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D-SAP: A New, Noncytotoxic, and Fully Protease Resistant Cell-Penetrating Peptide

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Protease resistant cell-penetrating peptides (CPPs) are promising carriers for drugs unable to cross the cell membrane. As these CPPs are stable in vivo for much longer periods of time compared to other classes of therapeutic peptides, noncytotoxicity is a property sine qua non for their pharmacological development. Described herein is a fully protease resistant CPP that is noncytotoxic at concentrations up to 1 mM. Proteolytic stability was obtained by chiral inversion of the residues of a known self-assembling CPP—from all L-amino acids to all D-amino acids—and then assessed against trypsin and human serum. Circular dichroism studies confirmed the enantiomeric structure of the analogue, and transmission electron microscopy (TEM) studies indicated that the new inverso analogue retains the ability of the original peptide to self-assemble. The results of uptake experiments indicate that the protease-stable (that is, D-amino acid) analogue of the peptide is internalised by cells to the same extent as the protease-susceptible (that is, L-amino acid) parent peptide. Also reported herein are the results of studies on the cellular internalisation mechanism of the all-D analogue, which reveal the steps followed by the peptide upon its entry into the cell.

Introduction

Given that most potential biomolecular drugs (for example, DNA, RNA, peptides, and proteins) are very often directed at intracellular targets, intracellular drug delivery is a focal point in drug development. Among the most promising strategies for this delivery is the use of peptides able to cross the cell membrane, known as cell-penetrating peptides (CPPs). Several families of CPPs exist, all of which are characterised by positively charged amino acids, hydrophobicity, and/or amphipathicity.[1] Moreover, CPPs have been employed to deliver various types of cargo, including low molecular weight compounds, antibodies, and nanoparticles.[2]

Drug delivery requires noncytotoxic carriers. However, the amount of data reported on the toxicity of CPPs is limited. Cytotoxicity has been described for Transportan,[3,4] SP family,[5,6] or MAP.[4] For Tat, no cytotoxicity was described for concentrations up to 100 μM at short incubation times (1–3 h), or at 10 μM concentration for longer treatments (24 h).[7–9] However, at higher concentrations (500 μM) of Tat and of Antp,[10] low cell-viability levels (40% and 11%, respectively) were observed at 24 h incubation.[11,12] For polyarginines, toxicity appears to depend on peptide length: 12 kDa polyarginines are toxic at concentrations higher than 0.8 μM.[13] Similar results have been obtained for polylysines.[14]

A second drawback of CPPs is proteolytic instability: they are quickly degraded by proteases upon entry into the bloodstream. A proteolysis-labile CPP is thus useless for in vivo administration regardless of its in vitro efficacy. Hence there is a pressing need to develop protease resistant CPPs. An attractive alternative that has been successfully applied to some CPPs[7,13,15–17] is to replace the L-amino acids with the corresponding D-amino acids. Of course, the viability of such an approach is related to the cellular-uptake mechanism and, if aggregation is an issue, to the aggregation behaviour. In the case of protease resistant CPPs, avoiding cytotoxicity is crucial, as even a minimal toxic effect would not only cause cell damage at the beginning of the treatment, but would continue to exert toxic effects until the peptide is completely eliminated.

We recently reported amphipathic Pro-rich peptides, a new family of CPPs which has been proven to efficiently cross the cell membrane without exhibiting cytotoxicity.[11] These peptides are designed with a Pro content of 50% to confer them with the 3.0 residue-per-turn, PPII structure. An amphipathic sequence can be obtained by placing hydrophobic residues at i/(i+2), (i+6)/(i+8), etc., and hydrophilic residues at (i+1), (i+7), etc. The most effective of these peptides is $(VRLPPP)_3$, named Sweet Arrow Peptide (SAP) owing to its safety—it is noncytotoxic at concentrations up to 1 mM—and efficacy.

