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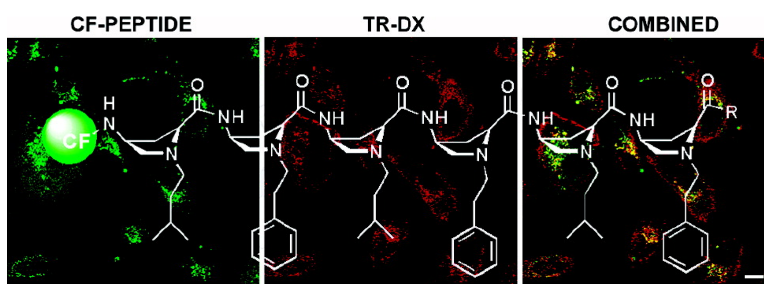


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# Cell-Penetrating *cis*-γ-Amino-L-Proline-Derived Peptides

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***Abstract:*** The synthesis of *cis*-γ-amino-L-proline oligomers functionalized at the proline α-amine with several groups that mimic the side chains of natural amino acids, including alanine, leucine, and phenylalanine, is herein described. These γ-peptides enter into different cell lines (COS-1 and HeLa) via an endocytic mechanism. The ability of these compounds to be taken up into cells was studied at 37 °C and 4 °C by plate fluorimetry, flow cytometry, and confocal microscopy. In addition to their capacity for cellular uptake, these unnatural short length oligomers offer advantages over the well-known penetrating TAT peptide, such as being less toxic than TAT and protease resistance.

## Introduction

In the past few years several peptides capable of crossing the cell membrane, namely cell-penetrating peptides (CPPs),[1] have been described in the literature.[2] This capacity suggests their potential application as new agents for cellular delivery of biomolecules. CPPs offer several advantages over other known cellular delivery systems,[3,4] including low toxicity, high efficiency toward different cell lines, and even inherent therapeutic potential. Peptides and proteins are nevertheless limited by low protease resistance and, sometimes, low membrane permeability.[5,6] Hence, compounds with greater proteolytic resistance, such as loligomers,[7,8] as well as biomolecular mimetics, such as β-peptides[9−12] (nonnatural peptides, formed by β-amino acids, that can adopt discrete and predictable well-defined secondary structures), have been evaluated as drug delivery agents.

Peptides capable of translocating the cell membrane can be classified into two groups: (i) cationic peptides with at least six charged amino acids (Lys or Arg) such as HIV-1, TAT peptide,[13] penetratin,[14] and chimeric transportan,[15] and (ii) hydrophobic peptides, such as those based on the H-region of signal-sequence proteins.[16] Bactericidal peptides are unusual in that they are both charged and contain hydrophobic regions in their primary or secondary structure.[17−21] As these features are implicated in both membrane permeabilization and pore-forming, functions which lead to microbicidal mechanisms, the

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(1) Abbreviations: Amp, *cis*-4-amino-L-proline or (2*S*,4*S*)-4-amino-pyrrolidine-2-carboxylic acid; Boc, *tert*-butoxycarbonyl; (2*S*,4*S*)-Boc-Amp(Fmoc)-OH, (2*S*,4*S*)-Fmoc-4-amino-1-Boc-pyrrolidine-2-carboxylic acid; CF, 5(6)-carboxyfluorescein; CPPs, cell-penetrating peptides; DCM, dichloromethane; DIEA, *N*,*N*-diisopropylethylamine; DIC, *N*,*N*′-diisopropylcarbodiimide; DMF, *N*,*N*-dimethylformamide; $Et_3N$, triethylamine; Fmoc, 9-fluorenylmethoxycarbonyl; HF, fluorhidric acid; HIV, human immunodeficiency virus; HOAc, acetic acid; HOBt, 1-hydroxy-1,2,3-benzotriazole; MALDI-TOF, matrix-assisted laser desorption ionization, time-of-flight; MBHA, *p*-methylbenzhydrylamine resin; MeCN, acetonitrile; RP-HPLC, reversed-phase high performance liquid chromatography; TBME, *tert*-butylmethyl ether; TFA, trifluoroacetic acid; TR-DX, Texas Red-Dextran.

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use of bactericidal peptides as delivery agents would appear to be limited. However, selective modulation of the bactericidal activity of these compounds has yielded new carriers.[22]

It has been reported that proline-rich peptides[22−24] and proline dendrimers[25] can be internalized by eukariotic cells. The most important advantage of proline-rich peptides in biological systems is their solubility in water. In this context, our group has described a nonnatural proline-derived $\gamma$-peptide, from *cis*-$\gamma$-amino-L-proline, with the ability to adopt secondary structure in solution.[26] Three different $\gamma$-peptide families were synthesized, $N^{\alpha}$-acyl-$\gamma$-peptides (polyamides on the side chains), $N^{\alpha}$-alkyl-$\gamma$-peptides (polyamines on the side chains), and $N^{\alpha}$-guanidilated-$\gamma$-peptides. In the present work, some of these peptide structures were selected to study their cell uptake properties (see Figure 1).

The amphipathic character of a compound determines its potential for internalization[24,27] and, in the case of the peptides at hand, varied with side chain structure. The modulation of this side chain structure offers a wide range of combinations that open the possibility of obtaining compounds with different hydrophobic/hydrophilic character and, consequently, with different properties as carriers. Hence acyl-peptides are more hydrophobic while other families are more hydrophilic or amphipathic. Additionally, the stability imparted by the $\gamma$-peptide skeleton of these compounds circumvents problems associated with protease liability, a major limitation of CPPs.[28] Cellular uptake studies for a new family of $\gamma$-peptides, utilizing plate fluorimetry and flow cytometry quantification techniques, confocal microscopy to determine subcellular localization, and the MTT assay to establish cytotoxicity are discussed herein.

## Results and Discussion

**Peptide Synthesis.** Various $\gamma$-peptides based on *cis*-$\gamma$-amino-L-proline (see Figure 1) were synthesized to assay their cellular uptake properties. These oligomers have a common backbone and distinct side chains introduced with different linkage types through the $\alpha$-amino group of the proline monomer. Based on the linkage type, three different peptide families were obtained and evaluated: $N^{\alpha}$-acyl-$\gamma$-peptides, $N^{\alpha}$-alkyl-$\gamma$-peptides, and $N^{\alpha}$-guanidilated-$\gamma$-peptides. The same Fmoc/Boc combined solid-phase strategy described in a previous work was used for the peptide synthesis.[26] Use of these two orthogonal protecting groups on solid phase for both amino functionalities of the *cis*-$\gamma$-amino-L-proline leads to a flexible strategy enabling the synthesis of a large number of diversely modified $\gamma$-peptides.

The $\gamma$-peptide backbone was prepared from protected amino acid (2*S*,4*S*)-Fmoc-4-amino-1-Boc-pyrrolidine-2-carboxylic acid [(2*S*,4*S*)-Boc-Amp(Fmoc)-OH] using DIC and HOBt as coupling agents. For $N^{\alpha}$-acyl-$\gamma$-peptides, the side chains were

