were grown in 24 well plate (2.5×10^4 cells/well) in complete medium for 18 h. MP-1 was treated to the cells at respective IC_{50} dose for 24 h. For measuring the MMP, post treatment with MP-1, cells were incubated with 5 μ M JC-1 dye (Thermo fisher Scientific, Waltham, MA, USA) for 30 min at 37 °C. Then cells were analysed under a fluorescence microscope (EVOS FL auto, Thermo fisher Scientific, UK) with an excitation wavelength of 488 nm and an emission range between 515/545 nm and 575/625 nm respectively.

5.11. Apoptosis (annexin V) assays

After treatment of cells with respective IC_{75} dose of MP-1 for 24 h, cells were trypsinised and washed with ice cold PBS and suspended in annexin binding buffer (Thermo fisher Scientific, Waltham, MA, USA). Annexin V- FITC (Thermo fisher Scientific, Waltham, MA, USA) at a final concentration of 2 μ g/ml was added to the cells and incubated for 15 min under dark conditions. Prior to flow cytometry, 2 μ g/ml 7-AAD (Thermo fisher Scientific, Waltham, MA, USA) was added and cells were analysed using a CytoFLEX flow cytometer (Beckman, California, USA). The data was analysed using the FlowJo software (version 10.6.1).

5.12. 3D spheroid assay

Low adherent round bottom 96 well plates were used to culture the spheroids. 1000 cells/well were added with 180 μ l of growth medium

supplemented with 10% FCS along with 2.5% Matrigel matrix (Corning, USA). The 96 well plates containing the cells were then centrifuged for 10mins at 350 g and then then incubated at 37 °C with 5% CO_2 for 48 h. Post incubation, the tumor spheroids were treated with IC_{50} dose of MP-1 for 24 h. To analyse the cytotoxicity of MP-1, the spheroids were incubated with 5 μ g/ml Hoechst 33342 for 20 min and further with 1.5 μ g/ml PI for 5mins before analysing under the confocal microscope (Nikon A1R LSM). 405 nm laser for Hoechst 33342 with excitation/emission wavelengths of 407 nm/450 nm and 488 nm laser for PI with excitation/emission wavelengths of 488 nm/525 nm respectively was used to analyse the spheroids under the confocal microscope.

CRediT authorship contribution statement

Andrew Booth: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Arindam Pramanik:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Dagmara Kobza:** Investigation, Formal analysis, Data curation. **Didi Derks:** Methodology. **Lekshmi Kailas:** Methodology, Investigation. **William J. Brackenbury:** Supervision, Methodology, Funding acquisition. **Simon D. Connell:** Writing – review & editing, Visualization, Supervision, Funding acquisition, Formal analysis, Conceptualization. **Thomas A. Hughes:** Writing – review & editing, Supervision, Funding acquisition, Formal analysis, Conceptualization. **Paul A. Beales:** Writing – review & editing, Supervision, Funding acquisition, Formal analysis, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jcis.2026.141642>.

Data availability

The data associated with this paper are openly available from the University of Leeds Data Repository. <https://doi.org/10.5518/1558>

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