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## Small Efficient Cell Penetrating Peptides Derived From the Scorpion Toxin Maurocalcine\*

Cathy Poillot<sup>1,2</sup>, Hicham Bichraoui<sup>1,2</sup>, Céline Tisseyre<sup>1,2</sup>, Eloi Bahemberae<sup>1,2</sup>, Nicolas Andreotti<sup>3</sup>, Jean-Marc Sabatier<sup>3</sup>, Michel Ronjat<sup>1,2</sup> & Michel De Waard<sup>1,2,4</sup>

<sup>1</sup>From Inserm U836, Grenoble Neuroscience Institute, Site santé Tronche, Chemin Fortuné Ferrini, BP170, 38042 Grenoble Cedex 9, France.

<sup>2</sup>Université Joseph Fourier, Grenoble, France.

<sup>3</sup>Inserm UMR1097, Luminy, France ; <sup>4</sup>Smartox Biotechnologies, Floralis, Biopolis, 5 Avenue du Grand Sablon, 38700 La Tronche, France.

Running title: *Small Cell Penetrating Maurocalcine Peptides*

To whom correspondence should be addressed: Dr. Michel De Waard, GIN, Inserm U836, BP170, Grenoble Cedex 09, France. Phone: +(33) 4 56 52 05 63 - Fax: +(33) 4 56 52 06 37 - E-mail: [michel.dewaard@ujf-grenoble.fr](mailto:michel.dewaard@ujf-grenoble.fr)

**Keywords:** maurocalcine; cell penetrating peptides; cell delivery; fluorescence

**Background:** This study aimed at developing a new set of maurocalcine-derived cell penetrating peptides from truncation.

**Results:** Several truncated peptides were designed and evaluated for Cy5 dye cell penetration.

**Conclusion:** All truncated peptides are competitive cell penetrating peptides, many of them comparing favorably well to TAT.

**Significance:** maurocalcine-derived truncated cell penetrating peptides differ in their properties enlarging the potential fields of applications.

### SUMMARY

Maurocalcine is the first demonstrated example of an animal toxin peptide with efficient cell penetration properties. While it is a highly competitive cell penetrating peptide (CPP), its relatively large size of 33 amino acids and the presence of three internal disulfide bridges may hamper its development for *in vitro* and *in vivo* applications. Here, we demonstrate that several efficient CPP can be derived from maurocalcine by replacing Cys residues by isosteric 2-aminobutyric acid residues and sequence truncation down to peptides of up to 9 residues in length. A surprising finding is that all the truncated maurocalcine analogues possessed cell penetrating properties indicating that the maurocalcine is a highly specialized CPP. Careful examination of the cell penetrating properties of the truncated analogues indicates that several

**maurocalcine-derived peptides should be of great interest for cell delivery applications where peptide size matters.**

Maurocalcine (MCA) is a 33-mer peptide that was initially isolated from the venom of a Tunisian chactid scorpion, *Scorpio maurus palmatus* (1). The toxin belongs to a family of peptide that folds according to an Inhibitor Cystine Knot (ICK motif), and thus contains three disulfide bridges with a Cys<sup>1</sup>-Cys<sup>4</sup>, Cys<sup>2</sup>-Cys<sup>5</sup> and Cys<sup>3</sup>-Cys<sup>6</sup> connecting pattern (2). The solution structure, as defined by <sup>1</sup>H-NMR, illustrates that MCA contains three  $\beta$ -strands (strand 1 from amino acid residues 9 to 11, strand 2 from 20 to 23, and strand 3 from 30 to 33). One distinctiveness of MCA is the fact that it is greatly enriched in basic amino acid residues. Out of the 33 amino acids that compose MCA, twelve of them are basic, most of them represented by Lys residues. Interestingly, the  $\beta$ -strands of MCA encompass most of the basic domains (see Fig. 1A). MCA turned to be of interest to our research group for several reasons. First, it is an exquisite pharmacological activator of the ryanodine receptor type 1 (RyR1) from skeletal muscle since it promotes high Po gating modes and long-lasting subconductance states of the ion channel (3,4). On myotubes, application of MCA rapidly induces Ca<sup>2+</sup> release from the sarcoplasmic reticulum (SR) (5), a result further confirmed by positive effect of MCA on the release of Ca<sup>2+</sup> from purified SR vesicles (3,5). The interaction of MCA

with RyR1 has been witnessed by increased [<sup>3</sup>H]-ryanodine binding onto purified RyR1 (3,5). The binding site for M<sub>Ca</sub> on RyR1 has also been mapped and shown to correspond to domain(s) that have a predicted localization within the cytoplasm (6). Second, similarly to imperatoxin A (7) for which this was first noted, M<sub>Ca</sub> has an interesting sequence homology with the II-III loop of the L-type calcium channel Ca<sub>v</sub>1.1 subunit over a domain that is slightly larger than the second β-strand of M<sub>Ca</sub> (see Fig. 1A) (5). This loop is predominantly involved in excitation-contraction coupling through direct molecular interactions with RyR1 (6,8). This homology has been a source of inspiration to understand how toxins may interfere with the process of excitation-contraction coupling (4,8,9). Third, and this is the scope of this manuscript, M<sub>Ca</sub> has been shown to act as a cell penetrating peptide (CPP) (10). This discovery stemmed from earlier criticisms that M<sub>Ca</sub> may not be an activator of RyR1 because peptide toxins were not known to cross the plasma membrane, which would be required here to bind to RyR1. Studies that were undertaken to demonstrate the ability of M<sub>Ca</sub> to reach its target showed that i) M<sub>Ca</sub> triggers Ca<sup>2+</sup> release from the sarcoplasmic reticulum a few seconds after its application in the extracellular medium (5), and ii) intracellular accumulation of fluorescent-streptavidin occurs if it incubated first with biotinylated M<sub>Ca</sub> (10). Since these pioneering studies, M<sub>Ca</sub> or analogues thereof proved powerful vectors for the cell entry of proteins, peptides (11), nanoparticles, or drugs such as doxorubicin (12-15). Although the mode of cell penetration of M<sub>Ca</sub> may vary according to cargo nature, cell type or chemical linkage employed, the data gathered so far suggest that the peptide may enter cells according to two priming steps onto the plasma membrane: first an interaction with proteoglycans with an affinity in the micromolar range, followed by a second interaction with negatively charged lipids which occurs with greater affinity (16,17). The mode of cell entry of M<sub>Ca</sub> is not altered by the absence of proteoglycans, but simply reduced quantitatively, suggesting that proteoglycans do not orient the mode of cell penetration. Two modes seem to concur to M<sub>Ca</sub> cell entry, as far as observed, one related to macropinocytosis and another to membrane translocation. The balance between both modes of entry was found correlated to cargo nature and the type of M<sub>Ca</sub> analogue used.

It is of great interest to pursue the study of M<sub>Ca</sub> as CPP in spite of the wealth of new CPP sequences that are discovered yearly. Among the competitive advantage of M<sub>Ca</sub> over other CPP sequences are the facts that it has almost no associated toxicity *in vitro* and *in vivo*, penetrates into cells at very low concentrations, and is extremely stable *in vivo* upon intravenous injection (over 24 hrs - manuscript in preparation).

While M<sub>Ca</sub> appears as an elaborate and efficient CPP, its pharmacological properties represent a serious hindrance while envisioning *in vitro* and *in vivo* applications. In addition, because of its length (33 amino acid residues) and the presence of three disulfide bridges, M<sub>Ca</sub> is a relatively difficult to synthesize CPP, comparatively to other CPP, and would benefit from a downsizing approach. Several strategies have been successfully employed in the past to overcome one or both of these issues. The first strategy was based on single point mutations of M<sub>Ca</sub> sequence. This strategy preserved the disulfide bridges and the 3D structure of the analogues. Overall, mutations affected more seriously the pharmacology of M<sub>Ca</sub> than the cell penetration properties (18). Many of the amino acids involved in RyR1 binding and pharmacology were located within the cluster of basic amino acids that presented sequence homology with the L-type Ca<sub>v</sub>1.1 channel. Some of these residues, but not all, were also important for cell penetration properties. Hence, several analogues could be defined that kept close to intact cell penetration properties while entirely losing their pharmacological action (M<sub>Ca</sub> R24A for instance). Some other analogues were actually better than M<sub>Ca</sub> itself for cell penetration suggesting that pairs of mutations, aiming at disrupting pharmacology and improving penetration, may be used in the future to define still better CPP analogues of M<sub>Ca</sub>. The second strategy, that has yield success, is based on the chemical synthesis of D-M<sub>Ca</sub>, an analogue entirely based on the use of D-amino acids. This peptide is a mirror image of the natural L-M<sub>Ca</sub> but, like other D-CPP, preserves its cell penetration properties, while losing entirely its ability to interact with RyR1 (19). This method has several advantages. It no longer is sensitive to proteases which may be an additional advantage for *in vivo* experiments where the half-life of the circulating peptide matters. It is also possible to improve this analogue by introducing point mutations shown previously to improve cell penetration (19). In

these two strategies, while being effective, one may argue that i) the peptides are still among the longest CPP known to date, implying increased costs of production, and ii) the yield of production of these peptides is hampered by the folding process. Also, the use of peptides with internal disulfide bridges, despite having advantageous features in terms of stability *in vivo*, makes chemical coupling of these CPP to cargoes more complicated (difficulty to add extra Cys residues to the peptides for instance without interfering with the correct folding process). The third strategy that was used to circumvent one of these criticisms was the chemical synthesis of an M<sub>Ca</sub> analogue in which all internal Cys residues were replaced by isosteric 2-aminobutyric acid residues (11). The resulting peptide was still 33-mer long but one step in production was saved by avoiding the folding process. In addition, an extra-Cys residue could be added to the N-terminus of the peptide in order to favor simplified cargo grafting on this CPP analogue. This peptide, termed here C-M<sub>Ca</sub><sub>UF1-33</sub> (C for extra-Cys, UF for unfolded, and 1-33 for its length, Fig. 1B) has no longer any secondary structures, but efficiently penetrates into cells. Interestingly also, the peptide completely lacks pharmacological activity indicating that folding and secondary structures are essential for binding onto RyR1. While this peptide is an efficient CPP, it remains less potent than M<sub>Ca</sub> in its folded version suggesting that further optimization should be brought to this analogue. Such optimization appears feasible on the basis of the fact that M<sub>Ca</sub> fulfills three different functions (pharmacology, obligation to resemble the L-type channel, and cell penetration). We reasoned that since only cell penetration was the quality we searched for, the peptide could be further simplified and novel analogues be designed.

In this study, we undertook to identify new more potent M<sub>Ca</sub> analogues with three criteria in mind. First, these analogues should be shorter than the folded version of M<sub>Ca</sub> (M<sub>Ca<sub>F</sub></sub>) or the unfolded version (M<sub>Ca<sub>UF</sub></sub>). Second, these peptides should be designed in order to better delimitate the domains of M<sub>Ca</sub> responsible for cell penetration. Third, we should be able to design analogues with extra free SH functions for cargo grafting. We present several new analogues that have highly potent cell penetration capabilities, while losing pharmacological activity, preserving lack of cell toxicity, and with facilitated cargo grafting. This new generation of M<sub>Ca</sub> analogues is predicted to

have bright futures for CPP applications *in vitro* and *in vivo*.

