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by

Narendra Ram[1,2], Emilie Jaumain[1,2], Michel Ronjat[1,2], Fabienne Pirollet[1,2] & Michel De Waard[1,2*]

[1] INSERM, U836, Team 3: Calcium channels, functions and pathologies, BP 170, Grenoble Cedex 9, F-38042, France

[2] Université Joseph Fourier, Institut des Neurosciences, BP 170, Grenoble Cedex 9, F-38042, France

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[*]To whom correspondence should be sent:
Dr. Michel De Waard
Tel.: +33 4 56 52 05 63
E-mail: michel.dewaard@ujf-grenoble.fr

ABSTRACT

Maurocalcine is a unique toxin in that its natural pharmacological target *in vivo*, the ryanodine receptor, is localized inside cells and not at the cell surface as commonly observed for most toxins. According to the membrane topology of the ryanodine receptor, the binding site of maurocalcine is localized within the cytoplasm. Application of maurocalcine to myotubes in culture induces calcium release via the ryanodine receptor within seconds indicating that the peptide reaches its binding site via a rapid and efficient diffusion through the plasma membrane. Analysis of the maurocalcine amino-acid sequence indicates that it is a heavily positively charged peptide, a property shared with many cell-penetrating peptides. A closer examination of the 3D structure of maurocalcine further illustrates that most of its positively charged residues are located on one face of the molecule according to a distribution that resembles that seen in Tat and penetratin, two cell penetrating peptides. Along with its unique cell penetrating properties, maurocalcine has also the ability to act as a vector for the intracellular delivery of various many cargo molecules or nanoobjects. Many key cell penetration properties of maurocalcine have been defined using a biotinylated version of the peptide that was coupled to a fluorescent streptavidin. Various structure-function strategies have been developed to isolate new maurocalcine analogues presenting the characteristic cell penetration properties without the undesired pharmacological activity. Examples of research and technological applications will be presented in which maurocalcine may prove a powerful delivery vector. By its amazing diversity of potential applications, this peptide opens a new trend of research in the toxin field.

INTRODUCTION

Venom toxins and applications

Venoms are an invaluable source of pharmacological agents, generally presented under the form of 10- to 70-mer peptides. Proteomic profiling of various animal venoms using MALDI-TOF mass spectrometry indicate that these venoms should contain no less than 100 peptides on average. More precise quantifications, by combining mass spectrometry analyses and genomic analyses, seem to indicate that venoms, at least spider ones, may contain up to 1000 different peptides (1). Considering that spider venoms may contain 200 unique peptides and that there are about 80,000 spider species, then spiders may provide no less than 16 million

different peptides. Although this peptide repertoire is considered less important in the case of cone snails (2) or scorpions (3), it remains an invaluable source of biologically active molecules. The scientific motivation for studying venoms and toxins has greatly evolved during the last two decades. If initial efforts were conducted mainly to study and identify the active principles involved in toxicity, and also to develop the means to counteract this toxicity, the current interest in peptides from venom sources rather lies in their enormous potential as pharmacological agents, drugs for therapeutics or pesticides for industrial use. As pharmacological agents, toxins target ion channels as well as G protein-coupled and ionotropic receptors, such as the nicotinic receptor. The search for peptides of biologically active significance was aided by the understanding that non-toxic venoms contain highly interesting molecules, and also by the emergence of technological means for synthetic methods of toxin production, overcoming the tedious tasks of peptide purification from crude venoms, venom collection and animal housing. Synthetic production of peptides appears as an invaluable asset to pharmacological development since natural peptides, previously characterized from a given venom source, could suddenly be optimized *in vitro* in order to obtain the desired characteristics, such as greater affinity for a given target, better selectivity or improved pharmacological effect. No doubts that the increasing use of bioinformatics for the analysis of the steadily growing list of orphan toxins is of tremendous help for the functional classification of these molecules and their potential use in pharmacological applications (4). With these latest developments, several toxin peptides made it this way to the clinics. This is for instance the case for several *Conus* peptides, identified in predatory cone snails, and that are used for medication in clinics for pain (Ziconotide, the synthetic form of ω-conotoxin MVIIA, a Ca^{2+} channel N-type blocker, from Elan Pharmaceuticals), epilepsy and other neuropathic disorders (5). Paralleling these latest developments, further applications regarding toxins are emerging and that are of technological nature. Hence, subcellular distribution of alpha7 nicotinic acetylcholine receptors can be studied with alpha bungarotoxin-gold nanoparticles (6). Surface immobilized toxins can be used for target protein purification or simply for the controlled activity of membrane channels by surface-attached molecules, as has been performed in the case example of muscimol, an agonist of $GABA_A$ and $GABA_C$ receptors (7). A 12 amino acid peptide sequence, Tet1, derived from tetanus toxin, in complex to polyethylenimine proved itself a very efficient vector for the specific delivery of DNA plasmids (8). A similar fragment of tetanus toxin, when placed in fusion to a reporter protein such as the green fluorescent protein GFP, proved itself valuable

for the mapping of synaptic connections during development of the mammalian central nervous system (9).

Herein, we will focus on maurocalcine, a scorpion toxin from *Scorpio maurus palmatus*. Maurocalcine is only the second discovered member of an ever growing family of new toxins active on ryanodine receptors (RyR). The first member of this family, imperatoxin A, was discovered in 1995 and initially purified from the venom of scorpion *Pandinus imperator* (10). This peptide was mainly characterized for its pharmacological activity on the RyR. Four more analogous peptides were subsequently discovered, first maurocalcine in 2000 from the scorpion *Scorpio maurus palmatus* (11), followed by both opicalcine 1 and 2 from the scorpion *Opistophthalmus carinatus* in 2003 (12), and hemicalcin from the scorpion *Hemiscorpius lepturus* in 2007 (13). The homology between these peptides ranges from 82% to 91%. All these peptides share common features that include a length of 33 amino acids, three disulfide bridges paired according to the motif Cys^1-Cys^4, Cys^2-Cys^5 and Cys^3-Cys^6 so common to the family of *scorpionidae* (**Figure 1**). A brief presentation of essential pharmacological features of maurocalcine will be performed before evidence is provided that maurocalcine is a new member of the family of cell penetrating peptides. Finally, examples of technological applications will be provided underscoring the huge potential of this uncommon peptide.

