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Massive glycosaminoglycan-dependent entry of Trp-containing cell-penetrating peptides induced by exogenous sphingomyelinase or cholesterol depletion

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Abstract

Among non-invasive cell delivery strategies, cell-penetrating peptide (CPP) vectors represent interesting new tools. To get fundamental knowledge about the still debated internalisation mechanisms of these peptides, we modified the membrane content of cells, typically by hydrolysis of sphingomyelin or depletion of cholesterol from the membrane outer leaflet. We quantified and visualised the effect of these viable cell-surface treatments on the internalisation efficiency of different CPPs, among which the most studied Tat, R₉, penetratin and analogues, that all carry the N-terminal biotin-Gly₄ tag cargo. Under these cell membrane treatments, only penetratin and R₆W₃ underwent a massive glycosaminoglycan (GAG)-dependent entry in cells. Internalization of the other peptides was only slightly increased, similarly in the absence or the presence of GAGs for R₉, and only in the presence of GAGs for Tat and R₆L₃. Ceramide formation (or cholesterol-depletion) is known to lead to the reorganisation of membrane lipid domains into larger platforms, which can serve as a trap and cluster receptors. These results show that GAG-clustering, enhanced by formation of ceramide, is efficiently exploited by penetratin and R₆W₃, which contain Trp residues in their sequence but not Tat, R₉ and R₆L₃. Hence, these data shed new lights on the differences in the internalisation mechanism and pathway of these peptides that are widely used in delivery of cargo molecules.

Ceramide-enriched domain / endocytosis / tryptophan-rich peptide / arginine-rich peptide / cationic peptide

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Introduction

Ever since their discovery two decades ago, extensive studies are carried out in order to understand the mechanisms of cellular internalisation of cell-penetrating peptides (CPPs) [1-3]. These are short peptides (5-30 residues), generally cationic, that have the capacity to cross cell membranes in a non-invasive way and can thus be used to deliver various cargoes, including therapeutics, inside living cells [4-6]. However, the final cellular localisation depends on the internalisation mechanism of the conjugated CPP-cargo conjugates, which stresses the importance of understanding their internalisation pathways. For all the described mechanisms of CPP internalization (endocytosis and direct translocation), cell entry must involve as a first step interaction with the plasma membrane (PM) bilayer. The latter has a very complex and dynamic structure, where the lateral organisation and segregation play a major role in trafficking processes, mainly through lipid rafts [7,8]. These rafts are reported as highly dynamic transient microdomains enriched in cholesterol and sphingomyelin (SM) in the outer leaflet of the PM, and thought to cluster into larger platforms in response to specific stimuli [8]. The implication of lipid rafts in the internalisation of CPPs in different cell lines was mostly studied by using various inhibitors of lipid raft-dependent endocytosis (mainly via cholesterol depletion) or by following different markers that would be sorted in such domains [9-13]. However, few are the studies that have assessed the implication of the SM/cholesterol interactions and the resulting PM organisation during internalisation. It was demonstrated that CPPs are taken up into mammalian cells more efficiently than into plant protoplast [14], probably because of the difference in sterol lipids and the absence of SM in plant cell membranes compared to mammalian PM. Moreover, the well-known CPP penetratin was shown to induce negative curvature, important for translocation, in fluid membrane domains but not in raft-like domains [15], while adding cholesterol to DOPG/DOPC vesicles was shown to increase the binding affinity of different analogues of penetratin to large unilamellar vesicles (LUVs) without evidence for specific peptide/cholesterol interactions [16]. A recent study suggested that high concentrations (> 15 μM) of some CPP sequences enhance internalisation by triggering the translocation of intracellular acid sphingomyelinase (SMase) from the cytosol to the outer leaflet of the plasma membrane, where it hydrolyses SM and produces ceramide-enriched domains [17].

In the present work, we explored the role of the two components of membrane microdomains, SM and cholesterol, on CPP internalisation in living cells, in the presence or absence of cell-surface glycosaminoglycans (GAGs). In fact, GAGs are present in the ectodomains of proteoglycans, which can be localised in cholesterol and SM-enriched plasma membrane microdomains. GAGs serve as high capacity regions for concentrating positively charged CPPs, and then transferring them to outer membrane lipids, or can directly internalise CPPs through clustering [18-20]. A recent study suggested that multivalent Tat peptide induces heparan sulfate proteoglycans clustering and recruitment to actin-associated lipid rafts, thus causing its internalisation by macropinocytosis [21].

To obtain basic knowledge about the internalisation mechanisms, we modified the membrane content of cells, typically by hydrolysis of sphingomyelin or depletion of cholesterol. The tested peptides were all modified at the N-terminus with a biotinyl-Gly₄ bait and quantitation tag sequence, which can be assimilated as a cargo moiety identical for all CPP sequences. We used five different CPPs (Table 1) -Tat, penetratin, R₉, R₆W₃ and R₆L₃ - and quantified the effect of these cell-surface treatments on their internalisation in a mutant GAG-deficient cell line (CHO-pgsA745, GAG^{neg}) compared to wild-type CHO (WT) cells. This GAG-deficient cell line has been widely employed before to analyse the impact of GAGs on the internalisation efficiency of various permeant molecules, and not only CPPs. It has been characterised both genetically (defect in xylosyltransferase) and biochemically (90-95 % GAG-deficient phenotype) [22] and is known to feature almost abolished endocytosis pathways for CPPs [23-26]. Thus we examined whether the effects of SMase or M β CD treatments are different when cells express different levels of cell-surface GAGs. The effect of SM hydrolysis was also visualised using confocal laser scanning microscopy for two representative CPPs, Tat peptide and penetratin. Further on, the effect of cholesterol depletion on the internalisation of these two CPPs was also studied, and their interaction with model systems mimicking lipid rafts was quantified. In this study, we have used peptide concentrations ranging from 1 μM up to 30 μM . One reason is that different internalisation pathways are involved according to the peptide concentration [24]. GAG-dependent internalization of penetratin is indeed activated at higher micromolar peptide concentration (> 3 μM) than for translocation [24]. The second reason is that some CPPs activate endogenous SMase release above 20 μM extracellular concentration [17]. This peptide concentration is high for delivery purposes, but local membrane concentrations could reach that value with accumulation of the peptide at the vicinity of the membrane after peptide administration *in vivo*.

Materials and Methods

Materials - Peptides (containing N-terminal biotin-G4 moiety and carboxamide at C-terminus) were obtained from PolyPeptide Laboratories (Strasbourg, France) or synthesized using the Boc-solid phase strategy. POPC and POPG were obtained as a powder from Genzyme (Switzerland). Cholesterol was purchased from Avanti Polar Lipids (Alabaster, AL). Ceramide, TRITC-streptavidin and avidin were purchased from ZYMED

laboratories (Invitrogen). Anti-ceramide mouse IgM was purchased from Glycobiotech (Borstel, Germany) and anti-Mouse IgG from Jackson ImmunoResearch Labs. Phalloidin-FITC, neutral sphingomyelinase from *Bacillus cereus*, heparinase I, heparinase III and chondroitinase ABC, methyl- β -cyclodextrin (M β CD), chicken egg yolk sphingomyelin (Fluka), FITC-dextran (4.4 kDa and 70 kDa) and all other reagents were from Sigma-Aldrich.