## Experimental procedures

**Reagents** – N- $\alpha$ -Fmoc-L-aminoacid, Wang-Tentagel resin and reagents used for peptide syntheses were obtained from Iris Biotech. Solvents were analytical grade products from Acros Organics. Cy5 maleimide mono-reactive dye was purchased from GE Healthcare.

**Solid-phase peptide syntheses** - Chemical syntheses of M<sub>Ca</sub> analogues were performed as previously described (19). Briefly, analogues of M<sub>Ca</sub> were chemically synthesized by the solid-phase method (20) using an automated peptide synthesizer (CEM® Liberty). Peptide chains were assembled stepwise on 0.24 mEq of Fmoc-D-Arg-Pbf-Wang-Tentagel resin using 0.24 mmol of N- $\alpha$ -fluorenylmethyloxycarbonyl (Fmoc) L-amino-acid derivatives. The side-chain protecting groups were: Trityl for Cys and Asn, *tert*-butyl for Ser, Thr, Glu and Asp, Pbf for Arg and *tert*-butylcarbonyl for Lys. Reagents were at the following concentrations: Fmoc-amino-acids (0.2 M Fmoc-AA-OH in dimethylformamide (DMF)), activator (0.5 M 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate in DMF), activator base (2 M diisopropylethylamine in N-methyl-pyrrolidone (NMP)) and deprotecting agent (5% piperazine / 0.1 M 1-hydroxybenzotriazole in DMF), as advised by PepDriver (CEM®). After peptide chain assembly, resins were treated 4 hrs at room temperature with a mixture of trifluoroacetic acid/water/triisopropylsilan (TIS)/dithiothreitol (DTT) (92.5/2.5/2.5/2.5). The peptide mixtures were then filtered and the filtrates were precipitated by adding cold *t*-butylmethyl ether. The crude peptides were pelleted by centrifugation (10.000  $\times$  g, 15 min) and the supernatants were discarded. M<sub>Ca</sub> analogues were purified by HPLC using a Vydac C18 column (218TP1010, 25 $\times$ 10 cm). Elutions of the peptides were performed with a 10-60% acetonitrile linear gradient containing 0.1% trifluoroacetic acid. The purified fractions were analyzed by analytical RP-HPLC (Vydac C18 column 218TP104, 25  $\times$  4.6 cm). All analogues were characterized by MALDI-TOF mass spectrometry.

**Labeling of peptide with Cy5** - Each peptide was labeled with Cy5 according to the manufacturer's protocol (GE Healthcare). Peptides were dissolved

at 1 mg/ml in 0.1 M Na<sub>2</sub>CO<sub>3</sub> buffer, pH 9.3. 300 µl of the solubilized peptides were added to Cy5-maleimide containing tubes. The mixtures were incubated during 2 hrs at room temperature and then purified by HPLC using an analytical Vydac C18 column. Elution of the Cy5-labeled peptides was performed with a 10-60% acetonitrile linear gradient containing 0.1% trifluoroacetic acid. The pure peak fractions were lyophilized and peptides quantified by UV spectrophotometer at 649 nm.

*Cell culture* - Chinese hamster ovary (CHO) and F98 cell lines (from ATCC) were maintained at 37°C in 5% CO<sub>2</sub> in F-12 nutrient medium (Invitrogen) supplemented with 10% (v/v, CHO) or 2% (v/v, F98) heat-inactivated fetal bovine serum (Invitrogen) and 100 units/ml streptomycin and penicillin (Invitrogen).

*MTT assay* - Cells were seeded into 96-well micro plates at a density of approximately 8×10<sup>4</sup> cells/well. After 2 days of culture, the cells were incubated for 24 hrs at 37°C with MCa analogues at a concentration of 10 µM. Control wells containing cell culture medium alone or with cells, both without peptide addition, were included in each experiment. 0.1% saponin was used as toxic agent for comparison. The cells were then incubated with 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) for 30 min. Conversion of MTT into purple colored MTT formazan by the living cells indicates the extent of cell viability. The crystals were dissolved with dimethyl sulfoxide (DMSO) and the optical density was measured at 540 nm using a microplate reader (Biotek ELx-800, Mandel Scientific Inc.) for quantification of cell viability. All assays were run in triplicates.

*Confocal microscopy* - For analysis of the subcellular localization of MCa-Cy5 analogues in living cells, cell cultures were incubated with the fluorescent peptides for 2 hrs, and then washed with phosphate-buffered saline (PBS) alone. The plasma membrane was stained with 5 µg/ml rhodamine-conjugated concanavalin A (Molecular Probes) for 5 min. Cells were washed once more. Live cells were then immediately analyzed by confocal laser scanning microscopy using a Leica TCS-SPE operating system. Rhodamine (580 nm) and Cy5 (670 nm) were sequentially excited and emission fluorescence were collected in z-confocal planes of 10–15 nm steps.

*Fluorescence Activated Cell Sorting* - CHO cells were incubated with various concentrations of Cy5-labeled peptides in F-12K culture medium

without serum at 37°C for 2 hrs. The cells were then washed with PBS to remove excess extracellular peptide and treated with 1 mg/ml trypsin (Invitrogen) for 5 min at 37°C to detach cells from the surface, and centrifuged at 200 g before suspension in PBS. For experiments with the macropinocytosis inhibitor, amiloride, CHO cells were initially washed with F-12K and preincubated for 30 min at 37°C with 1 mM amiloride (Sigma). The cells were then incubated for 2 hrs at 37°C with 1 µM of the Cy5-MCa analogues. For all these experimental conditions, flow cytometry analyses were performed with live cells using a Becton Dickinson FACS LSR II flow cytometer (BD Biosciences). Data were obtained and analyzed using FCS express software (De Novo). Live cells were gated by forward/side scattering from a total of 10,000 events.

*Preparation of heavy SR vesicles* - Heavy SR vesicles were prepared following the method of Kim et al. (21). Protein concentration was measured by the Biuret method.

*[<sup>3</sup>H]-Ryanodine binding assay* - Heavy SR vesicles (1 mg/ml) were incubated at 37°C for 2 hrs in an assay buffer composed of 10 nM [<sup>3</sup>H]-ryanodine, 150 mM KCl, 2 mM EGTA, 2 mM CaCl<sub>2</sub> (pCa = 5), and 20 mM MOPS, pH 7.4. Truncated MCa analogues were added prior to the addition of heavy SR vesicles. [<sup>3</sup>H]-ryanodine bound to heavy SR vesicles was measured by filtration through Whatman GF/B glass filters followed by three washes with 5 ml of ice-cold washing buffer composed of 150 mM NaCl, 20 mM HEPES, pH 7.4. [<sup>3</sup>H]-ryanodine retained on the filters was measured by liquid scintillation. Non-specific binding was measured in the presence of 80 µM unlabeled ryanodine. The data are presented as mean ± S.E. Each experiment was performed in triplicate.

*Statistical analyses* - All data are given as mean ± SD for n number of observations, and statistical significance (p) was calculated using Student's t test.

## RESULTS

### *Non folded truncated maurocalcine peptides are efficient CPP*

Figure 1A illustrates the primary structure of MCa with its secondary structures (β strands) and its pattern of disulfide bridges. This peptide will be termed MCa<sub>F</sub>, for folded (F) MCa. An earlier report has demonstrated that replacing the six internal

cysteine residues of M<sub>Ca</sub> by Abu residues results in a pharmacologically-inert and unfolded (UF) CPP (M<sub>Ca</sub><sub>UF1-33</sub>, Fig. 1B). This peptide loses its secondary structures (11). Since this project aims at identifying shorter CPP sequences based on M<sub>Ca</sub><sub>UF1-33</sub> sequence by the delivery of Cy5 cargo, we first determined where at the N-terminus (C-M<sub>Ca</sub><sub>UF1-33</sub>) or C-terminus (M<sub>Ca</sub><sub>UF1-33</sub>-C) the cargo could be best grafted after addition of an extra cysteine residue (C) (Fig. 1B). As shown, both vector/cargo complexes Cy5-C-M<sub>Ca</sub><sub>UF1-33</sub> and M<sub>Ca</sub><sub>UF1-33</sub>-C-Cy5 penetrated efficiently within CHO cells, as estimated by confocal microscopy (Fig. 1C) or by FACS (Fig. 1D). At 3  $\mu$ M, a slightly better cell penetration was observed with Cy5 localized at the C-terminus of M<sub>Ca</sub><sub>UF1-33</sub>, but this difference was not significant. Since chemical syntheses of truncated M<sub>Ca</sub><sub>UF1-33</sub> analogues was facilitated by adding the extra cysteine residue at the C-terminus of the sequence rather than at the N-terminus, we kept on working on the basis of M<sub>Ca</sub><sub>UF1-33</sub>-C sequence. Nevertheless, these data indicate for the first time that cargo grafting on the CPP M<sub>Ca</sub><sub>UF1-33</sub> can be performed likewise at both extremities of the sequence.

Next, we designed a series of truncated M<sub>Ca</sub><sub>UF</sub>-C peptides comprising either a C-terminal truncation (3 analogues: M<sub>Ca</sub><sub>UF1-20</sub>-C, M<sub>Ca</sub><sub>UF1-15</sub>-C and M<sub>Ca</sub><sub>UF1-9</sub>-C), a N-terminal truncation (7 analogues: M<sub>Ca</sub><sub>UF8-33</sub>-C, M<sub>Ca</sub><sub>UF11-33</sub>-C, M<sub>Ca</sub><sub>UF14-33</sub>-C, M<sub>Ca</sub><sub>UF18-33</sub>-C, M<sub>Ca</sub><sub>UF20-33</sub>-C, M<sub>Ca</sub><sub>UF22-33</sub>-C and M<sub>Ca</sub><sub>UF25-33</sub>-C), and Both N- and C-terminal truncations (2 analogues: M<sub>Ca</sub><sub>UF6-25</sub>-C and M<sub>Ca</sub><sub>UF14-25</sub>-C) (Fig. 2A). All of these analogues were then labeled with Cy5 to investigate their cell penetration properties. Every one of these peptides has been designed in such a way that the cargo would be removed from the peptide upon trypsin cleavage. This was useful for the FACS experiments in which the fluorescence associated to the cells is measured after trypsin treatment, thereby potentially removing the cargo from peptides that would eventually be associated to the outer part of the plasma membrane. The net positive charges of the peptides were drastically different, ranging from 0 (M<sub>Ca</sub><sub>UF1-15</sub>-C and M<sub>Ca</sub><sub>UF1-9</sub>-C) to +8 (M<sub>Ca</sub><sub>UF8-33</sub>-C). However, many of the peptides contained a percentage of positively charged residues equal (M<sub>Ca</sub><sub>UF25-33</sub>-C) or superior to M<sub>Ca</sub><sub>F</sub> or M<sub>Ca</sub><sub>UF1-33</sub> (8 out of twelve analogues). Three analogues had a lower percentage of basic residues than M<sub>Ca</sub><sub>F</sub> (all three C-terminal truncated analogues, M<sub>Ca</sub><sub>UF1-20</sub>-C, M<sub>Ca</sub><sub>UF1-15</sub>-C and M<sub>Ca</sub><sub>UF1-9</sub>-C).

