Replacement of a Proline with Silaproline Causes a 20-Fold Increase in the Cellular Uptake of a Pro-Rich Peptide

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Abstract: The results presented here show that elementary design enhancements have led to a 20-fold increase in the cellular uptake properties of a Pro-rich cell-penetrating peptide. These results are relevant not only due to the increasing interest in using CPPs as molecular shuttles for intracellular drug delivery but also because they illustrate the power of combining conformational analysis with rational design to modulate the behavior of biologically active compounds.

Introduction

Cell-penetrating peptides (CPPs) have been conceived as potential vectors to carry drugs that have low bioavailability across cell membranes.[1] Properties, such as amphipathicity,[2] a high guanidinium content (provided by arginines,[3] β-arginines,[4] or guanidinium peptoids[5]), and hydrophobicity,[6] have been claimed as essential for a peptide to be able to cross a cell membrane;[7] hence they should be considered in the design of a peptidic carrier. A new family of CPPs of the general formula $(VRLPPP)_n$, in which two of these features, amphipathicity and arginine groups, have been imprinted on a polyproline sequence, was recently reported by the group of Giralt.[8] The key to the design of these compounds was to maintain a proline content of at least 50% to ensure that the molecules adopt a left-handed polyproline II (PP II) helical structure in solution with a periodicity of 3.0 residues per turn. The remaining 50% of residues were optimized for amphipathicity of the PP II helix. More precisely, hydrophobic Val and Leu residues were placed at 1, 3, 7, 9, ... positions, while polar Arg residues were placed at 2, 8, ... positions. Ultimately, a new family of noncytotoxic peptides with good cellular uptake properties was obtained (Sweet Arrow Peptides[9]). We thus set about to determine if the amphipathicity of the PP II helix of these compounds could be further increased by manipulation of the Pro-containing regions. Specifically, it was thought that replacement of a Pro residue located at the hydrophobic face of a PP II helix with another amino acid might increase the amphipathicity and/or enhance the cellular uptake properties of a given CPP. The synthesis of γ-(dimethylsila)proline, or silaproline (Sip), as a proline derivative with enhanced hydrophobicity was recently reported by Cavelier et al. (Figure 1).[10] We thought that this non-natural amino acid could be used for such a purpose. The most efficiently internalized Pro-rich CPP [CF-$(VRLPPP)_3$, (**1**)] was chosen as reference.[8] Peptide **2** [CF-VRLPPSip$(VRLPPP)_2$] was designed by substituting Sip for Pro-6, which is located on the hydrophobic face of the amphipathic PP II helix.[11]

The present paper addresses the following questions: (i) would the introduction of Sip disrupt the PP II secondary structure of Pro-rich peptides? and (ii) if not, would this replacement increase the cellular uptake properties of the peptides?

Results and Discussion

Peptides were synthesized using a previously described Fmoc/*t*Bu strategy and labeled with 5(6)-carboxyfluorescein (CF) for confocal microscopy and flow cytometry studies.[8,12]

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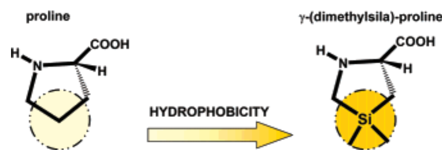

Figure 1. Structures of proline and γ-(dimethylsila)proline.

Hydrophobicity Studies. As previously reported, Sip is about 14 times more lipophilic than Pro, ascertained by the higher octanol−water partition coefficient (i.e., log $P = 1.3$ for Fmoc-Sip-OH compared to 0.094 for Fmoc-Pro-OH).[10] Thus, it could be anticipated that substitution of Pro-6 by Sip in peptide **1** would cause an increase in the global hydrophobicity of the peptide. RP-HPLC was used to compare the hydrophobicity of peptides **1** and **2**. As shown in Figure 2, a longer retention time (t_R) was observed for peptide **2** ($t_R = 7.5$ min) than for peptide **1** ($t_R = 6.8$ min). This difference was interpreted as evidence that the introduction of Sip led to an increase in peptide hydrophobicity. A longer, Pro-rich CPP [CF-(VRLPPP)$_4$, (**3**)] was then used for comparison. Despite the large increment in molecular weight, the retention time of **3** ($t_R = 6.7$ min) was very close to that of **1**.