Cell culture - Wild type Chinese Hamster Ovary CHO-K1 cells (WT) and xylose transferase- or GAG-deficient CHO-pgsA745 cells (GAG^{neg}) were obtained from the American Type Culture Collection (Rockville, MD). All cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), penicillin (100,000 IU/L), streptomycin (100,000 IU/L), and amphotericin B (1 mg/L) in a humidified atmosphere containing 5 % CO₂ at 37 °C.

Quantification of the internalised peptide - 10⁶ cells in suspension were incubated with 5 mM M β CD for 30 min at 37 °C. Cells were then washed with 1mL HBSS, then incubated with the peptide [biotinyl-(¹H)G₄ tagged] at the indicated concentration in 1 mL total DMEM volume for 70 min at 37 °C. Cells were treated with trypsin before their lysis in the presence of a known amount of the [biotinyl-(²H)G₄ tagged] internal standard peptide. Capture of the (¹H)- and (²H)-labelled peptides was carried out with streptavidin-coated magnetic beads. The amount of internalised peptide (captured on beads) was then quantified by matrix-assisted laser desorption/ionisation-Time of flight mass spectrometry (MALDI-TOF MS) as described [27,28]. To be accurate, quantification of the internalisation requires working with an identical number of cells upon different experimental conditions. Adherent cells are easier to handle. However upon M β CD treatment, we observed that some cells detached from the wells, without induced toxicity. Since washing steps are required for the preparation of the samples for MALDI-MS based quantifications (by direct aspiration of the buffered solution for adherent cells, or centrifugation steps to recover cells in suspension), we chose to perform internalisation with cells in suspension to avoid losing the detached cells. By contrast, in the case of SMase treatment, cells did not detach. Therefore, 10⁶ adherent cells were incubated with the peptides [biotinyl-(¹H)G₄ tagged] at the indicated concentrations in the presence of 1U/ml of neutral SMase from *Bacillus cereus*, in 1 mL total DMEM volume for 70 min at 37 °C. To remove cell-surface GAGs, cells were incubated for 45 min at 37°C in Hank's Balanced Salt Solution with heparinase I, heparinase III and chondroitinase ABC (every enzyme at 0.1 U/ 10⁶ cells /ml). Cells were washed and further incubated in the presence of penetratin (10 μ M) with or without SMase (1U/ml). The amount of internalised peptide was then quantified as described above. Experiments were repeated independently at least three times in triplicates.

Ceramide quantification in cells by flow cytometry - Cells were detached from the culture plate with 0.5 mM EDTA in PBS (5 min at 37°C). One million cells in suspension were incubated in the presence or absence of 0.05 U of SMase in 200 μ L DMEM for 30 min at 37°C. After washings by centrifugation (800g, 5 min, 4°C), cells were fixed in 3 % paraformaldehyde (4 °C, 10 min) and incubated with 10% FCS in PBS (20 °C, 30 min). Cells were incubated with a mouse anti-ceramide IGM antibody (1 μ g/ml) for 1h at room temperature. After PBS washes, cells were incubated with a secondary FITC-labeled anti-mouse antibody (SIGMA, 5 μ g/ml) for 1h at room temperature, then washed and kept in PBS for the flow cytometry analysis (FACS Calibur flow cytometer (BD Biosciences) using 488 nm laser excitation and a 515-545 nm emission filter. Each sample was analyzed for 10 000 events. The mean fluorescence values were obtained by subtracting the mean fluorescence without anti-ceramide antibody from the mean fluorescence of cells.

Fluorescence microscopy - 10⁵ cells were cultured, a day before, on cover slips in 24-well plates in DMEM supplemented with 10% FCS. After washing with culture medium, cells were incubated for 40 min with the biotinylated peptides at 37 °C, in the presence or absence of 0.05 U of SMase in 200 mL DMEM. Cells were washed three times with cold medium and incubated with unlabeled avidin (10 μ M) to block the membrane-bound peptide before fixation [29]. After washings, cells were fixed in 3 % paraformaldehyde (4 °C, 10 min), permeabilised with 0.1% TX-100 in phosphate- buffered saline (20 °C, 5 min) and incubated with 10% FCS in phosphate-buffered saline (20 °C, 30 min). Biotinylated peptides were detected with streptavidin-TRITC (2 μ g/ml) for 1h at room temperature. Cells were then incubated with phalloidin-fluorescein isothiocyanate (1/1000) (Sigma) for 30 min at room temperature, and finally incubated with DAPI (1.5 μ g/ml) (Pierce) for 10 min at room temperature. Cover slips were mounted in Fluoromount mounting medium for observation by confocal microscopy on an inverted Leica DMI 6000. Experiments were repeated several times for consistency and gave identical results.

Cell viability assays – Effects of SMase or M β CD on cell viability was evaluated with CCK8 kit (Dojindo Laboratories). Briefly 25,000-30,000 WT and GAG^{neg} cells were seeded into 96 multiwell plates 24h before cell viability assays. The culture medium (with FCS) was replaced by culture medium (without FCS) without (control) or with SMase or M β CD at the indicated concentrations and CCK8. Cells were incubated for 75 min to

130 min at 37°C before OD (450 nm and reference at 620 nm) measurement (PolarStar, BMG Labtech). The concentrations of SMase and M β CD were chosen to incorporate those used in MS quantification and imaging experiments. Data result from two independent experiments in triplicates.

Liposome preparation - Lipid films were made by dissolving lipids into chloroform or a mixture of chloroform and methanol (4/1 vol/vol). The solvent was then evaporated using a rotary evaporator and the dissolving/evaporation step was repeated three times. Lipid films were then hydrated with 50 mM Tris-Cl, 2 mM EDTA, 150 mM NaCl, pH 7.5. The films were then vortexed extensively at a temperature superior to the one of phase transition temperature of the lipid to obtain multilamellar vesicles (MLVs). To form LUVs, the MLVs were subjected to five freeze/thawing cycles. The homogeneous lipid suspension was passed 19 times through a mini-extruder (Avanti Alabaster, AL) equipped with two stacked 100 nm polycarbonate membranes.

Isothermal titration calorimetry experiments - ITC experiments were performed on a TA Instrument nano ITC calorimeter. Titrations were performed by injecting 10 μ L aliquots of peptide solutions (concentration between 250 and 500 μ M) into the calorimeter cell containing 10 mg/ml LUVs, with 10 min injection intervals. The experiments were performed at 25 °C, a temperature at which vesicles are in fluid phase. Data analysis was performed with the NanoAnalyse software provided by TA Instruments.

Results

Boosting the internalisation efficiency of Trp-containing CPPs upon sphingomyelin hydrolysis in the outer membrane leaflet of cells

Quantification of the internalised peptide - One convenient method for depletion of SM from cell membrane surfaces consists in their treatment with exogenous SMase. SM is then converted into ceramide, this leads to the accumulation of this latter, which alters the surface topography by enhancing membrane curvature [30] as well as inducing phase separation and formation of condensed ceramide-enriched domains [31,32]. We tested the effect of SM hydrolysis on the entry efficiency of CPPs into two model cell lines, WT and GAG^{neg} CHO cells, the latter being chosen to examine in addition the role of proteoglycans. The peptides were used at different extracellular concentrations: penetratin, R₆W₃, R₆L₃, R₉ and Tat peptide (Table 1). We quantified by MALDI-TOF MS the amount of internalised peptide after 70 min incubation (plateau of the internalisation kinetics) with one million cells, in the presence or absence of SMase (Figure 1). It should be noted that treatment of both cell types with SMase at the concentration used to study CPP internalization by MS (1 U SMase / mL / one million cells) did not induce any cytotoxic effect (Supplementary figure 1).

