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Brief Article

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# Cellular Translocation of a γ-AApeptide Mimetic of Tat Peptide

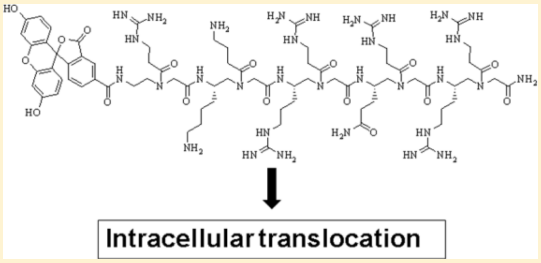
Youhong Niu,[†,‡] Ge Bai,[†,‡] Haifan Wu,[†] Rongsheng E. Wang,[†] Qiao Qiao,[†] Shruti Padhee,[†] Robert Buzzeo,[§] Chuanhai Cao,*[,∥] and Jianfeng Cai*[,†]

[†]Department of Chemistry, University of South Florida and [§]Department of Cell Biology, Microbiology and Molecular Biology, 4202 East Fowler Avenue, Tampa, Florida 33620, United States

[∥]USF College of Pharmacy, 4001 East Fletcher Avenue, Tampa, Florida 33613, United States

Supporting Information

**ABSTRACT:** Cell-penetrating peptides including the trans-activating transcriptional activator (Tat) from HIV-1 have been used as carriers for intracellular delivery of a myriad of cargoes including drugs, molecular probes, DNAs and nanoparticles. Utilizing fluorescence flow cytometry and confocal fluorescence microscopy, we demonstrate that a γ-AApeptide mimetic of Tat (48−57) can cross the cell membranes and enter the cytoplasm and nucleus of cells, with efficiency comparable to or better than that of Tat peptide (48−57). Deletion of the four side chains of the γ-AApeptide attenuates translocation capability. We also establish that the γ-AApeptide is even less toxic than the Tat peptide against mammalian cells. In addition to their low toxicity, γ-AApeptides are resistant to protease degradation, which may prove to be advantageous over α-peptides for further development of molecular transporters for intracellular delivery.




**KEYWORDS:** *γ-AApeptides, cellular uptake, peptidomimetics, Tat, cell penetrating peptide (CPP)*

## INTRODUCTION

The therapeutic values of a wide variety of polypeptides and oligonucleotides have been limited by their poor biomembrane permeability until the discovery of cell penetration peptides (CPPs). These short peptides facilitate the transport of hydrophobic/hydrophilic macromolecules into the cytoplasm and nucleus of living cells without disrupting membranes.[1] Among all available CPPs, the most eminent is the transcriptional activator (Tat) protein for human immunodeficiency virus, type 1 (HIV-1), and is composed of 86−102 amino acids.[1,2] Detailed structure−function analysis reveals that the sequence responsible for the cellular uptake and nuclear localization of Tat is amino acids 49−57 (RKKRRQRRR) in the basic region.[1−3] Further analysis of this short sequence indicated that the peptide backbone as well as the cationic charges brought by arginines contributes substantially to Tat's uptake. Truncation of the peptide or substitution of the arginines with other cationic amino acids resulted in diminished cellular uptake.[3]

The cellular-uptake mechanism of Tat still remains elusive, as both a passive transfer across the plasma membrane and an endocytic entry have been observed.[1,4] In the earlier 2000s, Gellman et al.[4] demonstrated using fluorescence confocal microscopy that the fluorescein-labeled $Tat_{47-57}$ achieved cell entry through an energy-dependent and endosome-mediated system which was inhibited by sodium azide.[4] Under endosome acidification, the peptide can then escape endosomal compartmentalization into the cytosol.[4] In practice, numerous biologically active proteins have been tethered to Tat and successfully delivered into intact cells. These proteins include the E2 repressor protein,[5] apoptosis-promoting caspase-3 protein,[6] CDK4/6 inhibiting peptide,[7] and ovalbumin.[8] Additionally, *in vivo* studies with Tat-fused β-galactosidase displayed an ability for the chimera to transduct to all tissues in mice, including the brain.[9]

