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# Binding of cell-penetrating penetratin peptides to plasma membrane vesicles correlates directly with cellular uptake

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ABSTRACT

Cell-penetrating peptides (CPPs) gain access to intracellular compartments mainly via endocytosis and have capacity to deliver macromolecular cargo into cells. Although the involvement of various endocytic routes has been described it is still unclear which interactions are involved in eliciting an uptake response and to what extent affinity for particular cell surface components may determine the efficiency of a particular CPP. Previous biophysical studies of the interaction between CPPs and either lipid vesicles or soluble sugar-mimics of cell surface proteoglycans, the two most commonly suggested CPP binding targets, have not allowed quantitative correlations to be established. We here explore the use of plasma membrane vesicles (PMVs) derived from cultured mammalian cells as cell surface models in biophysical experiments. Further, we examine the relationship between affinity for PMVs and uptake into live cells using the CPP penetratin and two analogs enriched in arginines and lysines respectively. We show, using centrifugation to sediment PMVs, that the amount of peptide in the pellet fraction correlates linearly with the degree of cell internalization and that the relative efficiency of all-arginine and all-lysine variants of penetratin can be ascribed to their respective cell surface affinities. Our data show differences between arginine- and lysine-rich variants of penetratin that has not been previously accounted for in studies using lipid vesicles. Our data also indicate greater differences in binding affinity to PMVs than to heparin, a commonly used cell surface proteoglycan mimic. Taken together, this suggests that the cell surface interactions of CPPs are dependent on several cell surface moieties and their molecular organization on the plasma membrane.



## 1. Introduction

Cell-penetrating peptides (CPPs) have received attention as intracellular delivery vectors due to their ability to enter cells and gain access to interior compartments even when in complex with macromolecular cargoes [1,2]. Uptake pathways of CPPs have been at center of attention ever since the first CPPs were identified more than 20 years ago [3–5]. Early studies focused on cell membrane penetration [6–11] and although this is still a suggested pathway for some arginine-rich CPPs [12–14], it has become increasingly clear that endocytosis is the predominant route of entry [2,12,15–17]. Recently, we and others have reported that CPPs are not only endocytosed passively upon cell-surface association, but instead also trigger their own uptake by specifically stimulating macropinocytosis [18–21]. Moreover, we have shown a qualitative relation between an increased rate of macropinocytosis and the extent of uptake of the CPP penetratin [21]. However, details of the molecular interactions that result in internalization remain poorly understood. CPP uptake appears independent of chiral receptors [22,23] but recent evidence indicate that cell surface proteoglycans (PGs), in particular heparan sulfates (HSPGs), play important roles[24–27], potentially acting as primary CPP binding sites [28,29]. CPP-induced clustering of HSPG chains may initiate intracellular signaling cascades resulting in endocytosis [30] and recently, Wittrup et al. identified a specific HSPG epitope that was necessary for endocytic stimulation to which the CPP Tat was able to bind [31].

Most CPPs are positively charged and interact readily with negatively charged cell surface moieties. However, positive charge as such is not sufficient for efficient internalization, but instead the type of charged residue is crucial. Peptides with a high content of arginines are generally more potent than corresponding peptides containing lysines [13,32]. This superior uptake-promoting ability of arginines has been ascribed to the guanidinium head-group which can form stable bidentate hydrogen-bonded ion-pairs with oxoanions on the cell surface [32–35]. We previously investigated the relative importance of arginine and lysine for uptake and intracellular

*Abbreviations:* CF, Carboxyfluorescein; CHO-K1, Chinese hamster ovarian cell; CPP, Cell-penetrating peptide; DTT, Dithiothreitol; FA, Formaldehyde; FITC, Fluorescein isothiocyanate; FM4-64, *N*-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl) hexatrienyl)pyridiniumdibromide; GABA, Gamma-amino-butyric acid; HBS, Hepes-buffered saline; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PMV, Plasma membrane vesicle

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distribution in live cells [14,21] for membrane affinity and bilayer integrity, induced peptide secondary structure, and hydrophobic partitioning using a set of designed CPP sequences [7,9,36–39]. Although more efficient uptake of arginine-rich CPP versions was confirmed, we saw only weak correlations between peptide-lipid interaction and uptake [14,21,36]. Furthermore, in a comparative study of amino acid affinity for heparin (a soluble HS mimic), it was reported that arginine has only a 2–3 fold stronger binding affinity than lysine. A similarly small difference in affinity was found when comparing a heptaarginine with a c-terminal tryptophan ($R_7W$) to the corresponding heptalysine ($K_7W$) [40]. These variations also appear to be insufficient to account for differences in cellular uptake for arginine- and lysine homopolymers of similar amino acid composition [13,32]. This prompted us to explore the interaction between CPPs and cell-derived plasma membrane vesicles as models of the surface of a living cell. The advantage of using such vesicles, compared to investigate cell-surface binding to whole cells, is that cell surface binding can be assessed independent of endocytosis and that the vesicles can be adapted to a wider range of biophysical experiments.