In WT cells, the internalisation of all CPPs increased upon SMase treatment but the effect was quantitatively different according to the peptide sequence and concentration. A strong increase in the amount of internalisation was measured for R₆W₃ (12-fold for 10 μ M extracellular peptide concentration) and penetratin (4, 6 and 8 fold increase for 1, 10 and 30 μ M extracellular peptide concentration, respectively). The effect was less important (2 to 3 fold) for R₉ (10 and 30 μ M), R₆L₃ and Tat. Interestingly, the impact of SM hydrolysis on the internalisation of R₆W₃ and penetratin was lower in GAG^{neg} cells. A 2-fold increase in internalisation was observed for R₆W₃ (10 μ M), whereas for penetratin an effect was observed only at high peptide concentration (3-fold increase at 30 μ M). On the other hand, there was a 2-fold increase in the internalisation of R₉ (10 and 30 μ M) but no effect of SMase treatment on the internalisation of Tat and R₆L₃. As mentioned before, SMase treatment did not perturb viability of WT and GAG^{neg} cells, discarding cytotoxicity as an explanation for the boosting effect of SMase on penetratin and R₆W₃ entry observed especially in WT cells. To confirm the direct implication of GAGs in the massive internalization observed for the two CPPs, penetratin and R₆W₃, we studied on penetratin whether an enzymatic removal of cell-surface carbohydrates before SMase treatment gave similar results as those obtained with GAG^{neg} cells. As shown in Table 2, the effect of SMase on penetratin internalization was similar in WT cells treated with a combination of heparinase I, heparinase III and chondroitinase ABC and GAG^{neg}, confirming the direct involvement of GAGs in the massive internalization of the peptide.

Thus, SMase treatment induced massive GAG-dependent endocytosis of penetratin and R₆W₃, while for R₉ the effect was similar in WT and GAG^{neg} cells, suggesting that SMase-mediated higher uptake of R₉ is independent of the presence of GAGs. Finally, the internalisation of Tat and R₆L₃ was weakly affected by SM hydrolysis and only in WT cells, suggesting that GAG-dependent endocytosis was slightly enhanced in this case.

Fluorescence staining - Because the major effect of SMase on peptide entry was measured in the presence of GAGs, we examined by confocal microscopy the internalisation, in WT and GAG^{neg} CHO cells, of two of the peptides that behaved differently: penetratin and Tat. In parallel, formation of ceramide was detected with monoclonal anti-ceramide antibodies, and quantified by flow cytometry (Figure 2) in non-permeabilised and fixed cells (Supplementary figure 2). As expected, treatment of cells with SMase increased the specific anti-ceramide antibody staining of the cell-surface from the two cell lines. Interestingly, ceramide-staining was

1 mostly punctuated with big patches visible at the cell-surface of the two cell types (Supplementary figure 2B,
2 2D) compared to the control (Supplementary figure 2A, 2C), a relevant indication of the segregation of
3 ceramide-enriched membrane domains. Flow cytometry (Figure 2) showed that ceramide production was even
4 more important in GAG^{neg} compared to WT cells after 30 minutes SMase treatment. This observation likely
5 results from the presence of GAGs at the cell-surface of WT compared to GAG^{neg} cells that should impede
6 access of SMase to the lipid bilayer.

7 Consistent with the massive increased intracellular peptide quantity measured by MALDI-MS, a massive
8 penetratin staining (cytosol and nuclei) was visible inside some WT cells (Figure 3B, upper, and Supplementary
9 figure 3) upon SMase treatment, compared to control cells (Figure 3A, and Supplementary figure 3).
10 Furthermore, in the absence of the massive peptide staining, we could clearly detect punctuations starting from
11 the plasma membrane (nucleation zones) and visible till the nucleus (Figure 3B). In the case of Tat peptide
12 (Figure 4A, 4B, and Supplementary figure 4) we never observed a massive staining, but few cells presented
13 nucleation zones on the plasma membrane (Figure 4B).

14 On the other hand, in GAG^{neg} cells the cytoplasmic fluorescent signal of penetratin staining became more
15 punctuated upon SM hydrolysis (Figure 3D) compared to untreated cells (Figure 3C). As for Tat, we could not
16 visualise any difference in the staining, in the presence or the absence of SMase (Figure 4C, 4D). Finally, at
17 higher extracellular concentrations of penetratin (30 μ M), the number of WT cells presenting massive staining
18 upon SM hydrolysis was significantly higher (Supplementary figure 5). More interestingly, stress fibers of actin
19 cytoskeleton were also much more visible (Supplementary figure 5). We could also detect an increase in the
20 fluorescence staining of the peptide in GAG^{neg} cells, sometimes with visible nucleation zones (Supplementary
21 figure 6).

22 **GAG-dependent endocytosis of penetratin and R₆W₃ is different from pinocytosis**

23 Altogether, the latter results show that the higher penetratin and R₆W₃ internalisation in WT cells compared to
24 GAG^{neg} cells following SM hydrolysis more likely corresponds to a boosting effect of endocytic processes
25 dependent on the presence of cell-surface GAGs. Because it was previously reported that GAG clustering might
26 be related to activation of macropinocytosis [33], we wanted to verify whether this is the case for these cell-
27 penetrating peptides.

28 Therefore, the selective role of GAGs in the massive internalisation of penetratin and R₆W₃ was further
29 examined for two chemically unrelated general endocytosis markers: FM1-43 for plasma membrane labeling,
30 and FITC-Dextran (4.4 kDa) as a bulk marker. Unlike CPPs, FM1-43 and FITC-Dextran showed similar
31 enhancement of internalisation during SM hydrolysis in both cell lines (Supplementary figure 7). In particular,
32 labelling of cells with the bulk marker FITC-Dextran was massive in all WT and GAG^{neg} cells. Furthermore, the
33 addition of any of the five CPPs (10 μ M) did not change the intensity and distribution of the endocytic markers,
34 be it with or without SMase treatment. Similar results were obtained with FITC-Dextran of higher molecular
35 weight (70 kDa). These results show that the entry pathway of penetratin and R₆W₃ in WT cells, is selectively
36 driven by the presence of GAGs and is likely distinct from macropinocytosis and general endocytosis.

37 We further tested this hypothesis by analysing if the internalisation in WT cells of a low-efficient CPP, R₆L₃,
38 could be affected by the co-presence of the highly efficient analogue R₆W₃. The high efficiency of internalisation
39 of R₆W₃ was indeed found related to its high binding affinity for heparin and chondroitin sulfate and its
40 propensity to induce GAG clustering, in contrast to R₆L₃ entry which is not dependent on cells-surface GAGs
41 [18] (Figure 1). After incubation of cells for 5-15 min with R₆L₃ (10 μ M) to allow its adsorption at the cell-
42 surface, R₆W₃ (2 or 10 μ M) was added, for additional 60 min incubation. For this experiment, only the R₆L₃
43 peptide contained a N-terminal biotin label to allow its capture with streptavidin-coated magnetic beads.
44 Quantification of R₆L₃ in these conditions showed that the presence of R₆W₃ decreases the entry of R₆L₃ in cells.
45 The presence of 2 μ M R₆W₃ evoked a two-fold inhibition of the entry ($0.15 \pm 0.05 \mu$ M versus $0.30 \pm 0.06 \mu$ M),
46 while increasing the concentration of R₆W₃ to 10 μ M prevented totally the intracellular detection of R₆L₃. These
47 results support the idea that R₆W₃ GAG-dependent entry is an internalisation pathway that is distinct from bulk-
48 phase pinocytosis (macropinocytosis), otherwise it would have stimulated or enhanced the entry of R₆L₃.