We first evaluated by FACS the fluorescence accumulation within CHO cells that occurred after 2 hrs incubation with 3  $\mu$ M of positively charged M<sub>Ca</sub> peptides (net charge  $\geq +5$ ; Fig. 2B). This first study revealed several unexpected findings. First, all of the charged peptides (8 tested) demonstrated CPP properties. These peptides had all the K<sup>22</sup>R<sup>23</sup>R<sup>24</sup> sequence in common, a cluster of basic amino acid residues shown to contribute to the dose-efficacy of cell penetration of M<sub>Ca</sub><sub>F</sub> in an earlier study (18). Interestingly, removing the last 8 C-terminal amino acids of M<sub>Ca</sub> had little impact on the cell penetration properties (if one compares M<sub>Ca</sub><sub>UF14-25</sub>-C with M<sub>Ca</sub><sub>UF14-33</sub>-C). Similarly, the removal of the amino acid region His<sup>6</sup>-Asn<sup>13</sup> did not drastically change cell penetration properties (M<sub>Ca</sub><sub>UF6-25</sub>-C *versus* M<sub>Ca</sub><sub>UF14-25</sub>-C). Second, all peptides appeared to behave better than the reference peptide M<sub>Ca</sub><sub>UF1-33</sub>-C, suggesting that sequence truncation of M<sub>Ca</sub><sub>UF</sub> may represent a potent strategy to define more efficient CPP. Less positively charged peptides were also tested for their ability to penetrate into CHO cells (Fig. 2C). No less surprisingly, all peptides showed CPP properties, including two peptides with no net positive charge (M<sub>Ca</sub><sub>UF1-9</sub>-C and M<sub>Ca</sub><sub>UF1-15</sub>-C). M<sub>Ca</sub><sub>UF1-9</sub>-C appeared as a better CPP than M<sub>Ca</sub><sub>UF1-15</sub>-C suggesting that the Abu<sup>10</sup>-Asp<sup>15</sup> region introduces no competitive advantage and confirming results shown in Fig. 2B. This may represent an inhibitory region because of the presence of Glu<sup>12</sup> and Asp<sup>15</sup>, two negatively charged residues. The finding that mutation of Glu<sup>12</sup> to Ala enhances cell penetration of both M<sub>Ca</sub><sub>F</sub> (18) and M<sub>Ca</sub><sub>UF</sub> (11) further supports this conclusion. M<sub>Ca</sub><sub>UF25-33</sub>-C turned out to have also CPP properties, even though this sequence did not confer a competitive advantage to other M<sub>Ca</sub> CPP analogues as shown in Fig. 2B. Two additional experiments were conducted to confirm the specificity of these findings. First, a truncated charybdotoxin peptide was synthesized (ChTx<sub>UF1-12</sub>-C) in which the internal Cys residue was replaced by Abu and an additional C-terminal Cys residue added for Cy5 labeling, as for our M<sub>Ca</sub><sub>UF</sub> analogues (Supplementary Fig. 1A). As shown by confocal microscopy, ChTx<sub>UF1-12</sub>-C was unable to deliver the Cy5 cargo at a higher concentration of 5  $\mu$ M. Second, we also designed two M<sub>Ca</sub><sub>UF</sub> peptides in which, instead of using Abu derivatives, we mutated internal Cys residues by Ala residues. As shown, both M<sub>Ca</sub><sub>UF1-9(Ala)</sub>-C and M<sub>Ca</sub><sub>UF14-25(Ala)</sub>-C worked well for Cy5 delivery into CHO cells

demonstrating that Abu residues were not responsible by themselves for the cell penetration properties of the peptides (Supplementary Fig. 1B,C). Ala residues were however not fully equivalent to Abu residues as dose-response curve was slightly better for  $\text{MCA}_{\text{UF14-25}(\text{Ala})}\text{-C}$  than for  $\text{MCA}_{\text{UF14-25}}\text{-C}$ , further arguing that Abu residues were not central to the cell penetrating properties of truncated  $\text{MCA}_{\text{UF}}$  peptides (Supplementary Fig. 1D). The overall message from this first study is that all truncated  $\text{MCA}_{\text{UF}}$  analogues can behave as CPP at the concentration tested. The findings suggest that MCA is a peptide fully specialized to achieve cell penetration including in domains that are not highly charged.

*The intracellular distribution of all truncated  $\text{MCA}_{\text{UF}}$  analogues bear resemblance with that of the full length  $\text{MCA}_{\text{UF}}$*

While all truncated derivatives of  $\text{MCA}_{\text{UF1-33}}$  show cell penetration properties according to the FACS analyses, we examined whether there were differences in intracellular distribution among these peptides. This question was investigated by confocal microscopy after 2 hrs of peptide accumulation into CHO cells (Fig. 3). Interestingly, all peptides showed very resembling intracellular distributions, although the degree of accumulated cell fluorescence varied somewhat with peptide sequences. In confirmation of the FACS results, the peptide that appeared to penetrate the least was the full length unfolded MCA,  $\text{MCA}_{\text{UF1-33}}\text{-C-Cy5}$  (Fig. 3A). The vast majority of the fluorescence appears in punctuate dots within the cells. In many cases, these dots appear at higher concentrations within one pole of the cell (see labeling of  $\text{MCA}_{\text{UF8-33}}\text{-C-Cy5}$ ,  $\text{MCA}_{\text{UF11-33}}\text{-C-Cy5}$ ,  $\text{MCA}_{\text{UF25-33}}\text{-C-Cy5}$ , and  $\text{MCA}_{\text{UF1-9}}\text{-C-Cy5}$  for instance). On various occasions also, all of the peptides tend to present a sub-plasma membrane distribution, forming a rim of smaller circumference than the concanavalin A labeling itself. This sub-plasma membrane rim localization was more evident for CHO cells labeled with  $\text{MCA}_{\text{UF14-25}}\text{-C-Cy5}$  as illustrated in Fig. 4A. Finally, more rarely, a direct plasma membrane labeling by the peptide-cargo complex was observable (Fig. 4B). This type of labeling could be observed with N-terminal truncated vectors exclusively and was most evident for  $\text{MCA}_{\text{UF22-33}}\text{-C-Cy5}$ . The staining of the plasma membrane was always diffuse in contrast to intracellular staining which was mainly punctuated. Diffuse membrane labeling was also observed for  $\text{MCA}_{\text{UF25-33}}\text{-C-Cy5}$  and  $\text{MCA}_{\text{UF20-33}}\text{-C}$

$\text{Cy5}$ , two peptides that differ from 2 to 3 amino acids of  $\text{MCA}_{\text{UF22-33}}\text{-C-Cy5}$ . It was difficult to evidence for the other vector/cargo complexes. We propose that this staining coincides with an alteration of the duration of peptide plasma membrane residency for these truncated  $\text{MCA}_{\text{UF}}$  analogues. The lower occurrence of this diffuse staining for the other truncated variants may reflect faster internalization by endocytosis and/or membrane translocation. Globally, these effects reflect cell entry and distribution tendencies that were hard to quantify and they should therefore be interpreted with caution.

In an attempt to better apprehend peptide behavior at the plasma membrane, we quantified the extent of  $\text{Cy5}$  / rhodamine staining colocalization. Rhodamine-positive staining was also  $\text{Cy5}$ -positive for 63% to 86% of the pixels (best performing peptides were  $\text{MCA}_{\text{UF14-33}}\text{-C-Cy5}$ ,  $\text{MCA}_{\text{UF18-33}}\text{-C-Cy5}$ ,  $\text{MCA}_{\text{UF20-33}}\text{-C-Cy5}$  and  $\text{MCA}_{\text{UF22-33}}\text{-C-Cy5}$ ; data not shown). This finding indicates that the peptides invade large membrane areas and that membrane interaction is not limited to small specialized surface areas. In contrast,  $\text{Cy5}$ -positive pixels were rhodamine-positive to far more variable extents (Fig. 4C). For instance,  $10.1 \pm 2.6\%$  of  $\text{MCA}_{\text{UF1-33}}\text{-C-Cy5}$ , the reference compound, was colocalized with the plasma membrane indicator. In spite of the fact that short plasma membrane staining times were used (few minutes), a fraction of the colocalization that is quantified also corresponds to intracellular staining following ongoing endocytosis. Nevertheless, this result indicates that this peptide does not remain stuck within the plasma membrane during its 2 hrs incubation with CHO cells. It indicates relatively fast cell penetration thus. Many of the other peptides however behaved differently from  $\text{MCA}_{\text{UF1-33}}\text{-C-Cy5}$ . Indeed, several peptides show surprisingly higher colocalization with rhodamine ( $21.3 \pm 2.6\%$  for  $\text{MCA}_{\text{UF11-33}}\text{-C-Cy5}$  and  $30.4 \pm 1.4\%$  for  $\text{MCA}_{\text{UF1-20}}\text{-C-Cy5}$  for instance). These higher values of colocalization indicate that some peptides remain for longer periods of time or at higher concentration within the plasma membrane. Alternatively, these peptides may rely more heavily on endocytosis for cell penetration and are present within intracellular organelles to which subsequent endocytotic vesicles that contain rhodamine labeling will fuse. Peptides most concerned by these behaviors were  $\text{MCA}_{\text{UF11-33}}\text{-C-Cy5}$  and  $\text{MCA}_{\text{UF14-33}}\text{-C-Cy5}$ , that contained two or one of the CPP inhibitory negative charges

(Glu<sup>12</sup> and Asp<sup>15</sup>), and M<sub>CaUF1-9</sub>-C-Cy5 and M<sub>CaUF1-20</sub>-C-Cy5, that were poorly charged peptides.

#### *Amiloride-sensitivity of the cell penetration of truncated M<sub>CaUF</sub> analogues*

In earlier studies, we have demonstrated that the cell entry of M<sub>CaUF1-33</sub> was largely sensitive to amiloride. Since amiloride is an exquisite blocker of macropinosomes (22-24), this suggested a predominant macropinocytosis mechanism for its cell penetration (17). However, it was likely that such a predominant reliance on macropinocytosis was also conferred by the cargo type transported (streptavidine in that report). We therefore conducted an in depth analysis of the amiloride-sensitivity of the various truncated M<sub>CaUF</sub> peptides with Cy5 as cargo and quantified by FACS the degree of cell penetration inhibition. Fig. 5A illustrates the amiloride-sensitivity of four different truncated peptides. As shown, amiloride inhibits the cell penetration of M<sub>CaUF1-33</sub>-C-Cy5 by 19.6% and of M<sub>CaUF1-15</sub>-C-Cy5 by 39%. The finding that amiloride blocks to a far lesser extent the penetration of Cy5 compared to that of streptavidin (17) when M<sub>CaUF1-33</sub> is the vector indicates the influence of the cargo nature on the mechanism of cell entry. Surprisingly, amiloride was found to enhance rather than inhibit the cell penetration of M<sub>CaUF20-33</sub>-C-Cy5 and M<sub>CaUF18-33</sub>-C-Cy5 (Fig. 5A). Preserving the plasma membrane from undergoing macropinocytosis may free surface areas for enhanced peptide translocation through the membrane. The effect of amiloride was always associated with a sharpening of the fluorescence intensity distribution in the x axis (see for instance M<sub>CaUF1-15</sub>-C-Cy5), reflecting reduced cell heterogeneity for the mechanisms underlying peptide penetration. The amiloride-sensitivity of cell penetration was further investigated for all truncated M<sub>CaUF</sub> peptides and the results presented in Fig. 5B. Four peptides showed higher amiloride-sensitivity than M<sub>CaUF1-33</sub>-C-Cy5 (M<sub>CaUF11-33</sub>-C-Cy5, M<sub>CaUF25-33</sub>-C-Cy5, M<sub>CaUF1-15</sub>-C-Cy5 and M<sub>CaUF14-25</sub>-C-Cy5). All other peptides showed reduced amiloride-sensitivities or a tendency for greater cell penetration under the effect of amiloride. We conclude that the Cy5 cargo does not promote macropinocytosis as the main route of peptide entry, and that truncation of M<sub>CaUF</sub> may lead to analogues that rely to a lesser extent on macropinocytosis for cell entry.

