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Research article

SialoPen peptides are new cationic foldamers with remarkable cell permeability

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ABSTRACT

The ability to access intracellular targets is of vital importance as the number of identified druggable intracellular targets increases every year. However, intracellular delivery poses a formidable barrier, as many potential therapeutics are impermeable to cell membranes, which hinders their practical application in drug development. Herein we present *de novo*-designed unnatural cell penetrating peptide foldamers utilizing a 2,3-Didehydro-2-deoxyneuraminic acid (Neu2en) scaffold. Conveniently, this scaffold is amenable to standard Fmoc-based solid-phase peptide synthesis, with the advantages of tunable secondary structures and enhanced biostability. Flow cytometry and live-cell confocal microscopy studies showed that these Neu2en-based peptides, hereinafter termed SialoPen peptides, have significantly superior uptake in HeLa and primary neuronal hippocampal cells, outperforming the classical cell permeable peptides penetratin and HIV-TAT.

1. Introduction

Access to intracellular targets poses a formidable obstacle for emerging therapeutics, as greater than 90% of druggable targets reside within the intracellular space (Rizzuti et al., 2015; Stanzl et al., 2013). The quickening pace of discovery of protein structures and cellular factor drug targets has accelerated the importance of accessing the intracellular compartment. For example, protein structure determinations have increased from ~11,000 in 2009 to ~147,600 in 2018 searchable protein structures available through the protein data bank (Berman et al., 2000; Burley et al., 2018). This dramatic increase in protein structure availability in addition to powerful 3-D modeling platforms are driving rationally designed therapeutics in the forms of small molecules, peptides, proteins, and genetic-based drugs. While rational design has led to a rapid increase in potential therapeutics, *in vivo* advancement of most leads has proven difficult (Waring et al., 2015). This becomes more evident as potential therapeutic molecule leads are discovered that deviate from the generalized rules for determining drug-likeness derived from Lipinski's rule-of-five (Lipinski, 2004). This deviation is particularly true in therapies for treating genetic disorders, or those aimed at intracellular targets, as quantitative structure-activity relationship analyses do not always generate ideal drug leads.

The inability of proposed drug-leads to access their intended intracellular target has created a need for technologies capable of translocating various cargos, including small molecules, peptides, genetic materials (sRNA's, DNA plasmids, etc.), and entire proteins, across the cell membrane. The desirable outcome of controlled intracellular delivery includes providing target access to many high value therapies,

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adding to the arsenal of new technologies to combat a wide range of diseases including cancers, genetic disorders, and intracellular bacterial, viral, and parasitic infections. Intracellular delivery would also create a new avenue for selective intracellular tagging and manipulation, aiding from fundamental research to *in vivo* applications (MacEwan and Chilkoti, 2013; McClorey and Banerjee, 2018; Stanzl et al., 2013).

One of the approaches to intracellular delivery includes the use of cationic cell penetrating peptides (CPPs). The extraordinary discovery of a novel 13 residue, cationic, and guanidinium rich sequence (residues 86-101, TATp) within the HIV-Trans-activator of Transcription (HIV-TAT) protein responsible for translocating HIV-TAT across cell membranes, led to the intense study of CPPs and related polymers as attractive non-viral intracellular delivery agents (Copolovici et al., 2014; Frankel and Pabo, 1988; Thoren et al., 2003). While CPPs have found some success with several entering clinical trials, including TATp based technologies, many obstacles for *in vivo* use remain, some of which are outlined below (Stanzl et al., 2013).

The original cationic and guanidinium rich CPPs held great promise for intracellular delivery, however, it quickly became evident that certain barriers would limit their use *in vivo* (Stanzl et al., 2013), which include poor stability in blood plasma, as α peptides are rapidly digested by proteases, cytotoxicity in many cases, especially for poly-arginines and poly-lysines, and the propensity for endosomal entrapment due to the nature of uptake (Futaki et al., 2013; Lundin et al., 2008; Madani et al., 2011). Despite these limitations, CPPs continue to be developed and improved via CPPs continue to be found in nature and through the generation of *de novo* amide bond based polymers created through various approaches (Horne, 2011; Potocky et al., 2007; Wender et al., 2000). Here we have designed a new class of unnatural cationic foldamers with defined secondary structures and proven biostability profiles to serve as CPPs.

Our previous studies using the α,δ-amino acid monomer Neu2en (compound **1** (Fmoc-Neu2en), Figure 1), a derivative of *N*-Acetylneuraminic acid (Sialic acid, NANA, Neu5Ac) that is amenable to widely used Fmoc-based solid phase peptide synthesis techniques, have identified a motif that leads to helical, bio-stable peptides (Saludes et al., 2009, 2010, 2013). These findings became the basis for the generation of a new class of CPPs herein called SialoPen peptides containing 6, 7, and 8 basic residues (SP6, SP7, and SP8 respectively; Figure 2). We sought to compare our new designs to the well characterized CPPs TATp and Penetratin (Pen) that possess a high number of cationic residues. This led to the design and synthesis of cationic SialoPen peptides (SP6, SP7, and SP8; Figure 2). consisting of 6, 7, and 8 repeating Neu2en-Arginine units (Mitchell et al., 2000; Wender et al., 2000). Our vision is to generate new intracellular carriers capable of outperforming existing CPP technologies in *in vitro* models, ultimately leading to new carriers suitable for *in vivo* applications.