49 **Cholesterol depletion enhances the internalisation of penetratin but not of Tat in the presence of cell- 50 surface GAGs**

51 Cholesterol strongly interacts with SM and is responsible for the formation of liquid-ordered (L_o) domains in the
52 cell plasma membrane. These L_o plasma domains are implicated in various clathrin-independent endocytic
53 routes. Partial removing of membrane cholesterol would thus be interesting to test the role of L_o domains in the
54 entry of CPPs. A simple method for depleting cholesterol relies on its extraction with methyl- β -cyclodextrin
55 (M β CD) [34]. Such M β CD treatments were previously reported to decrease the internalisation of Tat peptide
56 [35] and penetratin [36], a result interpreted as the inhibition of raft-dependent endocytosis of these peptides
57 [37,38].
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Using M β CD, we found herein the necessity to wash this compound out before adding peptides to the cells. We measured indeed a decrease in Tat and penetratin internalisation in the presence of M β CD (2 to 3 fold less internalised peptides) compared to the quantity internalised in the absence of M β CD treatment. This decrease was not due to the inhibition of raft-dependent endocytosis but likely to the binding and entrapment of the peptides into the hydrophobic cavity of the sugar ring through the aromatic side-chains of Phe, Tyr and Trp amino acids as reported [39], which leads to depletion of the free peptide from the extracellular milieu. Rodal *et al.* previously reported that upon M β CD treatment the uptake of transferrin was still inhibited by ~20% after 1h incubation even when serum or cholesterol was added; total recovery of cholesterol in the plasma membrane was reached 3 hours after treatment with M β CD [40]. Thus, the experimental conditions we used were to incubate cells with M β CD, and to wash cells quickly before further incubation with CPPs. On the other hand, we compared the effect of M β CD treatment in WT and GAG^{neg} cells, both cell lines having similar quantities of plasma membrane cholesterol [41,42]. M β CD treatment of cells did not influence cell viability (supplementary figure 8).

Using this protocol for cholesterol depletion, there was no significant effect on the internalisation of penetratin at low micromolar extracellular concentration (1 μ M) in WT and GAG^{neg} cells (Figure 4), a concentration at which the peptide directly translocates into cells and does not trigger GAG-dependent endocytosis [24,18]. However, at higher concentration of penetratin (10 μ M), a 50% increase in its internalisation was quantified in WT cells, while there was no significant difference in its internalisation in GAG^{neg} cells. In contrast, there was no significant difference in the internalisation of Tat in both cell lines after M β CD treatment even at 10 μ M extracellular peptide concentration. M β CD treatment has been associated to a fluidification of the PM and thus an increase in peptide translocation [12]. However, the fact that for penetratin we did not see the same effect in GAG^{neg} and WT cells indicates that its increased uptake upon M β CD treatment should be related to a GAG-dependent entry.

Binding of penetratin and Tat to LUVs mimicking cholesterol- and sphingomyelin-rich membrane domains

To further compare the interaction of the peptides with the membrane lipids, we measured the binding of penetratin and Tat peptide to large unilamellar vesicles (LUVs) mimicking cholesterol- and sphingomyelin-rich membrane domains, with or without additional ceramide. Various mixtures of cholesterol, SM, PC representative (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC)) and ceramide (N-palmitoylsphingosine, C₁₆-Ceramide) were used to form the liposomes [43]. PG (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG)) was added in small amounts (5% mole) as the single negatively charged lipid to minimise fusion of the vesicles. Control liposomes made of PC/PG (95/5) were also studied in interaction with the two peptides (Table 3). The binding constant measured by ITC is an apparent binding constant (K^{app}) reflecting three putative steps of the peptide binding to LUVs: adsorption of the peptide at the membrane surface (interaction with polar heads of phospholipids), hydrophobic penetration between fatty acyl chains and change of peptide conformation. Penetratin interacted better with LUVs mimicking rafts compared to pure PC/PG, and the presence of ceramide did not have any significant impact on the binding of the peptide to LUVs. As for Tat peptide, there was no difference between the interaction with the raft-like mixtures and pure PC/PG. But the binding of Tat increased deeply (~10 fold) in the presence of ceramide in the raft-like mixtures.

These results highlight the importance of both electrostatic and hydrophobic interactions in the binding of the peptides to lipid vesicles. Indeed, addition of ceramide to vesicles likely clustered or segregated the negatively charged PG, which increased the binding of Tat peptide (8 positive charges and no Trp) to the lipid vesicles. In the case of penetratin (7 positive charges and 2 Trp), the two tryptophans add a hydrophobic contribution to the interaction with LUVs, such that the global binding of the peptide did not change significantly in the presence or the absence of ceramide. These results also show that there is no direct relationship between the affinity of these peptides for ceramide-enriched membranes and their efficacy to internalise into cells.

Discussion

We found that SM hydrolysis (or ceramide generation) and cholesterol depletion in the cell plasma membrane increased the internalisation of cationic CPPs (Table 2). This increased efficiency of entry was much higher in cells containing chondroitin and heparan sulfate proteoglycans compared to cells depleted in these GAGs. This effect on the internalisation efficiency was also found higher for CPP sequences that contain tryptophan residues (penetratin and R₆W₃) compared to arginine-rich peptides with no tryptophan (R₉, Tat and R₆L₃) and confirmed previous results about the role of Trp in the interaction of peptides with GAGs [18].

The results also suggest that GAG-clustering is enhanced by conversion of SM into ceramide. Ceramide formation was proposed to lead to the reorganisation of membrane lipid domains into larger platforms, which could serve as a trap and cluster receptors [44,45]. For example, generation of ceramide through endogenous SMase action is used by some pathogens to enter host cells by receptor clustering [46]. Activation of

1 endogenous SMase was also proposed as a way for cell-penetrating peptides to enter cells when used at high-
2 concentrations [17].

3 Treatment of CHO cells with exogenous SMase has been found to induce rapid formation of endocytic vesicles,
4 which are 400 nm in diameter and not enriched in clathrin or caveolin, and that pinch off from the plasma
5 membrane and move into the cytosol [47]. Holopainen *et al.* attributed the endocytic budding vesicles to the
6 tendency of ceramide to separate into domains and to form negative spontaneous curvature, leading to membrane
7 invagination [48]. In this study, this mechanical energy-independent endocytosis (discussed below) was likely
8 more visible in GAG^{neg} cells, for which it was no longer masked by the important effect of GAG clustering.
9 SMase treatment induces lateral displacement of cholesterol in the plasma membrane of living cells (maximum
10 depletion 60 min after the addition of SMase) [49-52], probably due to the formation of ceramide [53] and
11 segregation of lipids. Following SMase treatment, recovery of cholesterol-rich plasma membrane domains is at
12 the same rate as SM is resynthesized from ceramide (partial recovery 180 min after the addition of SMase)
13 [51,52]. Hence, ceramide generation leads to the disruption of cholesterol-rich microdomains from the plasma
14 membrane [54], making it somehow similar to the effect of depleting cholesterol. For instance, the increase in
15 the internalisation of the cell-penetrating peptide R₈ upon MβCD treatment had already been reported [12], and
16 attributed to a fluidification of the plasma membrane leading to an increase in peptide translocation. We confirm
17 herein this previous assumption, since similar boosting effect of SMase treatment was observed on R₉ entry into
18 GAG^{neg} cells compared to WT cells, when R₉ entry in GAG^{neg} cells mostly relies on direct translocation across
19 the cell membrane [24].