PMVs can be released from cultured adherent cells upon chemical treatment [41–43]. They contain all major classes of naturally occurring cell surface components including lipids, membrane proteins, glycolipids, and glycoproteins. Therefore, their overall composition is representative of the plasma membrane from which they were derived [43]. We treated Chinese hamster ovary cells CHO-K1 with formaldehyde (FA) and dithiothreitol (DTT) to obtain micrometer-sized vesicles. Similarly prepared vesicles have been used to study membrane phase separation [44], membrane protein proteomics [45], lipid–protein interaction [46] or used as membrane source for nanotechnological applications [47].

Despite that a relation between the amount of CPP bound to the cell surface and its degree of internalization seems intuitively obvious, it is currently not clear whether this classical description of adsorptive endocytosis [48] also applies to CPPs. Drin et al. [49] proposed that uptake of penetratin may be governed by such mechanisms based on competition experiments where poly-lysine was added to block cell surface binding sites. However, other groups have subsequently suggested that a connection between cell-surface binding and uptake efficiency does not exist [50,51]. Here we compared the affinity of penetratin for PMVs to its total cellular uptake established in previous work [21]. To specifically address differences between arginines and lysines we further compared penetratin to its all-arginine and all-lysine versions PenArg and PenLys (Table 1) [14,21,36–38]. All experiments in this study were performed using FITC-labeled peptides. Confocal microscopy was used to visualize the binding of penetratin peptides to PMVs and to characterize the PMVs as a membrane model. Binding affinity was estimated by subjecting PMVs, incubated with various concentrations of peptide, to centrifugation to separate PMVs from buffer and thus obtain a measure of the amount of bound peptide. When comparing these data to the total cellular uptake we identified a virtually linear correlation between PMV binding and cellular internalization, which is mainly endocytic [14,21]. This indicates that internalization can be directly related to the amount of peptide associated with the cell surface, in accord with a classical adsorptive endocytic uptake mechanism. This finding provides a straight-forward explanation to one of the physico-chemical factors governing CPP efficacy and seen in a wider perspective, this could facilitate the design of new CPPs or improvements of existing ones, providing an engineering approach to the development of functional peptide-based drug delivery vectors.

**Table 1**
Peptide sequences.

| Peptide | Sequence |
|---|---|
| Penetratin | FITC-$_{GABA}$-**R**QIK IWFQ N**RR**M KWKK-$NH_2$ |
| PenArg | FITC-$_{GABA}$-**R**QI**R** IWFQ N**RR**M **R**W**RR**-$NH_2$ |
| PenLys | FITC-$_{GABA}$-KQIK IWFQ NKKM KWKK-$NH_2$ |

Arginines are marked in bold and lysines underlined.

## 2. Materials and methods

### 2.1. Cell culture

Chinese hamster ovarian cells CHO-K1 were cultured in Ham's F-12 media, with fetal bovine serum (10%) and L-glutamine (2 mM) (PAA Laboratories) in humidified atmosphere at 37 °C and 5% $CO_2$. At ~75–85% confluence, cells were trypsinized and re-seeded at a density of ~12,000 cells/cm$^2$ (every 3–4 days). For preparation of plasma membrane vesicles (PMVs), cells were cultured to confluence (typically 4.5 days). Cell media was replaced 8 h before addition of vesiculation-inducing chemicals to ensure cells being in good condition.

### 2.2. Preparation of plasma membrane vesicles

Vesiculation was induced by treating CHO-K1 cells with formaldehyde (FA) and dithiothreitol (DTT) (Sigma) essentially following the procedures described by Bauer et al. [45]. Prior to treatment, the cells were washed twice in HEPES buffered saline (HBS; 10 mM HEPES, 142 mM NaCl, 1.3 mM $CaCl_2$, pH 7.4). Vesiculation reagents were freshly dissolved and diluted in HBS buffer, the mixture was preheated to 37 °C and added to cell culture flasks so that cells were just covered in solution. The cells were then incubated for 15 h at 37 °C on a rocking table at 125 rpm. Plasma membrane vesicles (PMVs) were collected by pipetting off the vesiculation buffer, care taken not to detach cells from the culture flask. The PMV solution was filtered through a Sephadex G-25 de-salting column (GE Healthcare) to remove vesiculation chemicals. Based on the vesiculation rate estimated by Bauer et al. [45], the PMV concentration was in the order of $10^7$ vesicles/ml. For all experiments, the PMV solution was used without further dilution or subfractioning and on the day of preparation.

### 2.3. Confocal microscopy

Plasma membrane vesicles (PMVs) were deposited on a coverslip in a 50 μl droplet and allowed to settle for 15 min prior to addition of a small volume of FITC-labeled peptide (NeoMPS, purity >80%) dissolved in deionized water, to the top of the droplet. No mixing was done as this causes the vesicles to move and makes it impossible to image early events of CPP–PMV interaction. The peptide was instead allowed to mix with the sample by diffusion. The added amount of peptide corresponded to a final peptide concentration of 5 μM in the droplet. Vesicles were monitored continuously until peptide had diffused to the plane of the PMVs, typically taking 5–10 min. Confocal images were thereafter acquired using either a TCS SP (equipped with a HCX PL APO CS 63× objective) or a TCS SP2 RS (equipped with as APO CS 63×/1.2 W CORR objective) confocal laser scanning microscope system from Leica. The 488 nm line of an Ar ion laser was used to excite the FITC-labeled peptides, carboxyfluorescein (CF) (Sigma) and SYTO® RNASelect™ (Invitrogen). The 543 nm line from a HeNe laser was used to excite FM4-64(*N*-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl)hexatrienyl)pyridinium dibromide, Invitrogen). Fluorophores were added to the PMV samples as described for the peptides and concentrations are indicated in the figure legends. All experiments were repeated at least 3 times.