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An interesting property of SAP is its aggregation behaviour: in aqueous solution it forms tubular aggregates that can be visualised using TEM.[1]

Different SAP derivatives have recently been described. Pro-rich CPP derivatives bearing caproyl and myristyl fatty acyl moieties have been developed, and exhibit much higher rates of cellular uptake than does SAP.[18] In fact, the longer the fatty acyl chain, the greater the translocation of the lipopeptide through the cell membrane. Another promising SAP derivative is one in which a Pro of the hydrophobic face of the peptide is replaced by a Sip (γ-(dimethylsila)-proline), providing a 20-fold increase in the cell uptake level.[19]

In the present work, we set about answering the following questions: 1) Would an all-D version retain the cell-penetrating ability of SAP?; and 2) Would an all-D version retain the noncytotoxicity of the original? We ascertained that if both answers were positive, then the new analogue would be a powerful therapeutic tool. We thus anticipated the following questions regarding its mechanism of action: 1) What are the differences in the aggregation mode of D-SAP compared with L-SAP?; and 2) which is the preferred internalisation path for D-SAP?

Results and Discussion

SAP and D-SAP were synthesised by standard solid phase peptide synthesis (SPPS) protocols, and either with or without 5(6)-carboxyfluorescein (CF), a fluorescent label used to track cellular internalisation of the peptides. The secondary structure of both peptides was studied by circular dichroism (CD), which showed that D-SAP, when compared with the parent peptide, adopted mirror image secondary structures (see Figure 1); the spectra of the two peptides were perfect mirror images (that is, chiral inversion changes the handedness of the PPII helix from left to right).

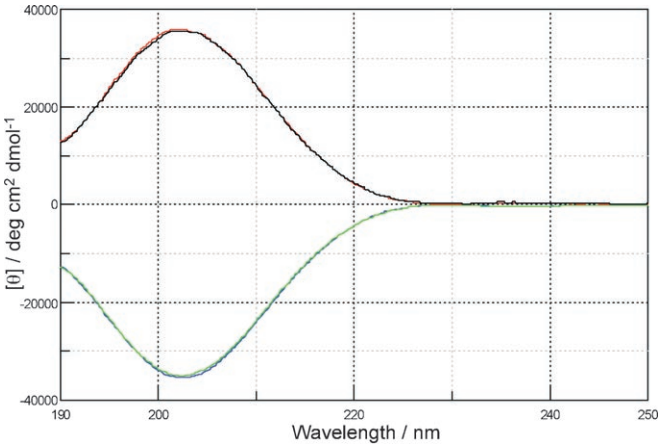

Figure 1. Circular dichroism spectra of SAP (50 μM in green, 100 μM in blue) and D-SAP (50 μM in black, 100 μM in red) in 10 mM phosphate buffer at 25 °C.

To quantify the internalisation of CF-SAP and of CF-D-SAP, cells were incubated with fluorescently labelled versions of either peptide and then analysed by flow cytometry. The mean fluorescence values from the two cell populations were equivalent (see Figure 5). The internalisation was further evaluated using CLSM (Confocal Laser Scanning Microscopy) (see Figure 2), which also revealed the same level of fluorescence from both peptides.

The interaction between a protein receptor and its ligand is normally stereospecific; hence, the enantiomer of the ligand will not bind. Therefore, the efficient internalisation of both CF-SAP and CF-D-SAP clearly indicates that the peptides are internalised via a receptor-independent mechanism.

Having demonstrated the efficient cellular uptake of CF-D-SAP, we set about to determine if it retained the noncytotoxici-