2. Results

2.1. Peptide design and synthesis

Fmoc-Neu2en (**1**) was synthesized as previously described (Gregar and Gervay-Hague, 2004; Paragas et al., 2015; Saludes et al., 2009, 2013). Fmoc-Neu2en was then used to synthesize the cationic SialoPen peptides SP6, SP 7, and SP 8. The rationale for the peptide design followed the novel class of sialic acid-derived, water-soluble α/δ hybrid peptide foldamers that we previously demonstrated to possess stable secondary structure when both the configuration of Glu and the peptide sequence are matched (Saludes et al., 2009). Following this design, we sought to generate helical foldamers containing similar charges to the archetypical cationic CPPs TATp and Pen by replacing Glu with Arg in an effort to generate CPPs with the desired properties for drug delivery. All peptides (Figure 2) were synthesized using standard Fmoc based microwave assisted solid phase peptide synthesis and ultimately conjugated to the cell impermeable far red fluorophore cyanine dye (Cy5) for use in flow cytometry and confocal microscopy studies. The peptides were purified via reversed phase HPLC and characterized using MALDI-TOF-MS. The purified peptides were concentrated and lyophilized to yield a fluffy blue powder as trifluoroacetate (TFA) salts. The peptides were then converted into chloride salts by dissolving the peptides in aqueous 2 mM HCl, followed by lyophilization to remove residual TFA before use in biological assays (Andrushchenko et al., 2007).

2.2. SialoPen peptides show remarkable uptake in HeLa and primary neuronal cells

SialoPen uptake efficiency was evaluated initially using HeLa cells via flow cytometry, and compared to Penetratin and to the HIV-TAT peptide transduction domain TATp as positive controls, and to a "scrambled" non-cell permeable KLA as a negative control, all conjugated to Cy5 a far red fluorophore (Monreal et al., 2015). Flow Cytometry studies indicated that SialoPen peptides are up to 5–7 fold more efficient in translocating across HeLa cell membranes as compared to penetratin and TATp at 5 μM (Figure 3). These initial studies prompted the evaluation of SialoPen peptide uptake efficiency using live-cell confocal microscopy to further evaluate internalization. We also sought to perform uptake studies using primary neurons, a cell type known to be impermeable to TATp (Simon et al., 2009). The expanded studies were conducted via live-cell confocal microscopy using HeLa cells and primary rat hippocampal neurons (Monreal et al., 2015; Wayman et al.). Confocal microscopy studies indicate that the uptake of SialoPen peptides ultimately leads to much higher internal fluorescence in HeLa cells as compared to TATp (Figure 4). These properties become more evident in the uptake profile of SialoPen peptides in primary neuronal cells where TATp failed to show appreciable uptake (Figure 4). Encouraged by these results we sought to investigate the potential mechanism(s) of cell entry.

2.3. SialoPen enters cells via an energy-dependent non-classical mode of endocytosis

After determination of SialoPen uptake via flow cytometry and live-cell confocal microscopy, we sought to determine if the mechanism of uptake was shared between penetratin, TATp, and the SialoPen series. We analyzed the peptide uptake at 4 and 37 °C at 1–5 μM to determine the energy requirements for uptake. Since it has been shown that some arginine rich CPPs are capable of translocation at low temperatures in an energy independent manner (Lundin et al., 2008; Nathan et al., 2010; Richard et al., 2003; Ter-Avetisyan et al., 2009). We found uptake to be drastically reduced at 4 °C (Figure 5), indicating that uptake likely occurs through an energy dependent pathway, expected for cationic CPPs at the tested concentrations (Futaki et al., 2013; Potocky et al., 2007).

These studies were followed by the use of chemical inhibitors of various endocytic pathways to determine the mechanism of cell entry of SialoPen peptides, similar to Penetratin and TATp (Copolovici et al., 2014; Madani et al., 2011; Vercauteren et al., 2010). These chemical inhibitors include chlorpromazine, wortmannin, nocodazole, and

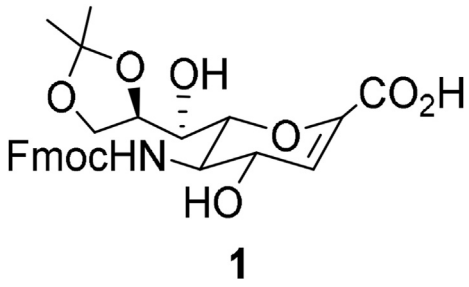

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Figure 1. Sialic acid derivative Fmoc-Neu2en. *N*-(9-fluorenylmethoxycarbonyl)-2,3-dehydro-8,9-isopropylidene neuraminic acid **1** the α-δ unnatural amino acid used to build the SialoPen peptides for use in Fmoc based SPPS.

Compound	Sequence
KLA	X-KLALKALKAALKLA-NH_2
Penetratin	X-RQIKIWFQNRRMKWKK-NH_2
TATp	X-GRKKRRQRRRPPQ-NH_2
$SialoPen_n$ (SP6,7,8)	X-(Neu2en-R)$_n$-Ahx-NH_2
X= Cy5 (fluorophore)	Neu2en = sialic acid residue

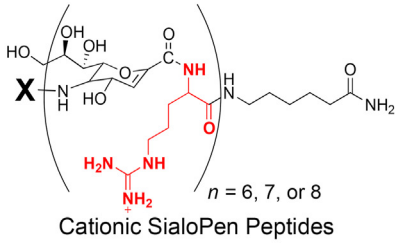


Figure 2. Chart showing the peptide design and fluorophore cargo attachment, and schematic showing structure of SialoPen peptides.

amiloride as probes of clathrin-mediated endocytosis, macropinocytosis via phosphatidylinositol-3-kinase, caveolae-mediated endocytosis, and macropinocytosis via sodium-proton exchange, respectively (Cleal et al., 2013; Copolovici et al., 2014; Khalil et al., 2006; Koivusalo et al., 2010; Sato et al., 1996; Spiro et al., 1996). The resulting uptake profile (Figure 6) indicates that SialoPen peptides do not follow the same mode of uptake as penetratin or TATp and is likely taken up via a unique non-classical pathway.