#### *Comparative dose-dependent cell penetration of the M<sub>CaUF</sub> analogues*

While we compared the properties of cell penetration of truncated peptides at rather mild concentrations, we also aimed at comparing the dose-dependence of cell penetration of these peptides by FACS (Fig. 6). One example of such an analysis is shown for peptide M<sub>CaUF8-33</sub>-C-Cy5 in Fig. 6A. 33  $\mu$ M was the highest concentration that could be tested on CHO cells and obviously cell penetration did not show any sign of saturation for cell incubation times with this peptide of 2 hrs. The dose-dependent cell penetration were compared for all N-terminal truncated peptides (Fig. 6B), C-terminal truncated peptides (Fig. 6C), and double truncated analogues (Fig. 6D) with the same settings. These analyses confirm that M<sub>CaUF1-33</sub>-C-Cy5 is the least-performing cell penetrating peptide. Most truncated peptides show detectable cell penetration at concentrations equal or above 1  $\mu$ M. One remarkable exception to this rule was noticeable. M<sub>CaUF1-9</sub>-C-Cy5 shows an unusual dose-dependent penetration with detectable cell penetration at 10 nM and only small progressive increases in fluorescence intensity with higher peptide concentrations (Fig. 6C). This peptide was therefore the best performing peptide for cell penetration at low concentrations. Finally, additional information that could be taken from these analyses is that the peptides differed significantly with regard to the maximal extent of cell penetration. Among the N-terminal truncated M<sub>CaUF</sub> analogues, M<sub>CaUF18-33</sub>-C-Cy5 performed drastically better than the other truncated peptides (Fig. 6B). The difference in cell penetration among M<sub>CaUF11-33</sub>-C-Cy5 and M<sub>CaUF18-33</sub>-C-Cy5 resides in the removal of KENKDAbuAbu sequence which we presume is inhibitory to some extent because of the presence of Glu<sup>12</sup> and Asp<sup>15</sup>. Among the C-terminal truncated peptides, M<sub>CaUF1-20</sub>-C-Cy5 was performing as well as M<sub>CaUF8-33</sub>-C-Cy5, and although not tested at higher concentrations, M<sub>CaUF1-9</sub>-C-Cy5 would be expected to perform still better. Finally, for N- and C-terminal truncated analogues, the best peptide turns out to be M<sub>CaUF14-25</sub>-C-Cy5 that yields the greatest fluorescence accumulation at 33  $\mu$ M compared to all other truncated M<sub>CaUF</sub> analogues. While all these peptides performed quite well, we were curious to compare them to a classical CPP under similar experimental conditions. Cell penetration was observed for TAT-C in CHO cells and dose-response curve was equivalent or even less favorable for TAT than for many M<sub>CaUF</sub> peptides (Supplementary Fig. 1E,F). This was



further largely confirmed when comparing the vector properties of TAT-C and M<sub>Ca</sub><sub>UF1-9</sub>-C in yet another cell type, the glioma F98 rat cell line (Supplementary Fig. 2). 3  $\mu$ M M<sub>Ca</sub><sub>UF1-9</sub>-C proved better than TAT-C at 3 and 10  $\mu$ M.

*Truncated M<sub>Ca</sub><sub>UF</sub> peptides lack pharmacological effects and are predominantly non toxic*

An earlier report has shown that M<sub>Ca</sub><sub>UF1-33</sub> is unable to interact with M<sub>Ca</sub>'s target, the ryanodine receptor RyR1 (11). This is due to the loss of secondary structures owing to the lack of internal disulfide bridging. We did therefore expect that truncated analogues of M<sub>Ca</sub><sub>UF</sub> should also be pharmacologically inert. This hypothesis was challenged by testing the ability of the Cy5-free peptides to stimulate [<sup>3</sup>H]-ryanodine binding (Fig. 7A). As shown, contrary to M<sub>Ca</sub><sub>F</sub> that contains secondary structures and disulfide bridges, none of the peptides we designed had an effect on [<sup>3</sup>H]-ryanodine binding.

Finally, the peptides were challenged for their toxicity by incubating CHO cells with 1 or 10  $\mu$ M peptide concentrations for an extended duration (24 hrs) that far exceeds the duration challenged for cell penetration (Fig. 7B). A 10  $\mu$ M peptide concentration was generally slightly more toxic than 1  $\mu$ M, except for M<sub>Ca</sub><sub>UF14-25</sub>-C. At 1  $\mu$ M, toxicity never exceeded 8% and significances of these effects were negligible. In contrast, toxicity could reach 20% at 10  $\mu$ M peptide concentration and these effects had higher significance. Most peptides behaved equally well or better than M<sub>Ca</sub><sub>UF1-33</sub>-C indicating that truncation did not enhance cell toxicity of the peptides.

## Discussion

M<sub>Ca</sub><sub>F</sub> is a rather large and complex CPP with its three disulfide bridges which makes its use in *in vitro* and *in vivo* applications more delicate because of production yield (peptide with correct disulfide bridging) and cargo coupling (thiol groups are for the moment difficult to use). One may also argue that the size of the peptide introduces additional synthesis cost compared to other popular in use CPP such as Tat and penetratin. While size and folding most likely adds a competitive advantage for *in vivo* applications because of peptide stability issues, it may be of interest to derive small CPP from M<sub>Ca</sub> that could have a broader use than M<sub>Ca</sub> itself. The study we conducted not only aimed at defining these smaller M<sub>Ca</sub>-derived CPP sequences, but also

provided interesting clues on how M<sub>Ca</sub> may have evolved for cell penetration. The most surprising finding from this study was therefore that all of the analogues we defined could be considered as CPP, which suggests that M<sub>Ca</sub> is a highly specialized sequence for the purpose of cell penetration. While evidently none of the peptides could compete with M<sub>Ca</sub><sub>F</sub> itself for cell penetration, it is nevertheless obvious that many of the truncated M<sub>Ca</sub><sub>UF</sub> peptides are better CPP than M<sub>Ca</sub><sub>UF1-33</sub> itself. These findings lead to two general conclusions. First, folding and disulfide bridging appears to be a prerequisite to properly optimize the CPP potential of all the M<sub>Ca</sub> domains. It is highly likely that the secondary structures triggered by disulfide bridging play a key role in that respect. We therefore believe that it would be worthwhile to design additional M<sub>Ca</sub> analogues presenting both truncations (like in the present study) and 1 to 2 disulfide bridges in order to regain some of the secondary structures that confer a competitive advantage to M<sub>Ca</sub><sub>F</sub> for cell penetration. Second, the fact that all the truncated sequences are CPP that possess different properties and efficacies, suggests that there is further room for M<sub>Ca</sub> cell penetrating optimization. In this study, our data indicate that the amino acid region Lys<sup>11</sup>-Ser<sup>18</sup> would be an ideal target for mutagenesis. Glu<sup>12</sup> mutation has already been shown to improve cell penetration on various analogues (11,18). We now sense that Asp<sup>15</sup> may also represent an excellent target for further optimization of M<sub>Ca</sub>'s properties. Double mutagenesis may be an option. However, it will be essential to determine whether the disulfide bridging and folding ability of the native peptide is not affected by this procedure. Truncated M<sub>Ca</sub><sub>UF</sub> peptides may also benefit from further mutagenesis of negatively charged residues, such as M<sub>Ca</sub><sub>UF1-20</sub>. It is noteworthy that another well behaving truncated CPP, M<sub>Ca</sub><sub>UF18-33</sub>, also contains a Glu residue at position 29 that could represent another target for mutagenesis and peptide optimization. This is also the case for M<sub>Ca</sub><sub>UF1-9</sub> that contains an Asp residue at position 2. Several other findings of this report merit some comments. First, we demonstrate that cargo coupling can occur at the N-terminus as well as the C-terminus of M<sub>Ca</sub> enhancing the flexibility of cargo coupling to our vectors. Second, while it is obvious that most of our truncated M<sub>Ca</sub><sub>UF</sub> analogues remain heavily basic, we also found out that poorly charged M<sub>Ca</sub> peptides could behave as

efficient CPP. This is the case for M<sub>CaUF1-9</sub> which is one of our best performing CPP, especially if one wishes to work with low CPP concentrations. Third, small size CPP can be derived from M<sub>CaUF</sub>, M<sub>CaUF1-9</sub> and M<sub>CaUF25-33</sub> being our smallest peptides. While we did not test greater truncation, it is not impossible that still smaller CPP might be designed on the basis of M<sub>Ca</sub> sequence. Fourth, the truncated M<sub>CaUF</sub> analogues differ somewhat in their mode of cell penetration, some being more prone to enter cells by macropinocytosis than others. Various peptides were even insensitive to amiloride application suggesting that macropinocytosis did not contribute at all to their entry. We can't exclude at this stage that these peptides rely on other modes of endocytosis than macropinocytosis. This is suggested by the punctuate nature of intracellular Cy5 fluorescence distribution. How reliably a punctuate distribution reveals endocytosis is however not known, and one can't rule out also that this distribution is due to peptide aggregation with or without lipid components. When plasma membrane distribution could be observed, large diffuse staining was evident indicating that when peptides encountered the membrane no such aggregation occurred. In any case, many of the peptides had distribution that mostly differed from plasma membrane labeling (including these membranes that had undergone endocytosis) indicating that peptide intracellular distribution may not necessarily colocalize with the plasma membrane components. Refined studies will be required however to define how much of the peptides enter cells by direct membrane translocation *versus* endocytosis. To conclude on this study, we identified several interesting lead CPP based on M<sub>CaUF</sub> truncation

strategy. This is the case for M<sub>CaUF18-33</sub> (macropinocytosis entry-independent), M<sub>CaUF1-9</sub> (penetrates better at low concentration), and M<sub>CaUF14-25</sub> (yields the greatest cell entry of the dye). These peptides are easy to produce, yield good cell penetration, and we should be able to further improve their cell penetrating characteristics by mutagenesis or by reintroducing one disulfide bridge to restore some of the secondary structures. While the scope of this study was not to compare our mini M<sub>Ca</sub> with other popular CPP, we were surprised to find out that many of the M<sub>CaUF</sub> vectors behaved far better than TAT for the transport of Cy5, both in CHO and F98 cells. It may be argued that the field doesn't require many more CPP sequences. However, a significant fraction of the ongoing research is based on the use of TAT, which no longer appears as the most competitive peptide. Also, increasing evidences suggest that CPP sequences differ in their cell-type targeting properties, which will undoubtedly represent an important feature to exploit when it comes to developing *in vivo* applications. Other essential parameters for *in vivo* applications will be the peptide stability upon intravenous or intra-peritoneal injection. In that respect, we found out that M<sub>Ca</sub> is a particularly stable peptide with and without disulfide bridges (unpublished observations). These still "hidden" aspects of the CPP features may be advantageously exploited later on if the number of known CPP is high. The advantages of using smaller CPP over M<sub>CaF</sub> remains to be investigated as one may of course argue also that they require higher concentrations and possibly longer incubation times for effectiveness, two factors that may lead to spurious signaling events *in vivo*.