The pharmacological target of maurocalcine has an atypical intracellular localization

Maurocalcine is an unusual scorpion toxin in that its main pharmacological target is an intracellular Ca^{2+} channel, RyR (14,15). RyR is a channel localized at the membrane of the endoplasmic reticulum with the bulk of its sequence localized in the cytoplasm. It controls the release of calcium from the endoplasmic reticulum and its activity is modulated by a wealth of factor including Ca^{2+} ions, ATP and protein interactions and namely the voltage-dependent Ca^{2+} channel of the plasma membrane, the dihydropyridine receptor of skeletal muscles (16). Application of maurocalcine to purified sarcoplasmic reticulum induces Ca^{2+} release, and converts low affinity [^{3}H]-ryanodine binding sites to high affinity ones (17). Using the [^{3}H]-ryanodine binding assay, it was indirectly estimated that maurocalcine binds to RyR with a K_D of about 10-20 nM (17). More direct proof that maurocalcine interacts with RyR comes from the observation that a biotinylated derivative of maurocalcine bound to streptavidin-coated beads pulls down purified skeletal muscle RyR (18). Finally, application of maurocalcine to RyR reconstituted in lipid bilayers produces a drastic increase in channel activity by a raise in opening probability and the occurrence of a long-lasting subconductance

state. This observation is in perfect agreement with the stimulatory effect of maurocalcine on Ca^{2+} release from sarcoplasmic reticulum (19). All combined, these data led to the identification of RyR as the pharmacological target of maurocalcine.

Three pieces of evidence indicate that maurocalcine reaches its pharmacological target probably by direct diffusion through the plasma membrane. First, maurocalcine has an effect on the channel activity from lipid bilayers only when applied to the cytoplasmic face of the channel (14). Second, the RyR binding site of maurocalcine has been mapped to a restricted domain predicted to be localized within the cytoplasm (18). Finally, it was observed that extracellular application of 100 nM maurocalcine produces a rapid rise (a few seconds) of intracellular calcium in myotubes (17). These data underline the succession of molecular events undergone by maurocalcine before Ca^{2+} gets released from the lumen of the sarcoplasmic reticulum: (i) rapid plasma membrane crossing, incompatible with endocytosis and late endosomal release, (ii) concentration elevation within the cytoplasm to levels above 10 nM in order to reach at least the K_D value for RyR, i.e. close to 10 nM, (iii) binding onto the RyR site, (iv) opening of RyR channels, and (v) Ca^{2+} release from the sarcoplasmic reticulum. The property of cell penetration of a peptide is a rather uncommon observation. This was one of the reasons why some researchers initially had doubts about the specificity of RyR as pharmacological target of maurocalcine. This question had to be solved by a specific study demonstrating the passage of maurocalcine from one side of the plasma membrane to the other (20). During this study, one additional property of maurocalcine could be highlighted. Maurocalcine not only enters into cells, but it also can be used a vector for the cell penetration of membrane-impermeable cargoes. This strongly suggested that Maurocalcine, apart its pharmacological effect on RyR, had original features of a member of the growing family of cell penetrating peptides or CPPs (21).

Properties of a CPP and mechanisms of cell penetration

Cell Penetrating Peptides have been reunited under the generic term of CPPs. In a non-exhaustive list, CPPs have been isolated from: Tat from the HIV-1 virus (22-24), the *Drosophila* transcription factor ANTP (encoded by the *antennapedia* gene) (25), also called penetratin, and the herpes simplex virus type 1 (HSV-1) VP22 transcription vector (26). CPPs have also been termed *Protein Transduction Domains* (PTD) when the structural domain responsible for cell penetration of a given protein can be isolated as an individual entity. Herein, we shall use the CPP term which is less restrictive than PTD. In spite of lack of

sequence homology between CPPs, these peptides possess a certain number of common functional features that are worth describing.

1- Structure-activities studies, in particular for Tat, indicated an important role for basic amino acids in transduction and led to the discovery that arginine-rich and synthetic homopoly-arginines were efficient cell penetrating vectors (22,27,28). Although the efficacy of poly-arginine peptides was shown to relate to a minimum number of 9 arginine residues, additional features appeared to be important as the guanidinium group of the arginine residue itself (28,29). Demonstration that amphipathic model peptides were good transducers points out that penetration may also depend of the spatial separation of hydrophobic and positively charged residues (30,31). Many other CPPs have since then be produced that possess little in common at the amino acid sequence level but share this peculiar global positive charge which is required for cell penetration (21).

2- CPPs are small peptides, generally not exceeding 20 amino acids. However, since they can be put in fusion to larger proteins to favor their cell penetration and that many CPPs are longer PTDs issued from natural proteins, this length issue is in fact more important for commercial and technical reasons. It is indeed easier to synthesize and handle small peptides, than larger ones. For many applications, especially *in vivo* one, it is highly desirable to bring the costs of peptide synthesis down when large scale production is envisioned. This economic value was a strong factor in the development of poly-arginine peptides as peptide carriers (29). The effect of CPP size minimization on its efficacy for cargo cell penetration is however poorly apprehended.

3- Generally, CPPs lack cell selectivity. They can thus enter numerous cell types (22,30,32). This is an interesting issue since it hints to the universality of the cellular mechanisms that preside to the cell penetration of CPPs. It also indicates that CPPs are poor cell targeting compounds besides being excellent vectors for the cell penetration. They can’t be used for cell target discrimination between a neuron or a glial cell for instance. Although it appears that CPPs can enter many different cell types, a quantitative comparison of the efficacy of penetration in various cell types would probably reveal some differences that might be worth investigating by adequate structure/function studies. A recent systematic evaluation by *in vivo* confocal microscopy of the transduction of fluorescent oligo-arginines has showed that difference of penetration between D- and L-forms of peptides was highly variable between cell types (33).

4- CPPs enter rapidly into cells. They do this efficiently and at weak concentrations. Cell penetration within a few minutes is a common observation, although quantitatively, this penetration can occur over longer periods of time, commonly a few hours before it reaches saturation. CPPs are also extremely efficient in that they penetrate into 100% of a given cell type. This is an important property hardly matched by other vectors. Finally, CCPs penetrate into cells at low concentrations, i.e. at micromolar concentrations and more rarely at submicromolar concentrations. Effective penetration at low concentrations ensures that reasonable quantities of peptides are used for any given application. This matter is essential, not just because of costs considerations, but also because of toxicity issues. The most effective cell penetrating concentration ought to be correlated with the minimal concentration that induces cell toxicity, in particular for vector applications. This index high transduction/low toxicity is however poorly defined for each known CPP to date, as it may vary with the application pursued. It will also depend of the CPPs stability inside cells, a criteria which is seldom address at the same time (33).

5- The cell penetration of CPPs does not require any specific membrane receptor. This has been proven for many CPPs since optical CPP stereoisomers, made of D-amino acids instead of the natural L-amino acids, are at least as efficient as their L-counterparts in crossing the plasma membrane (29,30). This lack of dependence on membrane receptors is further highlighted by the lack of structural similarities between the various CPPs identified so far. This does not mean of course that CPPs do no interact with any membrane component. Two types of cell surface components have been shown to interact with CPPs, glycoaminoglycans (GAGs) and negatively charged lipids (34,35). The basis of these interactions was shown to be electrostatic (36).