Despite the promising data accumulated from translocation studies, CPPs including Tat were found to undergo rapid degradation in biofluids, severely limiting most practical and/or clinical applications.[10] Though relatively more stable than other CPPs, $Tat_{47-57}$ still suffered from a short half-life in epithelial cells (9 h in Calu-3 cells).[10] In the past decade, peptidomimetics consisting of unnatural amino acids have been under extensive evaluation. These molecules comprise interesting biological activities by adopting protein-like secondary structures while, importantly, harboring resistance to proteolytic degradation. A number of peptidomimetics have been developed for cellular translocation purposes.[3,4,11−13] Umezawa et al.[11] constructed a β-amino acid analogue of $Tat_{47-57}$ and showed that its cellular delivery efficiency was comparable to that of $Tat_{47-57}$ at 4 °C, albeit a bit weaker at 37 °C.[11] A peptoid-based cationic N-hxg9 was also developed and showed favorable cellular uptake.[3] Nonetheless, the application of peptidomimetics is still hindered, and partially hampered by the limits of available frameworks.[14,15] Hence the exploration of new peptide mimics bearing novel framework and improved/

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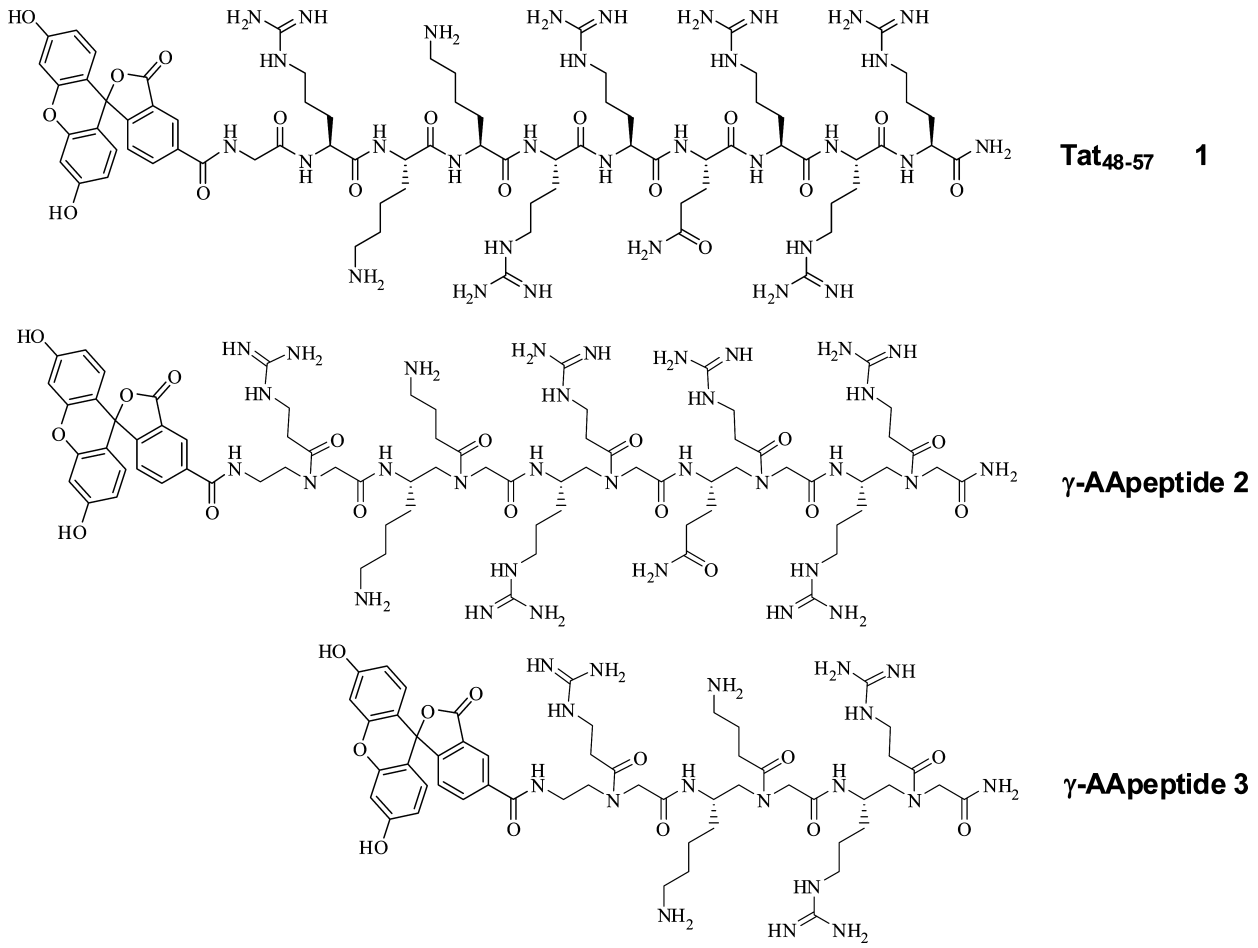


