BIOCHEMISTRY
including biophysical chemistry & molecular biology

Article

pubs.acs.org/biochemistry

Enhanced Cellular Uptake of Antisecretory Peptide AF-16 through Proteoglycan Binding

Maria Matson Dzebo,[†] Anna Reymer,[†,‡] Kristina Fant,[§] Per Lincoln,[†] Bengt Nordén,[†] and Sandra Rocha*[,†]

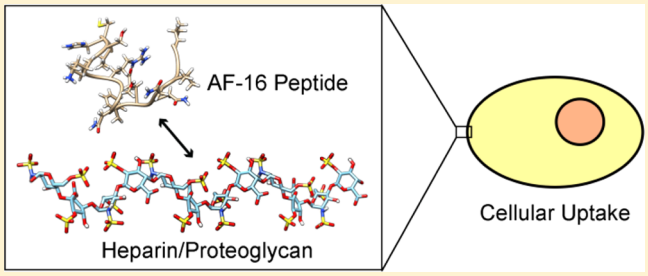
[†]Chemical and Biological Engineering, Physical Chemistry, Chalmers University of Technology, SE-412 96 Gothenburg, Sweden

[‡]Bases Moléculaires et Structurales des Systèmes Infectieux, Université Lyon 1/CNRS UMR 5086, IBCP, 7 passage du Vercors, Lyon 69367, France

[§]Chemistry, Materials and Surfaces, SP Technical Research Institute of Sweden, SE-50115 Borås, Sweden

Supporting Information

ABSTRACT: Peptide AF-16, which includes the active site of Antisecretory Factor protein, has antisecretory and anti-inflammatory properties, making it a potent drug candidate for treatment of secretory and inflammatory diseases such as diarrhea, inflammatory bowel diseases, and intracranial hypertension. Despite remarkable physiological effects and great pharmaceutical need for drug discovery, very little is yet understood about AF-16 mechanism of action. In order to address interaction mechanisms, we investigated the binding of AF-16 to sulfated glycosaminoglycan, heparin, with focus on the effect of pH and ionic strength, and studied the influence of cell-surface proteoglycans on cellular uptake efficiency. Confocal laser scanning microscopy and flow cytometry experiments on wild type and proteoglycan-deficient Chinese hamster ovary cells reveal an endocytotic nature of AF-16 cellular uptake that is, however, less efficient for the cells lacking cell-surface proteoglycans. Isothermal titration calorimetry provides quantitative thermodynamic data and evidence for that the peptide affinity to heparin increases at lower pH and ionic strength. Experimental data, supported by theoretical modeling, of peptide−glycosaminoglycan interaction indicate that it has a large electrostatic contribution, which will be enhanced in diseases accompanied by decreased pH and ionic strength. These observations show that cell-surface proteoglycans are of general and crucial importance for the antisecretory and anti-inflammatory activities of AF-16.



Antisecretory Factor (AF) is a 41 kDa protein possessing antisecretory[1,2] and anti-inflammatory[2−4] activity. The protein is natively found in an inactive form in most mammalian tissues, in neurons and in blood.[5−8] Upon exposure to bacterial enterotoxins, like cholera toxin, the protein expression increases and the protein is activated,[9−11] an effect that can also be induced by intake of specially processed food.[4,12,13] The sequence responsible for the protein activity has been identified as a minimum number of seven amino acids VCHSKTR near the N-terminus of the protein,[14] and from that a 16 amino acid peptide called AF-16 has been derived, which apart from comprising the active site is also resistant to proteolytic degradation (Figure 1). Both AF and AF-16 are able to reduce the diarrheal response induced by bacterial enterotoxins in human and animals[2,12] and are potential drug candidates for treatment of diseases with inflammation and/or secretory imbalances as main symptoms. In addition to diarrheal diseases, positive effect of AF therapy has been shown for a number of diseases including high intracranial pressure,[15] high tumor pressure,[16] inflammatory bowel disease,[4] matrisis,[17] and Ménière's disease.[18] The use of AF-16 as a peptide-based drug would be beneficial, since the peptide shares the activity with the native protein but is not dependent on gene up-regulation and activation. Although much is known about the physiological effects of AF and AF-16, their mechanism of action is still not known. Furthermore, there are only a few studies on their interactions with other biomolecules. The C-terminus of AF protein has been found to have binding sites for polyubiquitin,[19] whereas the N-terminus, carrying the antisecretory sequence, is interacting with flotillin-1.[20] In addition, AF-16 has been found in intracellular regions and colocalized with cholesterol and

Figure 1. Amino acid sequence of AF-16. Colored boxes highlight the following properties for the amino acids: green is hydrophobic, red is basic, and blue is acidic (categorized for pH range 5−7.4). The majority of the amino acids in the sequence are polar.

Received: August 19, 2014
Revised: September 18, 2014
Published: October 7, 2014

galactosylceramide in the cell membrane of rat pancreas after administration of the peptide.[21]

In diarrheal and inflammatory diseases, normal extracellular physiological conditions are usually disturbed, such as decreased pH and loss of electrolytes.[22−24] Understanding molecular interactions under these circumstances is therefore particularly relevant. In addition, proteoglycans, consisting of a protein core and branches of different glycosaminoglycans, have been shown to have an important role in inflammatory conditions,[25−27] both by recruiting lymphocytes to the inflammatory site and by acting as mediators for the inflammatory signal substances.[25−27] Proteoglycans have also been shown to be important for cellular uptake of some cell-penetrating peptides.[28−34] These peptides are often carrying multiple cationic residues and are hence attracted by the highly negatively charged environment provided by the glycosaminoglycans of the proteoglycans. The amino acids of AF-16 are mainly polar or aliphatic, and it has one acidic and two basic residues at pH 7.4 (Figure 1).

In this study, we investigate the interaction of AF-16 with proteoglycans and its cellular uptake to understand more about the mechanisms behind its biological activity on a molecular and cellular level. The affinity of the peptide to heparin (an analogue of the glycosaminoglycan heparan sulfate) was studied by isothermal titration calorimetry (ITC) at different pH, temperature, and salt concentrations to evaluate the influence of these factors. Moreover, the conformation of the peptide was characterized by circular dichroism (CD) spectroscopy and confirmed by de novo folding simulation through all-atom molecular dynamics. The cellular uptake in wild type and proteoglycan-deficient Chinese hamster ovary (CHO-K1) cells was studied using confocal laser scanning microscopy (CLSM) and flow cytometry.