6- The initial assumption has been that CPPs do not require metabolic energy for cell entry (37). Evidence for this comes from experiments in which CCP cell entry was preserved in cells maintained at 4°C (20) or in the presence of metabolic inhibitors (38). This property is however a hotly debated question because the metabolic-dependence or –independence of the cell penetration of CPPs is intimately linked to the mechanical view of cell penetration (39). Although methodological problems have been uncovered in analyzing cell penetration, several mechanisms of penetration have been proposed (40-43). In one process, penetration involves a reorganization of the plasma membrane which allows the peptide to move from the extracellular face of the

membrane to the intracellular one (44). By this mechanism, the peptide gets freely into the cytoplasm and makes CPPs valuable to deliver cargos targeted to soluble proteins, a challenge for many therapeutic applications (45). In another route to cell interior, many CPPs follow an endocytotic route, a process which requires energy (46). The type of endocytosis (macropinocytosis, clathrin-dependent, caveolin-dependent, or clathrin- and caveolin-independent) depends on cell type, CPP sequence, and cargo nature (47). In this case, CPPs mainly end up into late endosomes and are therefore still separated from the cytoplasm by a lipid membrane. Various experimental strategies are therefore pursued to favor the leakage of the CPP and attached cargo from the late endosomes to the cytoplasm (pH sensitive constructs, or addition of the lysosomotropic reagent chloroquine (48)). So far, three compartments are known to accumulate CPPs: endosomes, cytoplasm, and nucleus. Mitochondria for instance are not affected (46,49).

7- The foremost important property of CPPs is their ability to behave as vectors for a great variety of cargoes, all reputed for their inability to penetrate into cells by themselves (21,50). Most CPPs have no cell properties on their own, so their chemical coupling to functional entities of biological significance is highly desirable. The variety of cargoes, both in nature and in size, which have been coupled to CPPs and shown to enter into cells is simply amazing. So far, successful cell entry of DNA plasmids or mimics (51,52), oligonucleotides such as antisense, si or shRNA (53-55), peptide nucleic acids (PNA) (56), peptides, proteins (57), drugs (58), and nanoparticles (59) have been reported. New applications are emerging at an astonishing rate into the field of basic research, therapeutics (60), technology or medical imaging (61). CPPs can also be coupled to multiple cargoes in order to develop multitask functional complexes, for instance for therapy and imaging applications (62). The versatility of cargo nature obviously opens an unprecedented number of applications hardly matched by other cell delivery systems such as viruses or liposomes.

8- One last property displayed by some CPPs is their ability to deliver cargoes through the blood brain barrier, which also opens unprecedented possibilities for vector applications in neurosciences (63). The universality of this property for all CPPs, like the metabolic-dependence, has not yet been established.

Maurocalcine behaves as a CPP – Structural and functional evidences

1- Structural evidences

The 3-D solution structure of maurocalcine was determined by ^{1}H-NMR in 2000 (11). The peptide folds according to an inhibitor cysteine knot (ICK in short) also found in other peptides active on calcium channels such as ω-conotoxin GVIA that targets voltage-dependent N-type Ca^{2+} channels (64). With the characterization of this structure, it turned out to represent the first example of a scorpion toxin with an ICK fold (11). A careful examination of the electrostatic surface of maurocalcine reveals that the peptide is folded in such a manner that most of its basic residues are oriented towards one face of the molecule (**Figure 2**). Conversely, the other face of the molecule is hydrophobic indicating a strong dipole moment capable of orienting the peptide. The polarization of positive charge distribution of the peptide is a feature shared by several other peptides that have the ability to penetrate into cells (20).

2- Functional evidences

The very first proof that maurocalcine could act as a cell penetrating peptide came from a specific study in which a biotinylated derivative of maurocalcine was synthesized (20). Biotin is a convenient label since it can bind avidin or streptavidin. Biotinylated maurocalcine was coupled to a fluorescent derivative of streptavidin and the complex was assayed for cell penetration by direct incubation with a variety of cells. Intracellular accumulation of fluorescence and cell distribution of this fluorescence were followed by confocal microscopy, whereas the intensity of fluorescence was determined more quantitatively by fluorescence-activated cell sorting (FACS). According to these studies, maurocalcine-coupled fluorescent streptavidin has the ability to enter into all types of cells maintained in culture, including cell lines such as HEK293 cells, normal and glycosaminoglycan-deficient CHO cells, MCF7 cells, MDA-MB 231 cells and differentiated and non-differentiated myogenic L6 cells, or primary cultured cells, such as hippocampal CA1 neurons, dorsal root ganglion cells, astrocytes, and myotubes (20,65,66). An example of such a penetration is shown for a CA1 neuron maintained in culture (**Figure 3**). It is obvious from this example that the fluorescence distributes in all compartments of the neurons, including neurites. As expected for a CPP, cell penetration of streptavidin occurs in 100% of the cells in culture (**Figure 4**). The homogeneity of the cellular response to cell penetration by a CPP is an important asset in vector applications. Along with the fact that many cell types are concerned by cell penetration, it points to a great conservation of cellular mechanisms for peptide entry. Such a preservation of mechanisms is most likely due to the presence of well-conserved cell surface components that are required for CPP cell interaction and penetration.

The cell penetration of maurocalcine/streptavidin complexes is rapid since half-saturation in the cytoplasm is produced within 20 minutes (20). These values ought to be considered with caution however since the kinetics of cell penetration of maurocalcine is dependent on the nature of the cargo attached to it. Extracellular applications of free maurocalcine to myotubes produce a rise in intracellular calcium within seconds indicating that the real kinetics of cell entry of the vector is probably much faster when it is not coupled to any cargo. Thus, this parameter requires further evaluation once the design of novel fluorescent or radioactive analogues of maurocalcine will be achieved. Efforts are underway to evaluate the properties of an iodinated derivative of maurocalcine ([^{125}I]-Tyr-maurocalcine for quantitative purposes) or of an FITC-maurocalcine (for confocal microscopy analyses). Preliminary observations indicate that cell penetration of [^{125}I]-Tyr-maurocalcine is faster than maurocalcine/streptavidin complexes (unpublished observations).

Concerning maurocalcine/streptavidin complexes, the cell distribution of the penetrating complex follows a temporal gradient with the plasma membrane being the first locus of distribution, followed by endosomal compartments (67) and the cytoplasm (20). Subsequently, some nuclear distribution can be observed also but at a fractional level of the total penetration. This point needs clarification since the initial studies were performed with fixed cells instead of living material and the fixation procedure is now known to affect the observed distribution of CPPs (38). The cell distribution of intracellular maurocalcine cannot be dissociated from the mechanisms of cell uptake of the peptide. Two potential routes of diffusion of maurocalcine into the cytoplasm need to be investigated: (i) one that occurs through macropinocytosis with a subsequent slow and incomplete release from late endosomes to the cytoplasm, as shown for PDX-1 protein (68), and (ii) another one through direct translocation. The latter one is the favoured mechanism to rationally explain why and how extracellular application of maurocalcine to cells can produce such a rapid rise (a few seconds) in intracellular calcium (which can only occur through binding of maurocalcine to a cytoplasmic binding site of RyR). Translocation can possibly occur within seconds, but macropinocytosis and endosomal escape, not. FITC-maurocalcine appears to be exclusively distributed in the cytoplasm and this is also the case for a maurocalcine-cys-cys-peptide or a maurocalcine-maleimide-cys-nanoparticle complex (unpublished observations). In contrast, a major fraction of streptavidin-conjugated maurocalcine is taken up by macropinocytosis, at least in CHO cells (67), and a smaller fraction appears to be distributed outside late endosomes, mainly in the cytoplasm (20). The question remains whether this cytoplasmic distribution of maurocalcine/streptavidin complex results from translocation or from