**Figure 1.** The structures of oligomers used in the experiments: $Tat_{48-57}$ (Fl-GRKKRRQRRR) **1**, γ-AApeptide **2** (mimetic of Tat $_{48-57}$), and γ-AApeptide **3** (mimetic of Tat $_{48-53}$).

increased functionalities and stability is currently of urgent need.

In an effort to identify new peptidomimetics with novel framework, we recently developed a new class of peptide mimics termed "γ-AApeptides". γ-AApeptides were derived from γ-chiral PNAs, which are composed of γ-substituted N-acylated-*N*-amino-ethyl amino acids. They can either retain the functions of mimicked peptide or serve as potential inhibitors of protein/protein and protein/nucleic acid interactions.[16,17] For example, γ-AApeptide bearing the functionalities of the helical domain of p53 demonstrated effective inhibition of the p53/MDM2 interaction,[16] while γ-AApeptide mimicking $Tat_{48-57}$ exhibited similar binding affinity to TAR RNAs of HIV.[17] Remarkably, γ-AApeptides are highly stable to proteases,[16] thereby eliciting significant advantages over their natural counterparts for future biological applications. Since a comparable RNA binding activity was observed for the γ-AApeptide mimicking $Tat_{48-57}$,[17] it is essential to evaluate whether this γ-AApeptide can mimic the cellular translocation activity that $Tat_{48-57}$ features. Herein we demonstrate for the first time that a fluorescent γ-AApeptide mimetic of $Tat_{48-57}$ can efficiently translocate to cytoplasm and nucleus of cells with cell permeation ability comparable to or better than that of $Tat_{48-57}$ at all concentrations tested.

## EXPERIMENTAL SECTION

**Materials and Instrumentation.** Fmoc protected α-amino acids and Knorr resin were obtained from Chem-Impex International, Inc. All other reagents and solvents were provided by either Sigma-Aldrich or Fisher Scientific. NMR spectra of intermediates and γ-AApeptide building blocks were obtained on a Varian Inova 400. γ-AApeptide sequences were prepared on a Knorr resin in peptide synthesis vessels on a Burrell wrist-action shaker. The γ-AApeptides were analyzed and purified on a Waters HPLC installed with both analytical and preparative modules, respectively, and the desired fractions were lyophilized using a Labconco lyophilizer. Molecular weights of γ-AApeptides were identified on a Bruker AutoFlex MALDI-TOF mass spectrometer. Fluorescent flow cytometry was performed using a Zeiss fluorescent imaging system.