Conformational Analysis by Circular Dichroism. The effect of Sip on the secondary structure and aggregation state of the test peptide was first examined by circular dichroism (CD).

As shown in Figure 3a, the CD spectrum of peptide **2** (50 μM in pH 7 phosphate buffer) is characteristic of a PP II secondary structure[13] and is virtually identical to those of peptide **1** at the same concentration. Due to their amphipathic character, Pro-rich CPPs have a strong tendency to aggregate in solution.[14] In aqueous media, they adopt the amphipathic PP II structure, but being flexible peptides, this structure is in equilibrium with disordered nonamphipathic conformations. Once the peptide concentration is increased, the level of aggregation increases, displacing the equilibrium toward the ordered structure. This transformation can be monitored by recording CD spectra at different concentrations. Hence it was decided to establish if, and to what extent, said aggregation occurred for the corresponding Sip analogues. As shown in Figure 3b, the CD spectra of **2** at different concentrations indicate an aggregation pattern typical of amphipathic peptides, in which the aggregation increases from 5 to 50 μM and remains constant at higher concentrations.

Transmission Electron Microscopy (TEM). To confirm the CD results, drops of a 50 μM solution of **2** were freeze-fixed and freeze-dried to preserve the "in solution" structure and were covered with Pt over a coverslip for observation by TEM. This process allows preservation of any supermolecular structure present in the peptide solution.[15] TEM images of **2** (Figure 4) revealed fibrilar superstructures similar to that found both for (VRLPPP)$_3$ and for a native Pro-rich peptide (VHLPPP)$_8$ from the N-terminal domain of γ-zein.[14,16] The role of these aggregates in cellular internalization has yet to be determined.

Cellular Internalization Studies. The effect of Sip on the ability of the peptides to penetrate cells was tested by incubating them with HeLa cells and observed by confocal laser scanning microscopy (CLSM). Peptide **2**, which seemed to accumulate in small vesicles outside the nucleus, was efficiently internalized (Figure 5).

The punctuated pattern observed was similar to that described for the reference peptide **1** and suggests endocytic entry.[8,9] The internalization properties of the peptides were then quantified by flow cytometry.

Peptide **2**, in which Pro is substituted with Sip, demonstrated a 20-fold increase in internalization rate as compared to that of peptide **1** (Figure 6a). One of the more relevant properties of **1** from a therapeutic perspective is its nontoxicity, even at very high concentrations (1 mM), to cells.[8] The effects on this property as a result of replacing Pro with Sip were also determined. HeLa cell viability was assayed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The results are presented in Figure 6b and show that compound **2** is not cytotoxic after incubation for 24 h in concentrations up to 1 mM.

Endocytic cellular uptake is the major mechanism for most CPPs, including those reported in the present work.[14] Application of these compounds in intracellular drug delivery would require an efficient method for promoting endosomal release. Although this aspect is out of the scope of this article, encouraging results on endosomal release techniques have recently been published. The experiments employed endosome-disrupting peptides,[17,18] laser-triggered endosome aperture,[19] or an acid-labile linkage between the drug and the CPP.[20]

Conclusion

It has been shown that replacement of a Pro by Sip on the hydrophobic face of a Pro-rich amphipathic peptide does not perturb secondary structure, does not prevent peptide aggregation, and greatly enhances the cellular uptake of the peptide. In addition to highlighting the relevance of amphipathicity in CPP design, these results also demonstrate the utility of Sip as a new source of amphipathicity.

Experimental Section

Materials. Fmoc-Nα-protected amino acids were obtained from IRIS Biotech GmbH (Marktredwitz, Germany). The 2-chlorotrityl chloride resin was purchased from CBL-PATRAS (Patras, Greece). Coupling reagents: 7-azabenzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate, PyAOP, was obtained from Applied Biosystems (Foster City, CA); benzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate, PyBOP, was purchased from Novabiochem (Laüfelfingen, Switzerland); 1-hydroxy-7-azabenzotriazole (HOAt) was obtained from GL Biochem (Shanghai, China); 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) was purchased from Albatros Chem Inc. (Montreal, Canada). Trifluoroacetic acid (TFA) was purchased from Scharlab S.L. (Barcelona, Spain). Piperidine,