endosomal escape. The fact that penetration of maurocalcine/streptavidin is observed when cells are incubated at 4°C (65), at a temperature at which endocytosis is blocked, is an indication that cell translocation may contribute to a fraction of the penetration of the complex. Obviously, a point that will merit a closer look is the impact of cargo nature on the mechanism of cell entry. The basic idea that seems to progressively emerge from studying maurocalcine's properties is that translocation is involved in the absence of a cargo or when the peptide is conjugated to small cargoes, whereas, on the contrary, endocytosis is the main route of entry if the cargoes are of larger size or possess some yet unidentified physicochemical property incompatible with translocation.

Maurocalcine penetration is observed at concentrations as low as 10 nM and reaches saturation for concentrations close to 1 µM (65). Half-effects have been observed around 500 nM, but some maurocalcine analogues (i.e. maurocalcine E12A) display maximal penetration at 100 nM, making them the best CPPs known to date. Maurocalcine interacts both with GAGs (67) and several negatively charged lipids (65). Interaction was detected with heparin, heparin sulphate, and chondroitin sulphate. Incubating maurocalcine with soluble GAGs can inhibit up to 80% of the cell uptake of maurocalcine for two reasons: (i) soluble GAGs screen the positive charges of maurocalcine required for cell entry, and (ii) they compete for the interaction of maurocalcine with cell surface bounds GAGs. Interactions with GAGs are however not totally required for cell entry of maurocalcine since penetration is partially preserved in GAG-deficient CHO cells. However, GAGs contribute to cell penetration at the quantitative level by increasing the total amount of the cargo that enters into cells. Concerning lipids, interactions have been observed with gangliosides, such as GD3 (disialoganglioside NeuAcα2-8NeuAcα2-3Galβ1-4Glcβ1-Cer), phosphatidylinositol (PtdIns)(3)P, PtdIns(4)P, and PtdIns(5)P), phosphatidic acid and sulfatide, and more weakly with lysophosphatidic acid, PtdIns(3,5)P2 and phosphatidylserine. These interactions all take place with affinities in the submicromolar range (100 to 500 nM, lipids) to the low micromolar one (1-5 µM, GAGs). Because of the multiplicity of the intervening players, it is extremely difficult to precisely identify the cellular component(s) that contribute(s) the most to the cell entry of CPPs. This is also the case for maurocalcine. Obviously, the identification of these components would tremendously help the design of more efficient CPPs. This avenue of research definitively merits greater attention than it has so far. For the time being however, the preliminary results indicate that measuring binding affinities of maurocalcine or its analogues for cellular components is already an important route of investigation to develop new CPP analogues that penetrate at lower concentrations and with greater efficacies. Using

this methodology, it was determined that maurocalcine K20A interacts with weaker affinities on both GAGs and lipids and hence requires higher concentrations for cell penetration. Conversely, maurocalcine E12A was found to penetrate far better than maurocalcine itself because of its greater binding affinities for lipids. Thus, the precise identification of the key cellular components required for the cell penetration of maurocalcine will certainly help the design of still better cell-penetrating analogues.

Finally, an important observation is that maurocalcine shows almost no sign of cell toxicity as assessed by iodide propidium cell incorporation or an MTT cell survival assay (65,66). This toxicity can reach 20% when maurocalcine is assayed on primary cultures of neurons for 24 hours, thus far longer than the time required for cell penetration. This effect might be linked to the pharmacological impact of maurocalcine on neuronal RyR. Among the various analogues of maurocalcine designed so far, most appear far better than maurocalcine with regard to toxicity levels, probably because of modulation in pharmacological efficacy. Many analogues for instance show absolutely no toxicity after 24 hours of incubation with neurons and at concentrations up to 10 µM. The rather small cell toxicity of these analogues means that maurocalcine derivatives have excellent concentration ratio [cell penetration]/[cell toxicity] indexes. According to our analyses, they have the best indexes known so far for CPPs, at least when compared to penetratin, Tat or poly-R (unpublished data).

These studies are not exhaustive and much remains to be done to characterize the properties of maurocalcine, but these data are sufficiently conclusive to indicate that maurocalcine is a novel class of CPP molecule. Since it has homology with imperatoxin A, hemicalcin, and opicalcine 1 and 2, there are strong suspicions that these toxins may also be used as CPPs. These peptides can differ by the nature of the amino acid residues at only seven different positions, but the differences observed all respect a fixed number of positively charged residues (12 over the 33 amino acids). The net positive charge of these peptides is +8, a value globally conferred by lysine residues since arginine residues are mainly involved in electrostatic interactions with Glu or Asp residues. Variable positions 1 and 4 (respectively, amino acids 9 and 14) are interchangeable for one positively charged residue (Arg at position 1 or Lys at position 4). Similarly, variable positions 2 and 3 (respectively amino acids 12 and 13) are interchangeable for one negatively charged residue (Glu at position 2 and Asp at position 3). The impact of these variations on pharmacological and cell penetration properties still needs to be assessed. The sequence alignment of these toxins indicates that the extent of amino acid sequence variability undergone by this family of peptides is limited. In fact, these peptides respect three principal property constraints: (i) cell penetration, (ii) sequence

homology with a domain derived from a voltage-dependent calcium channel (17) (see chapter on the pharmacological properties of maurocalcine), and (iii) RyR binding and activation. Preserving all these criteria leaves little room for excessive sequence variation, but opens exciting research avenues for structure-function studies aimed at dissecting and isolating each function of these peptides. The existence of natural structural analogues of maurocalcine opens the route to new research programs aimed at discovering novel CPP molecules in the incredible rich sources of peptides that are venoms. Several strategies will be pursued: (i) determining novel toxins active on RyR by affinity column purification, (ii) screening for peptides that can enter into cells, and (iii) purifying peptides from venoms based on their basic nature. There are no doubts that many more peptide will be discovered in the future, all possessing technological potential for interesting applications. However, since toxin peptides are generally active on pharmacological targets, it is also important to perform an extensive structure-function research program aimed at dissociating the cell penetration property from the pharmacological one. Here is what has been performed for maurocalcine so far.