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## FOOTNOTES

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The abbreviations used are: CD: Circular Dichroism; CPP: Cell Penetrating Peptide; DMF: dimethylformamide; FACS: Fluorescence-Activated Cell Sorting; HPLC: high-performance liquid chromatography; MCa: Maurocalcine; NMP: N-methyl-pyrrolidone; RS: Sarcoplasmic reticulum; RyR: Ryanodine Receptor; TFA: trifluoroacetic acid.

## FIGURE LEGENDS

**Fig. 1.** Efficacy of cargo penetration as a function of grafting position on  $\text{MCA}_{\text{UF1-33}}$ . (A) Amino acid sequence of  $\text{MCA}_F$  in single letter code. The positions of half-cystine residues are highlighted in red. Cys residues are numbered and basic amino acids are highlighted in blue. Secondary structures ( $\beta$  strands) are indicated by arrows. The grey box is the sequence of homology of  $\text{MCA}$  with the dihydropyridine-sensitive  $\text{Ca}_v1.1$  channel. (B) Amino acid sequences of unfolded  $\text{MCA}$  analogues in single letter code. Cys residues are replaced by isosteric 2-aminobutyric acid residues (Abu, in red) to form  $\text{MCA}_{\text{UF1-33}}$ . An additional N-terminal (C- $\text{MCA}_{\text{UF1-33}}$ ) or C-terminal ( $\text{MCA}_{\text{UF1-33}}$ -C) Cys residue was added in two novel analogues competent for cargo grafting (shown in grey). (C) Confocal microscopy images illustrating cell penetration of Cy5-C- $\text{MCA}_{\text{UF1-33}}$  and  $\text{MCA}_{\text{UF1-33}}$ -C-Cy5 (green labeling). Plasma membranes are labeled with concanavalin-A-rhodamine (in red). CHO cells were incubated 2 hrs with 1  $\mu\text{M}$  peptide concentration. (D) Comparison of cell penetration efficacy between Cy5-C- $\text{MCA}_{\text{UF1-33}}$  and  $\text{MCA}_{\text{UF1-33}}$ -C-Cy5 as determined by FACS. CHO cells were incubated 2 hrs with 3  $\mu\text{M}$  peptide, washed, and treated 5 min by 1 mg/ml trypsin before quantification of intracellular fluorescence.

**Fig. 2.** Primary structure of truncated  $\text{MCA}_{\text{UF}}$  analogues and comparison of cell penetration efficacies. (A) Primary structures of truncated  $\text{MCA}_{\text{UF}}$ -C analogues and determination of their net positive charge and percentage of basic amino acid residues within the sequence. A total of 12 truncated  $\text{MCA}_{\text{UF}}$ -C analogues were produced (three with truncations in C-terminus, seven in N-terminus, and two in both N- and C-termini). Positively charged residues are in blue (His residues were not counted), whereas Abu residues that replace Cys residues are in red. (B) Comparative cell penetration efficacy of all  $\text{MCA}_{\text{UF}}$ -C-Cy5 truncated analogues that possess a net positive charge  $\geq +5$ . Code colors: red (net charge +8), blue (+7), pink (+6) and green (+5). The non-truncated  $\text{MCA}_{\text{UF1-33}}$ -C-Cy5 analogue is shown as reference (black line) for the efficacy of cell penetration of all analogues. Experimental conditions: CHO cell incubation with 1  $\mu\text{M}$  of each analogue for 2 hrs and fluorescence quantification by FACS. (C) Same as (B) but for truncated  $\text{MCA}_{\text{UF}}$ -C-Cy5 analogues with positive net charge  $\leq +2$ .

**Fig. 3.** All truncated  $\text{MCA}_{\text{UF}}$ -C-Cy5 vector/cargo complexes have resembling intracellular distributions. (A) Intracellular distribution of N-terminal truncated  $\text{MCA}_{\text{UF}}$  analogues. (B) Intracellular distribution of C-terminal truncated  $\text{MCA}_{\text{UF}}$  analogues. (C) Intracellular distribution of N- and C-terminal truncated  $\text{MCA}_{\text{UF}}$  analogues. CHO cells were incubated 2 hrs with 3  $\mu\text{M}$  of  $\text{MCA}_{\text{UF}}$ -C-Cy5 vector/cargo complexes, before extensive washing, membrane labeling with rhodamine-conjugated concanavalin A, and live cell confocal microscopy imaging. Cy5 is in blue, and rhodamine in red. White arrows illustrate a tendency for a preferential apical localization of the Cy5 dye. Yellow arrows illustrate the tendency for a sub-plasma membrane labeling of the Cy5 dye.

**Fig. 4.** Membrane staining is diffuse whereas intracellular staining is punctuated. (A) Lower magnification image of CHO cells stained with 3  $\mu\text{M}$   $\text{MCA}_{\text{UF14-25}}$ -C-Cy5 that illustrates a predominant sub-plasma membrane rim-like distribution. (B) Diffuse membrane staining of CHO cells by  $\text{MCA}_{\text{UF22-33}}$ -C-Cy5. White arrows indicate domains of the plasma membrane where the diffuse staining of the peptide/cargo complex is the most evident. (C) Extent of colocalization of the Cy5-labeled peptides with the rhodamine-labeled plasma membrane. NS, non significant; \*  $\leq 0.1$ ; \*\*  $\leq 0.05$ ; and \*\*\*  $\leq 0.001$ .

**Fig. 5.** Amiloride sensitivity of truncated  $\text{MCA}_{\text{UF}}$  peptide cell entry. (A) Representative FACS analyses of the effect of 5 mM amiloride on  $\text{MCA}_{\text{UF1-33}}$ -C-Cy5 (upper left panel),  $\text{MCA}_{\text{UF1-15}}$ -C-Cy5 (upper right panel),  $\text{MCA}_{\text{UF20-33}}$ -C-Cy5 (lower left panel) and  $\text{MCA}_{\text{UF18-33}}$ -C-Cy5 (lower right panel) entries. Numbers in red represent average decrease or increase in peptide entry upon amiloride treatment. Cells were treated 2 hrs with 3  $\mu\text{M}$  peptide concentration with or without 5 mM amiloride. (B) Average effect of amiloride on mean cell entry of the truncated peptides. Positive values reflect increase in cell entries, whereas negative values indicate reduction in cell penetration.

Fig. 6. Dose-dependent cell penetration of truncated M<sub>CaUF</sub> peptides. (A) Representative example of the dose-dependent cell penetration of M<sub>CaUF8-33</sub>-C-Cy5 in CHO cells as analyzed by FACS. The peptide was incubated 2 hrs with the cells before analyses. There was no saturation of cell entry for a concentration up to 33  $\mu$ M. (B) Dose-dependent cell penetration of N-terminal truncated M<sub>CaUF</sub> peptides compared to M<sub>CaUF1-33</sub>-C-Cy5 (open circle, dotted line). (C) Dose-dependent cell penetration of C-terminal truncated M<sub>CaUF</sub> peptides. (D) Dose-dependent cell penetration of N- and C-terminal truncated M<sub>CaUF</sub> peptides. Note the increase in scale for the penetration of these two peptides.

Fig. 7. Lack of pharmacological effects of the truncated peptides and reduced cell toxicity. (A) Effect of M<sub>CaF</sub>, M<sub>CaUF1-33</sub> and truncated M<sub>CaUF</sub> peptides on [<sup>3</sup>H]-ryanodine binding. Data were expressed as fold increase in binding induced by the peptides. (B) Effect of 1 and 10  $\mu$ M M<sub>CaUF1-33</sub> and truncated M<sub>CaUF</sub> peptides on CHO cell viability. Peptides were incubated 24 hrs with the cells *in vitro*.