EXPERIMENTAL PROCEDURE

Materials. AF-16 (VCHSKTRSNPENNVGL) and the Chinese hamster ovary (CHO-K1) cells were a kind gift from Prof. S. Lange, Gothenburg University. The proteoglycan-deficient mutant cell line CHO-pgsA745 was a kind gift from Prof. Ü. Langel, Stockholm University. Concentrations of the peptide were determined by weight, and the final concentrations used in the experiments were additionally checked using the Micro BCA protein assay kit (Thermo Scientific). Peptide samples were probed with the Measure-iT thiol assay kit (Molecular Probes) to determine whether disulfide bond formation occurs. It was established that the peptide molecules do not form disulfide bonds and thus the thiol group of the cysteine side chain remains free in solution. Heparin sodium salt, with MW of 13 000−15 000 Da, was from Serva (Heidelberg, Germany). The concentration of the heparin is reported in units of heparin disaccharides (MW 593 g/mol), and there are about 22−25 disaccharide units/heparin molecule. Sodium dodecyl sulfate (SDS), 2,2,2-trifluoroethanol (TFE), and buffer salts were from Sigma-Aldrich.

Isothermal Titration Calorimetry. Prior to the isothermal titration calorimetry (ITC) measurements, heparin was dissolved in buffer and dialyzed using Float-A-Lyzer G2 with cellulose ester membranes with a molecular weight cutoff of 500−1000 Da (Spectrum Laboratories, Inc.) during at least 15 h and with change of buffer three times. The peptide was dissolved directly in buffer previously dialyzed. ITC measurements were performed using a Microcal iTC200 calorimeter (GE Healthcare), with a sample cell volume of 206 μL and titration syringe volume of 40 μL. The sample cell was equilibrated with buffer few minutes before the experiment. The temperature was 25 °C, initial delay 60 s, reference power was 6 μCal/s, stirring speed was 1000 rpm, and high feedback/gain was used. Titrations of 3 μL, 6 s duration with spacing of 180 s and filtering time of 2s, were performed with different buffers (10 mM citrate-phosphate/10 mM NaCl (pH 5), 10 mM citrate-phosphate/50 mM NaCl (pH 5), 10 mM citrate-phosphate/150 mM NaCl (pH 5), 10 mM Na-phosphate/10 mM NaCl (pH 7.4) and 10 mM Na-phosphate/150 mM (pH 7.4)), and the concentration of the peptide and heparin was optimized for the individual conditions. At pH 5, titration of peptide to heparin was performed with starting concentration of, respectively, 0.5 mM (syringe) and 0.55 mM (sample cell). At pH 7.4, concentrations equal to or higher than 0.5 mM gave endothermic peaks when titrated into buffer. This could be explained by aggregated peptide in the syringe that upon dilution disaggregate, which is seen as heat consumption from the system. Therefore, the initial concentrations at pH 7.4 were adjusted to 0.4 mM of peptide (syringe) and 0.5 mM of heparin (sample cell). Additionally, the titration of AF-16 to heparin solution at pH 5 and low salt concentration (10 mM citrate-phosphate/10 mM NaCl) was also performed at 15 and 37 °C. For all titrations, a first aliquot of 0.5 μL was injected but not used in the analysis due to uncertainties in concentration of the first distributed volume. After 13 injections, the syringe was refilled, a new 0.5 μL aliquot injected (excluded from the analysis), and the titration resumed. The change in power during the titrations was registered in real time, and peaks were integrated to obtain the heat change per mole AF-16, ΔQ, for each titration using the Origin software supplied with the instrument. Integrated heats of peptide titrations into buffer were used as baseline and subtracted from the measurements before fitting. A single-site model was used for fitting the data, and the binding constant, K, binding enthalpy, ΔH, and number of binding sites per heparin unit, n, were obtained.

Circular Dichroism Spectroscopy. Circular dichroism (CD) of AF-16 (100 μM) was measured in a 1 mm cuvette with a Chirascan CD spectrometer (Applied Photophysics). Different buffers and solvents were used for the experiment: 10 mM citrate-phosphate/10 mM NaF (pH 5), 10 mM citrate-phosphate/10 mM NaF/50 mM SDS (pH 5), Na-phosphate/10 mM NaF (pH 7.4), and Na-phosphate/10 mM NaF/50 mM SDS (pH 7.4). Five spectra, recorded between 190 and 350 nm (large interval to ensure a stable baseline) with 1 nm steps, bandwidth of 1 nm, and time-per-point of 0.5 s, were averaged, and the spectra from the solvent alone were subtracted as baseline. Measurements were also performed in the presence of heparin (125 μM), from which the spectrum of heparin in buffer (weak signal in comparison to the one of the peptide at this concentration) was subtracted. NaF was used instead of NaCl, due to the strong absorption of Cl^- in the far UV-region. All spectra were recorded in millidegree units and converted to the mean residue ellipticity, θ_{MRW}, as described by Kelly et al.[35]

Molecular Modeling. De novo folding simulation of AF-16 peptide was performed through all-atom molecular dynamics using the Amber12 program package[36] with ff12SBforce field parameters. The simulation was initiated from a fully extended amino acids chain (unfolded state) solvated in TIP3P (three-point water model) allowing 10 Å of water from each of the ends of the peptide chain with periodic boundary conditions within a truncated octahedral cell in 20 mM NaCl. The number of ions was adjusted to ensure a zero net charge for the peptide-