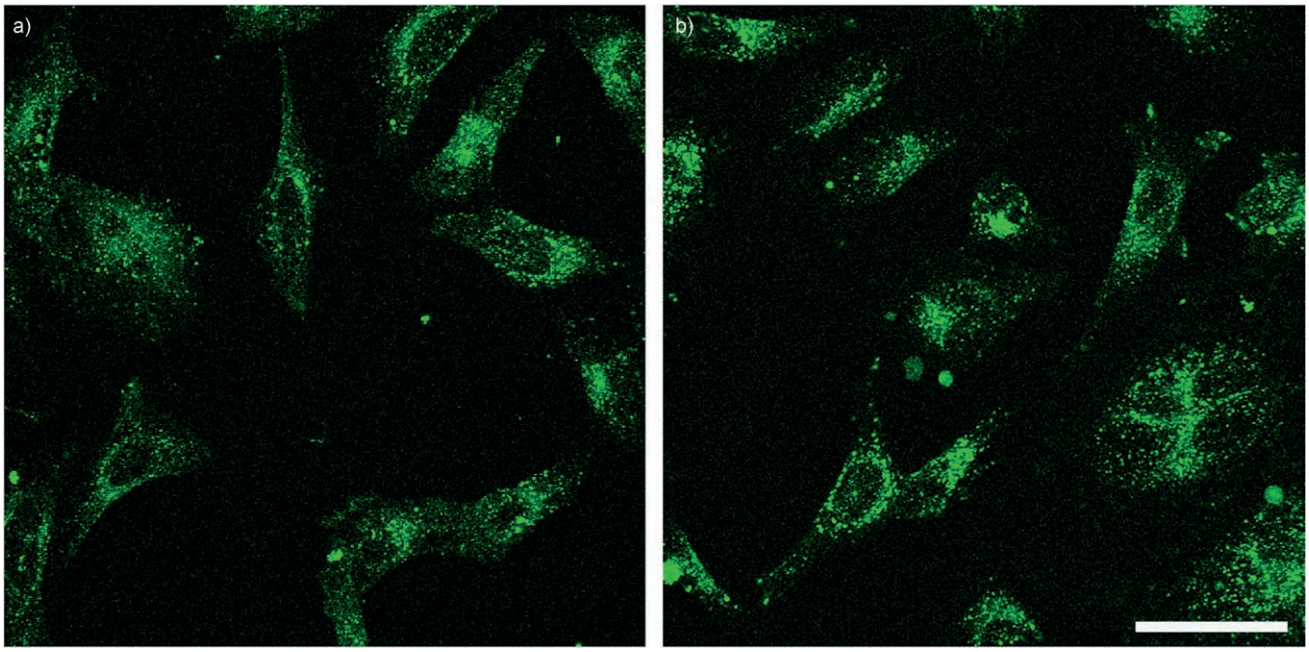

Figure 2. CLSM images of HeLa cells incubated with 50 μM a) CF-SAP or b) CF-D-SAP at 37 °C in 5% CO_2 for 3 h (scale bar = 50 μm).

ty of the parent compound, as these two properties would open the doors to its development for therapeutic applications. Thus, we next determined the cytotoxicity of D-SAP using a HeLa cell viability assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT).[20] As seen in Figure 3, D-SAP was noncytotoxic even at 1 mM for 24 h incubation.

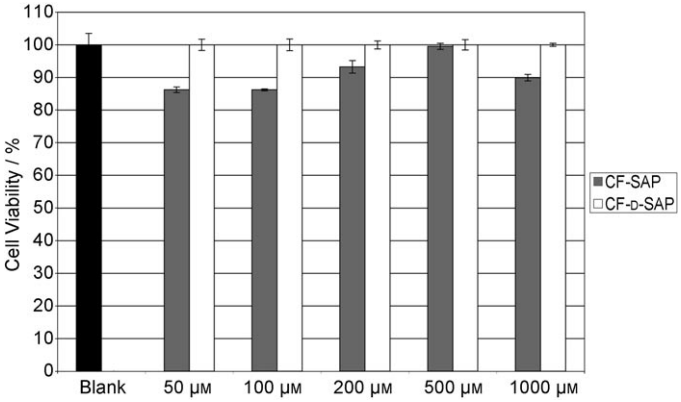

Figure 3. HeLa viability after 24 h incubation with SAP (in grey) and with D-SAP (in white).

Having established that D-SAP was efficiently internalised and noncytotoxic, we then decided to assess its stability to trypsin and to human serum. At a 100:1 peptide:trypsin ratio, D-SAP was completely stable, whereas SAP had a half-life of 6.3 ± 0.5 min (see Supporting Information). A similar result was obtained in a test using 90% human serum: D-SAP was completely stable, whereas SAP had a 15.4 ± 0.8 h half-life (see Supporting Information).

TEM was used to study the aggregation behaviour of D-SAP. More precisely, freeze-fixation and freeze-drying were used to preserve the on solution structure of the peptide. As shown in Figure 4, CF-D-SAP micrographs revealed tubular structures of different length and a width of approximately 16–20 nm very similar to those previously reported for SAP.[1] This means that neither the inversion of all the α-carbon stereogenic centres nor the consequent change in helix handedness affect the overall topology of the aggregate.