PMVs do not adhere well to a glass coverslip and to decrease movement of vesicles and thereby obtain an acceptable image quality we spin-coated coverslips prior to experiments with SU-8 (10%, MicroChem) at 2300 rpm for 60 s. Coverslips were thereafter baked

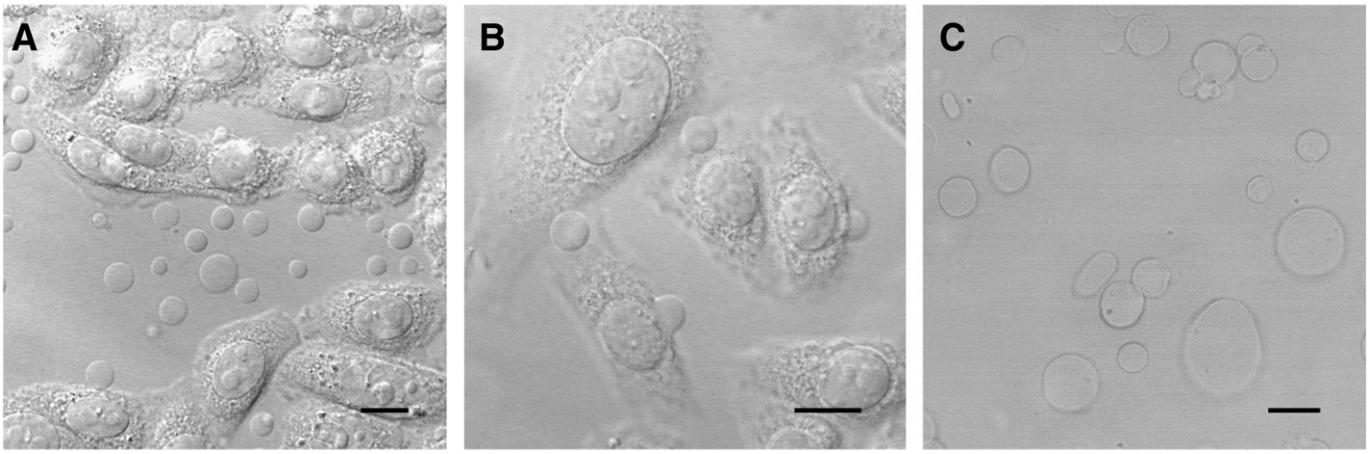


**Fig. 1.** Plasma membrane vesicles (PMVs). (A, B) CHO-K1 cells treated with vesiculation chemicals producing PMVs. (C) Gel filtration-purified PMVs harvested after 15 h of vesiculation. Scale bar = 10 μm.

on a hotplate at ~95 °C for 60 s, exposed to UV light (254 nm, ~100 mJ/cm$^2$) and baked a second time at ~95 °C for 60 s.

### 2.4. Quantitation of peptide binding to plasma membrane vesicles

The PMV solution was divided into 500 μl aliquots and FITC-labeled peptides (penetratin, PenArg or PenLys, 1, 5 or 10 μM) added to each sample. Samples were mixed gently and 250 μl was transferred to a 96-well plate as reference for the total amount of peptide in each sample ($c_{tot}$). Remaining volumes were incubated for 15 min at room temperature, followed by 20 min centrifugation at 50,000 *g* to separate bound (pellet) and free (supernatant) peptide. The supernatant was transferred to a 96-well plate. Pellets were redissolved in 250 μl HBS. A SpectraMax M2 plate reader (Molecular Devices) was used to read the FITC absorbance at 495 nm. All samples were corrected for background contributions by subtraction of the absorbance value of the buffer. The pellet fraction was not readily redissolved and the bound fraction was therefore taken as the difference between total amount of peptide and the peptide in the supernatant. Penetratin is known to have a tendency to adhere to plastics in tubes, pipette tips etc. [52], but control experiments in absence of PMVs, showed that peptide adsorption was not a problem.

## 3. Results and discussion

### 3.1. Characteristics of plasma membrane vesicles

Plasma membrane vesicles (PMVs) can be released from cultured cells by addition of formaldehyde (FA) and dithiothreitol (DTT) [45]. Fig. 1(A–B) shows CHO-K1 cells that release spherical, unilamellar PMVs with considerably less contrast than the cells, in agreement with that they contain cytosolic fluid but no organelles [43]. The PMVs bud off uniformly from the cell surface. Fig. 1C shows PMVs harvested from cells in culture after 15 h exposure to 25 mM FA and 2 mM DTT. The PMVs were separated from vesiculation chemicals, cell debris and other macromolecular structures on a Sephadex G-25 buffer exchange gel filtration column. Purified vesicles were spherical, unilamellar and approximately 5–10 μm in diameter, and the solution was virtually free from cell debris.