dx.doi.org/10.1021/bi5010377 | Biochemistry 2014, 53, 6566−6573

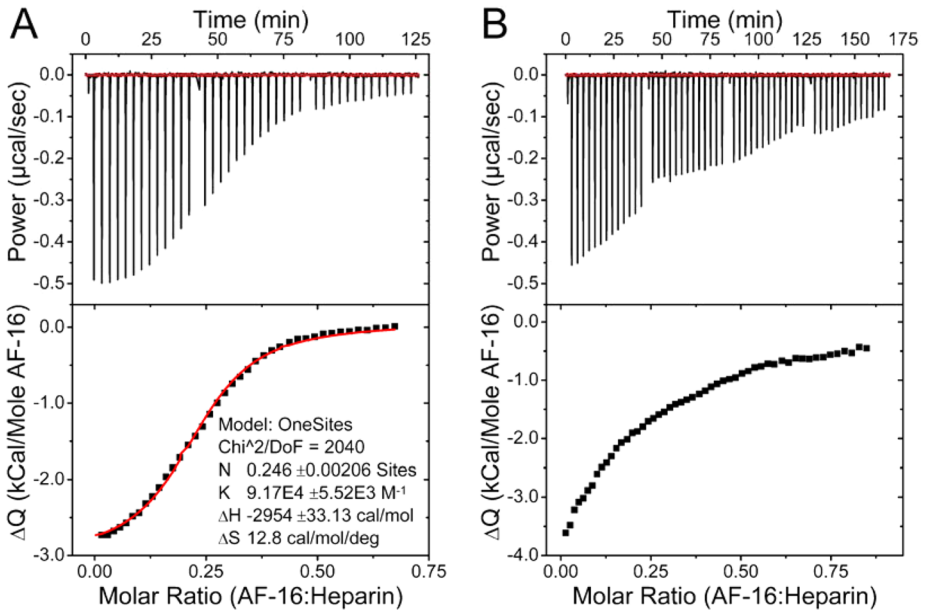


Figure 2. ITC measurement of (A) AF-16 (0.5 mM) titrated to heparin (0.55 mM) at pH 5 (10 mM citrate-phosphate/10 mM NaCl) and (B) AF-16 (0.4 mM) titrated to heparin (0.5 mM) pH 7.4 (10 mM Na-phosphate/10 mM NaCl). Top panels represent the power recorded during the titration, and bottom panels the integrated heats per mole peptide obtained by integration of the peaks. The binding affinity of AF-16 to heparin is significantly higher at pH 5.

counterion system. Electrostatic interactions were treated using the particle mesh Ewald method[37] with a real-space cutoff of 9 Å and cubic B-spline interpolation onto the charge grid with a spacing of ∼1 Å. Lennard−Jones interactions were truncated at 9 Å. Initial equilibration, involving energy minimization of the solvent, then of the solute−solvent system, followed by a slow thermalization to 300 K. The system was then equilibrated in NPT ensemble (T = 300 K) for 1 ns using the Berendsen algorithm[38] to control temperature and pressure, with a coupling constant of 5 ps for both parameters, then followed by simulation with alternating temperatures (T_1 = 350 K, T_2 = 300 K, duration between temperature alternations was 0.5 ns) to better explore conformational space. The total time of simulation was 100 ns. SHAKE[39] was used to restrain all chemical bonds involving hydrogen atoms to allow for a 2 fs time step. RMSD fluctuation of less than 0.5 Å of C_a atoms was used as a criterion for the folded state. A 20 units-long heparin chain was constructed using NMR structure (PDB ID: 1HPN) of 6 disaccharide units-long (6IDS and 6SGN units). Semiflexible docking, performed with Autodock Vina program,[40] was done in five stages, starting from docking of a small portion of the heparin chain (2.5 units) from one end of the heparin molecule. Then the heparin molecule was extended by another 2.5 units, while holding the end rigid in the conformation found in the previous step and allowing the new extension to move. This procedure was continued until all 20 heparin units were added, allowing a complete heparin chain to be docked to the peptide.

Cell Culturing. Two cell lines were used, Chinese hamster ovary (CHO-K1) cells and the proteoglycan-deficient mutant CHO-pgsA745 cells (denoted A745) lacking the xylosyltransferase needed for glycosaminoglycan synthesis. Both cell lines were cultured in HAM's F12 medium supplemented with 2 mM L-glutamine and 10% fetal bovine serum at 37 °C in a humidified atmosphere with 5% CO_2. For flow cytometry analysis, 10 000 cells/well were seeded in 96-well plates and cultured for 48 h. For confocal laser scanning microscopy, cells were seeded, with a density of 11 000 cells/cm^2 on glass bottom dishes ⌀ 30 mm (Willco Wells B.V.), 48 h prior to the experiment.

Peptide Labeling. For cellular uptake studies, AF-16 was labeled with Alexa Fluor 488 using Alexa Fluor 488 succinimidyl ester (Invitrogen). The supplier's instructions were followed with two exceptions: the AF-16/dye molar ratio was 1:1.2 and the buffer used was 10 mM sodium phosphate buffer (pH 7.8), avoiding too basic pH in order to mostly label the N-terminus and not the lysine.[41] After separation of the labeled peptide from the free dye, the peptide/dye molar ratio was determined to be 8:1. Concentrations in the flow cytometry and confocal imaging are referring to the concentration of peptide.

Flow Cytometry. Cells were washed with serum free HAM's F12 medium and then incubated with either 10 μM AF-16 (labeled with Alexa Fluor 488) in serum free medium during 25 min to 6 h at 37 °C or during 6 h with concentrations of 1−10 μM at 37 °C. The incubation was stopped by washing twice with buffer with 20 mM HEPES/150 mM NaCl, pH 7.4, and 100 μg/mL heparin was supplemented to the buffer the first time to wash away any surface associated peptide.[42] Cells were detached by 10 min trypsinization, and medium with serum was added. Analysis was done with a Guava EasyCyte 8HT flow cytometer (Merck Millipore). Live cells were gated using the forward and side scatter, and the mean fluorescence of 5000 cells was determined with Guava InCyte software of three technical replicates for each incubation condition. The autofluorescence of nontreated cells was subtracted from the data of cells incubated with AF-16. The mean fluorescence of three separate experiments was plotted with error bars corresponding to the standard deviation of mean.

Confocal Laser Scanning Microscopy. Prior to imaging, cells were washed once with serum free HAM's F12 medium and incubated with 10 μM AF-16 (labeled with Alexa Fluor 488) during 1 h at 37 or 4 °C in serum free medium (to maintain the pH outside the incubator, the medium at 4 °C was

dx.doi.org/10.1021/bi5010377 | Biochemistry 2014, 53, 6566−6573

supplemented with 10 mM HEPES). Incubation with 5 μM AF-16 at 37 °C during 6 h was also done. The imaging was performed with a Leica TCS SP confocal laser scanning microscope (Wetzlar) using a HCX PL APO CS 63 × 1.32 oil immersion objective. Excitation was done with an Ar laser of 488 nm, and the emission was collected at 500−550 nm. The gain of the detector was optimized for the CHO-K1 cells incubated at 37 °C and kept at the same level during the imaging of the other cells.

RESULTS AND DISCUSSION

Affinity of AF-16 to Heparin Is Dependent on pH and Ionic Strength. To study the affinity of AF-16 to proteoglycans, heparin, a disaccharide polymer similar to the glycosaminoglycan heparan sulfate, was used in ITC measurements. Figure 2A shows the titration of peptide into heparin solution in 10 mM citrate-phosphate/10 mM NaCl buffer, at pH 5. The titrations shown in the upper panel display negative peaks, which are characteristic of an exothermic process. The magnitude of the peaks is decreasing with the titration as a result of less and less peptide binding. All individual titration peaks were integrated yielding the heat change per mole peptide plotted against the molar ratio (AF-16/heparin units) in the lower panel. The obtained sigmoidal curve was fitted with a one-site model, and the binding constant and thermodynamic parameters found in Figure 2 were attained. The *n* value, number of peptide molecules per heparin unit, estimates that one peptide requires about four heparin disaccharide units as a binding site.