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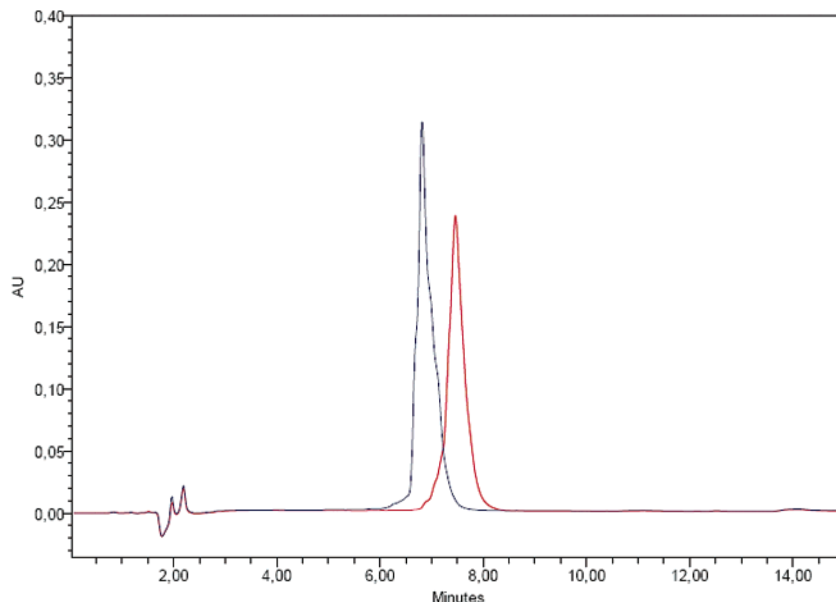

Figure 2. Analytical RP-HPLC chromatogram of **1** (in black) and **2** (in red); gradient = 20−60% B in 15 min (A = 0.045% TFA in H_2O, B = 0.036% TFA in acetonitrile, flow rate = 1 mL/min). For clarity, peptide **3** is not shown.

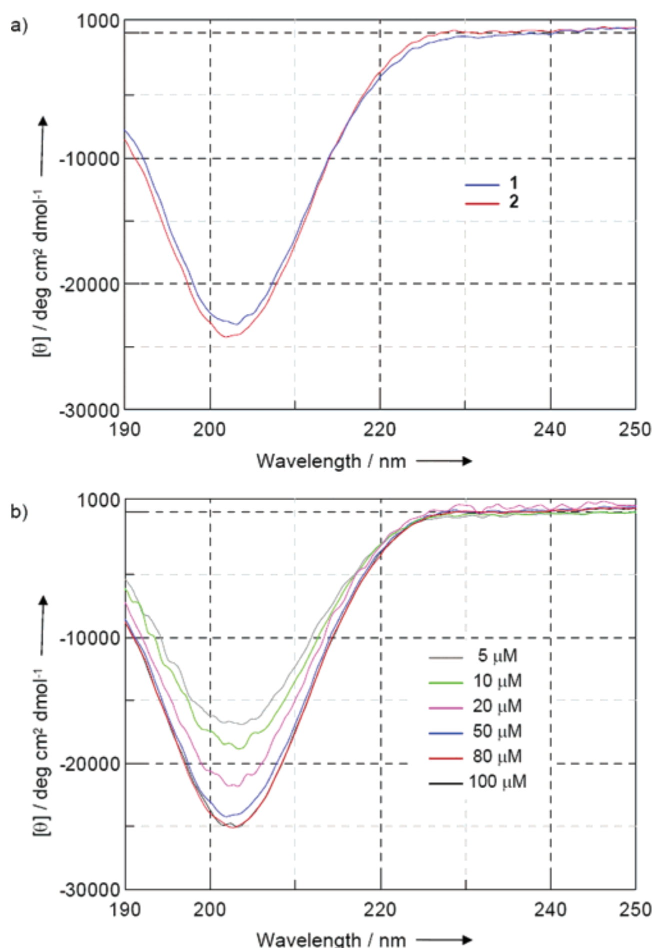

Figure 3. (a) Comparison of the circular dichroism spectra of a 50 μM solution of **1** and **2** in 10 mM phosphate buffer at pH 7. (b) CD spectra of **2** at varying concentrations ($c = 5-100$ μM).