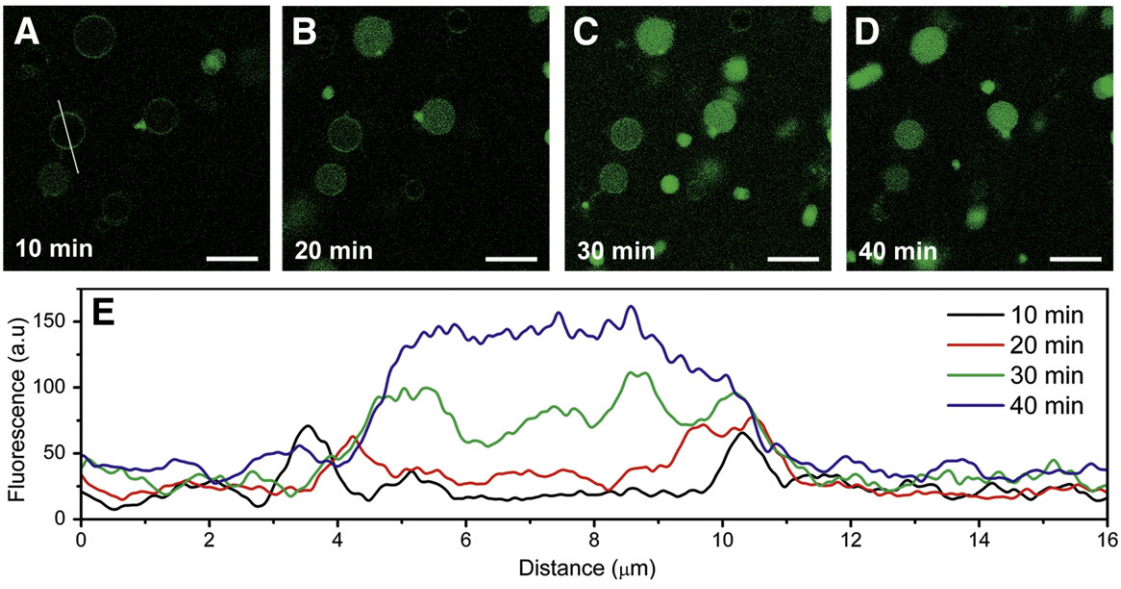


**Fig. 2.** Time-lapse confocal imaging of penetratin-PMV interactions. (A–D) Images acquired 10, 20, 30 and 40 min after addition of 5 μM penetratin to a droplet sample of PMVs. Mixing was by diffusion. Diffusion-limited peptide binding kinetics, but also peptide internalization and appearance of membrane fragments can be seen over time. (E) Intensity profiles along the line indicated in (A). Scale bar = 10 μm.

### 3.2. Confocal imaging of penetratin PMV interaction

We examined interactions between FITC-labeled penetratin and PMVs by confocal microscopy. A small aliquot of peptide was added to a droplet sample where PMVs had been allowed to settle onto a coverslip. Fig. 2 shows a representative series of confocal images, with corresponding intensity profiles for one vesicle, visualizing the time course of events following peptide addition. After 10 min, penetratin has diffused from the edge of the droplet to the site of the PMVs and membrane binding is then immediately observed. A few PMVs appear to directly internalize peptide. This may be due to membrane instability but could also be an apparent effect as all vesicles do not reside with their "equator" in the confocal plane and some contribution from membrane-associated fluorescence is thus expected. From 20 min onwards, the intensity from the membrane increases only marginally indicating that mixing by diffusion is complete and that equilibrium between bulk and membrane is established. Upon further incubation, accumulation inside the PMVs becomes more pronounced.

We noticed that vesicles occasionally detached from the coverslip during the time-course of the experiment, due to motion in the sample. Non-vesicular membrane fragments with bound peptide appeared after longer periods of incubation, indicating that penetratin may cause some vesicular lysis or PMV collapse. The number of PMVs in each frame of Fig. 2 increases because the PMVs do not firmly adhere to the coverslip and due to that additional vesicles settle onto the coverslip with time. To reduce initial image acquisition problems, due to vesicle mobility, we coated coverslips with hydrophobic SU-8. We cannot exclude that this may enhance destabilization of PMVs. Due to difficulties in imaging vesicles and in stringently distinguishing internalized from membrane-bound peptide, no quantitative information was extracted from these images.

### 3.3. PMVs are impermeable to fluid-phase markers and contain RNA

The observation of some penetratin inside the PMVs prompted us to investigate further the stability and intravesicular contents of PMVs. Staining with the cell-impermeable membrane probe FM4-64 which is only fluorescent when bound to membranes [53] resulted, in most cases, in bright fluorescence exclusively from the PMV membranes (Fig. 3). Occasionally, diffuse intravesicular fluorescence or lipid inclusions (~1 μm in diameter) were observed indicating that the PMV lumen may contain trace amounts of lipids. The occasional intravesicular FM4-64 staining suggests that the integrity of the PMV membrane is less strict than in a living cell, and that this, normally cell-impermeable, dye can in fact traverse these model membranes [53]. Vesicle leakiness was further tested by adding water-soluble carboxyfluorescein (CF) to monitor fluid-phase exchange over the PMV membrane (Figs. 3A–D). CF was not internalized in samples incubated for 10 min and only to a minor extent in samples incubated for 50 min. Presence of CF in concentrations similar to those used in the peptide experiments did not cause vesicle rupture as observed with the former (Fig. 2). Since penetratin was observed to accumulate inside PMVs they must contain molecules for which the peptide has some affinity. Staining with the RNA specific probe SYTO RNASelect (Figs. 3E–F) confirmed presence of RNA in agreement with that PMVs contain cytosolic fluid [43].