A similar titration was performed at pH 7.4 (10 mM Na-phosphate/10 mM NaCl-buffer), see Figure 2B, which also shows an exothermic process but the slope of the binding curve is smaller than at pH 5. The fitting of the binding curve of AF-16 titrated into heparin at pH 7.4 was rather poor as a consequence of the low binding affinity. The binding constant, with this rough estimation, was found to be about 60-fold less at pH 7.4 compared to the one at pH 5; see Supporting Information Figure S1 for details. The fact that the affinity is higher at pH 5 is reasonable, since the number of positive charges at this pH is higher and thus the electrostatic interaction with the negatively charged heparin (−4 per disaccharide unit) is stronger. The binding constant obtained at pH 5 is lower than that reported for other peptides such as penetratin,[43] HIV Tat-PTD,[43] melittin,[44] HIV-1 VPR(52−96),[32] and brain natriuretic peptide.[45] AF-16 is, however, not as charged as those peptides. Titrations in buffers with increased NaCl concentration (50 mM and 150 mM) were also done and showed a decreased heat evolvement and a decreased slope of the binding curves (see Supporting Information Figure S2). This indicates that the interaction between AF-16 and heparin is weaker at higher ionic strengths as expected for electrostatic interactions because of screening of the charges by NaCl. To further investigate the nature of the interaction between AF-16 and heparin, the temperature dependence of the binding enthalpy was studied. Hydrophobic effects are associated with a decrease in binding enthalpy with the temperature, which leads to a negative heat capacity,[43] whereas electrostatic interactions do not highly influence these parameters[46] or give rise to the opposite effect if charge neutralization is achieved.[47] For the interaction of AF-16 and heparin, the binding enthalpy did not change upon variation of the temperature (in the interval 15−37 °C); see Supporting Information Figure S3. From the observed dependence of binding on peptide charge, on ionic strength and temperature, we conclude that the interaction of the peptide with heparin has a large electrostatic contribution.

Secondary Structure of AF-16. CD spectroscopy was used to investigate whether heparin induces change in the secondary structure of the peptide. The spectrum of AF-16 in buffer at pH 5 shows a large negative peak centered at 199 nm and a weaker negative band at 222 nm (Figure 3). The

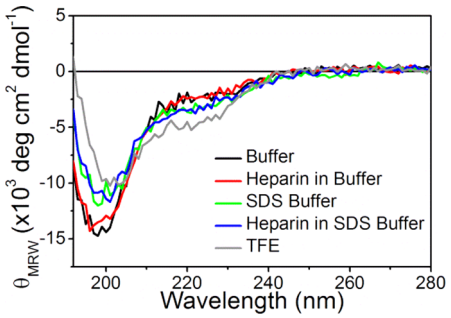


Figure 3. CD spectra of AF-16 (18 μM) in 10 mM citrate-phosphate/10 mM NaF buffer (pH 5) in the absence and presence of heparin (22.5 μM) and/or SDS (50 mM) as well as the CD spectrum of AF-16 (18 μM) in the more hydrophobic solvent TFE. Spectra are shown in units of mean residue ellipticity.

characteristic spectrum of a peptide with random coil conformation has a strong negative peak at around 200 nm and a smaller positive peak at around 220 nm, whereas in α-helical form the expected spectrum would contain a strong positive peak at around 195 nm and two negative peaks at 222 and 208 nm.[48] Thus, AF-16 in buffer has mainly an unordered structure with probably a small α-helical part. Many membrane-active peptides change conformation when they interact with glycosaminoglycans, commonly by having a random coil structure when free in solution and experiencing an increase in α-helical or β-sheet content when interacting with heparin.[28] The CD spectrum of AF-16, however, does not change upon addition of heparin, indicating that there is no significant conformational change of the peptide upon binding. α-Helices can be stabilized by hydrophobic environments, for instance, by exposure to micelles of SDS[49,50] or TFE[51] environment. When the buffer was supplemented with 50 mM SDS, the spectrum of AF-16 did indeed change, with an increased signal at 222 nm whereas the band at 199 nm decreases, suggesting an increase in α-helical content of the peptide when presumably exposed to the hydrophobic core of SDS micelles. The conformation of AF-16 in the presence of SDS did not further change upon addition of heparin. A similar, but even more pronounced, result was obtained for the peptide in TFE, which indicates that the α-helical content is increased even further in this more hydrophobic solvent. The conformation of the peptide alone and in the presence of heparin is conserved if the conditions are changed to pH 7.4 and/or physiological ionic strength. The interaction of AF-16 with glycosaminoglycans at the membrane surface is thus expected to not alter the peptide conformation.

Theoretical Modeling of AF-16-Heparin Molecular Interactions. To get some insight into the possible AF-16 peptide−heparin interactions at the atomistic level, a 20 disaccharide units-long heparin chain was docked to a 3D structure of AF-16 peptide using the Autodock Vina[40] program. The heparin chain was constructed based on an NMR structure (PDB ID: 1HPN). The 3D structure of AF-16 was obtained by a de novo folding simulation with all-atom molecular dynamics

dx.doi.org/10.1021/bi5010377 | *Biochemistry* 2014, 53, 6566−6573

using the Amber12 program package.[36] The peptide structure obtained has a central α-helical region (residues Lys5-Phe10), with the flanking residues being in a random coil configuration, which fits well with our CD data and with a previous structure prediction of the full protein.[20] During docking torsional angles of the peptide residues' side-chains as well as torsional angles of glycosidic bonds, sulfate and amide-sulfate groups of heparin were allowed to rotate freely. Docking analysis indicated plausible intramolecular interactions (Figure 4). The positively

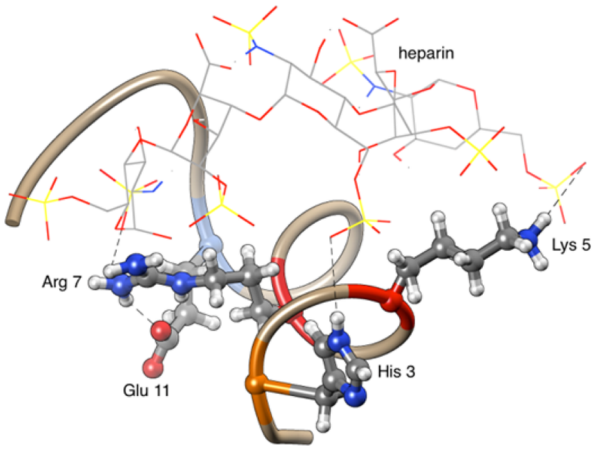


Figure 4. Plausible interactions between AF-16 peptide and heparin: positively charged Lys5 and Arg7 interact with negatively charged sulfate groups on heparin. At pH 5, His3 becomes protonated, further strengthening the electrostatic attraction between the two molecules. To avoid overcrowding, only 2.5 disaccharide units of heparin are included in the picture.