Deriving new CPP analogues from maurocalcine in order to avoid pharmacological activity

Using a venomous toxin as a vector for biological or technological applications has one obvious drawback: because of its associated pharmacological activity, it just can't be used in its original form for these applications. Hence, a complete structure-function analysis is required to develop analogues devoid of pharmacological activity but which preserve or enhance the cell penetration properties of the original molecule. For most CPPs, this type of study was not required because many of them have no biological activity on their own. In the case of maurocalcine, this type of research program has been tremendously facilitated by the identification of the pharmacological target and effect of the peptide. Several mutated analogues of maurocalcine have been produced, most by alanine substitution of positively charged amino acid residues (17,66). These analogues were compared to the wild-type sequence for (i) structure, (ii) RyR activation, (iii) cell penetration, (iv) interaction with GAGs and lipids, and (v) cell toxicity. Various analogues were thus isolated that are of interest either for direct use a vector or that can be used as lead to develop further sophisticated vectors. Hence maurocalcine R24A is of interest despite its lower cell penetration efficacy because it has fully knocked out the pharmacological potential of the peptide. Also, maurocalcine E12A turns out as a great lead molecule for the design of novel analogues since it is the best CPP known to date in spite of its greater pharmacological activity (66). Now, two new strategies are pursued. Firstly, the production of a disulfide-less peptide that should lead to abnormal

folding and impaired pharmacological activity, but that should preserve the cell penetration property. Preliminary data indicate that this is indeed the case (unpublished data). Secondly, we are in the process of producing a stereoisomer D-analogue of maurocalcine or of maurocalcine E12A that should be devoid of binding activity onto RyR, but that should fully preserve the cell penetration activity of the L-stereoisomer counterpart. Similar approaches have been used in the past for other CPPs. This research program has three benefits: (i) dissociate pharmacological and cell penetration structural determinants of maurocalcine, (ii) lead to a better understanding of the structural determinants (both maurocalcine and cell surface components) that contribute to an efficient cell penetration of maurocalcine, and (iii) produce better analogues than maurocalcine for cell penetration. In that respect, the initial goals concerning maurocalcine are already reached, but further analyses will improve our understanding of the molecular basis of cell penetration.

What does maurocalcine add to the field of cell penetration compared to other CPPs?

Considering that there are several CPPs on the market with proven biological applications, the question naturally arises about the add-on value of maurocalcine. Among the disadvantages of the molecule, one could cite the fact that maurocalcine is of greater backbone length than other CPPs making it more expensive to synthesize. Beside cost considerations, it is also more difficult to produce considering that it has to fold properly and organize with three well-defined disulfide bridges. The presence of these disulfide bridges (if such an analogue should be used) makes cargo coupling based on maleimide chemistry more hazardous than if no cysteine residue is present in the peptide. These considerations being stated, there are also interesting advantages to consider in the case of maurocalcine. First, the addition of a novel CPP to the CPP list should help refining the basic structural properties shared by all CPPs for an efficient cell penetration. Second, CPPs have in common the property of cell penetration, but might well be different in their mechanisms of cell penetration. Some may rely more heavily on endocytosis such as nonaarginine (69), whereas other may depend on translocation. This is not a minor issue because depending on the type of cell penetration used by a given CPP, the applications that can be envisioned are drastically different. There are only very little applications imagined for complexes that end up in late endosomes, whereas the number of applications for translocated complexes that accumulate in the cytoplasm are unlimited. The main asset of maurocalcine on this question is that the original free vector, in its non-complexed form, should enter cells through translocation. Cargo nature and size might induce a shift in the entry pathway, from translocation to endocytosis, but this point needs to

be clarified with appropriate studies. In the case of Tat for instance, even the type of endocytosis appears to be influenced by cargo type (47). Third, it is true that CPPs enter into all cell types, but the preliminary evidence that we gathered is that there are cell-specific differences in the efficiency of the penetration. In our hands, maurocalcine penetrates better into glial cells than in neurons (unpublished observations). It hints to the possibility that CPPs might eventually be designed in such a way to penetrate only into desired cell targets. The observation that CPPs may interact with different glycosaminoglycans species is an indication that they may be targeted to selective tissues that express specific glycoaminoglycan patterns at their surface (35). As a proof of concept, the observation that crotamine, a snake venom CPP, presents selective cell penetration into actively dividing cells is an indication that cell-selective CPPs are maybe not out of experimental reach (70,71). Fourth, cell toxicity of CPPs is an essential parameter to consider in any given application. For some CPPs, the concentration at which cell toxicity is observed is too close to the concentration required for cell penetration. We found that maurocalcine and its analogues behave quite well with regard to this issue. Fifth, disulfide bridges within toxins are required to stabilize the 3D structures of the peptides. They also contribute to a greater peptide stability, which may turn into a significant advantage for *in vivo* applications if a maurocalcine analogue is used as vector for cargo delivery into cells. For instance, the Tat CPP, that lacks disulfide bridges, was found to degrade rapidly even in the extracellular culture medium of epithelial cells (72). This point will be investigated in details when an adequate maurocalcine vector will be developed for versatile applications. Finally, thorough comparisons between CPPs need to be performed on several other essential issues which are for instance: (i) *in vivo* distribution (i.e., do they all cross the blood brain-barrier?), (ii) intracellular concentrations reached (what kind of intracellular concentration of each CPP is really found in cells and in what compartment?), (iii) *in vivo* toxicity, and (iv) *in vivo* pharmacokinetics.

Upcoming questions

One question appears crucially important for the development of novel vector-based applications: what kind of intracellular concentration does maurocalcine reach within the cell? Extracellular application of 100 nM maurocalcine on myotubes produces calcium release within seconds through activation of intracellular RyR. Since the K_d of maurocalcine for RyR is in the 10-20 nM range, these data suggest that maurocalcine very rapidly reaches these concentrations inside the cell (20). Such a rapid rise may indicate that the final concentration of maurocalcine in cells might get close to 100 nM or maybe higher. A first rapid estimation

using [^{125}I]-Tyr-maurocalcine lead us to conclude that the final concentration of maurocalcine within cells is 30-fold that of the original concentration (unpublished observation). Why would cells concentrate to this extent maurocalcine? All cells are negatively charged at the inner surface of the plasma membrane which represents an electrical force for positively charged molecules. Like other CPPs, maurocalcine is positively charged. If translocation is the route of entry of maurocalcine, then the peptide may be driven by two factors: (i) the concentration ratio, and (ii) the membrane potential of the cell. Like for ions, equilibrium in the fluxes of entry and exit may be found at the Nernst equilibrium potential of maurocalcine which is presumably quite high (above 50 mV). Predicatively, depolarizing the membrane potential of cells should decrease maurocalcine entry because it may rely only on the concentration gradient. This was indeed observed to some extent for the maurocalcine/streptavidin complex (65). A similar study needs to be performed with [^{125}I]-Tyr-maurocalcine to be more conclusive because this molecule is more likely to undergo translocation than the maurocalcine/streptavidin complex. Translocation is the preferred route of entry for maurocalcine if we envision developing specific applications. It ensures sufficient concentrations of the cargo and greater chance for nuclear delivery also. It was found that a doxorubicin-maurocalcine chimera molecule has antitumor activity on doxorubicin-resistant cells consistent with a nuclear function of doxorubicin, albeit the vast majority of the molecule is found in the cytoplasm (unpublished observations). If the route of cell entry is influenced by cargo nature (73), then the matter of maurocalcine/cargo concentration will need to be examined for each application. It is difficult to envision that endocytosis may lead to similar concentrations than translocation. In that case, it will also be necessary to exmine the fraction of penetrated complexes that has the chance to escape and reach the cytoplasm or the nucleus. Sometimes, this escape is so minimal that even oligonucleotide-based cell applications are impossible without a more drastic drug-induced endosomal rupture (48). Yet, on other occasions, this tiny amount might be sufficient for other biological applications, such as plasmid delivery (51).