The data above suggest that whereas the PMVs appear somewhat permeable to lipid-binding molecules such as the FM4-64 dye, they are not intrinsically leaky, since CF cannot enter and RNA is only observed inside the vesicles and thus efficiently confined. Penetratin binds avidly to the membrane and is apparently thereafter able to translocate. This is in accord with some previous observations in model systems [6,11,54] but in contrast with other [7–9]. We believe that the translocation into PMVs is exaggerated compared to translocation across the plasma membrane of a live cell because of

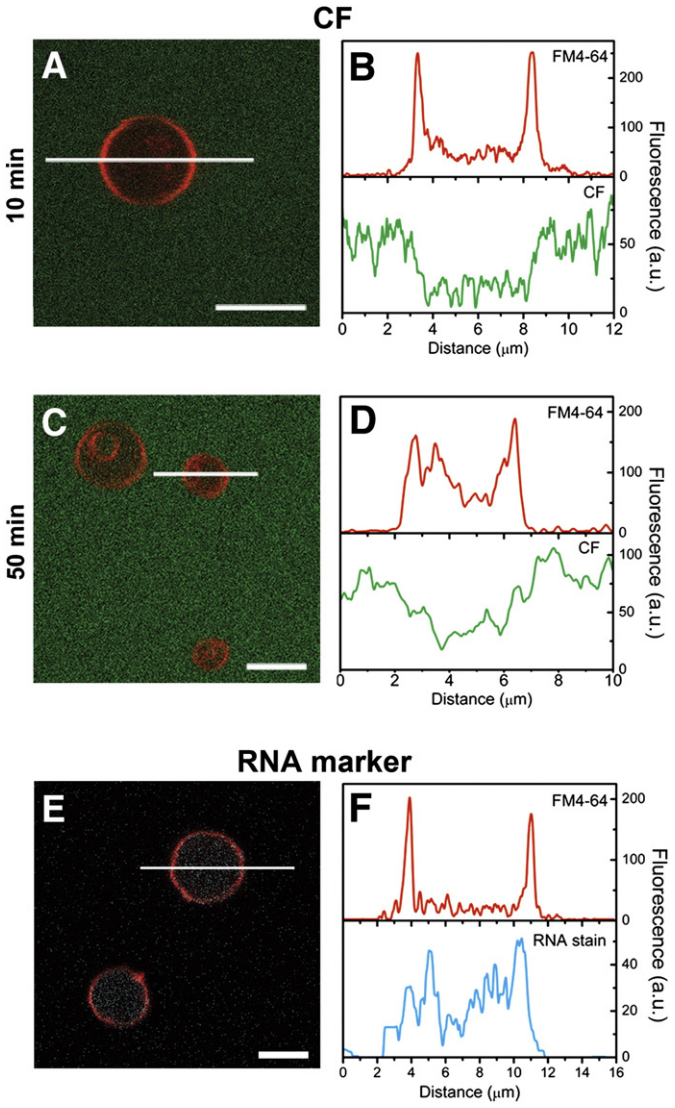


**Fig. 3.** PMV membrane integrity and contents. (A–D) Staining with membrane marker FM4-64 (20 μg/ml) and external addition of carboxyfluorescein (CF, 5 μM). Images acquired in two separate experiments 10 min (upper panels) and 50 min (middle panels) after addition of the dyes. (E, F) Staining with FM4-64 (20 μg/ml) and RNA-specific SYTO RNASelect (3 μM) acquired after 5 min incubation. Intensity profiles along the lines indicated in the confocal images are shown in panels to the right. Scale bar = 5 μm.

the metastable nature of the PMVs and it is unclear to us whether this phenomenon would be relevant to describe uptake of penetratin in live cells, which is, in addition, mainly endocytic [14,21]. However, the finding that penetratin has a tendency to disrupt PMV membranes with time (Fig. 2) indicates that the mechanism by which it translocates is invasive and that if this occurred in a live cell it would result in cytotoxicity. Except for PenArg added at high concentrations, penetratin variants are consistently reported to be nontoxic [21,55,56].

### 3.4. Influence of arginine and lysine on peptide binding to PMVs

The differences between arginines and lysines in terms of promoting cellular uptake are well established for penetratin as well as for other CPPs [13,14,21,32], but have previously not been quantitatively understood from biophysical experiments [7,9,36–40,57]. Fig. 4 shows representative confocal images of PMVs recorded 15 min after addition of FITC-labeled versions of penetratin. The all-arginine PenArg peptide and penetratin bind with an even distribution to PMV membranes in accord with previous findings [6–9]. The