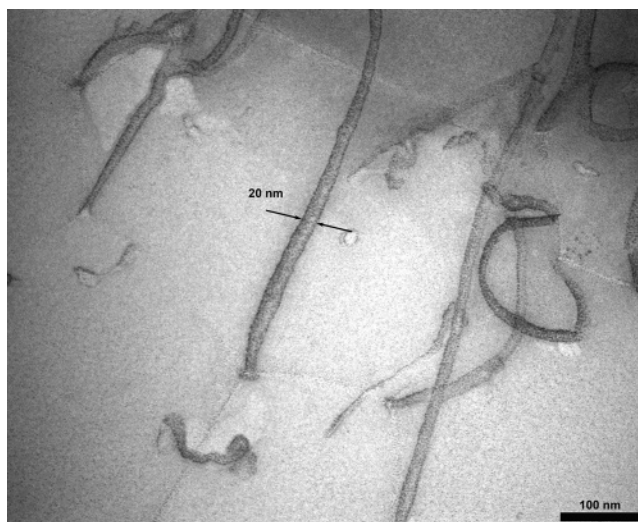

Figure 4. Transmission electron microscopy image of the replica obtained after freeze-fixation and freeze-drying a 50 μM solution of **2** in water over an uncoated coverslip.

dimethylformamide (DMF), dichloromethane (DCM), and acetonitrile were purchased from SDS (Peypin, France). 5(6)-Carboxyfluorescein (CF) was obtained from Acros (Somerville, NJ). Diisopropylcarbodiimide (DIC) and *N*,*N*-diisopropylethylamine (DIEA) were obtained from Merck (Darmstadt, Germany). Triisopropylsilane (TIS) was obtained from Fluka (Buchs, Switzerland).

Synthesis and Chromatography. Peptides were synthesized on 2-chlorotrityl chloride resin using a 9-fluorenylmethoxycarbonyl/*tert*-butyl (Fmoc/*t*Bu) strategy incorporating Nα-Fmoc-protected amino acids (2 equiv), TBTU (2 equiv), and DIEA (6 equiv). As a protecting group for the side chain of Arg, 2,2,4,6,7-pentamethyldihydrobenzofurane-5-sulfonyl (Pbf) was used. The Fmoc group was cleaved by treating the protected peptides with a solution of 20% piperidine in DMF (2 × 10 min).

For the incorporation of Fmoc-Arg(Pbf)-OH and its corresponding Fmoc-Silaproline derivative onto the growing peptide, TBTU was replaced by the more potent phosphonium salt PyBOP (2 equiv), employing a preactivation of 10 min prior to addition of the amino acid.

After completion of the peptide sequence, 5(6)-carboxyfluorescein (5 equiv), PyAOP (5 equiv), HOAt (5 equiv), and DIEA (10 equiv) were dissolved in a 9:1 mixture of DMF:DCM, preactivated for 10 min, and added to the peptidyl resin. The reaction mixture was stirred

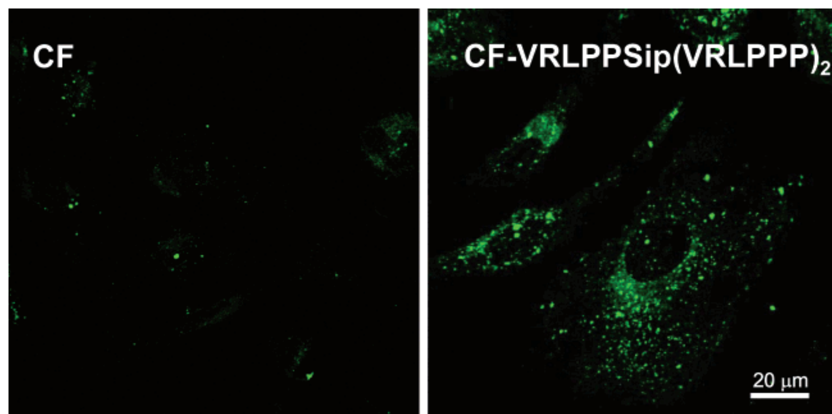

Figure 5. Confocal laser scanning microscopy images of HeLa cells incubated for 3 h at 37 °C with 50 μM CF as negative control (left) and CF-VRLPPSip-(VRLPPP)$_2$ (right).