What kind of research program can be developed with an efficient cell-penetrating maurocalcine analogue?

The number of applications that can be developed with CPPs is simply astonishing and limited only by imagination. For the purpose of illustration, one example is shown using maurocalcine as vector for the delivery of nanoparticules within cells (**Figure 5**). Nanoparticles are envisioned for many technological applications. They may serve as

platforms for the simultaneous chemical attachment of several ligands of biological interest. Many original applications can now be envisioned for maurocalcine. For instance, the peptide may help liposomal-induced drug delivery into cells, as observed for Tat and penetratin (59). It can be envisioned for the therapeutic delivery of proteins in fusion with the adequate CPP (22). Purification of such recombinant fusion proteins is easily achieved using a heparin affinity column owing to the heparin binding properties of CPPs (74). It holds great promises for the delivery of anti-tumour drugs for cancer treatment (39). CPPs have also been fused to the ectodomain of the coxsackievirus-adenovirus receptor to attach them to adenoviral fiber knobs, a strategy that overcomes the natural tropism of viruses by allowing an efficient infection in non-permissive cells (75). Now that maurocalcine is a proven and efficient CPP, it is on the track for biological, diagnostic and technological applications.

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FIGURE LEGENDS

Figure 1. Maurocalcine belongs to a larger family of scorpion toxins

Sequence alignment of maurocalcine with four analogous toxins, imperatoxin A, opicalcine 1, opicalcine 2 and hemicalcin. Maurocalcine, imperatoxin A and hemicalcin are three peptides known to be active on the ryanodine receptor. Opicalcine 1 and 2 have not been tested for pharmacological activity. All five toxins have the same number of positively charged amino acid residues (the N-terminal glycine residue, six or seven lysine residues, and five or four arginine residues as indicated in blue). Negatively charged amino acids are represented in red as well as sequence identity. Highlighted residues (seven possible positions) indicate variable identity among toxins. The year of discovery of each toxin is also provided in italics.

Figure 2. Basic face of maurocalcine

3-D structure of maurocalcine drawn by the Accelrys DS visualizer software and surface colored according to the electrostatic potential (blue and red for positively and negatively charged amino acid residues, respectively). The basic face depicts most positively charged residues of maurocalcine, i.e. Gly^{1}, Lys^{11}, Lys^{14}, Lys^{19}, Lys^{20}, Lys^{22}, Lys^{30} and Arg^{23}, Arg^{24} and Arg^{30}). The peptide backbone is depicted as a blue ribbon, whereas only the lateral chains of positively charged amino acid residues are indicated with scaled balls and sticks. Arg^{24}, a critical residue for the pharmacological effect of maurocalcine on RyR, is indicated in red and its lateral chain in yellow. For cell penetration studies, all chemical successful chemical coupling strategies were performed by modification or extension of the amino-terminus of maurocalcine.

Figure 3. Cell penetration of streptavidin-Cy5 induced by maurocalcine

Single plane confocal microscopy images of living hippocampal CA1 neurons at 18 days *in vitro* showing the intracellular distribution of streptavidin-Cy5/maurocalcine complexes (left panel), and the plasma membrane as labelled by concanavalin A (middle panel). The merge image (right panel) indicates the intracellular distribution of streptavidin-Cy5 including in neuritis.

Figure 4. Maurocalcine-induced cell penetration of streptavidin-Cy5 into CHO cells followed by confocal microscopy and FACS

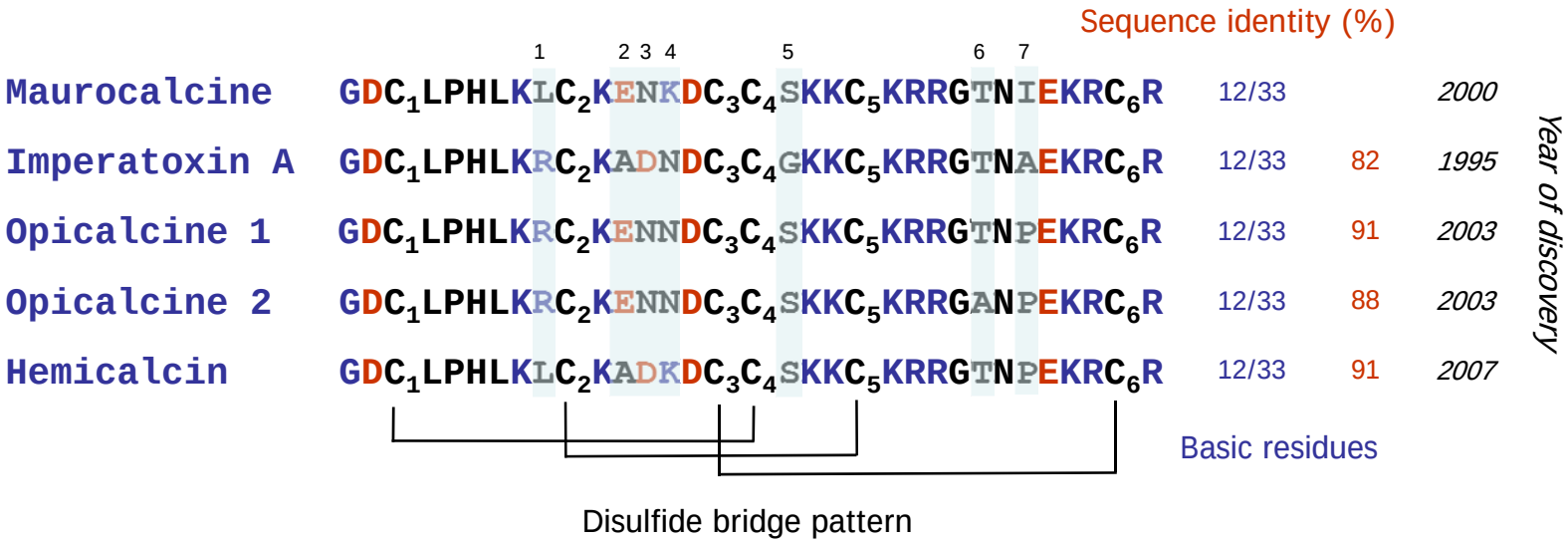
A- Single plane confocal microscopy image of a living CHO cell in culture showing the localization of streptavidin-Cy5/maurocalcine complexes. The punctuate distribution is indicative of endosomal distribution. Image taken immediately after 2 hrs incubation of the cells with 100 nM of complex.