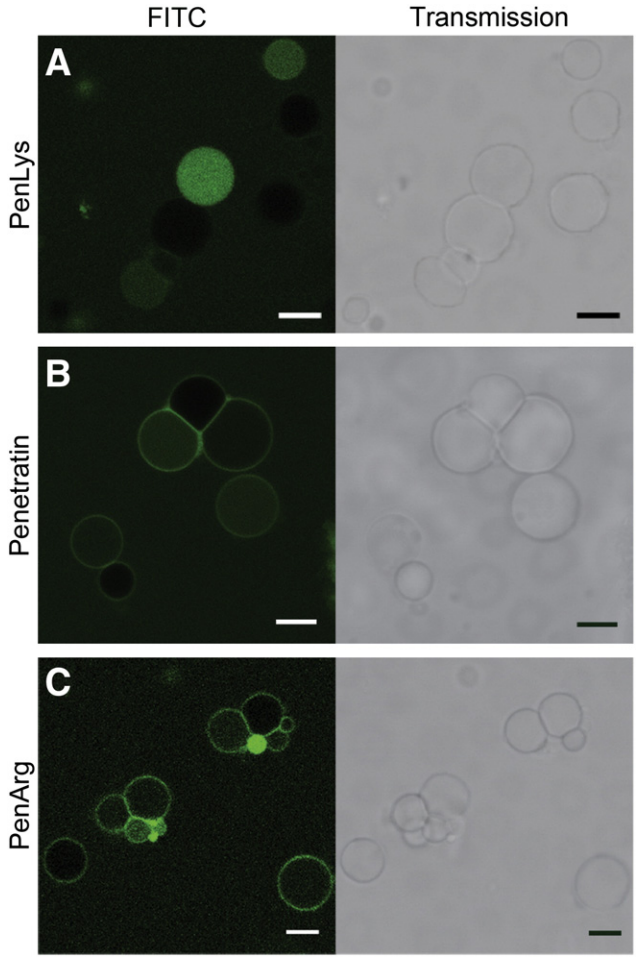


**Fig. 4.** Penetratin analogs displaying different patterns of binding to PMVs. (A) PenLys, (B) Penetratin, (C) PenArg. PenArg and penetratin show clear membrane association, while PenLys gives more diffuse fluorescence with no clear membrane localization. Images acquired 15 min after addition of peptide. Contrast and gain have been optimized for each image and the intensities are thus not directly comparable. Scale bar = 5 μm.

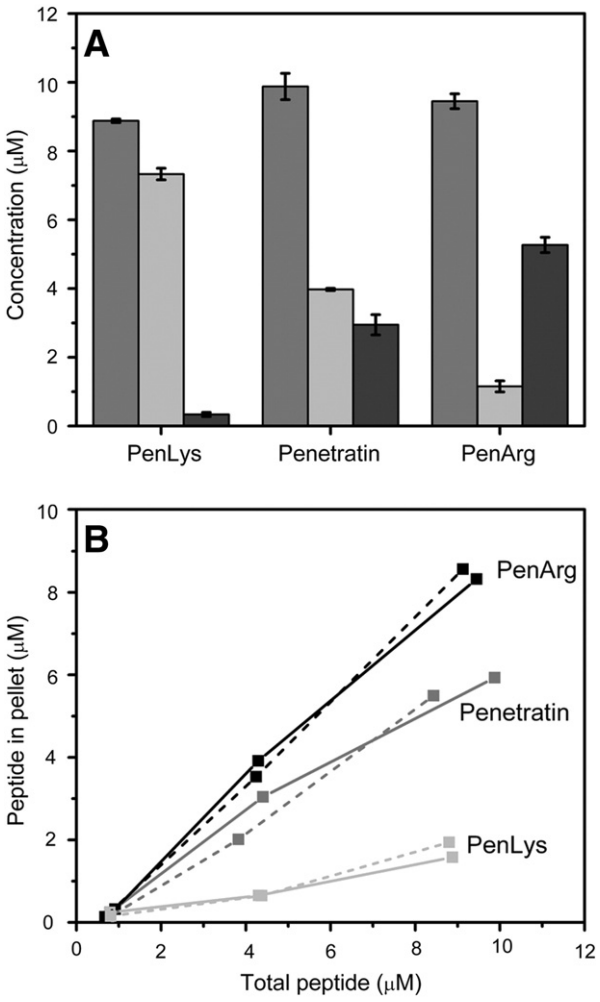


**Fig. 5.** Quantitation of peptide binding to PMVs. (A) Concentration of peptide prior to centrifugation (gray bars), in the supernatant (light gray bars) and in the pellet (dark gray bars). Error bars represent maximum and minimum values from experiments performed in duplicates. (B) Binding curves showing concentration in the pellet fraction (calculated as $c_{tot} - c_{supernatant}$) as a function of total peptide in each sample. Solid lines represent binding to PMV harvested and purified from CHO-K1 cells as described in the text. Dotted lines represent binding to PMVs that had been subjected to 5 cycles of freeze-thawing prior to purification on a gel filtration column and addition of peptide. The procedure removes intravesicular RNA.

all-lysine PenLys displays a much weaker binding and comparatively more diffuse intravesicular fluorescence, in agreement with its lesser ability to enter live CHO-K1 cells [21] and apparent inability to enter PC-12 or V79 cells [14].

While confocal imaging of peptide-PMV interaction gives a good qualitative view of how these peptides behave in presence of PMVs it was not feasible to obtain quantitative data of peptide binding from intensity analysis of these images (see above). Instead, we used centrifugal sedimentation to separate the PMVs from buffer and thereby estimate the amount of bound peptide. Sedimentation of the PMVs could be obtained at 5000 *g* as established by examining the supernatant for absence of vesicles and in agreement with similar data on PMVs from HEK293 cells [58]. However, to ensure efficient separation, samples were centrifuged at 50,000 *g* for 20 min (see Fig. S1 for images of FM4-64 stained pellet and supernatant fractions). For PenArg, the absolute concentration in the supernatant was very low showing that this peptide sedimented almost completely with the PMVs whereas samples containing PenLys, prepared under identical conditions, showed a significant amount of peptide in the supernatant (see below).