20 Cholesterol depletion induces aggregation of plasma membrane domains that contain signalling molecules and
21 are involved in endocytosis and in actin polymerisation [55,56]. Thus, penetratin and R₆W₃ interact strongly with
22 and cluster GAGs present in SM- and cholesterol-rich domains to induce endocytosis, a segregation process
23 expected to be enhanced when the cell membrane is enriched in ceramide or depleted in cholesterol.
24 Furthermore, our data support the idea that GAG-clustering and GAG-dependent endocytosis activated by these
25 CPPs, is distinct from bulk-phase pinocytosis. Indeed upon SMase treatment we found similar entry of bulk
26 phase endocytic markers in the two cell types. Moreover the concomitant incubation of a peptide that does not
27 cluster GAGs (R₆L₃) with a high-efficient one (R₆W₃) [18], led to a decreased entry of the former. In the case of
28 GAG-activated macropinocytosis it would have been expected that more R₆L₃ would have entered into cells.

29 On the other hand, in the absence of GAGs another route of entry is brought out, likely at the level of the lipid
30 bilayer. We previously reported that in the absence of GAGs, the main route of entry of R₉, penetratin and R₆W₃
31 is direct translocation across the plasma membrane [18,24]. Herein, formation of ceramide-enriched domains
32 also segregates and increases the clustering of charged lipids and thus the charge density in domains of the
33 plasma membrane. In addition to this propensity to promote phase separation, ceramide is known to induce
34 negative curvature and mechanical stress in membrane [57]. Hence R₉, penetratin and R₆W₃ could exploit the
35 negative curvature induced by ceramide formation to evoke mechanical endocytosis as reported in membrane
36 models for penetratin [15]. Concomitantly, CPP binding to cell membranes causes surface tension because of the
37 perturbation of polar head groups and acyl chains packing of the lipid bilayer. Because of the physico-chemical
38 properties of ceramide, the frequency of formation of defects is thereby enhanced when ceramide is produced in
39 the membrane. The increased frequency of events such as membrane thinning and formation of defects in the
40 bilayer [58], both decrease the energetic penalty necessary to form a transient pore that would allow
41 translocation of the CPP inside cells, thanks to the membrane potential driving-force [59].
42

43 **Conclusions**

44 To conclude, considering the results obtained with SMase and with cholesterol-depletion, we propose a model
45 for the effect of ceramide-generation on the internalisation of CPPs (Figure 5). An efficient cell-penetrating
46 peptide would bind, recruit and cluster specific membrane lipids, and thus evoke, stabilise locally and exploit
47 membrane thinning and defects to translocate. Alternatively, the CPP can bind and cluster GAGs that act as
48 autonomous receptors and follow a specific endocytosis pathway whose mechanism remains to be fully
49 understood [60, 61]. The kinetics of a given CPP to partition between cell-surface GAGs and in defect regions of
50 the lipid bilayer would determine the balance between endocytosis and translocation pathways of internalisation.
51

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57

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Table 1 - Sequence of the peptides (biotin-Gly₄ at the N-terminus, carboxamide at the C-terminus) used in this study. Net positive charge (Z), number of arginine $n_{(R)}$ and tryptophan residues $n_{(W)}$.

Peptide	Sequence	Z	$n_{(R)}$	$n_{(W)}$
R ₉	RRRRRRRRR	+9	9	0
Tat(47-57)	YGRKKRRQRRR	+8	6	0
R ₆ L ₃	RLLRRLRR	+6	6	0
Penetratin	RQIKIWFQNRRMKWKK	+7	3	2
R ₆ W ₃	RRWRRRWR	+6	6	3

Table 2 – Impact of cell-surface treatment on internalization of CPPs in wild type and GAG^{neg} cells – Cells were incubated with 10 μ M peptide. Data are normalized for every peptide to the quantity measured in control wild-type cells. SMase, sphingomyelinase; HI, heparinase I; HIII, heparinase III; ChABC, chondroitinase ABC.

Peptide	Wild-type				GAG-deficient		
	SMase	HI, HIII, ChABC	SMase, HI, HIII, ChABC	M β CD	control	SMase	M β CD
R ₆ L ₃	2	-	-	-	1	1	-
Tat	3	-	-	1	1	1	1
R ₉	3	-	-	-	0.35	0.65	-
penetratin	6	0.75	0.71	2	0.60	0.40	1
R ₆ W ₃	8	-	-	-	0.70	1.35	-

Table 3 - Binding of penetratin and Tat peptide to LUVs of different lipid content. PC/PG: 95/5 mol%; Chol/SM/PC/PG: 20/50/25/5 mol%; Chol/SM/Cer/PC/PG: 20/40/10/25/5. Chol, cholesterol; Cer, ceramide. For calculation of K^{app} , it was assumed that 50% of the total lipids were accessible to the peptide in LUV.

Lipid mixture	K^{app} (μ M)	
	Penetratin	Tat
PC/PG	9.0 \pm 5.0	12 \pm 7.5
Chol/SM/PC/PG	2.7 \pm 0.5	10 \pm 0.3
Chol/SM/Cer/PC/PG	1.7 \pm 1.1	0.8 \pm 0.2

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14 **Figure legends**
15

16 **Figure 1: Quantification of the internalisation of the CPPs, studied in the presence and the absence of**
17 **SMase, in WT and GAG^{neg} cells at 37 °C.** 10⁶ cells were incubated with the peptides in the presence or the
18 absence of 1U SMase, in 1 mL DMEM, at 37 °C for 70 min. The amounts of internalised peptides (μM) were
19 then quantified using a MALDI-TOF MS reported protocol[27,28]. Experiments were done in duplicate or
20 triplicate, and repeated at least three times independently. Significance was tested using a Welch's corrected t test
21 (ns p>0.05, * 0.05<p>0.01, ** 0.01<p>0.001, *** p<0.001).
22

23 **Figure 2: Ceramide quantification upon sphingomyelinase treatment of (A) WT cells or (B) GAG^{neg} cells.**
24 10⁶ cells in suspension were incubated with or without 0.05 U SMase at 37 °C for 30 min. Fixed cells were then
25 labeled with anti-ceramide (1mg/ml) mouse IgM and FITC-labeled anti-mouse IgM. Experiments were done in
26 triplicate. Significance was provided using a Kolmorov-Smirnov test: (A) D = 0.43, (B) D = 0.92.
27

28 **Figure 3: Confocal microscopy of penetratin internalisation** (10 μM extracellular concentration) in WT cells
29 in the absence (A) and the presence (B) of SMase, and in GAG^{neg} cells in the absence (C) and the presence (D)
30 of SMase. The peptide is labeled in red (streptavidin-TRITC), the actin cytoskeleton protein in green (phalloidin-
31 FITC) and the cell nuclei in blue (DAPI). Scale bar corresponds to 10 μm. Unmerged pictures are shown in
32 Supplementary figure 3.
33