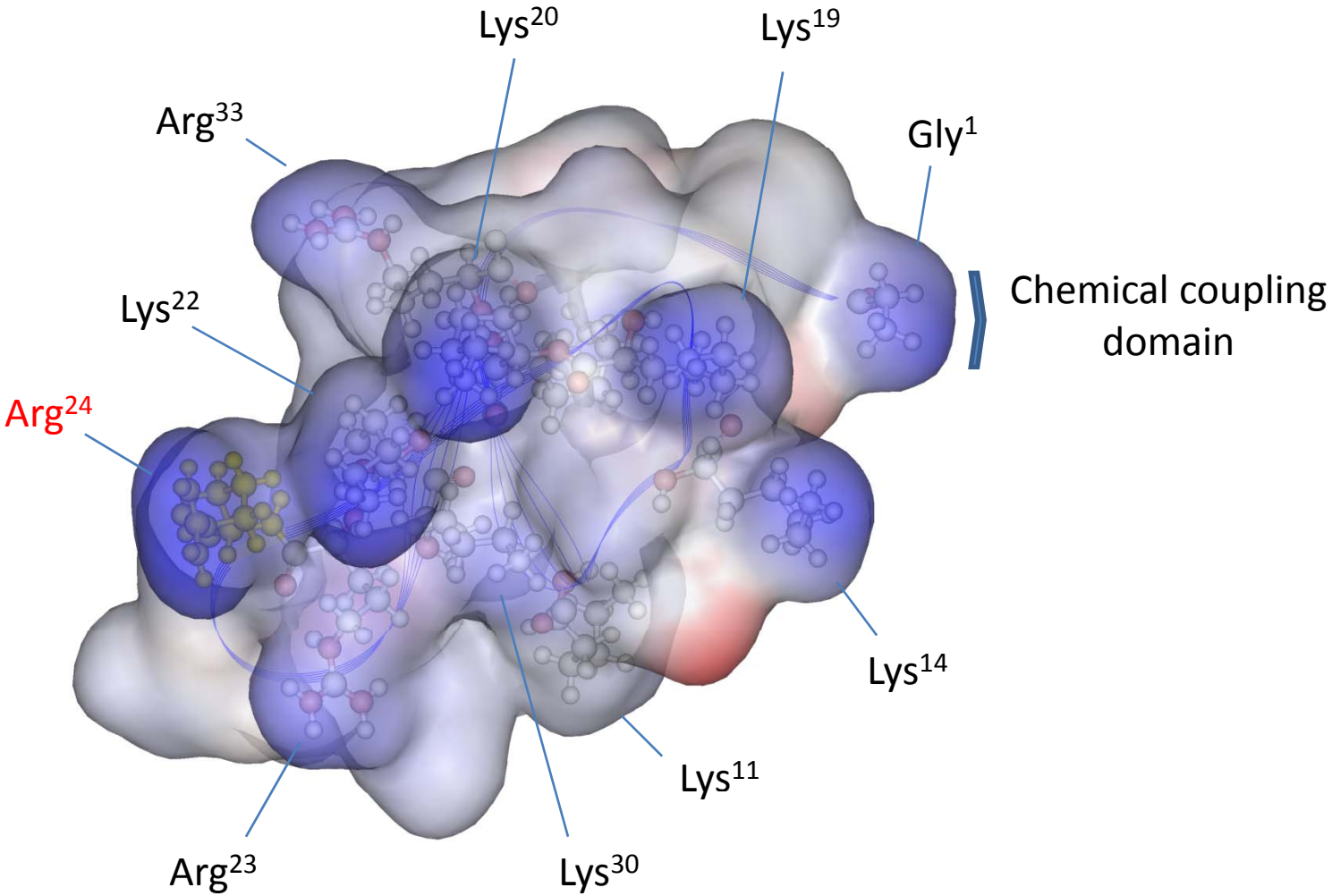
B- FACS analysis of the streptavidin-Cy5 (strep-Cy5) fluorescence in CHO cells after a 2 hour incubation with 1 µM strep-Cy5 alone (left Gaussian distribution) or in the presence of 4 µM maurocalcine (right Gaussian distribution). The shape of the distribution indicates that all cells have taken up the maurocalcine-bound strep-Cy5.

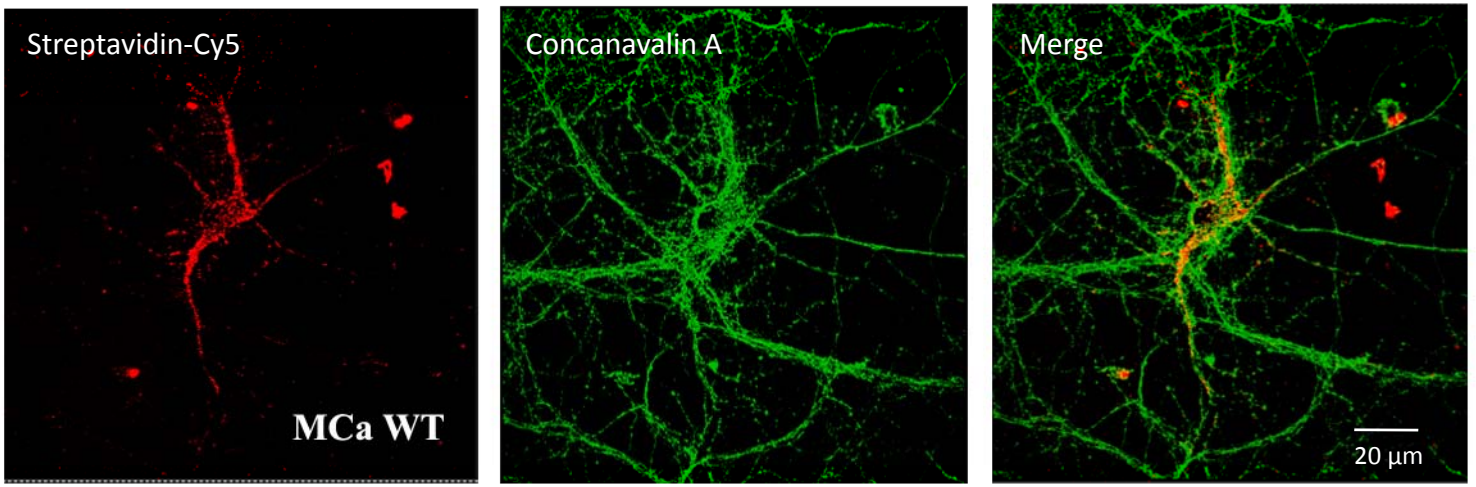
Figure 5. Maurocalcine is a good vector for the delivery of nanoparticles in cells

A- Architecture of a 10-15 nm diameter quantum dot. The core of the nanoparticle is made of cadmium selenide (CdSe), whereas the shell is made of zinc sphalerite (ZnS). The polymer coating is made of polyethyleneglycol to which streptavidin is attached. Biotinylated maurocalcine is linked to streptavidin. About 6-8 streptavidin molecules per nanoparticle. Peak fluorescence emission at 587 nm.