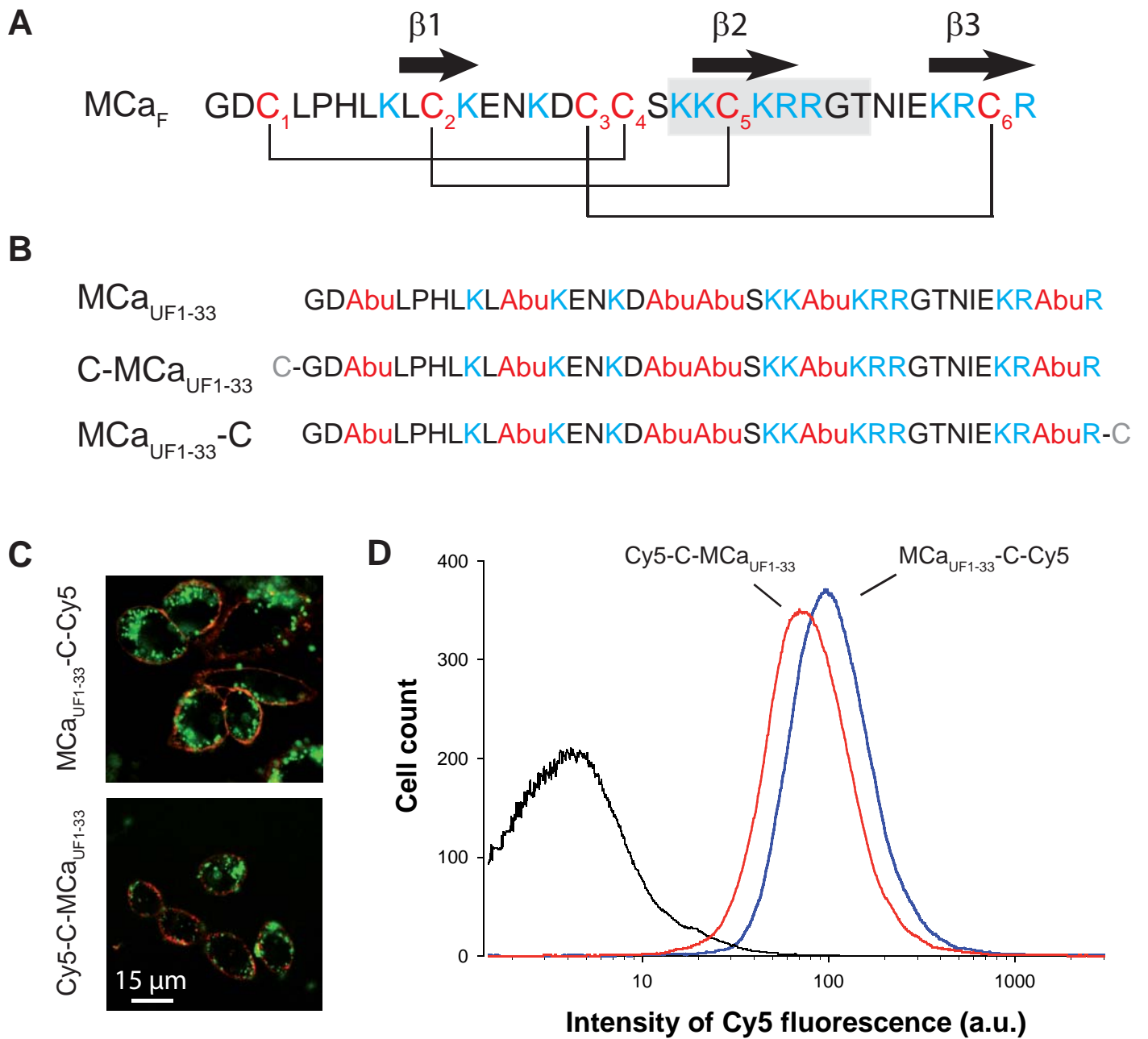


Figure 1

**A**

		Net charge (NC)	%
MCa <sub>UF1-33</sub> -C	GDAbuLPHLKLAbuKENKDAbuAbuSKKAbuKRRGTNIEKRAbuR-C	+7	33
MCa <sub>UF1-20</sub> -C	GDAbuLPHLKLAbuKENKDAbuAbuSKK-C	+2	25
MCa <sub>UF1-15</sub> -C	GDAbuLPHLKLAbuKENKD-C	0	20
MCa <sub>UF1-9</sub> -C	GDAbuLPHLKL-C	0	11
MCa <sub>UF8-33</sub> -C	KLAbuKENKDAbuAbuSKKAbuKRRGTNIEKRAbuR-C	+8	42
MCa <sub>UF11-33</sub> -C	KENKDAbuAbuSKKAbuKRRGTNIEKRAbuR-C	+7	43
MCa <sub>UF14-33</sub> -C	KDAbuAbuSKKAbuKRRGTNIEKRAbuR-C	+7	45
MCa <sub>UF18-33</sub> -C	SKKAbuKRRGTNIEKRAbuR-C	+7	50
MCa <sub>UF20-33</sub> -C	KAbuKRRGTNIEKRAbuR-C	+6	50
MCa <sub>UF22-33</sub> -C	KRRGTNIEKRAbuR-C	+5	50
MCa <sub>UF25-33</sub> -C	GTNIEKRAbuR-C	+2	33
MCa <sub>UF6-25</sub> -C	HLKLABuKENKDAbuAbuSKKAbuKRRG-C	+6	40
MCa <sub>UF14-25</sub> -C	KDAbuAbuSKKAbuKRRG-C	+5	50

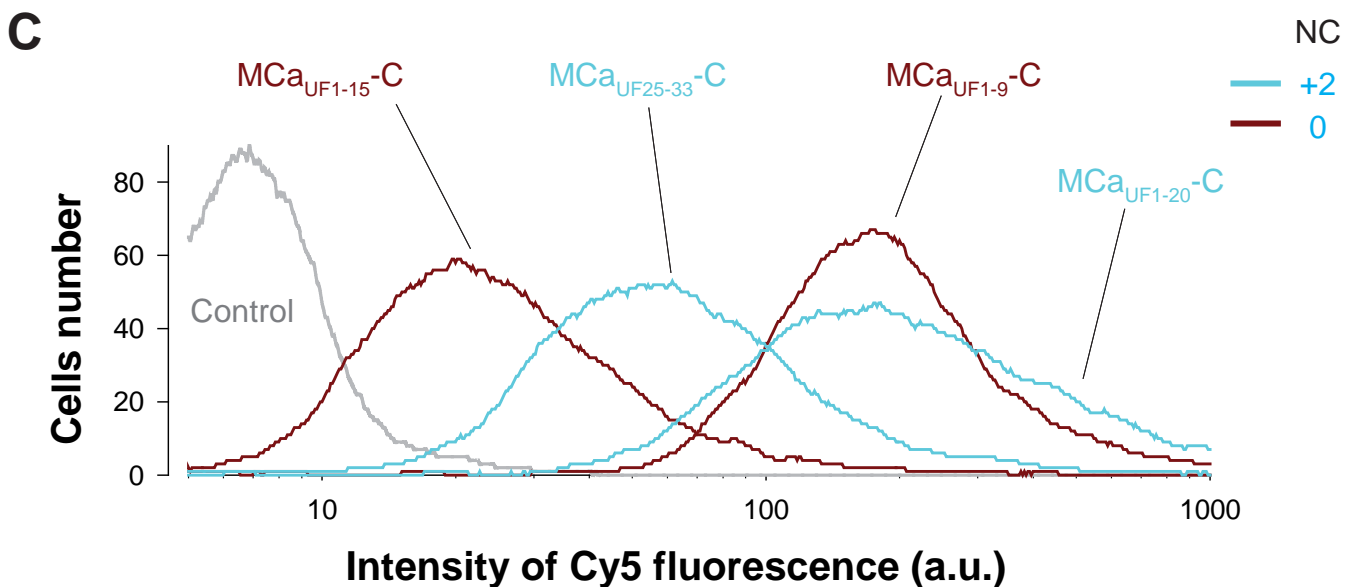
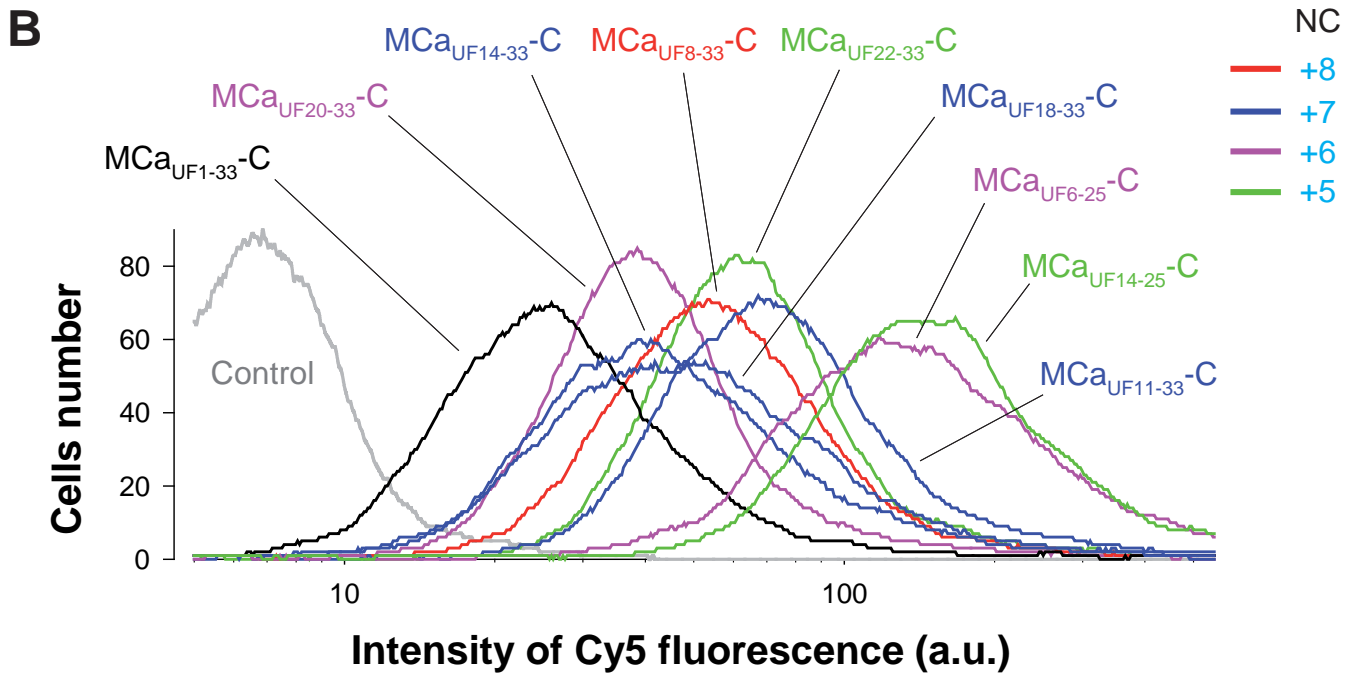