charged residues, Lys5 and Arg7, interact with the negatively charged sulfate groups of heparin. At pH 5, the histidine residue (His3), which is also in close proximity to heparin, becomes protonated and, hence, further strengthens the electrostatic interactions between AF-16 and heparin. The predicted interactions confirm our conclusions from the ITC data that suggest that the interaction between AF-16 and heparin has a predominant electrostatic contribution.

Endocytotic Cellular Uptake of AF-16 is Enhanced by Proteoglycans. To investigate if proteoglycans influence the cellular uptake of AF-16, wild type CHO-K1 cells and the proteoglycan-deficient mutant CHO-pgsA740 were compared. The cells were incubated with Alexa Fluor 488 labeled peptide for different periods of time (15 min to 6 h), and the uptake was measured as mean cellular fluorescence by flow cytometry. Linear uptake kinetics was observed for both cell lines, but the magnitude of the uptake was found to be higher for wild type cells compared to A740 cells (Figure 5A). This difference was statistically significant ($p < 0.05$ for two-tailed t test) at 4 h of incubation, and after 6 h of incubation the mean fluorescence in A740 cells was about 30% lower compared to the uptake in CHO-K1 cells. No saturation of the uptake is found during this time span. In addition, the concentration dependence of cellular uptake was investigated in the interval 1−10 μM; see Figure 5B. In this range, the uptake of AF-16 was found to be linearly dependent on the concentration for both cell lines with higher uptake observed for CHO-K1 cells. To further investigate the uptake, both wild type and proteoglycan-deficient cells were imaged after 6 h incubation with Alexa Fluor 488 labeled AF-16 using CLSM. Both cell types show

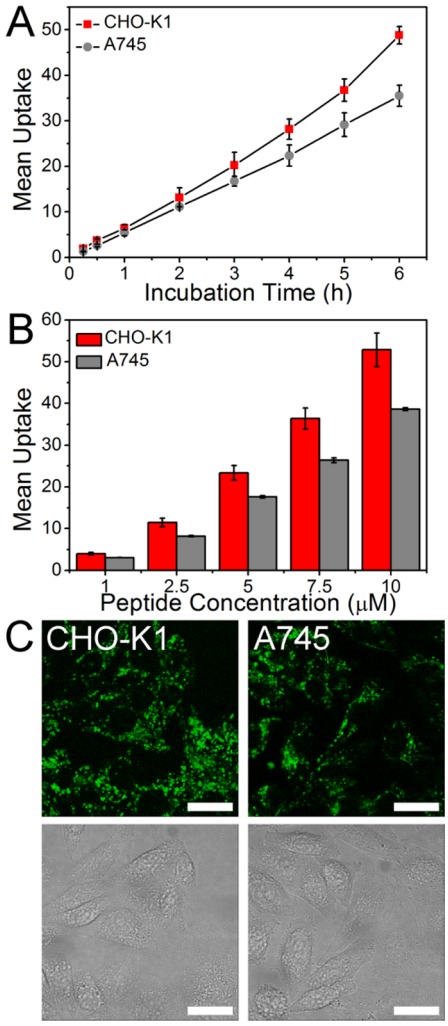


Figure 5. Cellular uptake of Alexa Fluor 488 labeled AF-16 in live wild type CHO-K1 and proteoglycan-deficient A745 cells (A) as a function of incubation time at a concentration of 10 μM (the lines are added as a guide for the eye) and (B) as a function of concentration after 6 h incubation at 37 °C. The uptake is measured as mean fluorescence by flow cytometry and the autofluorescence is subtracted from the data. Error bars are standard deviation of mean (n = 3). (C) Fluorescence images of live CHO-K1 and A745 cells incubated with AF-16 (5 μM) during 6 h (top panel) and the corresponding transmission images (bottom panel). Uptake of AF-16 is linearly dependent on incubation time and concentration for both cell types but is higher in wild type cells. Similar punctuate cellular staining is obtained for both cell types. Scale bar, 20 μm.

punctuate intracellular staining in the cytoplasm (Figure 5C), a staining pattern suggesting endocytotic uptake.

As the staining pattern seen in Figure 5C suggests a peptide uptake dependent on an endocytotic mechanism and since endocytosis is an energy dependent process which is inhibited at low temperatures, the uptake of AF-16 by cells incubated at 37 °C was compared to the uptake at 4 °C. In Figure 6, punctuate staining is seen in the cytoplasm of CHO-K1 cells incubated with 10 μM AF-16 during 1 h at 37 °C, whereas a dramatic decrease in uptake, to virtually no uptake, is seen for the cells at the lower incubation temperature. The same observation was attained for the proteoglycan-deficient cell line A745. In addition, the punctuate staining pattern is also observed for both cell types when incubated with AF-16

dx.doi.org/10.1021/bi5010377 | Biochemistry 2014, 53, 6566−6573

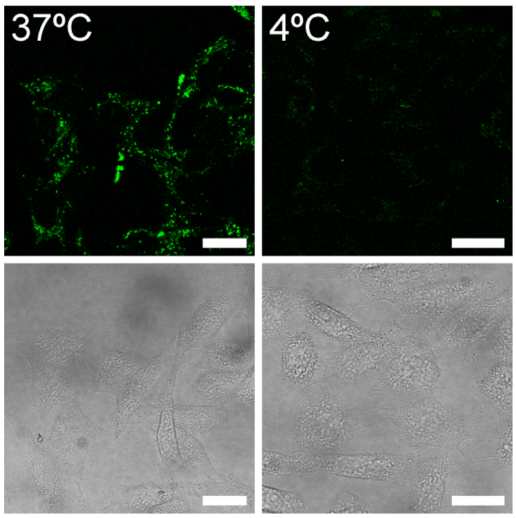


Figure 6. CLSM images of live CHO-K1 cells incubated with Alexa Fluor 488 labeled AF-16 (10 μM) during 1 h at 37 and 4 °C (top panel) and the corresponding transmission images (bottom panel). The uptake is highly reduced when the temperature is decreased. Scale bar, 20 μm.