**Cellular Uptake Assay by Fluorescent Flow Cytometry.**[3] The oligomers were each dissolved in PBS buffer (pH 7.4), and their concentration was determined by UV−vis spectrometer at 490 nm ($\varepsilon$ = 67,000, absorption of fluorescein). Jurkat cells (human T cell line) were used for cellular uptake experiments. Cells were seeded to a 96-well plate with $6 \times 10^5$ cells per well. Different volumes of oligomer stock solution (2% FBS in PBS solution) and PBS buffer were added to the 96-well plate (combined total of 200 μL) and incubated for 10 min at 37 °C. The cells were then centrifuged, and the cell pellet was washed with PBS buffer. Such process was repeated three times, then cells were resuspended in 0.02% propidium iodide in PBS


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buffer, and analyzed by using fluorescent flow cytometry. The data presented are the mean fluorescent signal for the 5,000 cells collected.

**Confocal Microscopy.**[4,18] Chamber slides were polylysine-coated by applying 0.01% poly-L-lysine in PBS. 160,000 HeLa cells were plated in 1 mL of DMEM with 10% FBS and 1% PSG onto one slot of a plate. Oligomers were diluted to 5 $\mu$M in 1 mL of appropriate DMEM in 1.5 mL microcentrifuge tubes. Media was aspirated from plates and replaced with 500 $\mu$L of complete DMEM containing peptide. Cells were incubated at 37 °C for 25 min and then washed gently with 2 mL of PBS. 1 mL of 4% PFA was added to all wells. The plate was covered, and cells were incubated at room temperature for 15 min, after which 10 $\mu$L of DAPI/mounting medium was added directly to cells in wells. The cells were extensively washed with PBS buffer and then imaged at 63× magnification in oil emersion.

## RESULTS AND DISCUSSION

The synthesis of $\gamma$-AApeptides was carried out on Knorr resin, following the standard Fmoc manual solid-phase synthesis, with building blocks prepared as described previously.[16,17] Two fluorescent $\gamma$-AApeptides were synthesized, with one (**2**) mimicking the full length of fluorescein-labeled $Tat_{48-57}$, and the other (**3**) mimicking the truncated piece $Tat_{48-53}$ (Figure 1). Fluorescein-conjugated $Tat_{48-57}$ **1** was prepared as a positive control. These synthesized oligomers were labeled with fluorescein at their N-terminus in order to allow for cellular uptake studies via flow cytometry and confocal fluorescence microscopy. The oligomers were subjected to HPLC purifications, with their identities confirmed by MALDI MS analysis (Table S1 in the Supporting Information).

Cellular uptake was then first evaluated by flow cytometry. Figure 2 shows the representative results of fluorescent flow cytometry for oligomers **1**–**3** in Jurkat cells. Whereas $\gamma$-AApeptide **2** (red bar) exhibited almost identical cellular uptake as $Tat_{48-57}$ **1** (black bar) when both peptides were incubated at a concentration of 12.5 $\mu$M for 10 min at 37 °C, $\gamma$-AApeptide **2** generally resulted in a higher cellular uptake compared to **1**, when both were administered at the same low concentrations. The more efficient intracellular translocation of **2** at low concentrations may result from the existence of tertiary amide bonds in $\gamma$-AApeptide backbone, consistent with previously reported cellular uptake of peptoids.[3] The reason for the same fluorescent intensity of **1** and **2**, observed when both were at 12.5 $\mu$M, was not clear. It may be due to the saturation of cell entry when both peptides were dosed at high concentrations. As a negative control, the truncated $\gamma$-AApeptide **3** (blue bar) almost did not exhibit cellular uptake, with little fluorescence detected when it was incubated with Jurkat cells at the concentrations of 0.39–3.13 $\mu$M. Even at the highest concentration of 12.5 $\mu$M, the fluorescence of **3** was only one-fifth of those from either **1** or **2**. This observation was consistent with previous reports that arginines 55–57 of Tat were critical for translocation[3] and $\beta$-peptide with depletion of these arginines failed to have any cell penetration.[11] Taken together, the flow cytometry study demonstrated the membrane crossing ability of $\gamma$-AApeptide **2**, with efficiency at least comparable to that of $Tat_{48-57}$ **1** itself.