34 **Figure 4: Confocal microscopy of Tat peptide internalisation** (10 μM extracellular concentration) in WT cells
35 in the absence (A) and the presence (B) of SMase, and in GAG^{neg} cells in the absence (C) and the presence (D)
36 of SMase. The peptide is labeled in red (streptavidin-TRITC), the actin cytoskeleton protein in green (phalloidin-
37 FITC) and the cell nuclei in blue (DAPI). Scale corresponds to 10 μm. Unmerged pictures are shown in
38 Supplementary figure 4.
39

40 **Figure 5: Quantification of the internalisation of penetratin and Tat peptide in the presence and the**
41 **absence of MβCD,** in WT and GAG^{neg} cells at 37 °C. 10⁶ cells were incubated with 5 mM MβCD at 37 °C for
42 30 min, cells were washed then incubated with the different peptides in 1 mL DMEM, at 37 °C for 70 min. The
43 amounts of internalised peptides (μM) were then quantified using a MALDI-TOF MS protocol [27,28].
44 Experiments were done in duplicate or triplicate, and repeated at least three times independently. Significance
45 was tested using a Welch's corrected t test (ns p>0.05, * 0.05<p>0.01, ** 0.01<p>0.001, *** p<0.001).
46

47 **Figure 6: Proposed mechanism for the enhanced internalisation of CPPs due to ceramide-enriched**
48 **domain formation.** The positively charged peptides interact with the negatively charged lipids and GAGs on the
49 cell surface. SMase hydrolyses extracellular leaflet SM, present in lipid rafts, which causes the generation of
50 ceramide-enriched domains. This enhances clustering and subsequent endocytosis of GAGs, which is massive in
51 the case of tryptophan-containing basic CPPs. On the other hand, arginine-rich CPPs can translocate directly into
52 the cytoplasm at the defective interfaces between ceramide-enriched domains and the bulk of the plasma
53 membrane.
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Figure 1
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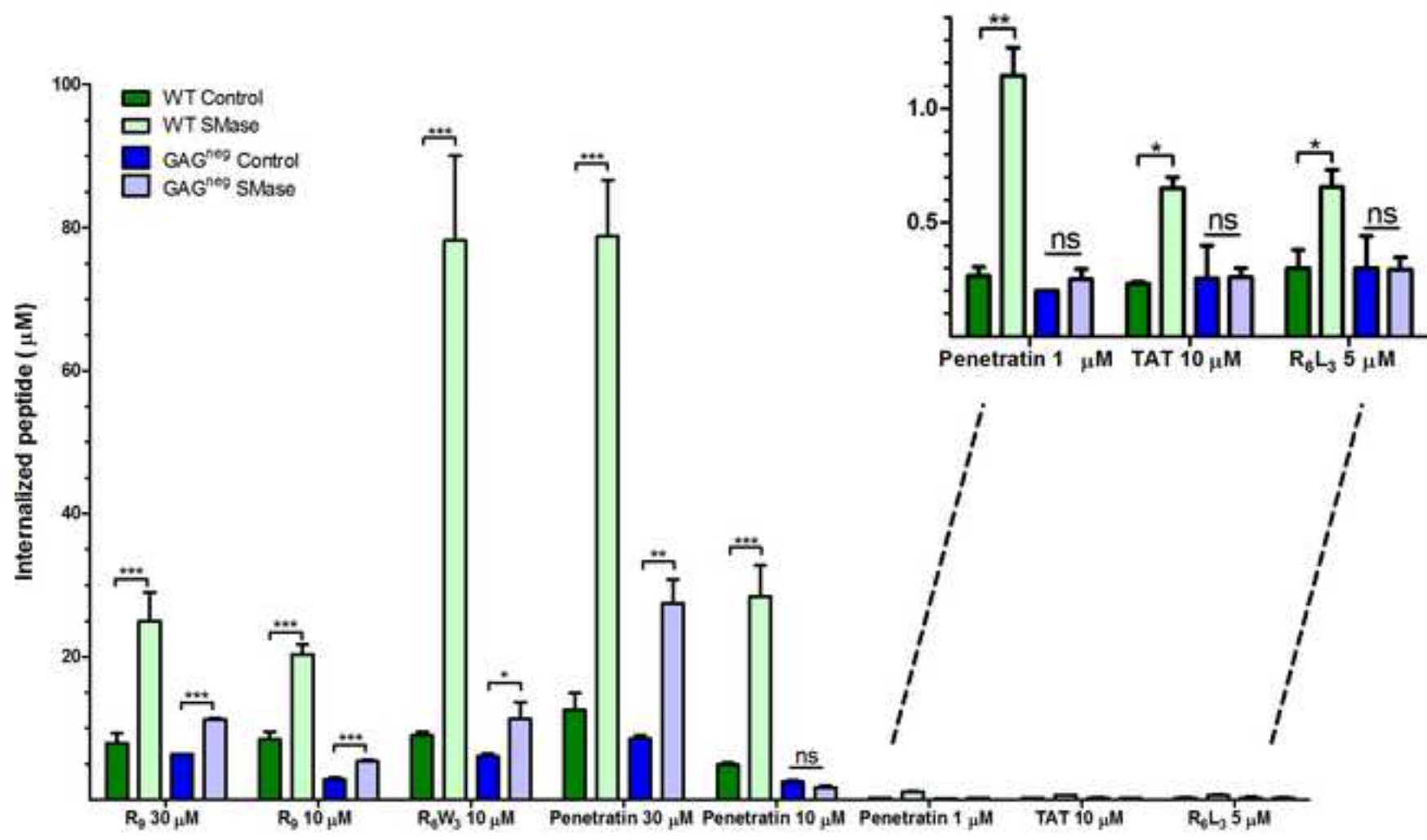


Figure 2

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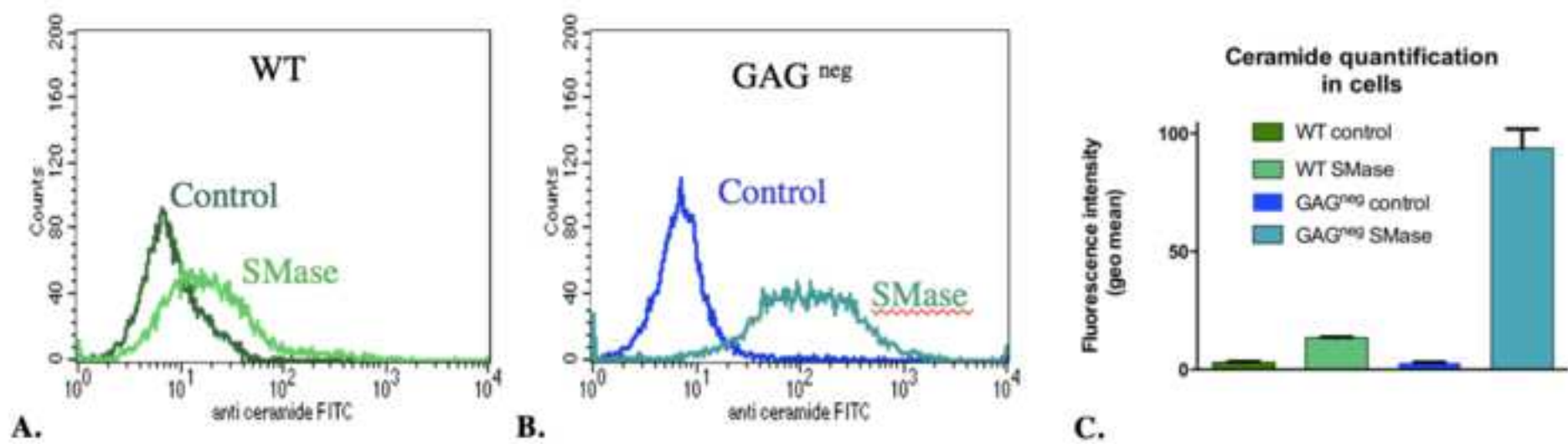