The peptide concentration in pellet and supernatant fractions was quantified spectrophotometrically from the absorbance of the FITC label. Fig. 5A shows the distribution of peptide between the supernatant and the pellet fraction upon addition of 10 μM peptide to a PMV sample. The concentration of peptide in the supernatant is in the order PenLys > penetratin > PenArg, and *vice versa* for the concentration of peptide in the pellet, as expected from previous affinity studies in lipid membranes [9,36,38]. The total amount of peptide in the supernatant and the pellet fractions do not add up to the total amount added to the sample. This is due to that the pellet could not be completely dissolved. This leads to an underestimation of the amount of peptide in the pellet fraction, a problem that we have encountered before, albeit using a different macromolecular system [59].We estimate that up to 25% of the total amount of added peptide could not be recovered. Therefore, the amount of peptide in the pellet fraction was determined indirectly as the difference between the total amount of peptide initially present in each sample and the amount of peptide in the supernatant ($c_{tot} - c_{supernatant}$).

Fig. 5B shows the calculated amount of peptide in the pellet fraction as function of total peptide concentration. As we did observe peptide translocation and subsequent accumulation in the PMVs (Fig. 2) we wanted to ensure that the data in Fig. 5 were representative of plasma membrane binding and that the amount of bound peptide was not overestimated due to intravesicular peptide binding (i.e. to RNA, see Fig. 3). Samples were therefore gently mixed to avoid potential vesicle rupture and then incubated for only 15 min

before quantification to minimize effects of time-dependent peptide internalization that we observed in Fig. 2. We also set aside half of the PMV suspension used to record the data in Fig. 5 and subjected these PMVs to five freeze-thaw cycles (liquid $N_2$/37 °C) followed by gel filtration prior to addition of peptide. Freeze-thawing will cause vesicles to open up and reseal and consequently release intravesicular content [60]. Furthermore, we observed that this procedure reduced the size of the PMVs (data not shown), which is consistent with a simultaneous release of contents. The gel filtration step thereafter removes released RNA. Binding curves from these experiments are shown as dashed lines in Fig. 5B and are within experimental error identical to those obtained from untreated vesicles. This shows that peptide internalization, as observed in the confocal experiments, does not significantly contribute to the material found in the pellet.

The data in Fig. 5B clearly shows that arginine residues have higher affinity for the PMVs than lysine residues. At the highest peptide concentration 90% of the added PenArg is bound to the vesicles while only 15% of PenLys is bound. Since neither the exact nature, nor the concentration of these binding sites is known, binding constants cannot be directly extracted from these data, but an estimation of a binding constant ratio ($K_{PA}/K_{PL}$) between PenArg (PA) and PenLys (PL) to PMV binding sites (B) can still be obtained for a generalized case based on a simple binding equilibrium:

$$\frac{K_{PA}}{K_{PL}} = \frac{[PA]_{bound}}{[PA]_{free}[B]_{free,PA}} \times \frac{[PL]_{free}[B]_{free}PL}{[PL]_{bound}}. \quad (1)$$

We used the experimentally determined bound and free peptide concentrations from Fig. 5B to estimate possible values for the quotient $K_{PA}/K_{PL}$ in Eq. (1). If the available binding sites on the PMV surface are in excess, this quotient will approach its lowest possible value which is $K_{PA}/K_{PL}$ ~34. If the available binding sites are instead limited the quotient will be even higher (see derivation in Supplementary information). The same is true for the relations between PenArg and penetratin or penetratin and PenLys and the lowest values of those binding constant quotients are $K_{PA}/K_{Pen}$ ~5 and $K_{Pen}/K_{PL}$ ~7 respectively. We and others have attempted to obtain a physico-chemical understanding of the differences in uptake efficiency of arginine- and lysine-rich cell-penetrating peptides by comparing their affinities for lipid vesicles of varying composition [9,36,38], their water–octanol/chloroform partitioning [32–34,37], or their ability to bind soluble fractions of heparan sulfates or heparin [40,61]. Whereas we observe that the difference in total cellular uptake is approximately 10-fold [21], the difference in lipid vesicle binding affinity between the three penetratin variants is less pronounced. The quotient between the surface partition coefficients for unlabeled PenArg and PenLys in partly negatively charged membranes is for example only around 3 [38]. In this study we used FITC-labeled peptides throughout and whereas we have seen that this will increase the lipid-mediated partitioning of all three penetratin variants into octanol, we also showed that their relative partitioning was independent of the label [37] suggesting that the more accentuated differences that we observe here cannot be caused by the label itself. In addition, we estimated the relative intrinsic affinity of the peptides for the lipid portion of the PMVs using the experimentally derived Wimley and White hydrophobicity scale [62]. The difference between PenArg and PenLys amounts to 1.26 kcal/mol which corresponds to a binding constant ratio ($K_{PA}/K_{PL}$) of ~8. This value is smaller than the ratio obtained above for the affinity for PMVs (≥34). Moreover, all peptides have net positive free energy changes for partitioning into POPC membranes according to the Wimley and Whites hydrophobicity scale and it has in fact been shown that penetratin variants do not interact significantly with purely zwitterionic membranes [63] indicating that negatively charged entities on the plasma membrane are needed for binding. A plausible explanation for these discrepancies is therefore that the penetratin peptides primarily associate with negatively charged heparan sulfates on the cell surface and that the relative magnitude of these interactions would be sufficient to explain the observed differences in uptake. However, according to measures in the literature, the dissociation constants for interaction with heparin differ only by a factor 2–3 between individual arginine and lysine residues [40]. Even though these values may not be directly comparable to the exact dissociation constants of penetratin, PenArg and PenLys, this gives a good indication that the differences in heparin affinity will be smaller than the differences in cell uptake. While it is difficult to directly compare data obtained in different model systems our findings therefore indicate that the complexity and the molecular organization of binding sites on the PMV surface may in fact be important to adequately describe the relation between cell surface affinity and uptake efficiency.