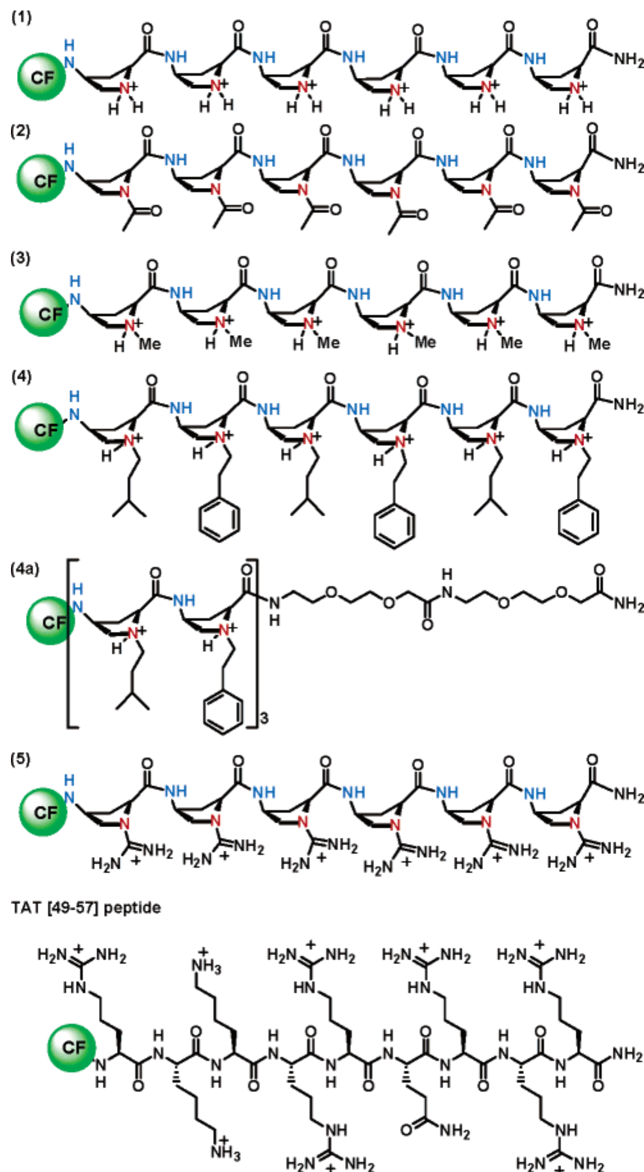


***Figure 1.*** $\gamma$-Aminoproline monomer based $\gamma$-peptides labeled with 5(6)-carboxyfluorescein.

introduced using the corresponding carboxylic acid and the same coupling reagents described above. $N^{\alpha}$-Alkyl-$\gamma$-peptides were obtained via reductive amination using the corresponding aldehyde in 1% HOAc in DMF for 30 min followed by addition of $NaBH_3CN$ dissolved in MeOH. Substitution of DMF with MeOH as described previously yielded cleaner crude oligomers in higher yield, as determined by HPLC (70% for peptide **3**, 66% for peptide **4**, and 67% for peptide **4a**). For peptide **5**, the $N^{\alpha}$-guanidylated-$\gamma$-peptide, the guanidinium group was introduced using *N*,*N*′-di-Boc-*N*”-trifluoromethanesulfonyl guanidine[29] in the presence of $Et_3N$ in DCM for 4 days. All reactions of the proline secondary amine were monitored by the chloranil test.

Taking into account that peptide **4** was not totally soluble in physiological media at high concentrations, peptide **4a**, containing two consecutive short polyethylenglycol chains (8-amino-3,6- dioxaoctanoic acid, Adoa) at its *C*-terminal, was synthesized

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as a more soluble analogue.[30] Peptide **4a** was obtained by coupling two molecules of Fmoc-8-amine-3,6-dioxaoctanoic acid to MBHA resin using DIC/HOBt as coupling reagents, followed by the synthesis described above for peptide **4**.

At the end of the synthesis, after Fmoc removal, the fluorescent label 5(6)-carboxyfluorescein (CF) was introduced onto the *N*-terminal $\gamma$-amino group using DIC/HOBt as coupling reagents, followed by piperidine washes just before cleavage of the peptide from the resin. These washes were required in order to remove overincorporated carboxyfluorescein.[31] When these washings were not carried out, compounds containing two and three extra units of carboxyfluorescein were observed in the crude product.

Peptides were ultimately cleaved from the resin by acidolytic treatment with anhydrous HF. The purity of the CF-$\gamma$-peptide crudes, as determined by HPLC, ranged from 65% to 90%. Compounds were purified to more than 95% homogeneity by preparative reverse-phase HPLC and characterized by electrospray and/or MALDI-TOF mass spectrometry.

**Enzymatic Stability.** Enzymatic stability was studied for peptide **4a** in trypsin and human serum and checked by HPLC, which showed total stability of the peptide, even during long incubation times (see Supporting Information). This result represents a major advantage of peptide **4a** over CPPs, which are labile to both trypsin and human serum and hence greatly limited for physiological applications. This precludes the use of trypsin for the removal of cell-surface-bound peptides before cytometry analysis (see below).

**Cellular Uptake of $\gamma$-Peptides.** The general idea that hydrophilic macromolecules penetrated cells by a classical endocytotic mechanism is extended.[32,33] These molecules are segregated into different endosomal compartments by the cell for their recycling or destruction, a fact that can hinder liberation of cargo in the cytoplasm.[34] In contrast, initial reports suggested that CPPs penetrate cells by an energy-independent route, and studies on the cellular entry of pAntp, Tat-derived peptides and VP22 reported that a nonclassical receptor, transporter, or endocytosis-mediated mechanism seemed to be involved.[35−38] These findings would suggest that the CPPs translocate membranes via an energy-independent mechanism. Nevertheless, recent reevaluations of the cellular translocation of some CPPs and cargo-conjugate CPPs suggest that endocytosis is indeed the primary internalization mechanism.[39,40] Thus, the exact nature of the mechanism of CPP across the cellular membrane remains unknown, as controversies exist among different studies published in the literature.[12,41,42] Despite some common features of CPPs, particularly their cationic nature, their structural diversity has fuelled the idea that the mechanisms that allow them to cross the membrane are not the same for CPPs of different types.[43]

A preliminary evaluation of the cellular uptake of the different $\gamma$-peptides was carried out in COS-1 and HeLa cells using plate fluorimetry. Cells grown on 96-well plates were incubated with a range of 0.01 to 25 $\mu$M of CF-peptide at 37 °C or 4 °C. After 2 h of incubation, CF-peptide containing medium was discarded and fluorescence was measured as described in the methods section. As shown in Figure 2A and B, peptide **4a** was the most efficient at crossing the cell membranes of COS-1 and HeLa cells at 37 °C. The fluorescence levels of cells incubated with peptide **4a** were significant in the case of HeLa cells and were higher for COS-1 cells. In addition, peptides **1**, **2**, **3**, and **5** were also taken up by both cell types, but the cells incubated with these CF-peptides flouresced 6 to 10 times less than those incubated with peptide **4a**. Similar results were obtained when the uptake experiments were performed for 8 h (see Supporting Information). To determine if the uptake mechanism of these peptides was energy-dependent, the same experiments were carried out at 4 °C (Figure 2C). Under these conditions, only peptide **4a** and, to a lesser degree, peptide **5** were taken up at a significant rate by HeLa cells. This result seemed to indicate that peptides **4a** and **5** entered into the cells via an energy-independent mechanism, although the possibility of the peptide binding to the plastic surface or to the cell membrane was not totally excluded. Evidence for these phenomena was observed in control wells (wells devoid of cells) and has been described in other studies.[44] Similar results to those obtained with peptide **4a** were previously obtained with peptide **4** (see Supporting Information). Having completed the aforementioned experiments, flow cytometry was then used to differentiate peptide uptake from cell surface and/or plastic surface binding.