The development of an efficient CPP also demands a precise understanding of the internalization process, such that the peptide can be fine tuned to improve cargo delivery. The first step of the internalisation of several CPPs seems to involve the interaction with cell-surface proteoglycans.[21] This interaction takes place between the negatively charged groups of proteoglycans (carboxylate, phosphate, and sulfate groups) and the positively charged residues of the CPP (mainly guanidinium groups). Most of the CPPs present a high content of arginines in the sequence, but SAP is an exception, presenting just 3 positive charges in its 18 amino acid-long sequence. Thus, competition assays with heparin present in the cell medium showed that CF-SAP and CF-D-SAP internalization was only slightly impaired, pointing to a minor involvement of the glycocalix in the first contact of SAP with the cell membrane or to a weak interaction and/or fast equilibrium between SAP and glycosaminoglycan polysaccharides (see Supporting Information).

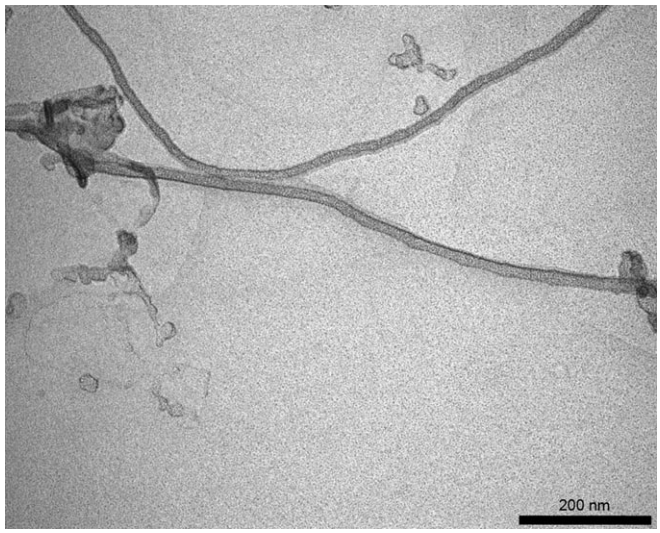

Figure 4. TEM image of the replica obtained after freeze-fixation and freeze-drying a 50 μM aqueous solution of CF-D-SAP onto an uncoated coverslip.

Once D-SAP is bound to the cell-membrane, it is endocytosed. This was deduced based on the fact that internalisation was blocked at 4 °C, and also under ATP-depletion conditions, created by adding 2-deoxy-D-glucose and sodium azide to the cell growth medium (see Figure 5). Colocalisation studies of

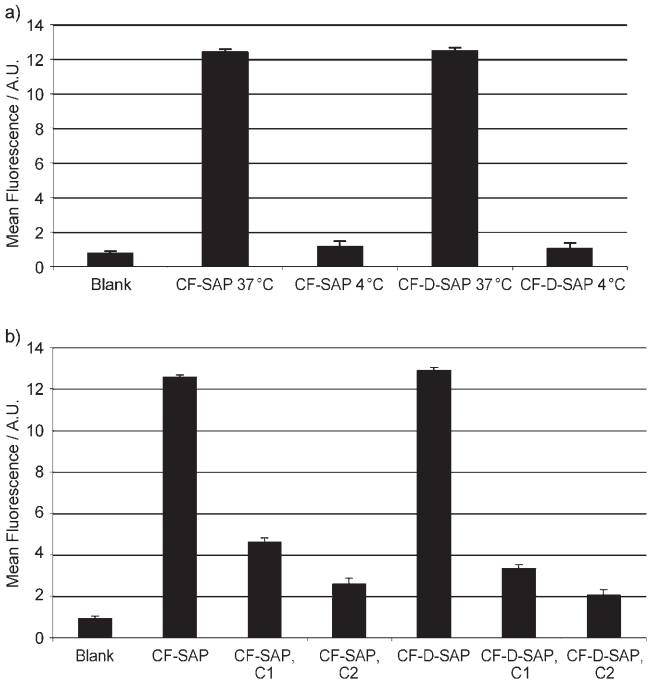

Figure 5. a) Flow cytometry results after incubating HeLa cells with 50 μM of CF-SAP or CF-D-SAP at 4 °C or 37 °C for 3 h; b) Flow cytometry results after incubating HeLa cells with 2-deoxy-D-glucose and sodium azide at different concentrations (C1: 6 mM 2-deoxy-D-glucose, 10 mM sodium azide; C2: 15 mM 2-deoxy-D-glucose, 15 mM sodium azide), at 50 μM of CF-SAP or CF-D-SAP and 37 °C in 5% CO_2 for 3 h.