2.4. SialoPen peptides yield no apparent cytotoxicity

To further validate the use of SialoPen peptides as carriers of pharmacologically-active compounds, we measured their cytotoxic effects using a colorimetric metabolism-based assay and membrane leakage assay via MTT and LDH assays (2002; Promega, 2009). Our findings from the MTT assay indicate no apparent cytotoxicity of SialoPen peptides up to 100 μM (Figure 7A). The LDH assay, which evaluates membrane integrity, was also performed up to 100 μM concentration of SialoPen peptides and yielded congruent results (Figure 7B). This indicates that SialoPen peptides are not cytotoxic or membranolytic up to 20 times the tested cell penetrating concentration. These studies further indicate that SialoPen peptides are suitable delivery agents to pursue *in vivo,* as these do not exhibit obvious deleterious effects *in vitro*.

2.5. Secondary structure determination

One of the goals of the SialoPen design is to generate peptides with a stable helical secondary structure. The secondary structure of Neu2en based peptides has previously been described in detail (Saludes et al., 2009, 2010, 2013). Based on these previous findings, we found that SialoPen peptides follow previously reported trends of increasing helicity with length, as observed via circular dichroism (Figure 8) and calculation of peak to trough difference using the mean residue ellipticity at 245 and 211 nm, respectively. Previous NMR studies of Neu2en homo/hetero polymers show that the positive cotton effect at ~242 nm is unique to helical foldamers of Neu2en based peptides (Saludes et al., 2009, 2013). This is in contrast to the random coils that are observed when using Daa's (D-amino acids, enantionmers of natural L-amino acids) which give a positive cotton effect at 223 nm. This is also in contrast to the double cotton effect seen at ~218 and 230 nm when the order of Neu2en-Laa (L-amino acids) are reversed indicative of weak hydrogen bonds and possible interconversion between stable secondary and random coil conformations. We also found that the SialoPen peptides followed a similar trend where helicity increased with length as determined via peak-trough measurements (Figure 8). Further, our work is consistent with our prior findings that Neu2en-Laa peptides are viable templates for the rational design of foldamers (Saludes et al., 2009) and validated that

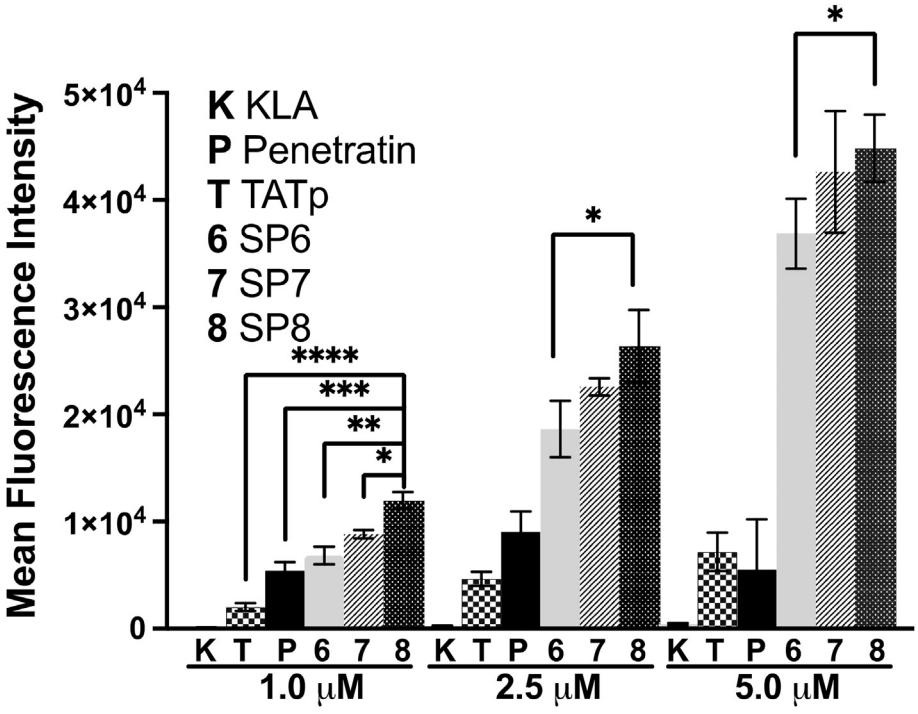


Figure 3. SialoPen peptides translocate HeLa cells efficiently as assessed by flow cytometry. Mean fluorescence intensity (MFI) of HeLa cells indicating that SialoPen peptides (**6, 7, 8**) show enhanced uptake *vs* peptides Pentratin **P** and TATp **T** especially at 5.0 μM. Error represented as ±SD, $n = 3$ biological replicates (triplicate wells per condition per replicate). Significance determined using unpaired t-tests reporting two-tailed p-values (95% confidence interval) additional values found in SI (Table S1). *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001.

the stability of their secondary structure is directed by the peptide sequence and the configuration of Laa, independent of the charge of Laa.

3. Conclusions

Cell penetrating peptides, and their related amide-based polymers, represent a promising non-viral method for delivering high value therapeutics that require access to the intracellular space. The design and synthesis of helical SialoPen CPPs (unnatural α,δ-cell penetrating peptides) modeled after existing cationic CPPs (Yang et al., 2019) has yielded a promising new class of intracellular delivery agents described here for the first time.

The flow cytometry results indicate that SialoPen peptides SP6, SP7, and SP8 have enhanced uptake versus penetratin and TATp, especially at 5 μM. This finding, coupled with the significantly enhanced uptake of SP7 and SP8 compared to penetratin at 1 μM is promising as Penetratin is the CPP of choice at lower concentrations and is known for its cytotoxicity at higher concentrations (Monreal et al., 2015). While initial flow cytometry experiments yielded exciting results they give no indication as to the location of the peptides leading us to pursue live-cell microscopy experiments.