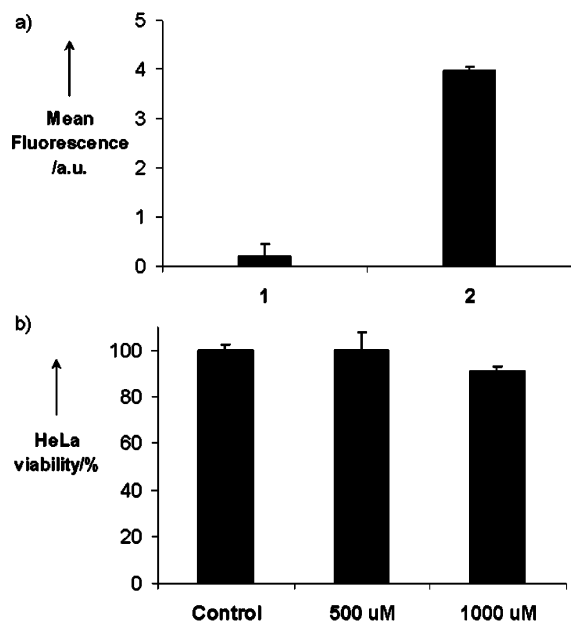

Figure 6. (a) Flow cytometry results after incubating HeLa cells with **1** and with **2** at 50 μM peptide concentration for 3 h in the presence of CO_2. (b) HeLa viability after 24 h incubation with **2**.

for 1.5 h. The labeled peptides were cleaved from the resin by treatment with 95% TFA, 2.5% TIS, and 2.5% water for 1 h. The peptides were analyzed by RP-HPLC [Waters 996 photodiode array detector (λ = 443 nm) equipped with a Waters 2695 separation module (Milford, MA), a Symmetry column (C18, 5 μm, 4.6 × 150 mm), and Millennium software; flow rate = 1 mL/min, gradient = 5−100% B over 15 min (A = 0.045% TFA in H_2O, and B = 0.036% TFA in acetonitrile)].

The carboxyfluoresceinated peptides were purified by semipreparative RP-HPLC [Waters 2487 Dual λ Absorbance Detector equipped with a Waters 2700 Sample Manager, a Waters 600 Controller, a Waters Fraction Collector, a Symmetry column (C18, 5 μm, 30 × 100 mm), and Millennium software; flow rate = 10 mL/min, gradient = 5−20% D in 5 min, 20−70% D in 30 min, 70−100% D over 5 min (C = 0.1% TFA in H_2O, D = 0.05% TFA in acetonitrile)].

The target products were characterized by MALDI-TOF mass spectrometry (PE Biosystems Voyager-DE RP MALDI-TOF with an N_2 laser at 337 nm, Foster City, CA). CF-(VRLPPP)$_3$, (**1**), [M + H$^+$] = 2357.3; CF-VRLPPSip(VRLPPP)$_2$, (**2**), [M + H$^+$] = 2401.2; CF-(VRLPPP)$_4$, (**3**), [M + H$^+$] = 3016.1.

For the comparison of retention times of compounds **1**−**3**, an analytical RP-HPLC system with the following gradient was used: 20−60% B over 15 min (A = 0.045% TFA in H_2O, B = 0.036% TFA in acetonitrile, flow rate = 1 mL/min).

Circular Dichroism. Circular dichroism spectra were recorded with a Jasco 810 spectropolarimeter (spectral bandwidth = 1 nm, time response = 4 s, scan speed = 10 nm/min, step resolution = 0.1 nm). Each spectrum was the average of three accumulations. Spectra were measured at concentrations ranging from 5 to 100 μM and were recorded at 25 °C. The blank was subtracted from each peptide spectrum. Molar ellipticities at each concentration are expressed in decimol of residue.

Transmission Electron Microscopy (TEM). Drops of 50 μM aqueous solutions of the peptides were deposited over uncoated cover slips. Cover slips were freeze-fixed by projection against a copper block, cooled by liquid nitrogen (−196 °C) using a Cryoblock (Reichert-Jung, Leica, Germany). The frozen samples were stored at −196 °C in liquid nitrogen until subsequent use. Samples were freeze-dried at −90 °C and coated with platinum and carbon using a freeze-etching unit (Model BAF-060, BAL-TEC, Liechstenstein). Rotary shadowing of the exposed surface was made by evaporating 1 nm platinum−carbon at 6° above the horizontal, followed by 10 nm of carbon evaporated at 90°. The replica was separated from the cover slip by immersion in concentrated hydrofluoric acid, washed twice in distilled water, and digested with 5% (v/v) sodium hypochlorite for 5−10 min. The replicas were washed several times in distilled water and collected on Formvar-coated copper grids for electron microscopy. All electron micrographs were obtained using a JEOL JEM 1010 MT electron microscope (Tokyo, Japan) operating at 80 kV. Images were obtained on a Megaview III CCD camera (ISIS, Münster, Germany). Samples were prepared in triplicate following the sample procedure, and results obtained by TEM imaging were reproducible.