CF-SAP or CF-D-SAP with Alexa Fluor 555-labelled transferrin (Tfn) and cholera toxin subunit B (Ctx), indicated that both peptides had colocalised with Ctx (see Figure 6 for CF-D-SAP and Supporting Information for CF-SAP). This finding suggests that uptake of CF-SAP and CF-D-SAP occurs via lipid-rafts or caveolae, and not via clathrin-mediated endocytosis. Moreover, we ruled out a macropinocytosis mechanism after observing that macropinocytosis inhibitors[22–24] (amiloride, cytochalasin D, or nocodazole) failed to block CF-SAP or CF-D-SAP uptake (see Supporting Information).

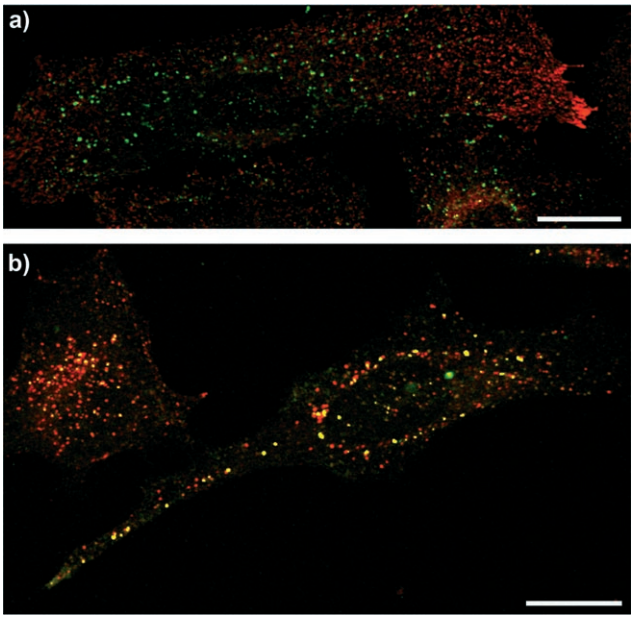

Figure 6. CLSM image of HeLa cells co-incubated with 50 μM CF-D-SAP and a) AlexaFluor555-Tfn at 50 μg mL^{-1} or b) AlexaFluor555-Ctx at 10 μg mL^{-1} and 37 °C in 5% CO_2 for 1 h (scale bar = 20 μm).

Regarding the fate of CF-D-SAP once inside the cell, colocalisation studies with ER-Tracker Red (BODIPY TR glibenclamide) suggest that it does not end up in the endoplasmic reticulum (see Supporting Information).

Conclusions

In summary, D-SAP is a relatively efficient cell-penetrating peptide that it is internalised in HeLa cells by clathrin-independent endocytosis. It is noncytotoxic and protease resistant, making it a solid candidate not only for cell biology experiments but also for human therapeutic use. Nonetheless, therapeutic application of D-SAP is pendant upon further investigation into how it is internalised in different cells, tissues, and biological conditions, as well as its in vivo distribution and toxicology.

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-(1Hbenzotriazol-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, dimethylformamide (DMF), dichloromethane (DCM), and acetonitrile were purchased from SDS (Peypin, France). *N*,*N*-diisopropylethylamine (DIEA) was obtained from Merck (Darmstadt, Germany). Triisopropylsilane (TIS) was obtained from Fluka (Buchs, Switzerland). 5(6)-Carboxyfluorescein (CF) was obtained from Acros (Somerville, NJ), heparin sodium salt from porcine intestinal mucosa (M.W. = 17–19 KDa) from Sigma (St Louis, MO) and Cholera toxin subunit B (recombinant) Alexa Fluor 555 conjugate, Transferrin from human serum Alexa Fluor 555 conjugate, ER Tracker red (glibenclamide BODIPY TR), and AlexaFluor 488 carboxylic acid, succinimidyl ester from Invitrogen (Carlsbad, CA).