To get a more precise view of the behavior of the $\gamma$-peptide derivatives, new cell uptake experiments in COS-1 cells using flow cytometry were performed. In our opinion flow cytometry provides more reliable quantitative data than plate fluorimetry. The main source of imprecision in plate fluorimetry when measuring the cell uptake of highly hydrophobic compounds such as peptide **4a** is an artifactual increase in the apparent uptake caused by nonspecific binding of the ligand to the plastic surface of the plate.[44] Although the preliminary flow cytometry experiments were carried out using the entire set of $\gamma$-peptides, focus was ultimately shifted on the two most promising compounds, **4a** and **5**, employing the well-known cell-penetrating peptide TAT 49−57 (CF-RKKRRQRRR-$NH_2$) as a positive control. Flow cytometry analysis can quantify cellular association of CF-tagged peptides excluding any unspecific binding of the peptides to the plastic surface of the plate. Although the uptake levels of peptides **4a** and **5** were lower than those found using plate fluorimetry, suggesting that they had been partially bound to the plate plastic, Figure 3A illustrates that the compounds were indeed internalized by COS-1 cells. Peptide **4a** was taken up very efficiently by COS-1 cells and even

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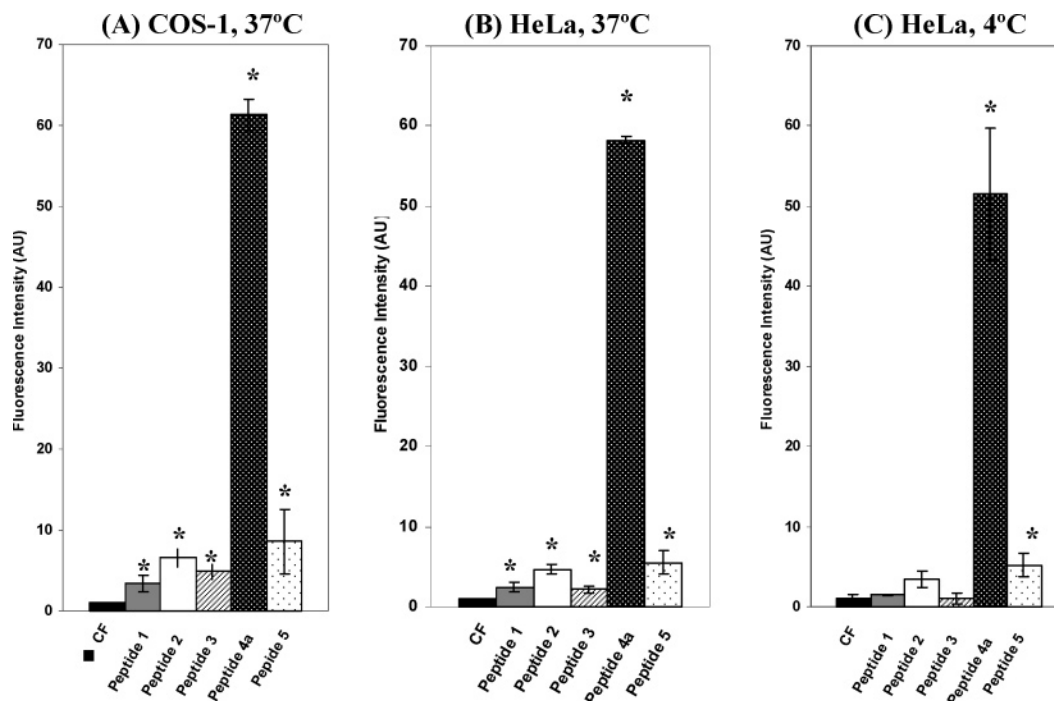


***Figure 2.*** Fluorimetry quantification of the cellular uptake of different peptides tested (A) in COS-1 cells and (B and C) in HeLa cells. Cells were incubated with the peptide at a final concentration of 10 $\mu$M for 2 h at 37 °C (A and B) and at 4 °C (C). Results were normalized by subtracting the autofluorescence control value and dividing by the CF-fluorescence value for the same experiment. Error bars represent standard deviation (SD) from the mean value of three independent experiments of each condition.*, significant $p < 0.05$.

seemed to be taken up at 4 °C, while peptide **5** was internalized less, especially at 4 °C. In comparison with **TAT** peptide, cells incubated with peptide **4a** flouresced 3.5 times less than those incubated with **TAT**.

Flow cytometry does not, however, discriminate between extracellular membrane-bound and internalized CF-peptide. To determine if there was any contribution of surface-bound CF-peptides in the peptide uptake measurements, we performed additional flow cytometry experiments. The treatment of cells with trypsin in order to remove cell-surface-bound peptide before flow cytometry analysis has been reported.[40] However, $\gamma$-peptides are not trypsin-sensitive as discussed above; hence enzymatic digestion could not be employed. An alternative method based on the pH-dependence of 5(6)-carboxyfluorescein fluorescence emission in receptor-endocytosis studies has been previously described.[45] As shown for COS-1 cells incubated with CF-peptide **4a** (Figure 3B) or with **TAT** peptide (Figure 3C) for 2 h, flow cytometry analysis revealed that the amount of fluorescence was very similar at pH 7.4 and pH 6. Only a small decrease of the fluorescence was observed in both peptide **4a** and **TAT** peptide, indicating that the fluorescence detected by the cytometer was mainly due to internalized peptides.[46]

A cell uptake time-course experiment in COS-1 cells was also carried out for peptide **4a**. The progressive increase uptake of peptide **4a** demonstrated that it was endocytocized by the cells, as opposed to having attached to the membrane, a process that occurs rapidly and is temperature-independent (see Supporting Information).

**Intracellular Distribution of $\gamma$-Peptides.** The intracellular distribution of CF-peptides was examined by confocal microscopy in both fixed and unfixed COS-1 cells recording optical sections that allowed 3D reconstruction of the cell. The carboxyflouresceinated peptides were located inside the cells and were not attached to the membrane. Figure 4 shows the distribution of each CF-peptide tested in fixed COS-1 cells costained with the nucleic acid marker PI. In cells treated with peptides **1**, **2**, and **3** at 37 °C, a slight vesicular distribution of the peptides was observed. No differences with the CF were observed when cells were incubated with these peptides at 4 °C (data not shown). This vesicular distribution, similar to the localization of the endocytotic marker used (Figure 5), together with the results obtained at 37 °C and 4 °C, suggested that an endocytotic mechanism was involved. However, peptides **5** and **TAT** were found throughout the cytoplasm and in the nuclei of the cells with no punctuated pattern observed. Similar distribution of both peptides was found when cells were incubated at 4 °C (data not shown). In those cells treated with peptide **4a**, a similar but more intense punctated distribution was observed, as well as a diffuse distribution throughout the cytoplasm.

It has recently been reported that cellular distribution of **TAT** can vary for live or paraformaldehyde-fixed cells.[12] Confocal microscopy pictures of **TAT** internalized in paraformaldehyde-fixed cells show distribution in the nucleus and endocytotic vesicles of the cytoplasm, whereas live-cell pictures suggest that endocytosis is the predominant uptake mechanism of this peptide. The difference in cellular distribution can be attributed to fixation of the cells with paraformaldehyde. Arginine-rich

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(46) A minor contribution from any unquenched residual carboxyflouresceinated peptide attached to the membrane at pH 6 could still exist (at pH 6 around the 80% of the carboxyfluorescein fluorescence is quenched), and as a consequence, only a small amount of fluorescence could be due to the cell-surface-bound peptides. These experiments could not be carried out at a pH lower than 5−5.5 (where carboxyfluorescein is completely quenched) because of the rapid cell death observed under our experimental conditions.