concentrations over a large range (0.1−25 μM) at 37 °C. This indicates that the uptake mechanism is not concentration dependent in this range as seen for other peptides. In the case of cell-penetrating peptides, for example, the uptake mechanism is still unclear.[52−54] The predominant mechanism of entry is endocytosis,[52,53,55] but direct penetration has also been suggested as a route of entry[53,56] or a combination of the two pathways. The mechanism seems to depend on peptide properties and also on experimental conditions such as peptide concentration and incubation time.[52,54] For instance, WR9[30] and the penetratin analogue PenArg[29] are internalized by pure endocytotic mechanisms at low micromolar concentration, but at a threshold of >5 μM also direct translocation is seen. The peptide R6W3, on the other hand, shows the opposite behavior with direct penetration at low concentration and at increased concentration also endocytotic mechanisms are active.[28] Peptides that facilitate an energy-independent uptake mechanism are generally highly cationic and often also dependent on interaction of hydrophobic amino acids with the membrane interior,[52,54] properties which are not found for AF-16 (see Figure 1).

Both cell lines seem to facilitate endocytosis for uptake of AF-16, and the uptake is enhanced by the presence of cell surface proteoglycans. Some studies have shown that the proteoglycan dependent endocytotic uptake of penetratin and its analogues,[29] as well as a number of arginine and tryptophan rich peptides,[28] seems to be facilitated by proteoglycan clustering. These peptides efficiently aggregate glycosaminoglycans in solution, and it seems that the presence of tryptophan is important for the clustering to occur.[28] AF-16 did not show any tendency to aggregate heparin as no aggregates were detected by dynamic light scattering experiments, performed as described by Åmand et al.[29] with charge ratios of 0.25, 0.5, and 1 (+/−) at pH 5 as well as pH 7.4; see Supporting Information Figure S4. On the other hand, since AF-16 is not as highly positively charged and has a weaker binding affinity to heparin, it is not able to neutralize the highly negative charge of heparin to the degree where it can start to fold into aggregates. In addition, the peptide does not contain any tryptophan residue to stabilize the clustering. Further, the uptake of AF-16 is only partly reduced in proteoglycan-deficient cells, and thus, the peptide is also likely using another type of endocytotic route not dependent on the proteoglycans. Diarrheal and inflammatory conditions are usually accompanied by disturbances in pH and electrolyte concentrations; often these factors are decreased in the extracellular matrix.[22−24] Since the binding constant for heparin interaction increases with decreasing pH and salt concentration, the uptake of AF-16 might be even higher in cells with proteoglycans at their surfaces because of a locally higher concentration of the peptide at the cell membrane. There is, however, not always a correlation of membrane affinity and cellular uptake for peptides.[29,31,57]

AF-16 seems to have similarities to cell-penetrating peptides, since it is able to enter cells and, as for some cell-penetrating peptides, does so in a proteoglycan-dependent manner. Yet there are also some significant differences. Many other peptides show saturation in uptake with time, whereas uptake of AF-16 is linear with time up to at least 6 h. Second, the uptake seems to be purely endocytotic at both low and high micromolar concentrations. Third, the proteoglycans are important for the uptake even if the peptide seems to not facilitate a mechanism dependent on clustering of proteoglycans. Finally, the peptide is not highly cationic nor does it have amphipathic character with alternating hydrophobic and hydrophilic residues which seems to be two common features for many of the cell-penetrating peptides that show intracellular uptake.[52,58] Other peptides with the same mean charge per residue are either of amphipathic or hydrophobic nature,[58] whereas AF-16 has a more polar nature. In this perspective, the peptide is unique not only because of its antisecretory and anti-inflammatory activity but also for its features regarding cellular uptake.

■ CONCLUSION

We have shown that the antisecretory and anti-inflammatory peptide AF-16 is dependent on endocytotic mechanisms for the cellular uptake and that this uptake is increased by the presence of proteoglycans. The proteoglycans are, however, not the only key to cellular uptake, since cells lacking them on their surface also show peptide uptake, probably using other endocytotic pathways. Higher affinity for heparin at lower pH and lower ionic strength suggests a possible enhancement of peptide uptake during diarrheal and inflammatory conditions in which pH values and electrolyte concentration usually decrease compared to the healthy state. The peptide was found, by both circular dichroism and de novo folding simulation, to have a conformation with majority random coil structure and some α-helix content, which does not change upon binding to heparin. In summary, our study suggests that proteoglycans have a role not only in the initiation of the inflammation by the immune system, but also in the antisecretory and anti-inflammatory defense provided by AF-16 by promoting its cellular uptake. Our results provide new insights into the cellular uptake of the peptide that can be of importance for revealing the mechanism behind its antisecretory and anti-inflammatory effect.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Fitting of a one-site model to Figure 2B (Figure S1), ITC measurements at different NaCl concentrations (Figure S2) and at different temperatures (Figure S3), as well as the

dx.doi.org/10.1021/bi5010377 | *Biochemistry* 2014, 53, 6566−6573

correlogram from DLS measurements of samples with AF-16 and heparin (Figure S4). This material is available free of charge via Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*E-mail: sandra.rocha@chalmers.se. Tel: +46 317722815. Fax: +46 317723858.

Funding

This work was funded by the European Research Council (ERC Senior Advanced grant to B.N.).

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

Prof. S. Lange, Prof. I. Lönnroth, E. Johansson, I. Jonson, and Prof. E. Jennische from Gothenburg University are acknowledged for our fruitful discussions and the gift of the AF-16 peptide and CHO-K1 cells. Prof. Ü. Langel, Stockholm University is acknowledged for the gift of CHO-pgsA745 cells.

REFERENCES

(1) Johansson, E., Lönnroth, I., Lange, S., Jonson, I., Jennische, E., and Lönnroth, C. (1995) Molecular cloning and expression of a pituitary gland protein modulating intestinal fluid secretion. *J. Biol. Chem. 270*, 20615−20620.

(2) Johansson, E., Jennische, E., Lange, S., and Lönnroth, I. (1997) Antisecretory factor suppresses intestinal inflammation and hypersecretion. *Gut 41*, 642−645.

(3) Davidson, T., and Hickey, W. (2004) Antisecretory factor expression is regulated by inflammatory mediators and influences the severity of experimental autoimmune encephalomyelitis. *J. Leukocyte Biol. 74*, 835−844.