Synthesis and chromatography. Peptides were synthesised by solid phase synthesis using the 9-fluorenylmethoxycarbonyl/tert-butyl (Fmoc/*t*Bu) strategy. 2-Chlorotrityl resin, Nα-Fmoc-protected amino acids (2 equiv)/TBTU(2 equiv), and DIEA(6 equiv) were used. As protecting group for the side chain of Arg, 2,2,4,6,7-pentamethyldihydrobenzofurane-5-sulfonyl (Pbf) was used. The Fmoc protecting group was cleaved by treatment with a solution of 20% piperidine in DMF (2×10 min). For incorporation onto the growing peptide resin of the Fmoc-Arg(Pbf)-OH, the TBTU coupling reagent was replaced with the more potent phosphonium salt PyBOP (2 equiv), which was preactivated for 10 min before addition of the amino acid to the peptide resin. Peptides were cleaved from the resin by treatment with 95% TFA, 2.5% TIS, 2.5% water for 1 h 30 min and detected at $\lambda = 220$ and/or 443 nm (last λ for the carboxyfluoresceinated peptides) by analytical RP-HPLC [Waters 996 photodiode array detector equipped with the Waters 2695 separation module, the Symmetry column (C_{18}, 5 μm, 4.6×150 mm) and the Millenium software; Flow = 1 mL min^{-1}; Gradient = 5–100% B in 15 min; A = 0.045% TFA in H_2O, B = 0.036% TFA in acetonitrile)]. 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 [C_{18}, 5 μm, 30×100 mm] and Millenium chromatography manager software). HPLC conditions: Flow = 10 mL min^{-1}. Gradient = 5–20% D in 5 min; 20–70% D in 30 min; 70–100% D in 5 min; C = 0.1% TFA in H_2O, D = 0.05% TFA in acetonitrile. Peptides were characterised by MALDI-TOF mass spectrometry (Vogayer-DE RP MALDI-TOF, PE Biosystems with a N_2 laser of 337 nm).

Circular dichroism. Circular dichroism spectra were recorded with a Jasco 810 UV-Vis spectropolarimeter, a Peltier CDF 426S/426L and a temperature control JULABO F25 programme. The spectra were obtained in a wavelength range of 190 to 250 nm at a spectral bandwidth of 1 nm, with a time response of 4 s, a scan speed of 10 nm min^{-1}, and a step resolution of 0.1 nm. Each spectrum was the average of three accumulations. Spectra were measured at 50 and 100 μM concentration of peptide solved in 10 mM phosphate buffer at pH 7 and were recorded at 25 °C. The blank was subtracted from each peptide spectrum. Molar ellipticity is expressed per decimal residue.

Transmission electron microscopy (TEM). Drops of 50 μM aqueous solutions of the peptides were deposited on 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, BALTEC, Liechstenstein). Rotatory shadowing of the exposed surface was performed 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 to 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 (Japan) operating at 80 KV. Images were obtained on a CCD camera Megaview III (ISIS), Münster, Germany. Three samples were prepared 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^{-1} glucose, Biological Industries) containing 10% fetal calf serum (FCS), 2 mM glutamine, 50 U mL^{-1} penicillin, and 0.05 g mL^{-1} 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 21.4×103 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 to 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 labelled 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 in DMEM medium at 37 °C under 5% CO_2 with a known concentration of CF or CF-peptide.

Confocal laser scanning microscopy (CLSM). HeLa cells on coverslips were incubated for 3 h at 37 °C under 5% CO_2 with CF (as negative control), CF-SAP, and CF-D-SAP at a concentration of 50 μM. 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 to 530 nm. Experiments with live cells were also performed using a Deltavision Imaging System.

Flow cytometry. For each assay, 21.4×10^3 cells cm^{-2} were seeded and cultured for 24 h on plastic dishes. The culture medium was then discarded, 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^{-1}). Fluorescence analysis was performed with a Coulter XL flow cytometer. Cells stained with propidium iodide were excluded from further analysis. At least 10000 events per sample were analysed twice. The results shown are the average of two measures in the flow cytometer, and bars indicate the standard deviation.

MTT assay. HeLa viability in the presence of SAP and all D-SAP were 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^{-1}. 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.