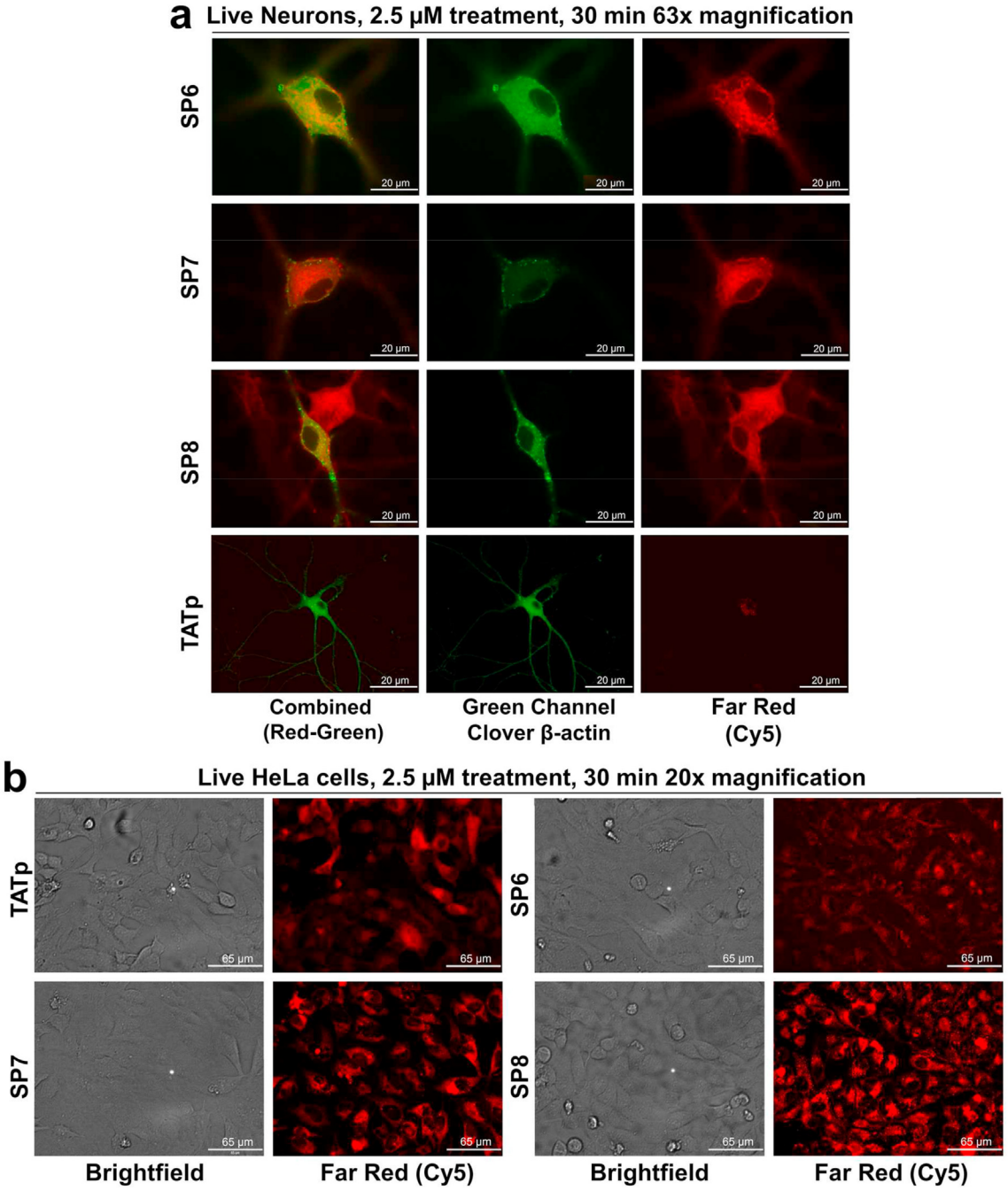


Figure 4. SialoPen peptides are taken up efficiently in primary and immortalized cell lines. Confocal microscopy images taken after 30 min incubation at 37 °C in imaging buffer showing uptake of SialoPen in primary rat hippocampal neurons and HeLa cells compared to TATp, all conjugated to the fluorophore Cy5. Representative images shown were taken at identical settings with no post processing. (a) Primary hippocampal neuronal cells incubated with TATp and SialoPen 6–8 taken at 63x magnification show no uptake of TATp by cells, (b) HeLa cells incubated with TATp and SialoPen 6–8 taken at 20x magnification. Three biological replicates (duplicate wells per replicate) per condition.

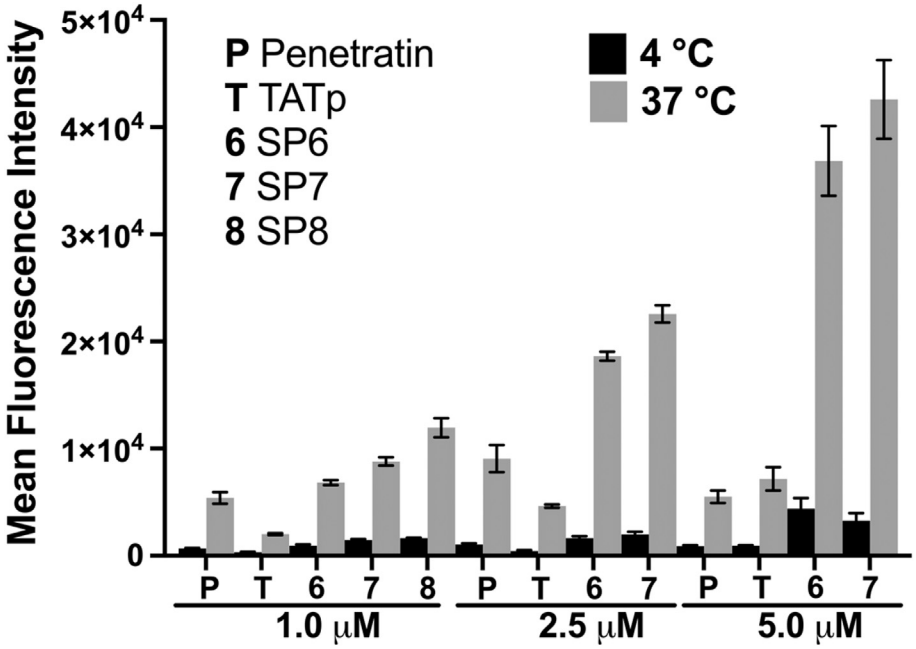


Figure 5. SialoPen peptides enter cells via an energy dependent pathway. Flow cytometry profile of HeLa cells incubated at 4 and 37 °C showing that peptides are likely taken up via an energy dependent endocytic pathway. Mean fluorescence intensity chart of parallel experiments showing normalized fluorescence intensity of HeLa cells treated with Cy5 labeled peptides at various concentrations. Error bars represented as ± SD, $n = 3$ biological replicates (triplicate wells per condition per biological replicate).