(4) Björck, S., Bosaeus, I., Ek, E., Jennische, E., Lönnroth, I., Johansson, E., and Lange, S. (2000) Food induced stimulation of the antisecretory factor can improve symptoms in human inflammatory bowel disease: a study of a concept. *Gut 46*, 824−829.

(5) Lange, S., Jennische, E., Johansson, E., and Lönnroth, I. (1999) The antisecretory factor: synthesis and intracellular localisation in porcine tissues. *Cell Tissue Res. 296*, 607−617.

(6) Lange, S., and Lönnroth, I. (2001) The antisecretory factor: synthesis, anatomical and cellular distribution, and biological action in experimental and clinical studies. *Int. Rev. Cytol. 210*, 39−75.

(7) Lönnroth, I., and Lange, S. (1986) Purification and characterization of the antisecretory factor: a protein in the central nervous system and in the gut which inhibits intestinal hypersecretion induced by cholera toxin. *Biochim. Biophys. Acta 883*, 138−144.

(8) Johansson, E., Lönnroth, I., Jonson, I., Lange, S., and Jennische, E. (2009) Development of monoclonal antibodies for detection of Antisecretory Factor activity in human plasma. *J. Immunol. Methods 342*, 64−70.

(9) Lönnroth, I., and Lange, S. (1984) Purification and characterization of a hormone-like factor which inhibits cholera secretion. *FEBS Lett. 177*, 104−108.

(10) Lönnroth, I., Lange, S., Jennische, E., Johansson, E., Jonson, I., and Torres, J. (2003) Cholera toxin protects against action by Clostridium difficile toxin A. The role of antisecretory factor in intestinal secretion and inflammation in rat. *APMIS 111*, 969−977.

(11) Lönnroth, I., Lange, S., and Skadhauge, E. (1988) The antisecretory factors: inducible proteins which modulate secretion in the small intestine. *Comp. Biochem. Physiol. 90*, 611−617.

(12) Zaman, S., Mannan, J., Lange, S., Lönnroth, I., and Hanson, L.-A. (2007) B 221, a medical food containing antisecretory factor reduces child diarrhoea: a placebo controlled trial. *Acta Paediatr. 96*, 1655−1659.

(13) Johansson, E., Lange, S., and Jennische, E. (2011) Specially processed cereals diet increases plasma levels of active antisecretory factor and up-regulates rat hepatic glutathione S-transferase mu. *Nutrition 27*, 949−954.

(14) Johansson, E., Lange, S., and Lönnroth, I. (1997) Identification of an active site in the antisecretory factor protein. *Biochim. Biophys. Acta 1362*, 177−182.

(15) Jennische, E., Bergström, T., Johansson, M., Nyström, K., Tarkowski, A., Hansson, H.-A., and Lange, S. (2008) The peptide AF-16 abolishes sickness and death at experimental encephalitis by reducing increase of intracranial pressure. *Brain Res. 1227*, 189−197.

(16) Al-Olama, M., Wallgren, A., Andersson, B., Gatzinsky, K., Hultborn, R., Karlsson-Parra, A., Lange, S., Hansson, H.-A., and Jennische, E. (2011) The peptide AF-16 decreases high interstitial fluid pressure in solid tumors. *Acta Oncol. 50*, 1098−1104.

(17) Svensson, K., Lange, S., Lönnroth, I., Widström, A.-M., and Hanson, L. (2004) Induction of anti-secretory factor in human milk may prevent mastitis. *Acta Paediatr. 93*, 1228−1231.

(18) Hanner, P., Jennische, E., Lange, S., Lönnroth, I., and Wahlström, B. (2004) Increased antisecretory factor reduces vertigo in patients with Ménière's disease: a pilot study. *Hear. Res. 190*, 31−36.

(19) Young, P., Deveraux, Q., Beal, R. E., Pickart, C. M., and Rechsteiner, M. (1998) Characterization of two polyubiquitin binding sites in the 26 S protease subunit 5a. *J. Biol. Chem. 273*, 5461−5467.

(20) Johansson, E., Jonson, I., Bosaeus, M., and Jennische, E. (2008) Identification of flotillin-1 as an interacting protein for antisecretory factor. *Regul. Pept. 146*, 303−309.

(21) Lange, S., Malmberg, P., and Nygren, H. (2013) Binding of the VCHSKT peptide to ceramide-rich and cholesterol-rich domains of cell membranes in rat pancreatic glands. *Surf. Interface Anal. 45*, 268−272.

(22) Cieza, J., Sovero, Y., Estremadoyro, L., and Dumler, F. (1995) Electrolyte disturbances in elderly patients with severe diarrhea due to cholera. *J. Am. Soc. Nephrol. 6*, 1463−1467.

(23) Gennari, F. J., and Weise, W. J. (2008) Acid-base disturbances in gastrointestinal disease. *Clin. J. Am. Soc. Nephrol. 3*, 1861−1868.

(24) Lardner, A. (2001) The effects of extracellular pH on immune function. *J. Leukocyte Biol. 69*, 522−530.

(25) Parish, C. R. (2006) The role of heparan sulphate in inflammation. *Nat. Rev. Immunol. 6*, 633−643.

(26) Poole, A. R. (1986) Proteoglycans in health and disease: structures and functions. *Biochem. J. 236*, 1−14.

(27) Delehedde, M., Allain, F., Payne, S. J., Borgo, R., Vanpouille, C., Fernig, D. G., and Deudon, E. (2002) Proteoglycans in inflammation. *Curr. Med. Chem.: Anti-Inflammatory Anti-Allergy Agents 1*, 89−102.

(28) Bechara, C., Pallerla, M., Zaltsman, Y., Burlina, F., Alves, I. D., Lequin, O., and Sagan, S. (2013) Tryptophan within basic peptide sequences triggers glycosaminoglycan-dependent endocytosis. *FASEB J. 27*, 738−749.

(29) Åmand, H. L., Rydberg, H. A, Fornander, L. H., Lincoln, P., Nordén, B., and Esbjörner, E. K. (2012) Cell surface binding and uptake of arginine- and lysine-rich penetratin peptides in absence and presence of proteoglycans. *Biochim. Biophys. Acta 1818*, 2669−2678.

(30) Ziegler, A., and Seelig, J. (2011) Contributions of glycosaminoglycan binding and clustering to the biological uptake of the nonamphipatic cell-penetrating peptide WR9. *Biochemistry 50*, 4650−4664.

(31) Alves, I. D., Bechara, C., Walrant, A., Zaltsman, Y., Jiao, C.-Y., and Sagan, S. (2011) Relationships between membrane binding, affinity and cell internalization efficacy of a cell-penetrating peptide: penetratin as a case study. *PLoS One 6*, e24096.