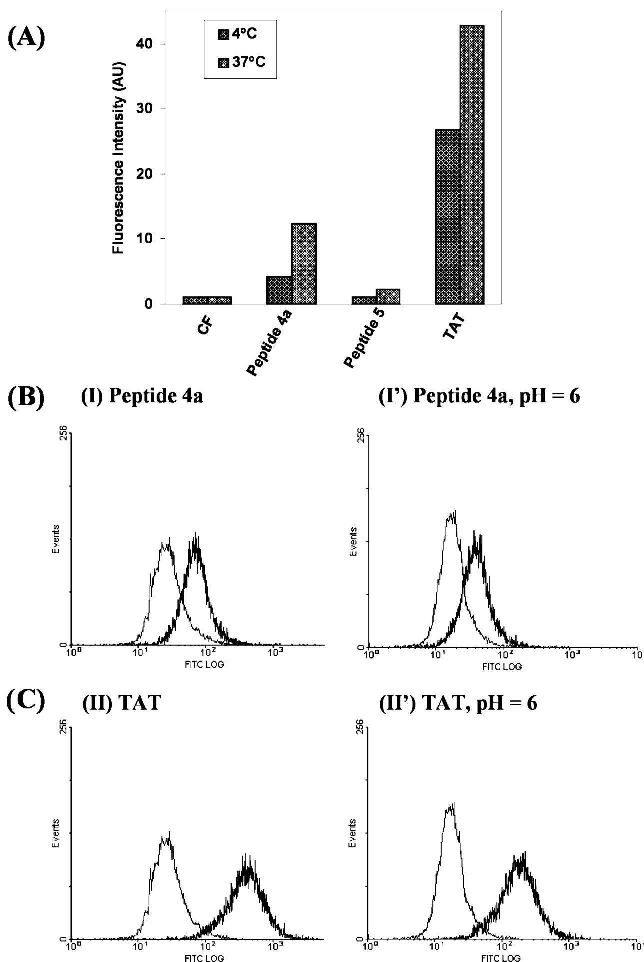


***Figure 3.*** (A) Cell uptake quantification of peptides **4a**, **5**, and **TAT** with flow cytometry at 4 and 37 °C (10 $\mu$M, 2 h). Although the uptake levels of **4a** and **5** peptides were lower than those found by plate fluorimetry, the results demonstrate that they were indeed taken up by COS-1 cells. (B and C) Flow cytometry analysis to quantify cellular association of CF-tagged peptides while avoiding nonspecific plastic surface binding of the peptide. To quantify plasmatic-membrane-attached peptide ((B) for peptide **4a** and (C) for **TAT** peptide), fluorescence of CF-peptide-incubated COS-1 cells (10 $\mu$M, 2 h, 37 °C) was measured by flow cytometry before (I, II) and after (I′, II′) acidic buffer (pH = 6) quench of extracellular fluorescence. Graphics B and C have the peptide quantification and the corresponding CF negative control.

peptides such as **TAT** and $(Arg)_9$ arbitrarily redistribute in the nucleus in some common cell fixation protocols.[12]

To determine whether the CF-peptides were actually endocytozied or if they diffused across the cell membrane, time-course uptake experiments using the fluid-phase endocytotic marker Texas Red-Dextran (TR-DX) were performed. HeLa cells were simultaneously incubated with CF-peptides (10 $\mu$M, 37 °C) and the endocytotic marker TR-DX. Figure 5 shows results obtained when cells were incubated with two distinct peptides (peptides **2** and **4a**) for 15 min or 2 h at 37 °C. To avoid formaldehyde-induced changes in their distribution, confocal images of live cells were obtained. As shown in the upper panel of Figure 5, even after 15 min of incubation, peptide **2** almost totally colocalized with TR-DX, indicating a fluid-phase endocytosis mechanism. In contrast, peptide **4a** had also colocalized in TR-DX-containing vesicles after 15 min of incubation, but it was also distributed in the nuclei and the cytoplasm. After 2 h of incubation, there were more vesicles containing both peptide **4a** and TR-DX, but they were more concentrated in the perinuclear area of the cells. Cytoplasmic and punctuated or vesicular distribution of peptide **4a** was also observed. Similar results were obtained when the same experiments were carried out in COS-1 cells (see Supporting Information). Although peptide **4a** does not concentrate exclusively in vesicular structures, it cannot be stated that it translocates across the cell membrane. An alternative explanation could be that peptide **4a** was taken up by endocytosis and later released from the vesicles into the cytoplasm in a manner similar to that which has been demonstrated for the human antimicrobial peptide LL-37[47] and other cell-penetrating peptides.[48]

**Cytotoxicity of γ-Peptides.** Cell viability assays were performed to determine the toxicity of the proline derived γ-peptides (Figure 6). The toxicities of each of the peptides to COS-1 and HeLa cells were determined by MTT assays and estimated to be lower than that of **TAT** peptide at the same concentration. The viability of COS-1 cells after their treatment with γ-peptides for 24 h was always higher than 95%, even at the highest concentration used (25 $\mu$M). Only peptide **5** exhibited slight cytotoxicity at 25 $\mu$M (89−92%) in HeLa cells. The viability of COS-1 cells after 2 h of incubation with **TAT** peptide was reduced even when cells were treated at lower concentrations (viability at 1 $\mu$M was 81 ± 2% and at 25 $\mu$M was 70 ± 2%). Similar results were observed when cells were incubated at concentrations up to 500 $\mu$M with γ-peptides **1**, **2**, **3**, and **4a** for 2 and 8 h (data not shown).

## Concluding Remarks

These results reveal that peptides with the same proline mimetic skeleton but distinct side chains are taken up into cells via different mechanisms. Although proline has a hydrophobic skeleton, proline-rich peptides are surprisingly soluble in water as well as a broad range of organic solvents. γ-Amino-L-proline has an extra amino group (in the γ position of the proline) which makes it more hydrophilic than the corresponding natural parent amino acid. While the different γ-peptide constructs share a common part (the cyclic proline backbone), they vary in the type and linkage of their side chains, which can be hydrophobic or hydrophilic. Hence the combination of skeleton, side chain type, and side chain linkage endows each peptide with a distinct hydrophobic or hydrophilic character. Peptide **2** is thus clearly hydrophobic due to the combination of a hydrophobic skeleton and the noncharged side chain linkage to its backbone. Peptides **1**, **3**, **4**, **4a**, and **5** can be considered special amphipathic peptides as a result of their backbone and their charged side chain linkages. In all cases, the side chain hydrophobicity determines the cell uptake properties. Hydrophobic peptides are typically associated with passive diffusion while hydrophilic and amphipathic peptides can move by both endocytosis and passive diffusion. Examples of the latter phenomena include the hydrophilic peptide **TAT** and the amphipathic peptide penetratin, which are endocytocized by the cell (a mechanism that is inhibited by peptide incubation at low temperature), while passive diffusion of these compounds through the lipid bilayer at a biologically meaningful rate seems unlikely (although an alternative mechanism cannot be totally excluded). The γ-pep-

(47) Sandgren, S.; Wittrup, A.; Cheng, F.; Jönsson, M.; Eklund, E.; Busch, S.; Belting, M. *J. Biol. Chem.* **2004**, *279*, 17951−17956.
(48) Foerg, C.; Ziegler, U.; Fernandez-Carneado, J.; Giralt, E.; Rennert, R.; Beck-Sickinger, A. G.; Merkle, H. P. *Biochemistry* **2005**, *44*, 72−81.