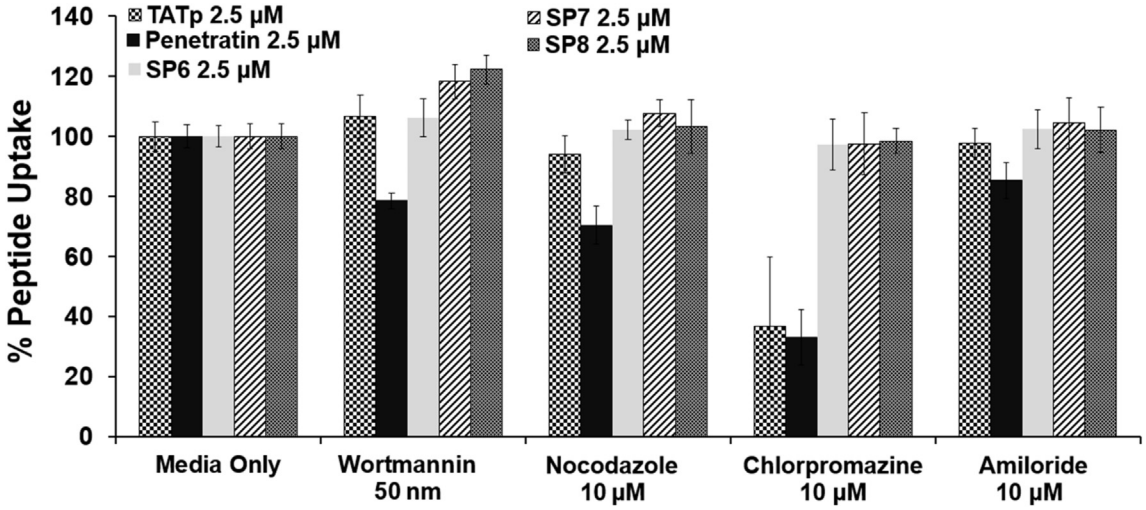


Figure 6. SialoPen peptides enter cells via non-classical endocytosis. Effects of endocytosis inhibitors on uptake of CPPs at 2.5 μM showing the SialoPen peptides are taken up via non-classical endocytic pathways compared to penetratin and TATp. Error represented as ±SD, n = 3 biological replicates (triplicate wells per condition per biological replicate). Uptake normalized to cells incubated in media only measured via flow cytometry. Further statistical analysis can be found in SI (Table S1).

The initial live-cell confocal microscopy experiments were carried out using HeLa cells, which confirmed the uptake of TATp and SialoPen peptides into the intracellular space wherein both appeared to be localized as puncta within cells after a 30 min incubation at 2.5 μM. Following the HeLa cell studies, we proceeded to investigate the ability of SialoPen peptides to enter primary rat hippocampal neuronal cells. These cells were specifically chosen as primary neurons are known to be resistant to induced uptake by TATp and other CPPs as well as to standard transfection protocols (Khalil et al., 2006). We found that SialoPen peptides containing 6–8 basic residues (SialoPen 6-8) quickly and efficiently entered neuronal cells whereas peptide TATp did not show appreciable uptake under the same conditions, even at 8 μM (data not shown). This observation is especially evident in the live-cell uptake videos (Videos in Supporting Information), revealing that incubation with TATp at 2.5 μM for 30 min did not show appreciable uptake as compared to SialoPen.

Supplementary content related to this article has been published online at https://doi.org/10.1016/j.heliyon.2020.e05780.

With the promise of SialoPen peptides becoming evident, we sought to investigate their possible mechanism of entry. TATp and Penetratin have well established uptake profiles at these concentrations[4] with TATp primarily dependent on clathrin-mediated endocytosis and penetratin known to take advantage of both clathrin, and caveolae dependent endocytosis (Alves et al., 2010; Kumari et al., 2010). The inhibition profile indicates that fluorophore conjugated SialoPen peptides at these concentrations are not inhibited by inhibitors of clathrin-mediated endocytosis, caveolae-dependent endocytosis, or macropinocytosis mediated endocytosis, which is both surprising and promising. This suggests the possibility that SialoPen enters cells via a clathrin- and caveolae-independent (non-classical) endocytic pathway as seen for other CPPs including p18 and p28 azurin fragments, transportan, and low molecular weight protamine/siRNA conjugate (Ruseska and Zimmer,