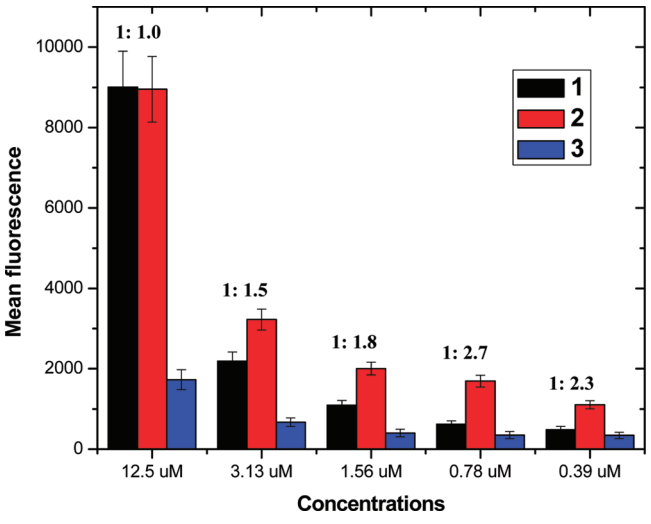


**Figure 2.** FACS cellular uptake assay of $Tat_{48-57}$ (Fl-GRKKRRQRRR) **1**, $\gamma$-AApeptide **2** (mimetic of Tat 48–57), and $\gamma$-AApeptide **3** (mimetic of Tat 48–53). Jurkat cells were incubated with different concentrations of oligomers for 10 min at 37 °C. The ratios shown in the figure are the ratios of FACS cellular uptake between **1** and **2**.

As further verification and to visually confirm the cellular uptake as well as to understand the cellular distribution of $\gamma$-AApeptide **2**, confocal fluorescence microscopy images were obtained for all oligomers, utilizing 5 $\mu$M of each oligomer with HeLa cells at 37 °C for 25 min. As shown in Figure 3A, the control peptide $Tat_{48-57}$ effectively penetrated the cell membrane and can be unambiguously detected in both punctate and diffuse patterns within the cytoplasm and nucleus. These observations are in accordance with previous reports,[4] in which punctate structures represent a distribution consistent with endosome sequestration. Compared to $Tat_{48-57}$, $\gamma$-AApeptide **2**, however, not only can translocate to cytoplasm and nucleus but also appears localized to the nucleolus (Figure 3B), thereby suggesting its future potential to deliver agents to specific nuclear regions. Noteworthy, a similar observation has been previously documented for a $\beta$-peptide majorly composed of arginines,[4] suggesting that the mechanism of localization to nucleolus may be related to the unnatural backbones of $\gamma$-AApeptide and $\beta$-peptide. In addition, although dominated by a diffuse pattern of uptake, punctate fluorescence was also present in the confocal images of $\gamma$-AApeptide **2**, indicating that