(32) Greiner, V. J., Shvadchak, V., Fritz, J., Arntz, Y., Didier, P., Frisch, B., Boudier, C., Mély, Y., and de Rocquigny, H. (2011) Characterization of the mechanisms of HIV-1 Vpr(52−96) internalization in cells. *Biochimie 93*, 1647−1658.

(33) Mano, M., Teodósio, C., Paiva, A., Simões, S., and Pedroso de Lima, M. C. (2005) On the mechanisms of the internalization of S4(13)-PV cell-penetrating peptide. *Biochem. J. 390*, 603−612.

(34) Poon, G. M. K., and Gariépy, J. (2007) Cell-surface proteoglycans as molecular portals for cationic peptide and polymer entry into cells. *Biochem. Soc. Trans. 35*, 788−793.
(35) Kelly, S. M., Jess, T. J., and Price, N. C. (2005) How to study proteins by circular dichroism. *Biochim. Biophys. Acta 1751*, 119−139.
(36) Case, D. A., Darden, T. A., Cheatham, T. E., III, Simmerling, C. L., Wang, J., Duke, R. E., Luo, R., Walker, R. C., Zhang, W., Merz, K. M., Roberts, B., Hayik, S., Roitberg, A., Seabra, G., Swails, J., Götz, A. W., Kolossváry, I., Wong, K. F., Paesani, F., Vanicek, J., Wolf, R. M., Liu, J., Wu, X., Brozell, S. R., Steinbrecher, T., Gohlke, H., Cai, Q., Ye, X., Hsieh, M.-J., Cui, G., Roe, D. R., Mathews, D. H., Seetin, M. G., Salomon-Ferrer, R., Sagui, C., Babin, V., Luchko, T., Gusarov, S., Kovalenko, A., and Kollman, P. A. (2012) *AMBER 12*, University of California, San Francisco.
(37) Essmann, U., Perera, L., Berkowitz, M. L., Darden, T., Lee, H., and Pedersen, L. G. (1995) A smooth particle mesh Ewald method. *J. Chem. Phys. 103*, 8577−8593.
(38) Berendsen, H. J. C., Postma, J. P. M., van Gunsteren, W. F., DiNola, A., and Haak, J. R. (1984) Molecular dynamics with coupling to an external bath. *J. Chem. Phys. 81*, 3684−3690.
(39) Ryckaert, J. P., Ciccotti, G., and Berendsen, H. J. C. (1977) Numerical Integration of the Cartesian equations of motion of a system with constraints: Molecular dynamics of n-alkanes. *J. Comput. Phys. 23*, 327−341.
(40) Trott, O., and Olson, A. J. (2010) AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization and multithreadingNo Title. *J. Comput. Chem. 31*, 455−461.
(41) Gaudriault, G., and Vincent, J.-P. (1992) Selective labeling of α- or ε-amino groups in peptides by the bolton-hunter reagent. *Peptides 13*, 1187−1192.
(42) Kaplan, I. M., Wadia, J. S., and Dowdy, S. F. (2005) Cationic TAT peptide transduction domain enters cells by macropinocytosis. *J. Controlled Release 102*, 247−253.
(43) Ziegler, A., and Seelig, J. (2008) Binding and clustering of glycosaminoglycans: a common property of mono- and multivalent cell-penetrating compounds. *Biophys. J. 94*, 2142−2149.
(44) Klocek, G., and Seelig, J. (2008) Melittin interaction with sulfated cell surface sugars. *Biochemistry 47*, 2841−2849.
(45) Hileman, R., Jennings, R., and Linhardt, R. (1998) Thermodynamic analysis of the heparin interaction with a basic cyclic peptide using isothermal titration calorimetry. *Biochemistry 37*, 15231−15237.
(46) Gallagher, K., and Sharp, K. (1998) Electrostatic contributions to heat capacity changes of DNA-ligand binding. *Biophys. J. 75*, 769−776.
(47) Gonçalves, E., Kitas, E., and Seelig, J. (2005) Binding of oligoarginine to membrane lipids and heparan sulfate: structural and thermodynamic characterization of a cell-penetrating peptide. *Biochemistry 44*, 2692−2702.
(48) Nordén, B., Rodger, A., and Dafforn, T. (2010) *Linear dichroism and circular dichroism - A textbook on polarized-light spectroscopy*, pp 89−96, RSC Publishing, Cambridge.
(49) Wu, C., and Yang, J. (1978) Conformation of naturally-occurring peptides in surfactant solution: Its relation to the structure-forming potential of amino acid sequence. *Biochem. Biophys. Res. Commun. 82*, 85−91.
(50) McLeish, M. J., Nielsen, K. J., Wade, J. D., and Craik, D. J. (1993) A peptide corresponding to the N-terminal 13 residues of T4 lysozyme forms an alpha-helix. *FEBS Lett. 315*, 323−328.
(51) Buck, M. (1998) Trifluoroethanol and colleagues: cosolvents come of age. Recent studies with peptides and proteins. *Q. Rev. Biophys. 31*, 297−355.
(52) Madani, F., Lindberg, S., Langel, U., Futaki, S., and Gräslund, A. (2011) Mechanisms of cellular uptake of cell-penetrating peptides. *J. Biophys. 2011*, 414729.
(53) Futaki, S., Nakase, I., Tadokoro, A., Takeuchi, T., and Jones, a T. (2007) Arginine-rich peptides and their internalization mechanisms. *Biochem. Soc. Trans. 35*, 784−787.
(54) Bechara, C., and Sagan, S. (2013) Cell-penetrating peptides: 20 years later, where do we stand? *FEBS Lett. 587*, 1693−1702.
(55) Fotin-Mleczek, M., Fischer, R., and Brock, R. (2005) Endocytosis and cationic cell-penetrating peptides—A merger of concepts and methods. *Curr. Pharm. Des. 11*, 3613−3628.
(56) Ter-Avetisyan, G., Tünnemann, G., Nowak, D., Nitschke, M., Herrmann, A., Drab, M., and Cardoso, M. C. (2009) Cell entry of arginine-rich peptides is independent of endocytosis. *J. Biol. Chem. 284*, 3370−3378.
(57) Burlina, F., Sagan, S., Bolbach, G., and Chassaing, G. (2005) Quantification of the cellular uptake of cell-penetrating peptides by MALDI-TOF mass spectrometry. *Angew. Chem., Int. Ed. Engl. 44*, 4244−4247.
(58) Milletti, F. (2012) Cell-penetrating peptides: classes, origin, and current landscape. *Drug Discovery Today 17*, 850−860.