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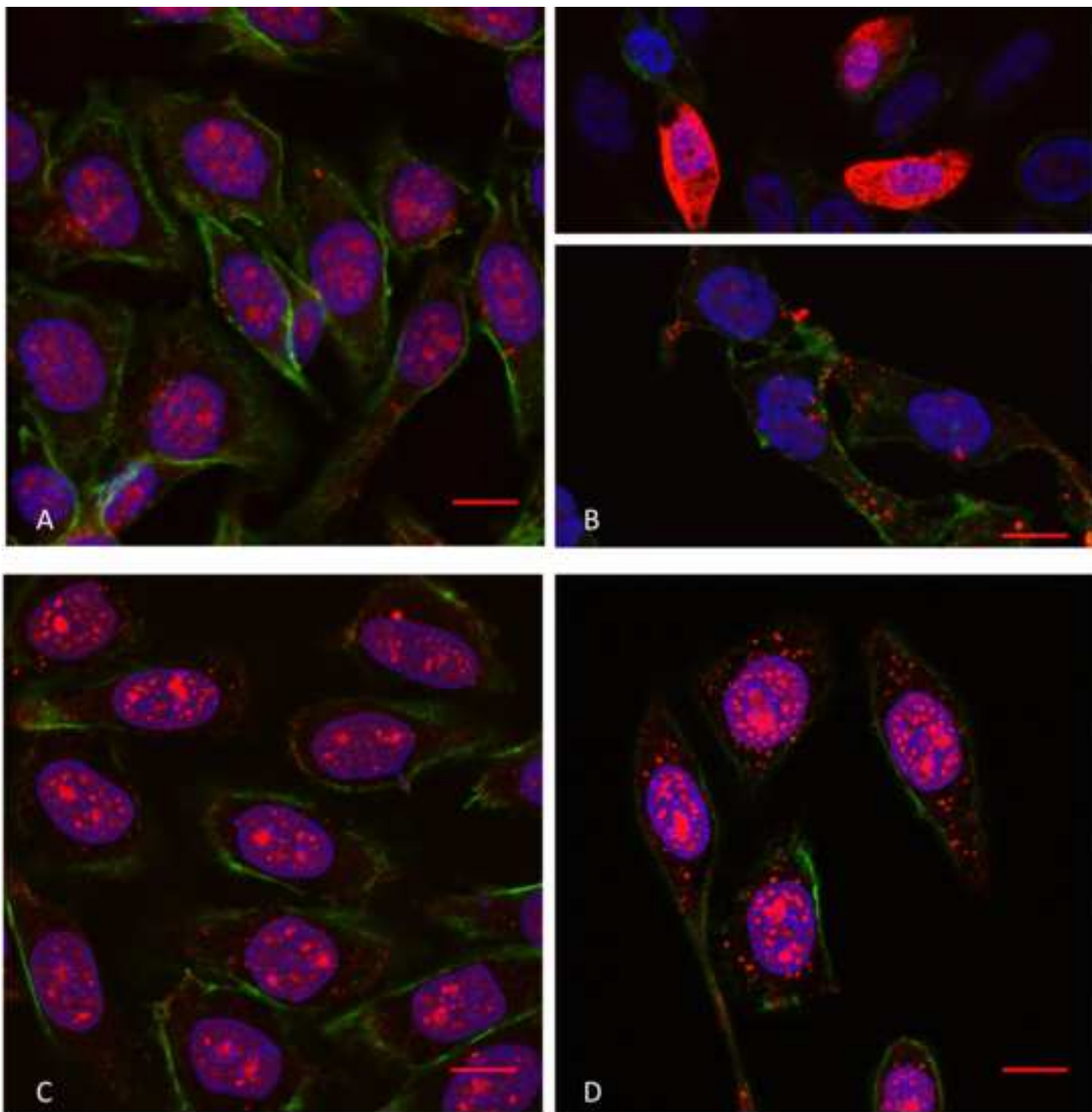