Figure 2

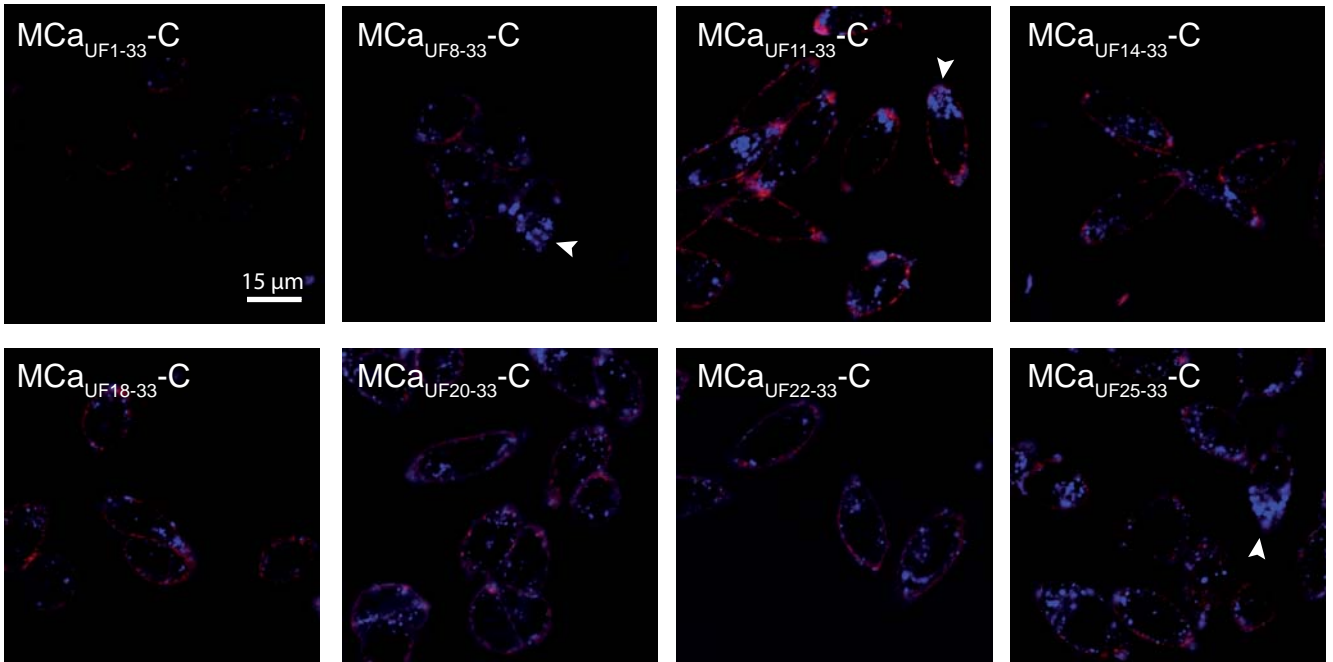
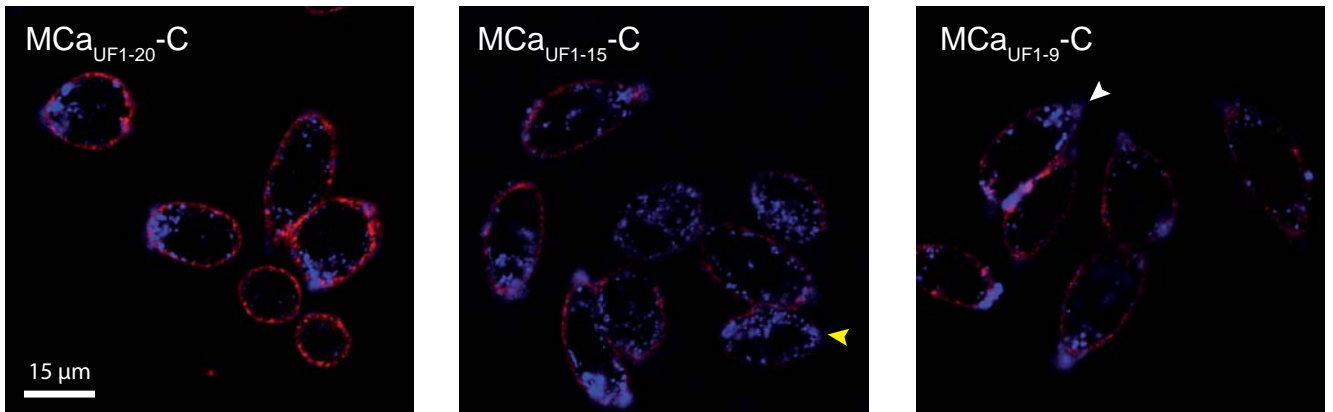
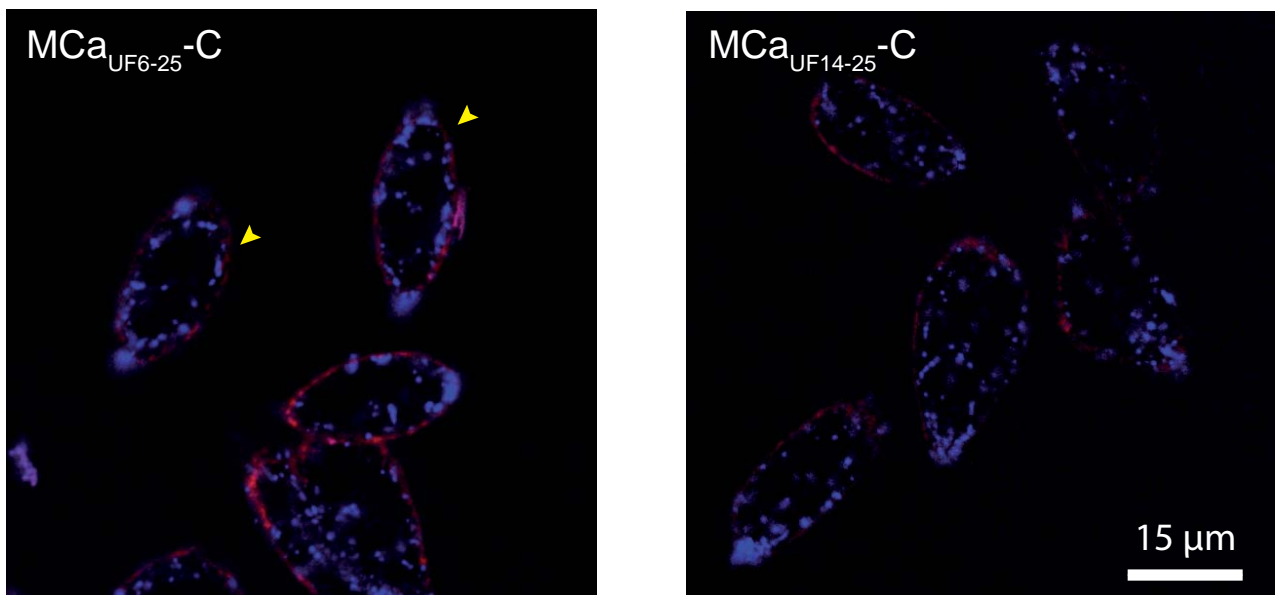
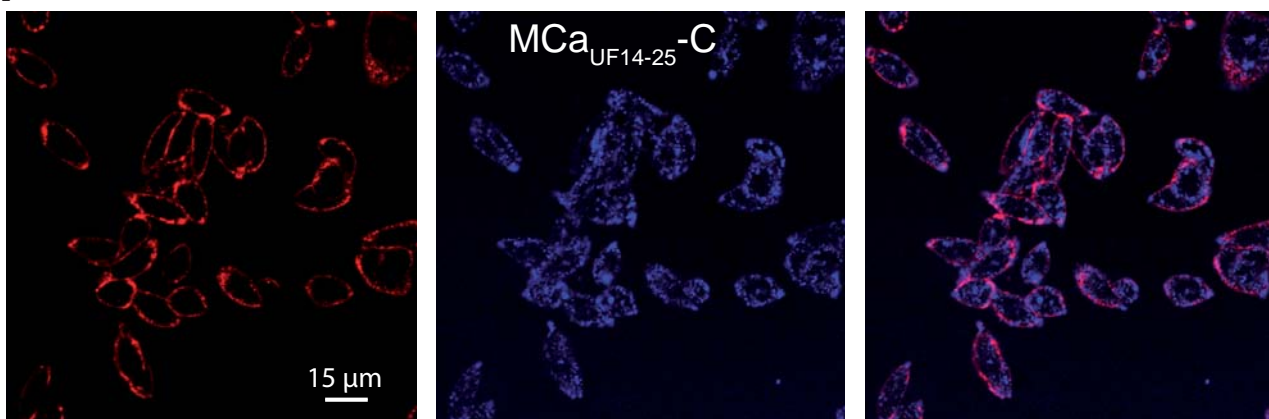
**A****B****C**