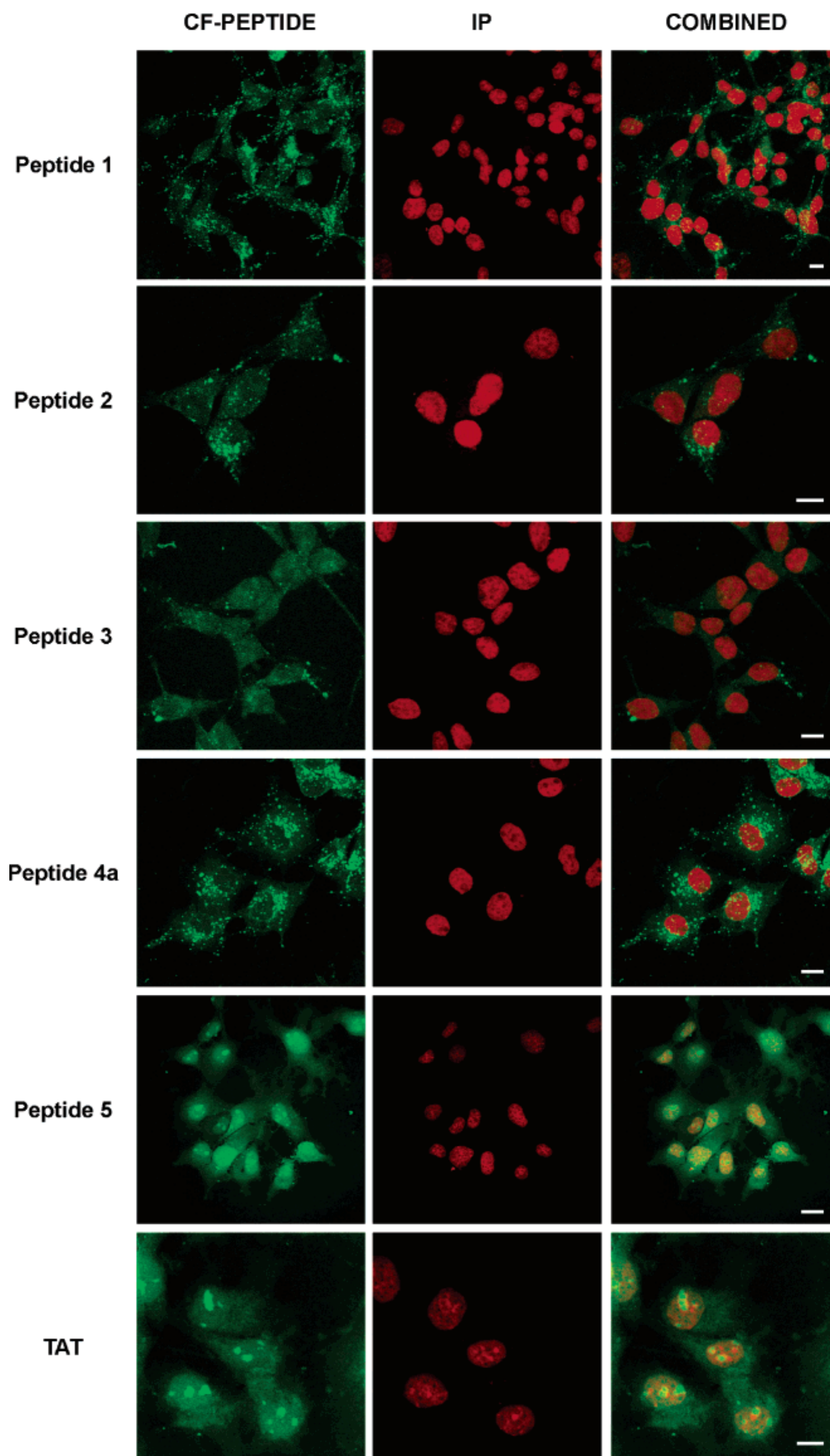


***Figure 4.*** Confocal images of COS-1 cells illustrating the internalization of all peptides tested. After 2 h of incubation with peptides (10 $\mu$M), cells were fixed with 3% PF and nuclei were stained with IP. Scale bars, 10 $\mu$m.

tides synthesized for this work are remarkably amphipathic; they can enter into the cell following both mechanisms depending on the contribution of the side chains to hydrophobicity in the secondary structure. Thus, CF-peptide incubation in both COS-1 and HeLa at 4 °C cells suggest that some of the $\gamma$-peptides (**4a**, and to a lesser degree, **5**) can translocate across the cell membrane, indicating that the internalization process of **4a** and **5** is not completely dependent on the endosomal pathway. A possible explanation for these results is that the $\gamma$-peptide family is capable of cellular internalization via different mechanisms. While all of the compounds can be endocytocized by the cell, peptides **4a** and **5** can also translocate across the cell membrane (passive diffusion), suggesting that at least two different mechanisms are involved in the entry of these peptides. When peptide **4** was modified with a PEG tail in order to improve its solubility at physiological pH, the resulting analogue (peptide **4a**) exhibited increased solubility and conserved cellular uptake.

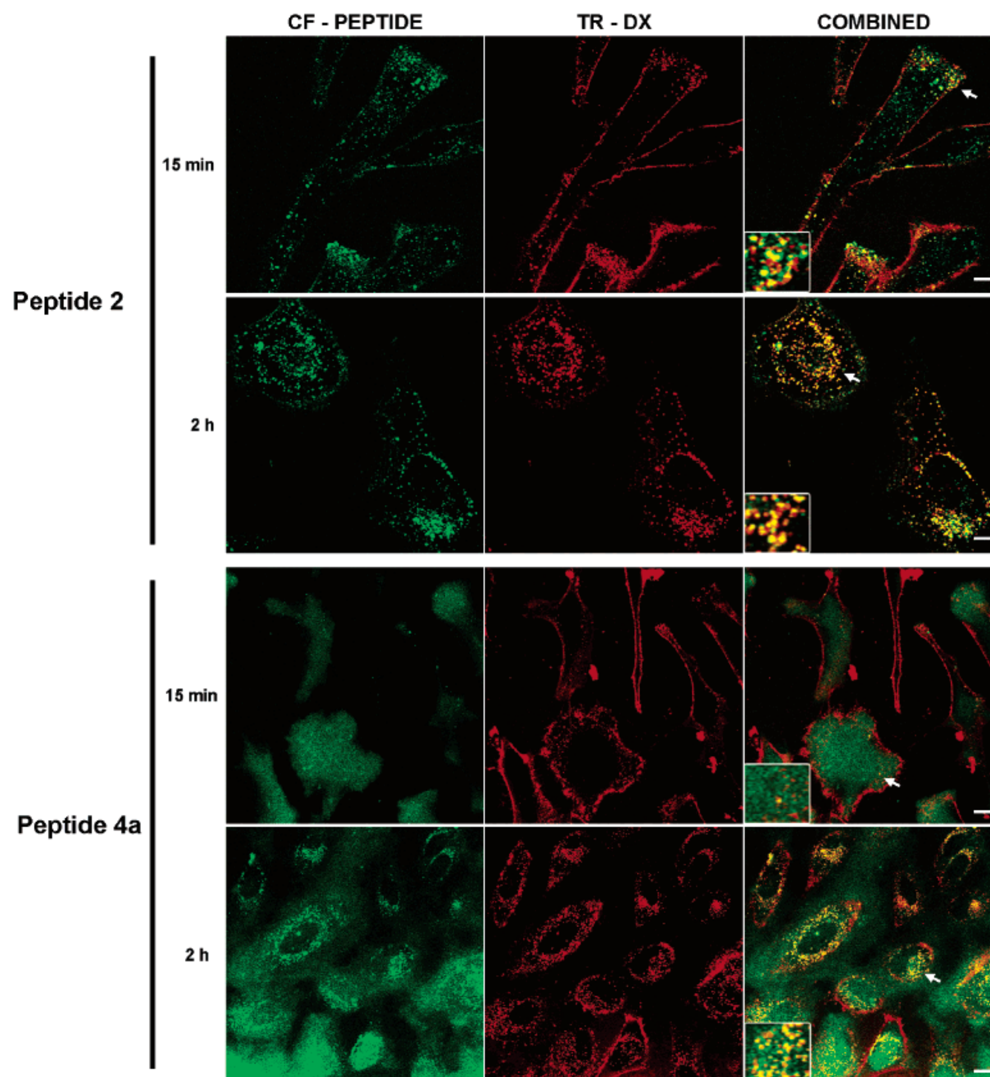


***Figure 5.*** Live-cell imaging of CF-peptide uptake. Internalization experiments of CF-peptide (10 $\mu$M, 37 °C) in HeLa cells were carried out with the fluid-phase (endocytotic) marker Texas Red-Dextran (TR-DX). xy confocal sections obtained from the middle part of cells. Scale bars, 10 $\mu$m. Inserts: 3.2×.