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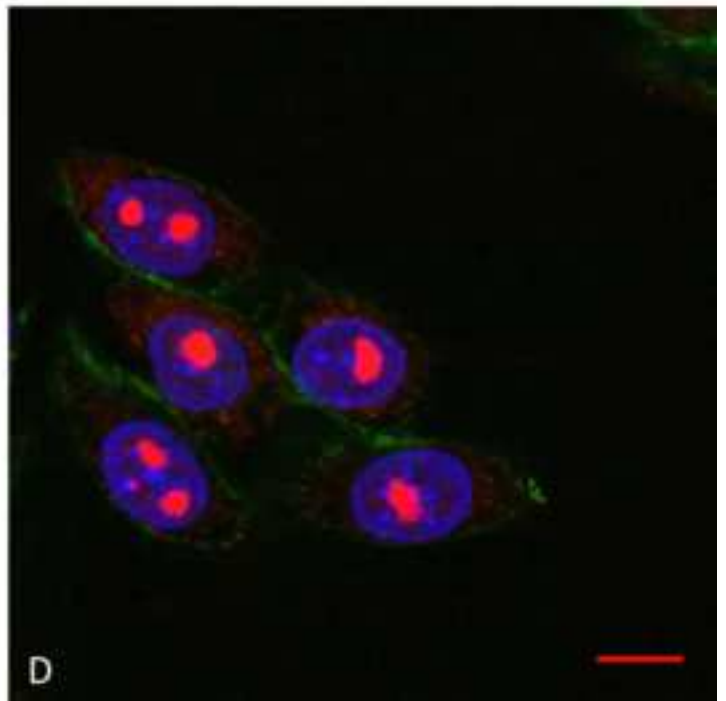
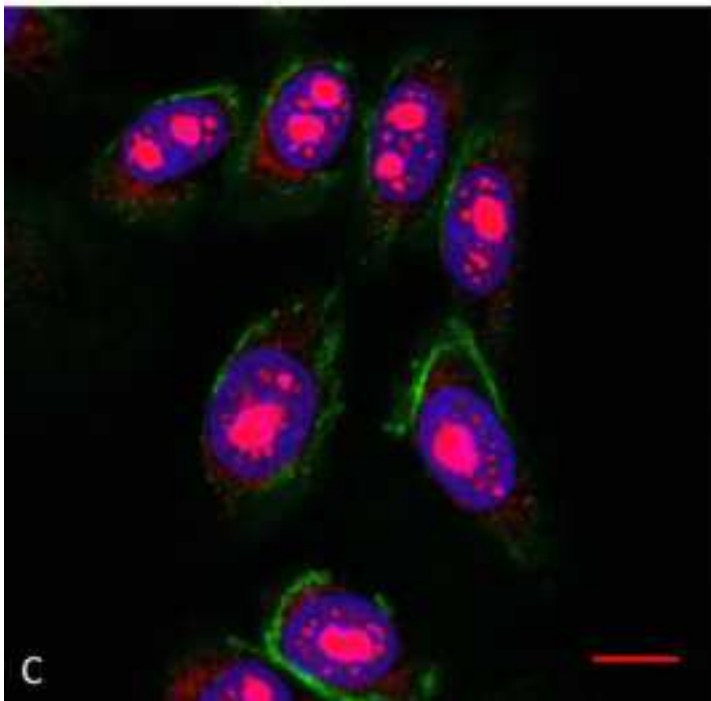
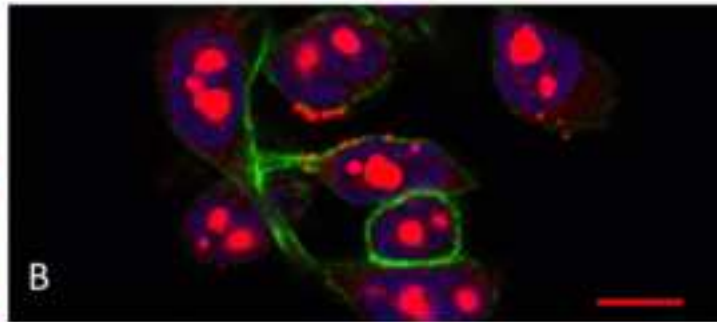
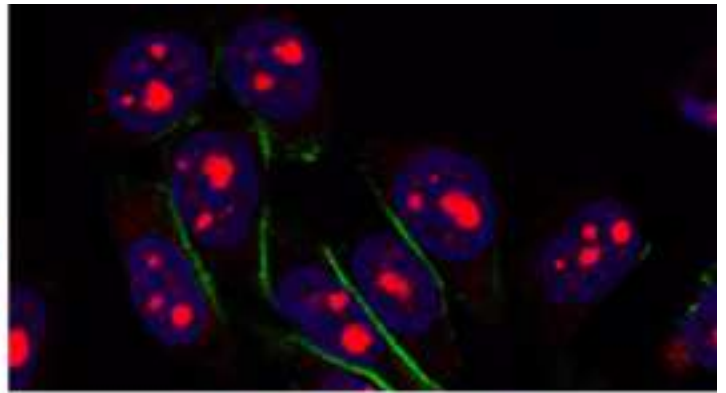
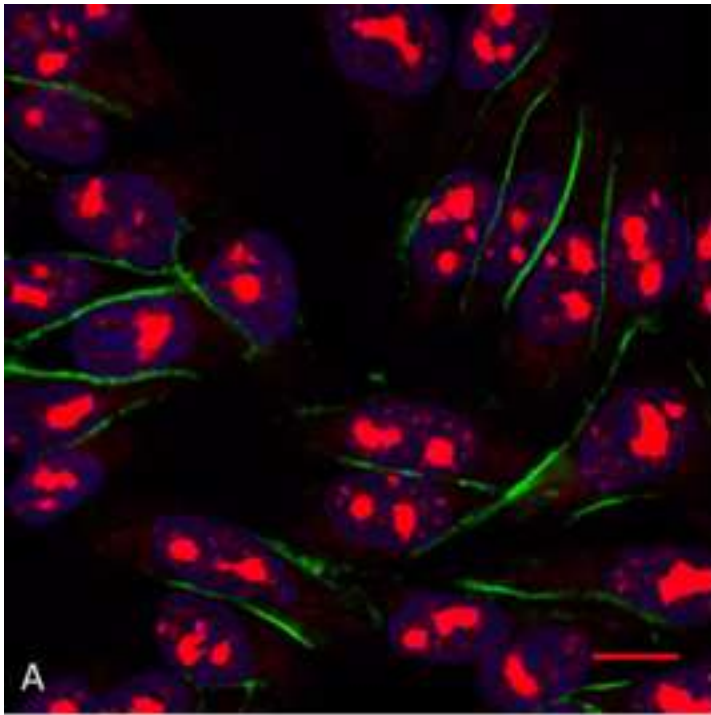


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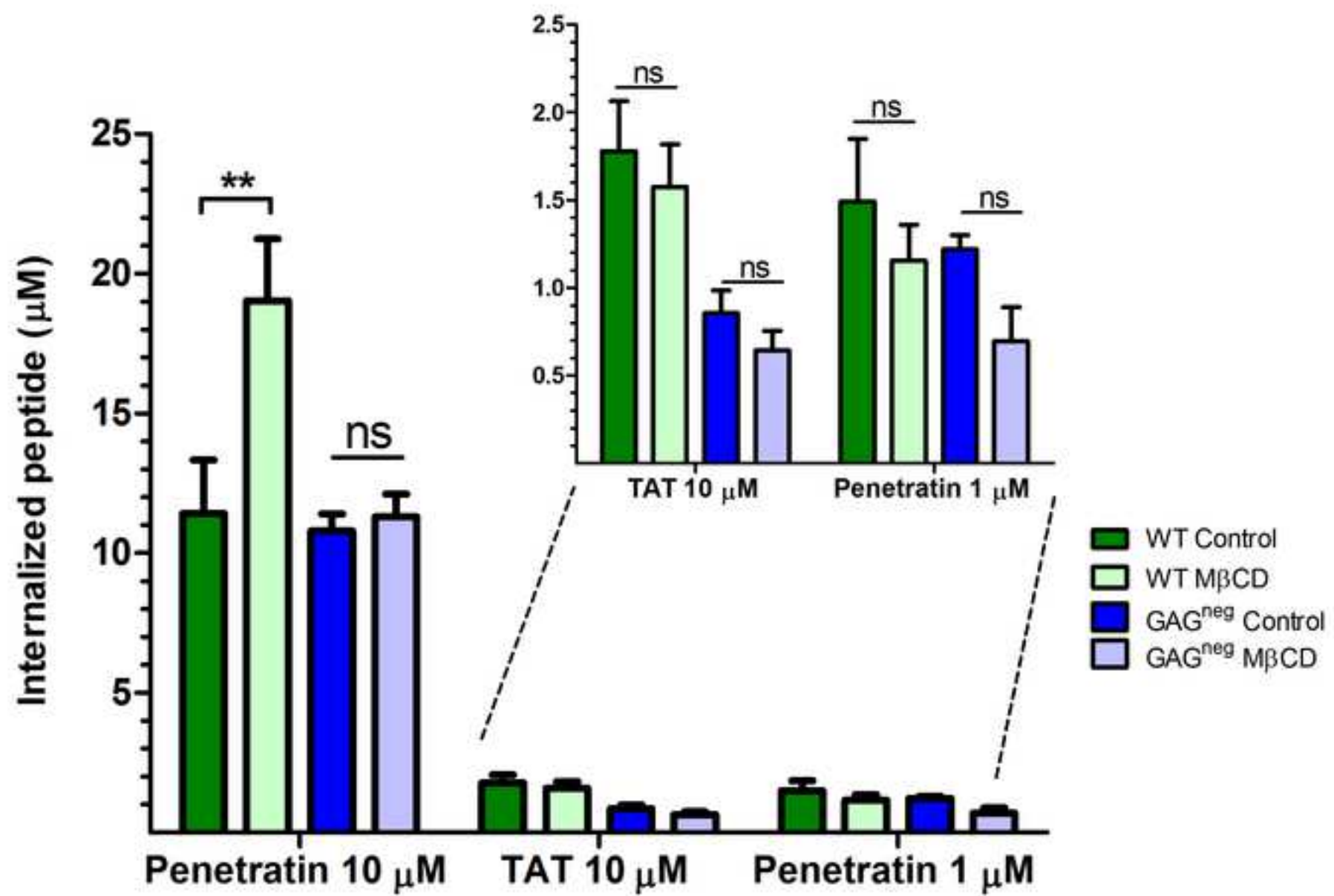


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