Since PMVs do differ somewhat in composition from the cell surface potentially displaying a slight enrichment in cholesterol and sphingomyelin [43] and some alteration in transbilayer distribution of phosphatidylethanolamine (PE) [64], and since the preparation method involves low concentrations of cross-linking agents (5% of what is normally used for cell fixation) which could cause membrane protein denaturation, we wished to confirm that the PMVs would still be representative models of the cell surface. To this end, we measured the affinity of the penetratin peptides to the cell surface of CHO-K1 cells kept at 4 °C to avoid endocytosis and compared these data to the affinity for PMVs. We found that there is a linear relationship between cell surface binding and PMV affinity (Fig. S2) which in turn suggests that the composition of the PMV surface is sufficiently similar to that of the plasma membrane of the live cells from which these vesicles were derived.

We then compared the amount of peptide in the pelleted fractions (calculated as the difference between applied peptide and peptide in the supernatant, Fig. 5B) to the total cellular uptake in CHO-K1 cells after 1 h incubation [21]. At this time-point internalization had reached a plateau (Fig. S3). We obtained similar linear relationships between these two parameters for each of the three peptides examined (Fig. 6). This suggests that cell uptake of penetratin is indeed occurring through adsorptive endocytosis and that cell surface affinity is a major determinant for cell uptake efficiency. This conclusion is in contrast to a claim that no such relations exist [50,51] but in accord with a recent study of the Tat peptide [65] and early observations by Drin et al. [49]. Still, we have previously shown that penetratin and its two analogs can stimulate macropinocytosis

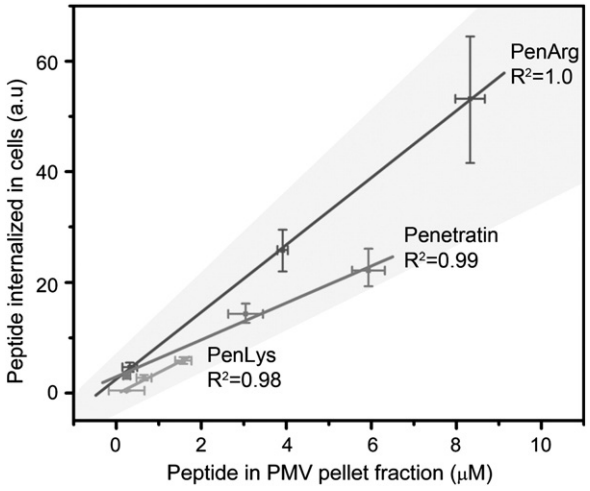


**Fig. 6.** Relation between peptide uptake and membrane binding. Total uptake in live cells as a function of peptide in the pellet fraction (Fig. 5B). Cells and PMVs were subjected to 1, 5 or 10 μM peptide. Error bars represent minimum and maximum values in each experiment. Linear correlation is seen for all three peptides, with importance of arginines emphasized for both uptake and binding. Cell uptake data was retrieved from Reference [21].

[21]. Such triggering of uptake is not immediately consistent with the present finding and a possible explanation to this could be that whereas a certain subset of the interactions are responsible for initiating endocytosis, the bulk of internalized peptide is simply co-internalized because it is bound to the cell-surface. One specific CPP binding site has indeed recently been proposed [31] suggesting that particular heparan sulfate interactions trigger endocytosis. It is possible, given the strong electrostatic nature of CPP-target interactions, that an increase in overall membrane affinity also results in increased binding to such particular "triggering sites", but from our data we cannot exclude that efficient binding of the penetratin peptides requires simultaneous interactions with several binding sites. Further, our data support that the superior uptake-promoting capability of arginine compared to lysine to a large extent is related to its higher cell-surface affinity.

## 4. Conclusions

This study evidences that penetratin peptides with different arginine content bind to a plasma membrane vesicle model of the CHO-K1 cell surface to an extent that correlates quantitatively with uptake in these cells. Our findings substantiate the intuitive, but previously unconfirmed view that strong affinity for the cell surface is a key property for extensive CPP internalization. This suggests that the degree of endocytic stimulation these peptides evoke is directly related to the amount of peptide tightly bound to the cell surface. The study demonstrates the usefulness of plasma membrane vesicles as cell membrane models in biophysical studies of cell surface interactions and that a top–down approach in design of cell-mimicking membrane models has the potential to provide novel and detailed molecular understanding of how CPPs interact with the cell membrane.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.bbamem.2011.03.011.

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