B- Cell penetration of maurocalcine-bound nanoparticles into CHO cells. Nanoparticles appear in red, whereas the nucleus is stained in green. Because of the presence of streptavidin, the nanoparticles are taken up in endosomes.

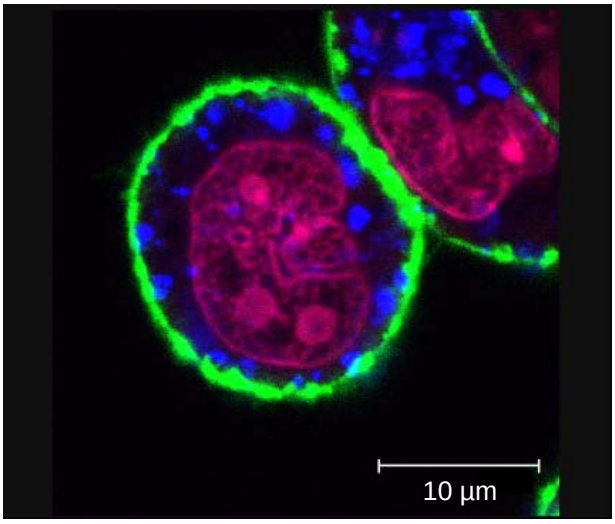

Sequence identity (%)
1 2 3 4 5 6 7
Maurocalcine GDC1LPHLKLC2KENKDC3C4SKKC5KRRGTNIEKRC6R 12/33 2000
Imperatoxin A GDC1LPHLKRC2KADNDC3C4GKKC5KRRGTNAEKRC6R 12/33 82 1995
Opicalcine 1 GDC1LPHLKRC2KENNDC3C4SKKC5KRRGTNPEKRC6R 12/33 91 2003
Opicalcine 2 GDC1LPHLKRC2KENNDC3C4SKKC5KRRGANPEKRC6R 12/33 88 2003
Hemicalcin GDC1LPHLKLC2KADKDC3C4SKKC5KRRGTNPEKRC6R 12/33 91 2007
Year of discovery
Basic residues
Disulfide bridge pattern


Lys20
Lys19
Arg33
Gly1
Lys22
Arg24
Chemical coupling domain
Lys14
Lys11
Arg23
Lys30


Streptavidin-Cy5
MCa WT
Concanavalin A
Merge
20 µm

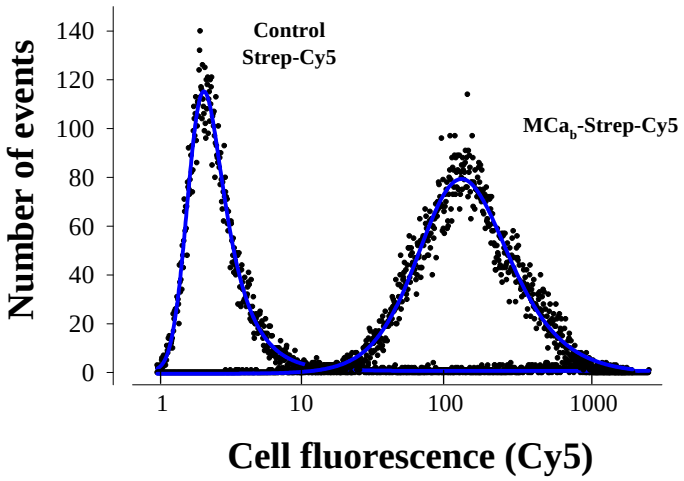
A

Confocal microscopy

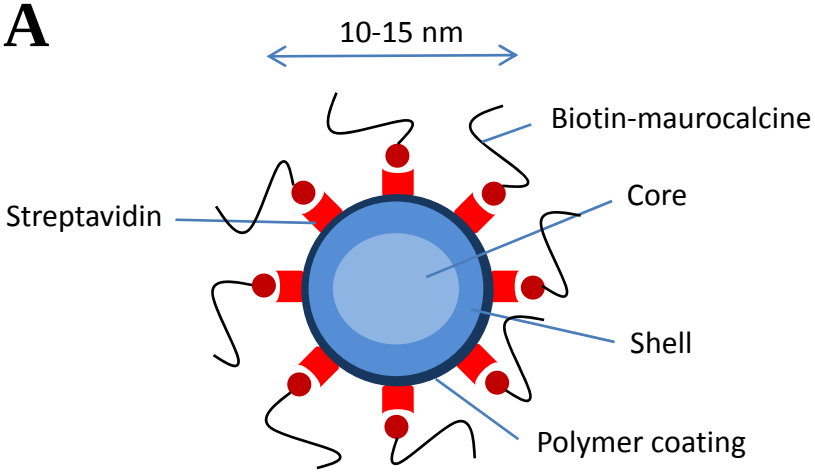


B

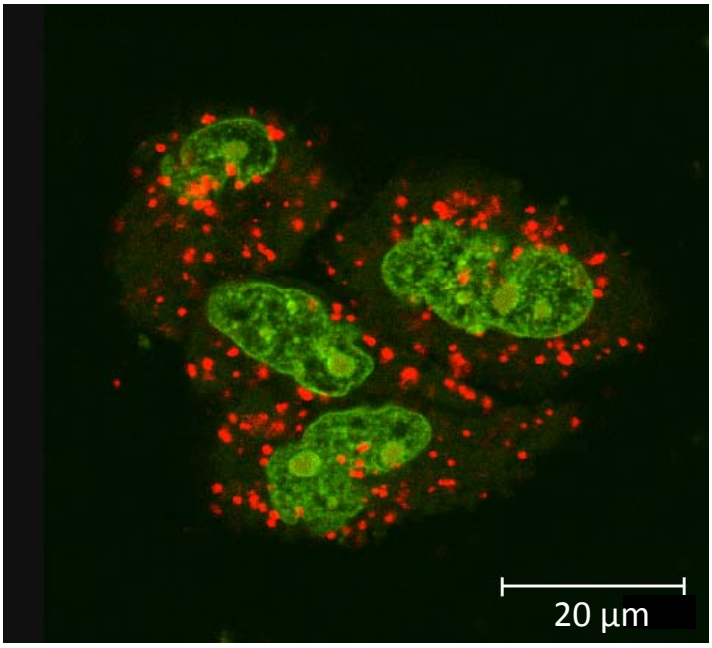
FACS



A


10-15 nm
Biotin-maurocalcine
Core
Streptavidin
Shell
Polymer coating

B


20 µm