With respect to subcellular localization, confocal microscopy revealed that all of the peptides congregate in the cytoplasm, distribute throughout vesicles when incubated at 37 °C (peptides **1**, **2**, **3**, **4a**, and **5**), or are widespread in the cytoplasm when they are incubated at 37 °C and 4 °C (peptides **4a** and **5**). This punctuated distribution implies that the $\gamma$-peptide family is endocytocized at 37 °C. Furthermore, the widespread pattern found in the cytoplasm at 37 °C for peptides **4a** and **5** suggests that either they translocate across the cell membrane or they are taken up by endocytosis and thereafter escape from the vesicles, being released to the cytoplasm. The translocation observed in the flow cytometry quantifications at 4 °C reinforced the idea that peptide **4a** could translocate across the cell membrane. In conclusion, two different routes seem to be involved in the transduction mechanism of peptide **4a**, including endocytosis and another energy-independent mechanism. Nevertheless, the possibility that the $\gamma$-peptides enter into the cell via endocytosis and are thereafter released from the vesicles into the cytoplasm cannot be completely excluded.

Furthermore, peptides **4a** and **5** apparently reached the nucleus, suggesting that they could be used in the delivery of molecules that act in the nucleus. Even though these peptides were not taken up in cells to the degree of TAT peptide, they offer certain advantages. While TAT peptide, as consistent with literature reports,[49] exhibited cytotoxicity even at low concentrations, $\gamma$-aminoproline oligomers were nontoxic at concentrations up to 500 $\mu$M and only peptide **5** exhibited slight cytotoxicity. Furthermore, $\gamma$-peptide oligomers appear to be protease-resistant as deduced from the results of experiments in the presence and absence of serum proteases. This fact has been proven by analyzing the protease stability of peptide **4a** in human serum by HPLC.

Although various cell-membrane translocating peptidomimetics have been reported (e.g., $\beta$-peptides), this is the first communication of a $\gamma$-peptide family with said capability. The aforementioned compounds are among the smallest cell-penetrating peptidomimetics described to date, and their low toxicity, proteolytic stability and, in general, good aqueous solubility suggest their potential utility as agents for drug delivery. $\gamma$-Amino-L-proline is a very convenient building block for the preparation of these cell-penetrating $\gamma$-peptides. The use

(49) Pooga, M.; Elmquist, A.; Langel, Ü. In *Cell-penetrating peptides*, *Processes and applications*; Langel, Ü., Eds.; CRC Press: Boca Raton, Florida, 2002; Chapter 11, pp 245−261.

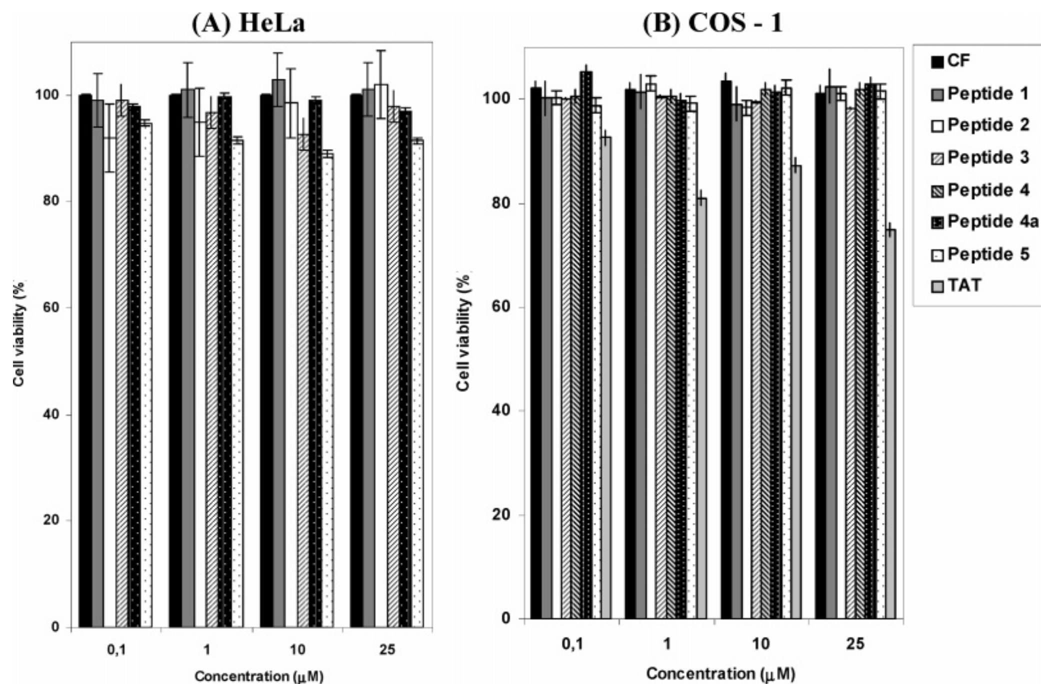


***Figure 6.*** Cytotoxicity of different $\gamma$-peptides as monitored in (A) HeLa and (B) COS-1 cell lines. Cell death was quantified using the MTT assay after 1 day of incubation using different peptide concentrations.

of two orthogonal protecting groups for both amino functionalities of the building block leads to a flexible synthetic strategy for diverse substituted $\gamma$-peptides, providing the possibility of incorporating a covalently linked drug in the last step of the synthesis. With the hope of gaining a better understanding of the translocation of peptides across cell membranes, the synthesis of new analogues employing a wide range of functional groups is in progress.

## Experimental Section

**Materials and Methods.** Protected amino acids were obtained from Neosystem (Strasbourg, France), and MBHA resin (0.7 mmol/g) was supplied by Calbiochem-Novabiochem AG. Diisopropylcarbodiimide (DIC) was obtained from Fluka Chemika (Buchs, Switzerland) and HOBt from Luxembourg (Tel Aviv, Israel). 5(6)-Carboxyfluorescein (CF) was obtained from Acros (New Jersey, USA). Solvents for peptide synthesis and RP-HPLC equipment were obtained from Scharlau (Barcelona, Spain). Trifluoroacetic acid was supplied by KaliChemie (Bad Wimpfen, Germany). Other chemicals were obtained from Aldrich (Milwaukee, WI) and were of the highest commercially available purity. All commercial reagents and solvents were used as received. HF was obtained from Air Products and Chemicals, Inc. (Allentown, Canada), and related equipment was obtained from Peptide Institute Inc., Minoh, Osaka, Japan. Analytical RP-HPLC was performed using Waters (Milford, MA) chromatography systems with reversed-phase Symmetry $C_{18}$ (150 × 4.6 mm$^2$) 5 $\mu$m columns with UV detection at 220 nm. Semipreparative RP-HPLC was performed on a Waters (Milford, MA) chromatography system using Symmetry $C_8$ (3 × 10 cm$^2$, 5 $\mu$m) columns. Compounds were detected by UV absorption at 220 nm. Mass spectra were recorded on a MALDI Voyager DE RP time-of-flight (TOF) spectrometer (Applied Biosystems, Framingham). DHB was used as a matrix and was purchased from Aldrich.

**General Procedures. Solid-Phase Synthesis.** The synthesis of peptides for this work employed a combined Fmoc/Boc solid phase strategy on MBHA resin. Peptide syntheses were performed manually in a polypropylene syringe fitted with a polyethylene porous disk. Solvents and soluble reagents were removed by suction. Washings between deprotection, coupling, and subsequent deprotection steps were carried out with DMF (5 × 1 min) and DCM (5 × 1 min) using 10 mL of solvent/g of resin for each wash.

**Fmoc Group Removal.** (i) DMF (5 × 1 min); (ii) piperidine/DMF (2:8) (1 × 1 min + 2 × 15 min); (iii) DMF (5 × 1 min).

**Boc Group Removal.** (i) DCM (5 × 1 min); (ii) TFA/DCM (4:6) (1 × 1 min + 1 × 30 min); (iii) DCM (5 × 1 min); (iv) DIEA/DCM (5:95) (3 × 3 min); (v) DCM (5 × 1 min).