Cell Culture and Incubation with CF Peptides. HeLa cells were obtained from ATCC (Manasas, VA) and cultured in DMEM (1000 mg/L glucose, Biological Industries) containing 10% fetal calf serum (FCS), 2 mM glutamine, 50 U/mL penicillin, and 0.05 g/mL streptomycin. Exponentially growing HeLa 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 glass cover slips, 4-well Lab-Teck chambered coverglass, or plastic dishes (Nalge Nunc International, Rochester, NY), depending on the experiment. Experiments were carried out 24 h later, at approximately 60−70% confluence. Stock solutions of CF compounds were dissolved in PBS (phosphate buffered saline) and passed through 0.22 μm filters (Millex-GV, PVDF, Durapore, Millipore, Billerica, MA). The labeled peptides and 5(6)-carboxyfluorescein (CF) stock solutions were then diluted in the cell culture medium. Nonadherent cells were washed away, and attached cells were incubated at 37 °C under 5% CO_2 in DMEM medium with a known concentration of CF or CF peptide.

Confocal Laser Scanning Microscopy (CLSM). HeLa cells were incubated for 3 h at 37 °C under 5% CO_2 with CF (as negative control),

1, **2**, and **3** at 50 μM concentration. Cells were then rinsed three times in PBS and fixed in a 3% paraformaldehyde solution in 0.1 M PBS containing 60 μM saccharose for 15 min. Cells were washed in PBS for 5 min and mounted with Mowiol-Dabco medium. Confocal laser scanning microscopy was performed using an Olympus Fluoview 500 confocal microscope with a 60X/1.4 NA objective. CF fluorescence was excited with the 488 nm line of an argon laser, and its emission was detected over the range of 515−530 nm.

Flow Cytometry. For each assay, 21.4×10^3 cells/cm^2 were seeded and cultured for 24 h on plastic dishes. After 24 h, the culture medium was exchanged and the cells were incubated for 3 h at 37 °C under 5% CO_2 with fresh medium containing CF peptides or CF as a negative control. Cells were washed in PBS, treated with trypsin for 5 min at 37 °C, and collected in plastic tubes in cold medium. After centrifugation (1000 rpm, 4 °C, 4 min), the trypsin-containing solution was discarded, and the cells were resuspended in 25 mM Hepes-buffered cell culture medium containing propidium iodide (5 μg/mL). Fluorescence analyses were performed with a Coulter XL flow cytometer. Cells stained with propidium iodide were excluded from further analysis. At least 10 000 events per sample were analyzed twice. The results shown are the average of two measures in the flow cytometer, and the standard deviation is indicated by bars.

MTT Assay. HeLa viability in the presence CF-VRLPPSip-(VRLPPP)$_2$ was tested using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. For each assay, 7×10^3 cells/cm^2 were seeded on a 96-well plate (Nalge Nunc) and cultured for 24 h. Compounds were added at concentrations ranging from 50 μM to 1 mM. Cells were incubated for 24 h at 37 °C under a 5% CO_2 atmosphere. After 22 h, MTT was added to a final concentration of 0.5 mg/mL. The cells with peptide and MTT were incubated for a further 2 h, and the medium was then discarded. 2-Propanol was added to dissolve the formazan product, and absorbance was measured (λ = 570 nm) after 30 min. Cell viability percentages were calculated by dividing the absorbance value of cells treated with a given compound by the absorbance value of untreated cells.

Acknowledgment. The authors acknowledge Dr. Carmen López-Iglesias, Dr. Susana Castel, and Dr. Jaume Comas for technical advice on TEM, CLSM, and flow cytometry, respectively, as well as financial support from MEC-FEDER (Bio2005-00295 and NAN2004-09159-C04-02), the European Union (QLK2-CT-2001-01451), MCYT-FEDER (Bio2002-2301), and Generalitat de Catalunya (CeRBa and SGR).