Proteolytic stability. Trypsin (trypsin from bovine pancreas, E.C. 3.4.21.4, Roche) stock solution was stored at −20 °C in glycerol 50% in Tris·HCl 100 mM. Peptides were incubated at 37 °C dissolved in Tris·HCl 100 mM at 150 μM, and trypsin was added at the desired concentration. To obtain a peptide:trypsin ratio of 100:1, trypsin at a final concentration of 35.8 mg mL^{-1} (3.94 U mL^{-1}, U: Chromozym Try as a substrate) was added. Aliquots of 50 μL were extracted at different times, and 150 μL HCl 1 N at 4 °C was added to stop the enzyme degradation. Every sample was analysed by RP-HPLC (flow = 1 mL min^{-1}; gradient = 0–50% B in 15 min; A = 0.045% TFA in H_2O, B = 0.036% TFA in acetonitrile). The kinetics analysis was performed by plotting the log% A from the HPLC peak versus time using the least-squares method. Determining the slope of the straight-line half-life was calculated for every peptide. Experiments were performed in triplicate.

For the human serum study, peptides at a final concentration of 150 μM in HBSS were incubated at 37 °C in the presence of 90% human serum (from human male AB plasma, sterile-filtered, Sigma). 50 μL aliquots were extracted with time and precipitating serum proteins with 200 μL MeOH at 4 °C stopped the degradation process. After 30 min at 4 °C, the samples were centrifuged at 10000 rpm for 15 min at 4 °C. As SAP and D-SAP are completely soluble in MeOH, the supernatant was analysed by RP-HPLC (flow = 1 mL min^{-1}; gradient = 0–50% B in 15 min; A = 0.045% TFA in H_2O, B = 0.036% TFA in acetonitrile).

Internalisation mechanism studies. To assess the implication of cell-surface proteoglycans in the internalisation mechanism, cells were pretreated for 1 h with heparin at different concentrations (from 50 to 400 μg mL^{-1}) and treated by coincubating CF-SAP or CF-D-SAP at 50 μM with the corresponding heparin concentration for 3 h. After incubation, the cells were treated for flow cytometry analysis.

For the experiments at 4 °C, CLSM and flow cytometry were performed with the same protocol as described above for 37 °C and 5% CO_2 experiments, except that the cells were kept at 4 °C in Hepes buffer 1 mM. For the flow cytometry experiments with ATP depletion, cells were pretreated with 6 mM 2-deoxy-D-glucose and 10 mM NaN_3 or 15 mM 2-deoxy-D-glucose and 15 mM NaN_3 and treated for 3 h with the same conditions plus CF-SAP or CF-D-SAP at 50 μM concentration.

In the case of the macropinocytosis inhibitors study, cells were pretreated for 15 min either with amiloride at 10 μM, cytochalasin D at 5 μM or nocodazole at 10 μM. Then cells were treated for 3 h at the same concentration of inhibitor and at 50 μM concentration of CF-SAP or CF-D-SAP. After the incubation period, cells were prepared for the flow cytometry analysis.

Colocalisation assays by CLSM. HeLa cells were seeded in coverslips and co-incubated for 30 min or 1 h with solutions of CF-SAP or CF-D-SAP and either AlexaFluor555-Cholera toxin (10 $\mu g\,mL^{-1}$) or AlexaFluor555-Transferrin (50 $\mu g\,mL^{-1}$). Cell fixation was performed in the same conditions as for the assays with only CF-SAP or CF-D-SAP, except that in the CLSM observation, the channel for AlexaFluor 555 was added. This entailed exciting the samples with the HeNe laser (543 nm), and then detecting the emission from 560 nm. To avoid cross-talk, emission signals were recorded sequentially.

To perform the colocalisation assays with ER-Tracker Red (glibenclamide BODIPY TR), D-SAP was labelled with Alexa Fluor 488. Cells seeded in coverslips were incubated with AlexaFluor488-D-SAP for 3 h, the last 15 min adding ER-Tracker Red. Cell fixation and observation were performed as described for cholera toxin and transferrin.