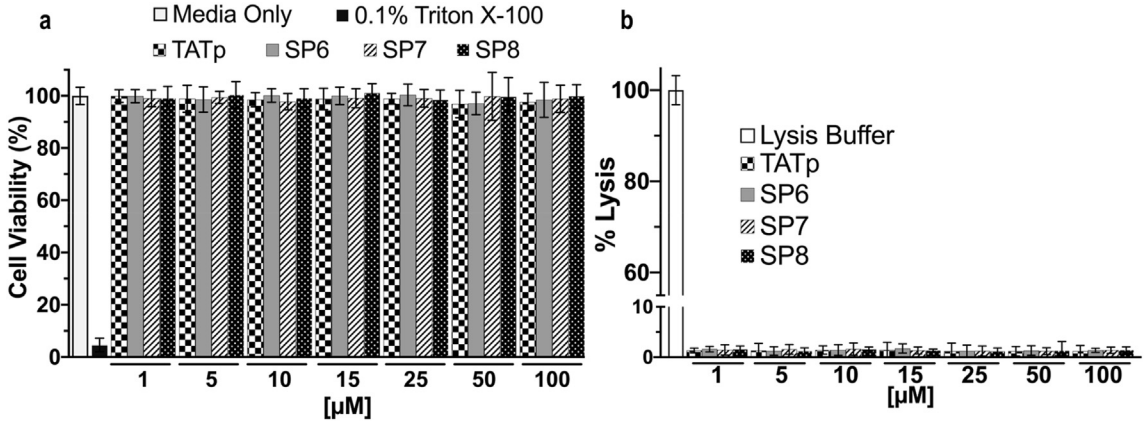
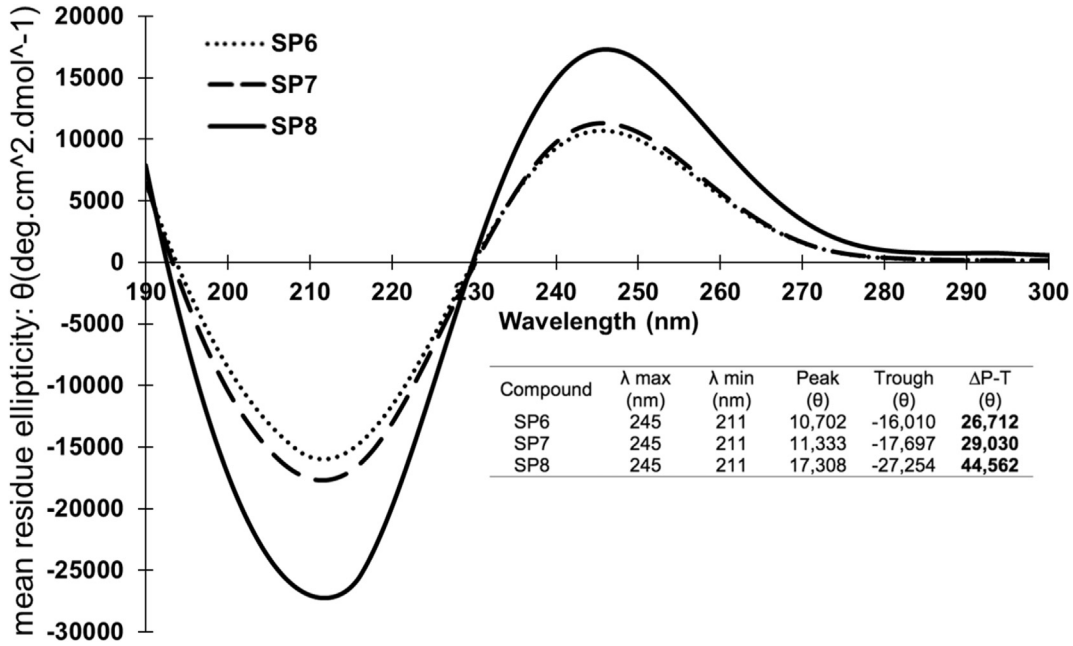


Figure 7. SialoPen peptides show no apparent cytotoxic or lytic effects: MTT assay, and Membrane integrity: LDH assay. A.) Chart showing that SialoPen peptides are not toxic to HeLa cells up to 20 times (100 μM) the maximum tested concentration used in the flow cytometry assay. **B.)** Chart showing that SialoPen peptides have an insignificant effect on membrane integrity of HeLa cells up to 100 μM. Error represented as ± SD, $n = 3$ biological replicates (triplicate wells per biological repliate).



Compound	λ max (nm)	λ min (nm)	Peak (θ)	Trough (θ)	ΔP-T (θ)
SP6	245	211	10,702	-16,010	**26,712**
SP7	245	211	11,333	-17,697	**29,030**
SP8	245	211	17,308	-27,254	**44,562**

Figure 8. Circular dichroism studies show SialoPen peptides adopt helical conformations. Mean residue ellipticity (MRE) graph of SialoPen peptides showing SialoPen peptides are helical foldamers. Insert: Peak to trough analysis of SialoPen peptides showing that the helicity of SialoPen peptides increase with length.

2020). It is additionally possible that the nature of Neu2en (a sialic acid derivative and possible sialidase inhibitor) is participating in receptor mediated (sialidase) interactions or possibly uptake, in addition to the helicity of these peptides playing a role. However, since the increase seems to correlate with charge more than helicity (Figures 3 and 8) we speculate that as with other cationic CPPs initial membrane or glycan/-proteoglycan (heparin sulfate or otherwise) mediated interactions followed by internalization likely play a large role in uptake pathways (Kauffman et al., 2015; Kolmel et al., 2014; Ruseska and Zimmer, 2020). This finding will prompt further studies including any possible selectivity for specific cell types to determine the mechanism of uptake including the use of larger cargos as this has been shown to affect mechanism of uptake (Kauffman et al., 2015; Lundin et al., 2008). These studies are encouraged by the fact that SialoPen peptides do not show any cytotoxicity or membranolytic activity up to 100 μM concentrations.