**Solid-Phase $\gamma$-Peptide Backbone Elongation.** All syntheses were carried out with MBHA resin (1 g) by an Fmoc/Boc combined solid-phase strategy. Couplings of Boc-Amp(Fmoc)-OH (1.58 g, 5 equiv) were carried out with DIC (540 $\mu$L, 5 equiv) and HOBt (472 mg, 5 equiv) in DMF for 2 h at 25 °C. The resin was washed with DMF (5 × 1 min) and DCM (5 × 1 min) after each coupling. Couplings were monitored by the Kaiser test.[50]

In the synthesis of peptide **4a**, 2 equiv of Fmoc-8-amine-3,6-dioxaoctanoic acid were first coupled to the resin using the same coupling reagents mentioned above. Peptide synthesis then continued for **4a** as described for the other compounds.

**Synthesis of Homo $N^{\alpha}$-Acyl-$\gamma$-hexapeptides.** After the $N^{\alpha}$-Boc groups had been removed, acylation of the α-amino groups was carried out using RCOOH (30 equiv, 5 equiv for each amine), DIC (540 $\mu$L, 30 equiv) and HOBt (472 mg, 30 equiv) in DMF for 2 h at 25 °C. The resin was washed with DMF (5 × 1 min) and DCM (5 × 1 min). The acylation was monitored by the chloranil test.[51]

**Synthesis of Homo $N^{\alpha}$-Alkyl-$\gamma$-hexapeptides.** After the $N^{\alpha}$-Boc groups had been removed, alkylation of the α-amino group was performed by on-resin reductive amination using RCHO (30 equiv, 5 equiv for each amine) in 1% HOAc in DMF for 30 min and then treating the resin with $NaBH_3CN$ (232 mg, 30 equiv) in MeOH for 2 h. After that, the resin was washed with DMF (5 × 1 min) and DCM (5 × 1 min). The alkylation was monitored by the chloranil test.

**Synthesis of Hetero $N^{\alpha}$-Alkyl-$\gamma$-hexapeptides.** Once the monomer had been introduced by coupling of the corresponding protected

(50) The $\gamma$-amino functions of the resin-bound building blocks give a clear positive Kaiser ninhydrin test; hence the test is useful for controlling the synthesis of these kinds of peptides. Kaiser, E.; Colescott, R L.; Bossinger, C. D.; Cook, P. I. *Anal. Biochem.* **1970**, *34*, 594−598.

(51) Christensen, T. *Acta Chem. Scand.* **1979**, *33*, 760−766.

monomer, the $N^{\alpha}$-Boc protecting group was removed and the alkylation was performed as above. After removal of the $N^{\gamma}$-Fmoc protecting group, the reactive sequence was repeated.

**Synthesis of $N^{\alpha}$-Guanidyl-γ-hexapeptide.** After the $N^{\alpha}$-Boc groups had been removed, peptide **5** was guanidilated using *N*,*N*′-di-Boc-*N*″-trifluoromethanesulfonyl guanidine[52] (5 equiv) and $Et_3N$ (5 equiv) in DCM for 4 days at room temperature. After the reaction, the resin was washed with DCM (5 × 1 min). The guanidilation was monitored by the chloranil test.

**5(6)-Carboxyfluorescein $N^{\gamma}$-Terminal Labeling.** CF was coupled to the $N^{\gamma}$-terminal group using DIC/HOBt (5 equiv/5 equiv) for 2 h. To avoid over incorporation of CF, two 30-min treatments with 20% piperidine−DMF were carried out before the cleavage of the peptide from the resin.[31]

**Acidolytic Cleavage with HF.** The peptide resin was washed with MeOH (3 × 1 min), dried, and treated with HF in the presence of 10% anisole for 1 h at 0 °C. Peptides were precipitated with cold anhydrous MTBE, dissolved in HOAc, and then lyophilized.

**CF-(γAmp)$_6$-NH$_2$ (1):** The crude peptide was purified by preparative HPLC using a linear gradient (from 0 to 20% of MeCN in 30 min) of MeCN (containing 1% of TFA) and $H_2O$ (containing 1% of TFA). The purity of each fraction was verified by analytical HPLC and MALDI-TOF and showed that the peptides were 99% pure. MS calcd for $C_{51}H_{61}N_{13}O_{12}$ $[M + H]^+$: 1048.11. MALDI-TOF found: 1048.38 $[M + H]^+$, 1070.45 $[M + Na]^+$, and 1086.37 $[M + K]^+$.

**CF-[γAmp($N^{\alpha}$-Ac)]$_6$-NH$_2$ (2):** The crude peptide was purified by preparative HPLC using a linear gradient (from 20 to 80% of MeCN in 30 min) of MeCN (containing 1% of TFA) and $H_2O$ (containing 1% of TFA). The purity of each fraction was verified by analytical HPLC and MALDI-TOF and showed that the peptides were 96% pure. MS calcd for $C_{63}H_{73}N_{13}O_{18}$ $[M + H]^+$: 1300.33. MALDI-TOF found: 1301.22 $[M + H]^+$, 1322.23 $[M + Na]^+$, and 1338.21 $[M + K]^+$.

**CF-[γAmp($N^{\alpha}$-Me)]$_6$-NH$_2$ (3):** The crude peptide was purified by preparative HPLC using a linear gradient (from 0 to 20% of MeCN in 30 min) of MeCN (containing 1% of TFA) and $H_2O$ (containing 1% of TFA). The purity of each fraction was verified by analytical HPLC and MALDI-TOF and showed that the peptides were 95% pure. MS calcd for $C_{57}H_{73}N_{12}O_{12}$ $[M + H]^+$: 1132.26. MALDI-TOF found: 1131.60 $[M + H]^+$ and 1153.63 $[M + Na]^+$.

**CF-{γAmp[$N^{\alpha}$-CH$_2$CH$_2$CH(CH$_3$)$_2$]-γAmp($N^{\alpha}$-CH$_2$CH$_2$Ph)}$_3$-NH$_2$ (4):** The crude peptide was purified by preparative HPLC using a linear gradient (from 0 to 50% of MeCN in 30 min) of MeCN (containing 1% of TFA) and $H_2O$ (containing 1% of TFA). The purity of each fraction was verified by analytical HPLC and MALDI-TOF and showed that the peptides were 99% pure. MS calcd for $C_{90}H_{115}N_{13}O_{12}$ $[M + H]^+$: 1570.95. MALDI-TOF found: 1570.46 $[M + H]^+$ and 1592.91 $[M + Na]^+$.

**CF-{γAmp[$N^{\alpha}$-CH$_2$CH$_2$CH(CH$_3$)$_2$]-γAmp($N^{\alpha}$-CH$_2$CH$_2$Ph)}$_3$-PEG-NH$_2$ (4a):** The crude peptide was purified by preparative HPLC using a linear gradient (from 0 to 40% of MeCN in 30 min) of MeCN (containing 1% of TFA) and $H_2O$ (containing 1% of TFA). The purity of each fraction was verified by analytical HPLC and MALDI-TOF and showed that the peptides were 99% pure. MS calcd for $C_{112}H_{137}N_{15}O_{18}$ $[M + H]^+$: 1861.26. MALDI-TOF found: 1863.18 $[M + H]^+$ and 1885.15 $[M + Na]^+$.

**CF-[γAmp($N^{\alpha}$-C(NH)(NH$_2$))]$_6$-NH$_2$ (5):** The crude peptide was purified by preparative HPLC using a linear gradient (from 0 to 20% of MeCN in 30 min) of MeCN (containing 1% of TFA) and $H_2O$ (containing 1% of TFA). The purity of each fraction was verified by analytical HPLC and MALDI-TOF and showed that the peptides were 99% pure. MS calcd for $C_{57}H_{73}N_{25}O_{12}$ $[M + H]^+$: 1300.35. MALDI-TOF found: 1302.01 $[M + H]^+$ and 1324.36 $[M + Na]^+$.