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Keywords: cell-penetrating peptides · D-amino acids · drug delivery · proteolytic resistance · self-assembly

[1] S. Pujals, J. Fernandez-Carneado, C. Lopez-Iglesias, M. J. Kogan, E. Giralt, *Biochim. Biophys. Acta Biomembr.* **2006**, *1758*, 264–279.
[2] U. Langel, *Handbook of Cell-Penetrating Peptides*, CRC, Boca Raton, **2007**.
[3] M. E. Lindgren, M. M. Haellbrink, A. M. Elmquist, U. Langel, *Biochem. J.* **2004**, *377*, 69–76.
[4] M. Hällbrink, A. Floren, A. Elmquist, M. Pooga, T. Bartfai, U. Langel, *Biochim. Biophys. Acta Biomembr.* **2001**, *1515*, 101–109.
[5] E. Dé, L. Chaloin, A. Heitz, J. Mery, G. Molle, F. Heitz, *J. Pept. Sci.* **2001**, *7*, 41–49.
[6] L. Chaloin, E. De, P. Charnet, G. Molle, F. Heitz, *Biochim. Biophys. Acta Biomembr.* **1998**, *1375*, 52–60.
[7] S. Futaki, T. Suzuki, W. Ohashi, T. Yagami, S. Tanaka, K. Ueda, Y. Sugiura, *J. Biol. Chem.* **2001**, *276*, 5836–5840.
[8] T. Suzuki, S. Futaki, M. Niwa, S. Tanaka, K. Ueda, Y. Sugiura, *J. Biol. Chem.* **2002**, *277*, 2437–2443.
[9] M. Zhao, R. Weissleder, *Med. Res. Rev.* **2004**, *24*, 1–12.
[10] D. Derossi, A. H. Joliot, G. Chassaing, A. Prochiantz, *J. Biol. Chem.* **1994**, *269*, 10444–10450.
[11] J. Fernández-Carneado, M. J. Kogan, S. Castel, E. Giralt, *Angew. Chem.* **2004**, *116*, 1847–1850; *Angew. Chem. Int. Ed.* **2004**, *43*, 1811–1814.
[12] C. García-Echeverría, L. Jiang, T. M. Ramsey, S. K. Sharma, Y. N. P. Chen, *Bioorg. Med. Chem. Lett.* **2001**, *11*, 1363–1366.
[13] D. J. Mitchell, D. T. Kim, L. Steinman, C. G. Fathman, J. B. Rothbard, *J. Pept. Res.* **2000**, *56*, 318–325.
[14] L. J. Arnold, Jr., A. Dagan, J. Gutheil, N. O. Kaplan, *Proc. Natl. Acad. Sci. USA* **1979**, *76*, 3246–3250.
[15] P. A. Wender, D. J. Mitchell, K. Pattabiraman, E. T. Pelkey, L. Steinman, J. B. Rothbard, *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 13003–13008.
[16] D. Derossi, S. Calvet, A. Trembleau, A. Brunissen, G. Chassaing, A. Prochiantz, *J. Biol. Chem.* **1996**, *271*, 18188–18193.
[17] A. Elmquist, U. Langel, *Biol. Chem.* **2003**, *384*, 387–393.
[18] J. Fernandez-Carneado, M. J. Kogan, N. Van Mau, S. Pujals, C. Lopez-Iglesias, F. Heitz, E. Giralt, *J. Pept. Res.* **2005**, *65*, 580–590.
[19] S. Pujals, J. Fernandez-Carneado, M. J. Kogan, J. Martinez, F. Cavelier, E. Giralt, *J. Am. Chem. Soc.* **2006**, *128*, 8479–8483.
[20] T. Mosmann, *J. Immunol. Methods* **1983**, *65*, 55–63.
[21] M. Tyagi, M. Rusnati, M. Presta, M. Giacca, *J. Biol. Chem.* **2001**, *276*, 3254–3261.
[22] I. Nakase, M. Niwa, T. Takeuchi, K. Sonomura, N. Kawabata, Y. Koike, M. Takehashi, S. Tanaka, K. Ueda, J. C. Simpson, A. T. Jones, Y. Sugiura, S. Futaki, *Mol. Ther.* **2004**, *10*, 1011–1022.
[23] I. Nakase, A. Tadokoro, N. Kawabata, T. Takeuchi, H. Katoh, K. Hiramoto, M. Negishi, M. Nomizu, Y. Sugiura, S. Futaki, *Biochemistry* **2007**, *46*, 492–501.
[24] I. M. Kaplan, J. S. Wadia, S. F. Dowdy, *J. Controlled Release* **2005**, *102*, 247–253.

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