We also designed the SialoPen peptides to generate helical foldamers based on the expectation that reversing the charge of previously reported helical Nu2en-Glu peptides (Saludes et al., 2009, 2010, 2013) would not alter the secondary structure. We followed the established motif of (Neu2en, L-amino acid)$_n$-Ahx anticipating the same circular dichroism spectra of helical Neu2en peptides (Saludes et al., 2013). We observed that these peptides continued the trend of increasing helicity with increasing length. Conformational Neu2en peptide studies indicate that all L-amino acids (Laa) will have appropriate interactions with Ne2en to generate helical foldamers so long as the proper order of (Neu2-en-Laa)$_n$-Ahx is followed (Saludes et al., 2009). This secondary structure is in part due to the rigidity offered by the flattened pyranose ring of Neu2en effectively acting as a dipeptide. Neu2en peptide helical foldamers are also induced by interactions identified through NMR and CD that include interactions of the amide proton of the Laa with the Neu2en proton on carbon 8, as well as the amide proton of the Neu2en bond to the proton on the alpha carbon of the Laa. These interactions are also ideal, as all the interactions required for helicity are present in locations that allow for functionalization of side chains and are not dependent on the properties of the Laa's allowing for a high degree of modularity when designing Neu2en peptides that will yield helical foldamers. This could

allow for the development of zwitterionic or amphipathic peptides as well and can be controlled or made in a modular fashion. It has also been shown that the helical Neu2en based peptides are resistant to proteases with half-lives approaching 2 weeks in full human blood plasma at least in part due to the angle of helicity which does not allow for the peptide to fit in the enzymatic pocket (Saludes et al., 2010).

Herein we present a new class of cationic helical Neu2en-based cell penetrating family of peptides called SialoPen. SialoPen peptides show promising membrane permeability profile and will be used to investigate the delivery of functional cargos to measure the efficiency of delivery in practical applications. This will include covalently attaching small molecule drugs such as doxorubicin, and larger genetic materials such as plasmids for transfections via covalent attachment as well as the creation of polyplexes through ionic interactions. Additionally, SialoPen utilizes standard fmoc based SPPS (solid-phase peptide synthesis) chemistry and therefore can be subjected to standard techniques of conjugation and even cyclization through the inclusion of commercially available propargylglycine and azido-lysine among other strategies as shown previously by our lab (Saludes et al., 2013). CPPs have shown promise as carriers for chemotherapeutics, nucleic material, and protein delivery to overcome the bottleneck caused the inability of therapeutics to cross the cell membrane (Habault and Poyet, 2019). It is our belief that these peptides represent an exciting new series of foldamer CPPs with ideal properties that will allow for successful use *in vivo*.

4. Methods

4.1. Synthesis of sialic acid derivative Fmoc-Neu2en N-(9-fluorenylmethoxycarbonyl)-2,3-dehydro-8,9-isopropylidene neuraminic acid (Neu2en, 1)

Neu2en (**1**) was prepared in 8 steps following our previously published methods with slight modification with an overall yield of 24% (Gregar and Gervay-Hague, 2004; Monreal et al., 2015; Paragas et al., 2015; Saludes et al., 2009, 2014).

4.2. Solid-phase peptide synthesis

Peptides were synthesized using Biotage Initiator + SP Wave microwave-assisted solid-phase peptide synthesizer (Biotage, Charlotte, North Carolina) on Rink amide resin following standard Fmoc chemistry (Monreal et al., 2015). Typically, 0.05 mmol resin (Rink: 0.56 meq/g) was used, swollen in DMF for 30 min at 50 °C with vortexing set to 800 rpm. Fmoc deprotection was performed using 1.6 mL of 6% piperazine in DMF, irradiation to 75 °C for 5 min. Coupling steps were performed by adding 4 eq. amino acid (0.2 mmol), 4 eq. HBTU (0.2 mmol) or PyBOP in the case of SialoPen Peptides, and 8 eq. DIPEA (0.4 mmol) in 1.6 mL total volume according to the peptide design in (Figure 2), followed by irradiation to 75 °C for 5 min. The efficiency of coupling and deprotection processes was monitored using Kaiser Test (Reagent A: 0.4 mL of 0.001 M KCN in 20 mL pyridine; Reagent B: 0.5 g ninhydrin in 10 mL EtOH). SialoPen peptides were synthesized as stated above using 3 eq. of Neu2en monomer (0.15 mmol) 3 eq. PyBOP (0.15 mmol) and 6 eq DIPEA (0.30 mmol). Peptides were subsequently Fmoc deprotected and coupled to the carboxylic acid terminus of Cy5 (0.5 eq.) using HATU (0.5 eq.) and DIPEA (1.0 eq.) in DMF at rt for 3 h. Peptides were cleaved using 95/2.5/2.5 TFA/TIS/H2O (KLA, TATp) or 90/2.5/2.5/2.5/2.5 TFA/EDT/TIS/Thioanisole/H_2O (Penetratin) under Ar blanket for 2 h. SialoPen peptides were cleaved using 30/67.5/2.5 TFA/DCM/TIS 1 h. Peptides were subsequently precipitated in ice-cold Et_2O, purified via reverse phase HPLC, and lyophilized as TFA salts. Ultimately, the peptides were dissolved in 2 mM HCl and lyophilized as chloride salts before use in cell culture.

4.3. Flow cytometry studies

Flow cytometry assays were performed following a previously published procedure (Monreal et al., 2015). Briefly, HeLa cells ATCC (Manassas, VA) were incubated and passaged in 10 cm plates cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 IU of penicillin/mL, 50 μg of streptomycin/mL, and 2 mM glutamine. HeLa cells were seeded in a 96-well plate to ~60% confluency, then incubated with 100 μL of Cy5 labeled peptides at desired peptide concentrations in triplicate. The cells were incubated at 37 °C for 1 h. Post incubation, the media was aspirated followed by washing with 1x Dulbecco's phosphate buffered saline (DPBS, GIBCO, Life Technologies) before adding 100 μL of 1x Trypsin-EDTA (0.25% Trypsin-EDTA, GIBCO, Life Technologies) per well and incubating at 37 °C for 30 min. The cells were then centrifuged at 500 RCF for 8 min followed by aspiration of the supernatant. The cells were then resuspended in PBS followed by centrifugation, and washed a total of three times. The cells were resuspended in 200 μL of 0.5% paraformaldehyde solution in PBS and >10,000 cells were analyzed/well using the Guava EasyCyte 8HT flow cytometer (EMD Millipore, Germany) with detection set for fluorescence at 661 nm. Studies were repeated three times. Representative data of one trial shown. Flow cytometry data was processed using GuavaSoft 2.6. In the low temperature experiments, two 96-well plates were prepared, one treated as described above and the other pre-incubated at 4 °C in a temperature regulated cooler for 30 min, followed by peptide treatment and incubation at 4 °C for 1 h while the first plate was incubated at 37 °C. Post incubation, both plates were treated and analyzed as described above.