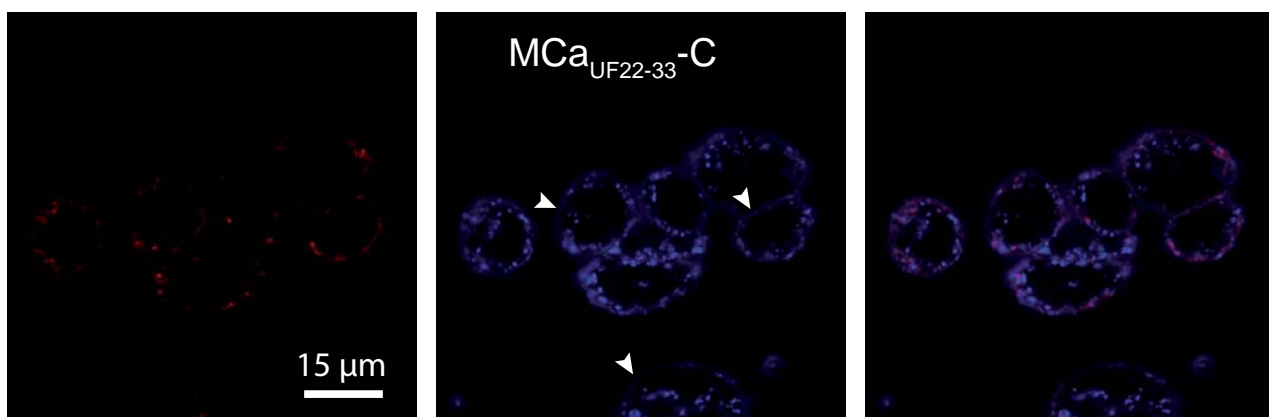


Figure 4

**A**



**B**



**C**

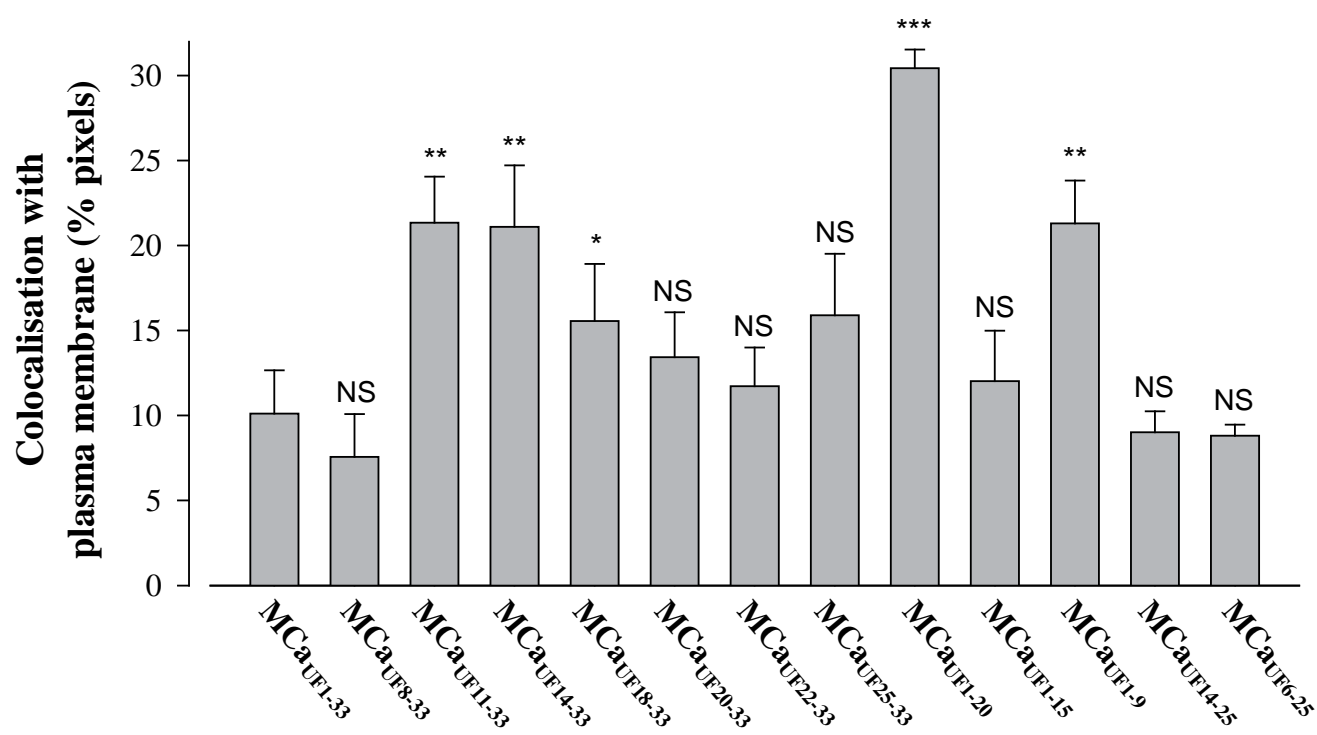
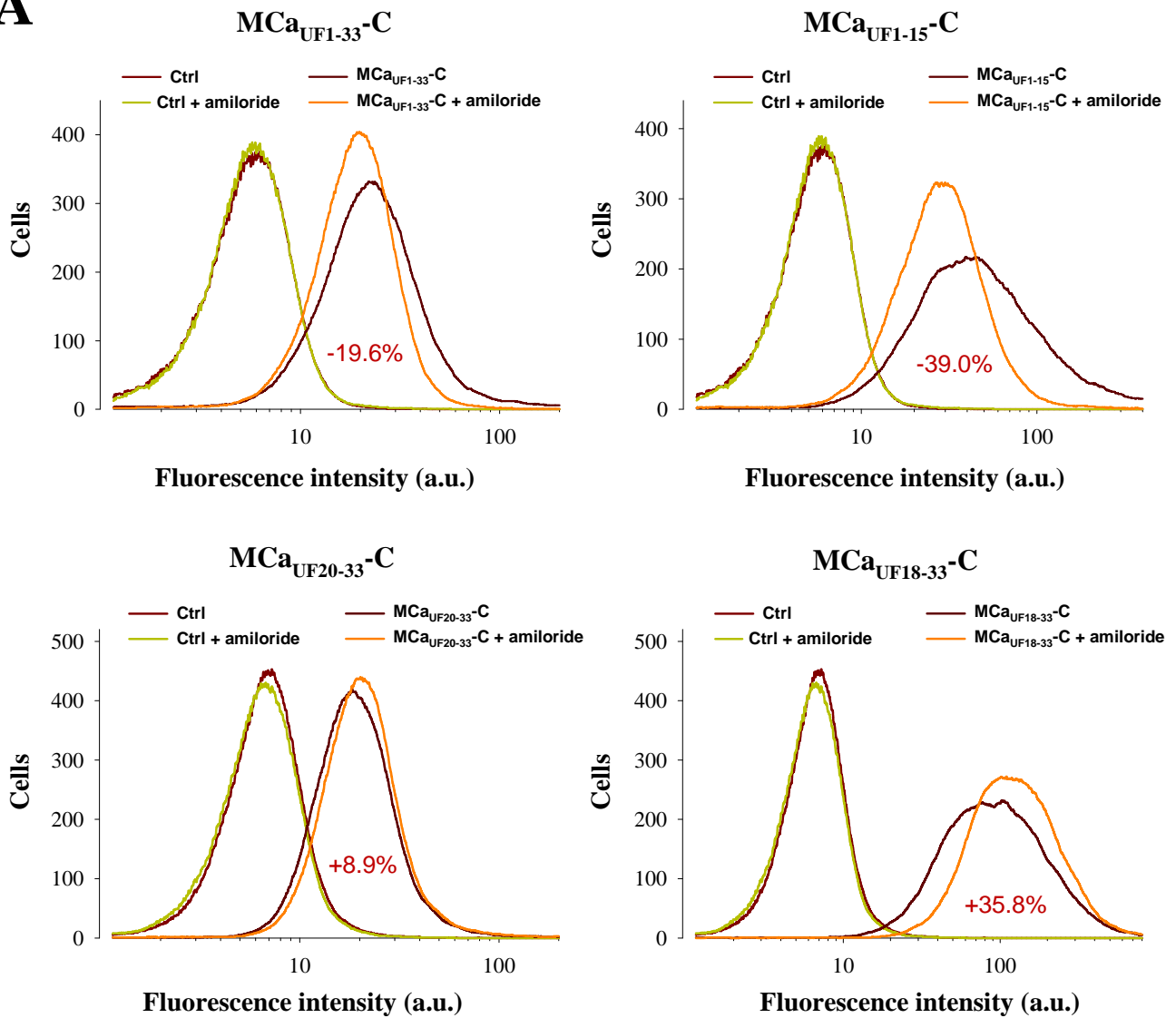
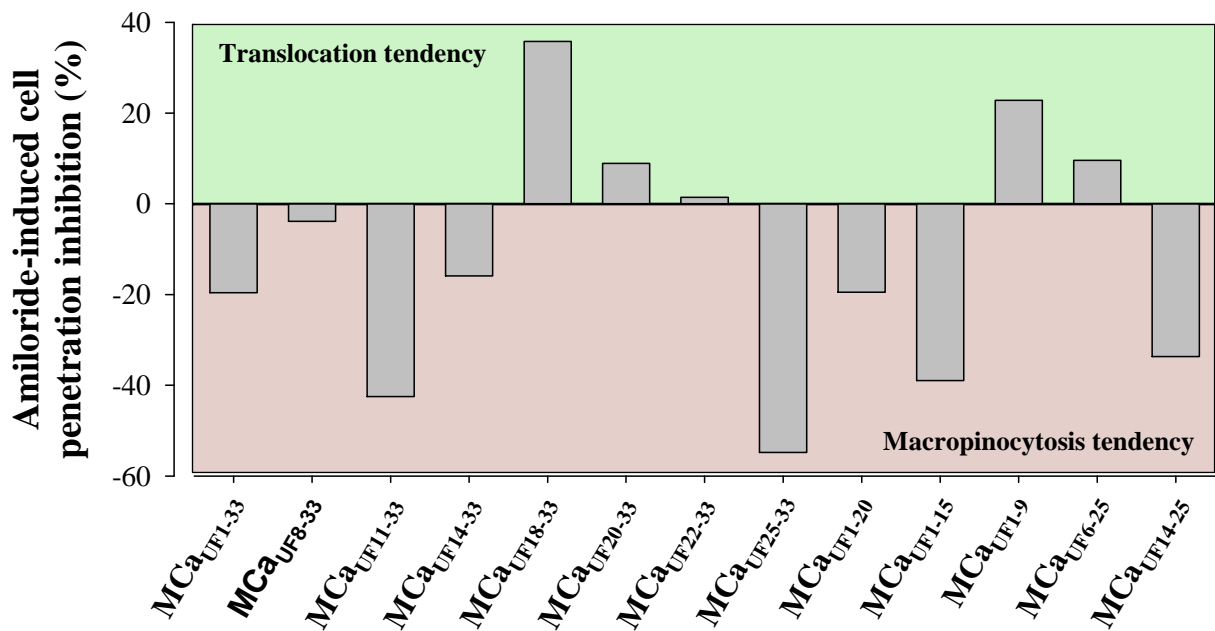


Figure 5

**A**



**B**



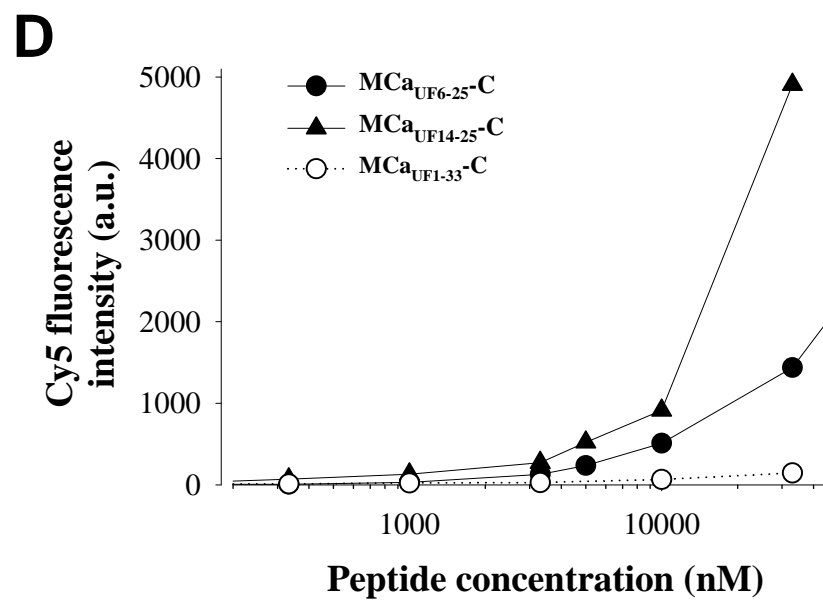
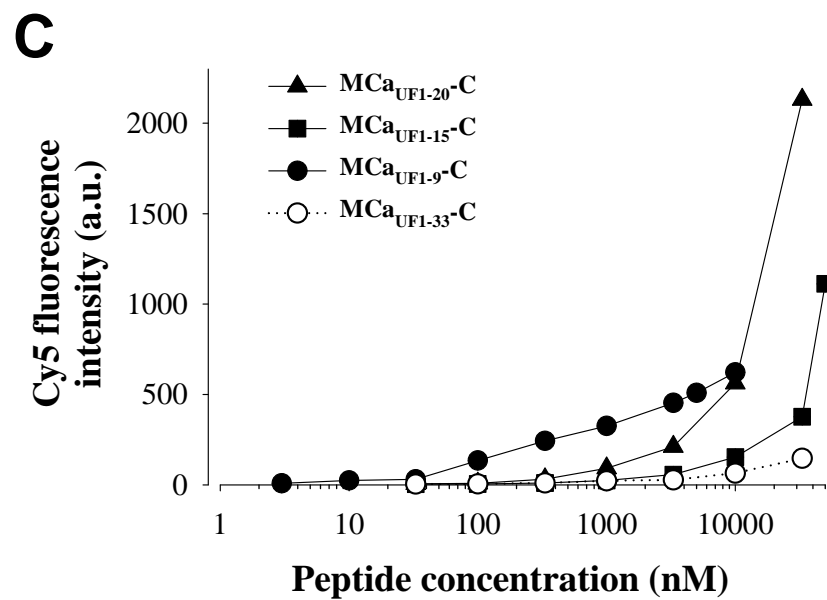
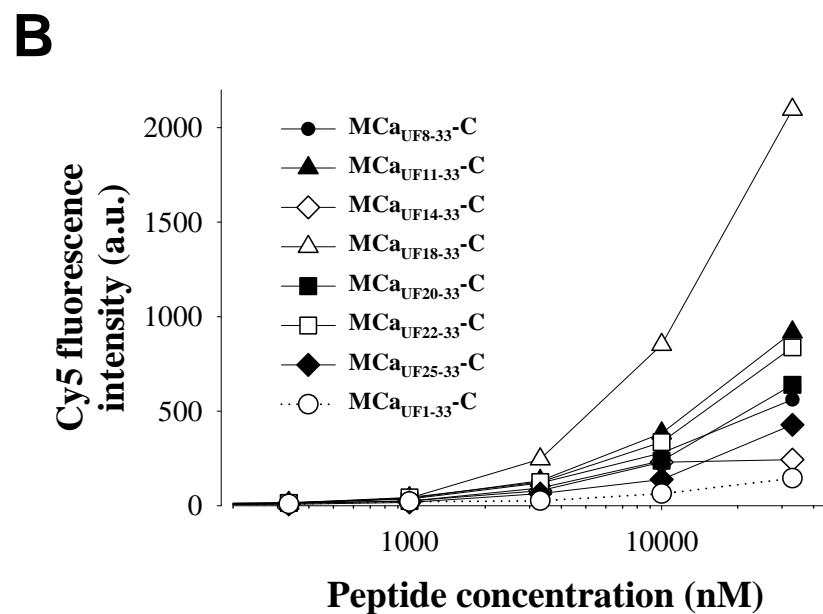
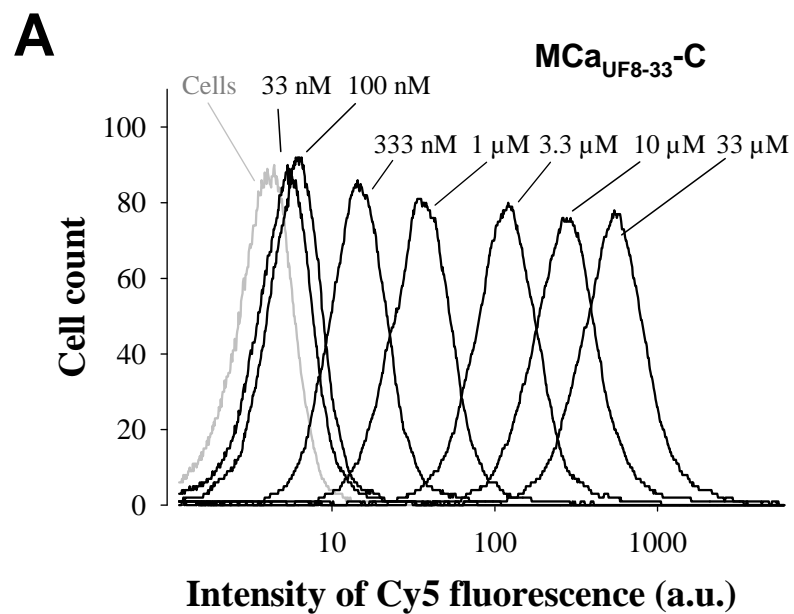


Figure 6

Figure 7