**Cell Culture and Peptide Treatments.** HeLa and COS-1 cells were maintained in DMEM (1000 mg/mL glucose for HeLa and 4500 mg/mL for COS-1) culture medium (Biological Industries) containing 10% fetal calf serum (FCS), 2 mM glutamine, 50 u/mL penicillin, and 0.05 g/mL streptomycin. For all experiments, exponentially growing cells were detached from the culture flasks using a trypsin−0.25% EDTA solution, and the cell suspension was seeded at a concentration of 21.4 × $10^3$ cells/cm$^2$ onto plastic dishes, glass coverslips, 8-well Lab-Teck chambered coverglass or 96-well plates (Nalge Nunc International, Naprville, USA), depending on the experiment. Experiments were carried out 24 h later, at a confluence level of approximately 60%−70%. The carboxyfluoresceinated compounds were dissolved in PBS and sterilized with 0.22 μm filters (Millex-GV, PVDF, Durapore, Millipore). The peptides and 5(6)-carboxyfluorescein stock solutions were diluted with the cell culture medium. Nonadherent cells were washed away and attached cells were incubated with the peptides in DMEM at 37 °C in $CO_2$ atmosphere or in 25 mM Hepes Buffered DMEM at 4° C.

**Enzymatic Stability.** Enzymatic degradation using trypsin was carried out by incubation at 37 °C of peptide **4a** with the enzyme in 100 mM Tris-HCl at pH 8. The trypsin−peptide ratio was 1:100, using 3.43 μL of a solution of trypsin from bovine pancreas E.C. 3.4.21.4 (Roche, Basel) in glycerol−$H_2O$ (1:1) (6.25 mg/mL). Aliquots (50 μL) were periodically taken at 2 h to 120 h, 150 μL of 1 N HCl were added, and the resulting solution cooled with ice. Degradation was monitored by HPLC.

Enzymatic degradation using human serum (Aldrich, Milwaukee) was carried out by incubation at 37 °C of peptide **4a** with the serum (diluted 9:1 in HBSS buffer). The ratio peptide−serum was 9, and peptide was used at a final concentration of 125 μM (added to the serum dissolved). Aliquots (50 μL) were periodically taken at 2 h to 120 h, poured into 200 μL of MeOH to precipitate the proteins, and cooled on ice. After 30 min, the sample was centrifuged and the supernatant was analyzed by HPLC.

**Uptake Measurements by Plate Fluorimetry and Flow Cytometry.** COS-1 and HeLa cells were seeded onto 96-microwell plates at a concentration of 21.4 × $10^3$ cells/cm$^2$ for 24 h. The culture medium was discarded and replaced by new medium containing different CF-peptides concentrations (0.01 μM, 0.05 μM, 0.1 μM, 1 μM, 5 μM, 10 μM, and 25 μM), using TAT peptide as a positive control and CF as a negative control. Cells were then incubated for 2 or 8 h at 4 °C and 37 °C. After incubation, cells were washed three times with PBS containing 1.1 mM $CaCl_2$ and 1.3 mM $MgCl_2$, then lysed by adding 200 μL of lysis buffer (0.1% Triton X-100 in 50 mM Tris, pH 8.5) to each well. Internalized peptides were quantified by measuring the fluorescence intensity of the supernatant using a FL600 microplate fluorescence reader (Bio-Tek). Fluorescence was measured at $\lambda_{excitation}$ = 485/20 nm and $\lambda_{emission}$ = 530/25 nm. Triplicates of each measurement were performed, and the fluorescence emitted from the blanks was subtracted.

To analyze the internalization of CF-peptides by flow cytometry, COS-1 and HeLa cells were seeded onto 35-mm plates at a concentration of 21.4 × $10^3$ cells/cm$^2$. After 24 h, cells were incubated with CF-peptides as previously described for fluorimetry assays. After various incubation times, cells were washed 3 times with PBS, detached with tripsine−EDTA 0.25%, centrifuged at 800 × *g*, and washed again. Finally, they were resuspended in PBS containing 0.1 mM of propidium iodide (PI). To remove fluorescence of CF or CF-peptides bound to the plasma membrane, the pH of the PBS/PI solution was brought down to 6 by the addition of 1 N HCl just before measuring fluorescence. At pH = 6, extracellular fluorescence of CF is quenched without altering cell mechanisms.[45]

Fluorescence analysis was performed with an Epics XL flow cytometer (Coulter). Cells stained with PI were excluded from further analysis. Triplicates of each sample were performed for each condition, and results from independent experiments were normalized by subtrac-

(52) Feichtinger, K.; Zapf, C.; Sings, H. L.; Goodman, M. *J. Org. Chem.* **1998**, *63*, 3804−3805.

tion of the autofluorescence control value from each value and dividing by the fluorescence value obtained from the CF control under the same experimental conditions

**MTT Cytotoxicity Assay.**[53] The viability of COS-1 and HeLa cells in the presence of the peptides was tested using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. To avoid saturation in cell growth after 24 h of peptide incubation, $7 \times 10^3$ cells/well were seeded on a 96-well plate (Nange Nunc) for each assay. After 24 h, the culture medium was discarded and replaced by a new medium containing different CF-peptide concentrations. Cells were incubated for 2 h, 8 h, and 24 h at 37 °C under 5% $CO_2$ atmosphere, and MTT (0.5 mg/mL) was added 2 h before the end of incubation. After 2 h of incubation with MTT, the medium was discarded by aspiration and 2-propanol was added to dissolve formazan, a dark blue colored crystal observed in the wells. Absorbance was measured at 570 nm in a spectrophotometric Elx800 Universal microplate reader (Bio-Tek), 30 min after the addition of 2-propanol. Cell viability is expressed as a percent ratio of cells treated with peptide to untreated cells, which were used as a control.

**Confocal Laser Scanning Microscopy.** COS-1 and HeLa cells were seeded onto glass coverslips at $21.4 \times 10^3$ cells/cm$^2$ and, after 24 h, were incubated with CF-peptides as described above. For endocytosis experiments, Texas Red-Dextran (TR-DX, 3 mg/mL, MW = 10.000, Molecular Probes) was incubated together with CF-peptide. After CF-peptide incubation, cells were washed 3 times with PBS and fixed in 3% para-formaldehyde−2% sucrose in 0.1 M phosphate buffer (PB) for 15 min, washed 3 times in PBS, and mounted in Mowiol with 2.5% DABCO. PI (1 $\mu$g/mL) staining was performed at room temperature for 15 min in the presence of RNAsa in PBS (1 mg/mL). As a fixation control, similar experiments were performed in cells plated onto glass bottom Lab-Tek chambers for live-cell imaging. After 2 h of incubation, cells were washed for 3 times with PBS containing 1.1 mM $CaCl_2$, 1.3 mM $MgCl_2$, and 25 mM Hepes. Images were subsequently acquired within the next 30 min.

Confocal laser scanning microscopy was performed with an Olympus Fluoview 500 confocal microscope using a 60×/1.4 NA plan-apochromatic objective. The carboxyfluorescein fluorescence was excited with the 488-nm line of an argon laser, and its emission was detected in a range of 515−530 nm. The microscope settings were maintained identical for each peptide and dose. PI and TR-DX were excited at 543 nm and detected with a 560-nm long pass filter. To avoid crosstalk, the two-fluorescence scanning was performed in a sequential mode.

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**Supporting Information Available:** HPLC chromatograms and supplemental confocal microscopy images. This material is available free of charge via the Internet at http://pubs.acd.org

JA051648K

(53) (a) MTT-cell proliferation assay, Cell Biology: A Laboratory Handbook, 2nd ed.; Academic Press, 1998; Vol. 1. (b) Mechanism of cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction: Liu, Y.; Peterson, D. A.; Kimura, H.; Schubert, D. *J. Neurochem.* **1997**, *69*, 581−593.