4.4. Confocal laser scanning microscopy studies

HeLa cells were maintained as described above. Primary neuronal hippocampal cells were prepared and imaged as described previously (Monreal et al., 2015; Wayman et al., 2008). Briefly, primary neuronal cells harvested from rat brains were grown in Neurobasal A media supplemented with 2% B27 and 1% 100x GlutaMAX (Invitrogen, Grand Island, NY) and transfected with Clover-β actin (green fluorescent protein) at DIV 6 using Lipofectamine 2000 (Invitrogen, Grand Island, NY) according to the manufacturer's protocol (Dhar et al., 2014), yielding 3–5% transfection efficiency that allowed visualization of select neurons via observed green fluorescence. Both cell types were plated on poly-L-lysine coated coverslips in 24-well plates. Coverslips were prepared in an incubation chamber at 37 °C to which 150 μm live imaging buffer was added (145 mM NaCl, 2.5 mM KCl, 10 mM glucose, 10 mM HEPES, 2 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4) with set concentrations of peptides. Microscopy studies were done using a Leica DMI6000 SD confocal microscope (Buffalo Grove, IL) equipped with a Yokogawa CSU-X1 spinning disk and the images were captured using a Hamatsu-R2 CCD camera. The samples were excited using ILE/4 integrated laser engine (Spectral Applied Research, Ontario, Canada) with excitation set to 488 nm, 150 mW max (green channel) and 640 nm, 100 mW max (far red channel) and laser power set to 10–25% power and exposure times of 3–500 ms. The cells were treated with various concentrations of TATp and SialoPen Peptides, and peptide uptake was monitored by continuously imaging the live cells for 30 min at 30 s intervals. To measure the fluorescence intensity of the intracellular domain, the cytoplasm of HeLa cells and soma of neuronal cells were analyzed after 30 min incubation, as described above, in separate studies. Intracellular fluorescence intensity was measured in triplicate and determined using MetaMorph software from Molecular Devices (Sunnyvale, CA).

4.5. Mechanism of entry studies

HeLa cells were prepared in 96-well plates as described above. Following overnight incubation of HeLa cells, the media was replaced with 90 μL of fresh full media followed by addition of 10 μL of inhibitor

stock solutions to a final concentration of 10 μM chlorpromazine, 50 nM wortmannin, 10 μM nocodazole, and 10 μM amiloride. HeLa cells were incubated with the inhibitors for 30 min at 37 °C prior to addition of TATp, penetratin, and SialoPen peptides at 2.5 μM, followed by 1 h incubation at 37 °C. The cells were then washed, resuspended in buffer and analyzed by flow cytometry as described above. Studies were done in triplicate measuring 10,000–20,000 cells per well and percent uptake determined by normalizing the mean fluorescence intensity from cells treated with inhibitors to cells incubated with peptides only.

4.6. Cytotoxicity assays: 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetra-zolium-bromide (MTT) and membrane integrity lactate dehydrogenase (LDH)

Cellular toxicity of TATp and SialoPen peptides was evaluated against HeLa cells colorimetrically using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazoliumbromide (MTT) following an established protocol (Vybrant, 2002). HeLa cells (1.0×10^4/well) were seeded into 96-well plates and incubated overnight. 10 μL of a 12 mM MTT stock solution was added to each well in phenol red free DMEM. The cells were incubated at 37 °C for 4 h with varying concentration of peptide. After incubation 100 μL of a 0.01 M HCl 1% SDS solution (w/v) was added to each sample mixed thoroughly and incubated for 2 h. Following SDS incubation, the samples were thoroughly mixed and absorbance was measured at 570 nm using a Tecan Infinite M1000 PRO microplate reader (Männedorf, Switzerland). A solution of 0.1% Triton X-100 served as positive control. All tests were done in triplicate. Membrane integrity was assayed using CytoTox-ONE™ Homogeneous Membrane Integrity Assay from Promega (part# G7891) following the supplier provided protocol (Promega, 2009). HeLa cells were plated overnight as described above using phenol red free media. Cells were then treated in triplicate to TATp and SialoPen peptides at varying concentrations for 4 h at 37 °C. LDH release was then measured by incubating the media for 30 min with the provided reagents including blank cell free media for background correction, and media attained from lysed cells using the provided lysis buffer as the maximum LDH release. Fluorescence was then measured using a Tecan Infinite M1000 PRO microplate reader (Männedorf, Switzerland) with excitation set to 560 nm and emission wavelength measured at 590 nm. Percent cytotoxicity was measured by dividing measured fluorescence minus background by maximum LDH release minus background x100.

4.7. Circular dichroism studies

Cy5 labeled SialoPen peptides were dissolved in 10 mM phosphate buffer pH 7.4 and evaluated using a JASCO J-815 CD spectrometer temperature regulated at 25 °C. Peak to trough measurements were taken by calculating the mean residue ellipticity at 245 nm and 211 nm. These findings were compared to previously published results using Neu2en homopolymers and $(Neu2en\text{-}Glutamate)_n$-ahx peptides (Saludes et al., 2009, 2010, 2013).

Other methods

Any remaining experimental procedures and further details are described in the Supplementary Information.

Author Contribution Statement

I. Abrrey Monreal: Conceived and designed the experiments; performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Erik M. Contreras, Gary A. Wayman: performed the experiments; Analyzed and interpreted the data.

Hector C. Aguilar, Jonel P. Saludes: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

No data was used for the research described in the article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

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