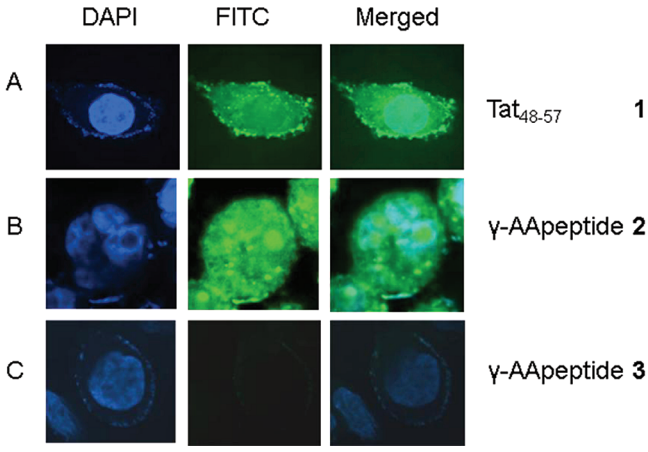


**Figure 3.** Confocal images of HeLa cells treated with 5 $\mu$M of $Tat_{48-57}$ (Fl-GRKKRRQRRR) peptide **1** (A), $\gamma$- AApeptide **2** (B), and $\gamma$-AApeptide **3** (C) for 25 min at 37 °C.


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its cellular uptake may also be endosome-mediated and energy-dependent (Figure 3B). Regarding the negative control, as expected, at the concentration of 5 $\mu$M, no cellular translocation of the truncated $\gamma$-AApeptide **3** was detected by confocal microscopy (Figure 3C), which was believed as a result of the removal of key arginine groups.[3] Taken together, the confocal images are highly consistent with the uptake data obtained utilizing fluorescent flow cytometry.

To begin to evaluate the mechanism of uptake, cells before the incubation with peptides were pretreated with 0.5% sodium azide that is considered as an energy poison and can block the endosome-uptake pathway. The corresponding confocal images manifested that both $Tat_{48-57}$ **1** (Figure 4A) and $\gamma$-AApeptide **2**

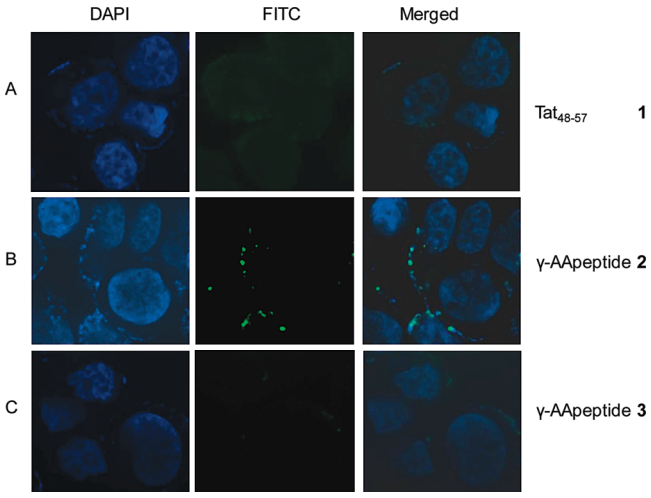


**Figure 4.** Confocal images of HeLa cells pretreated with $NaN_3$, and then 5 $\mu$M $Tat_{48-57}$ (Fl-GRKKRRQRRR) peptide **1** (A), $\gamma$-AApeptide **2** (B), and $\gamma$-AApeptide **3** (C) for 25 min at 37 °C.

(Figure 4B) abolished their cellular translocation capability and failed to cross cell membranes, with their results similar to the negative control $\gamma$-AApeptide **3** (Figure 4C). All together, these results further confirmed that $\gamma$-AApeptide **2** undergoes cellular translocation through energy-dependent endocytosis, in a way similar to natural $Tat_{48-57}$ peptide **1.**

The endocytic uptake mechanisms of $\gamma$-AApeptide **2** were further investigated by incubating **2** with HeLa cells that had been pretreated with filipin or sucrose, respectively (Figure 5). $Tat_{48-57}$ peptide **1** was used as a control. It is well-known that both filipin and sucrose are endocytosis inhibitors.[18] Filipin can bind to cholesterol and inhibit caveolae-mediated endocytosis; while sucrose can block clathrin recycling via clathrin-coated pit mechanisms due to the hyperosmolarity conditions generated by high sucrose concentrations. Consistent with previous research findings,[19] our experiments show that the treatment of either filipin or sucrose dramatically decreased the uptake of Tat peptide **1** (Figures 5A and 5B), strongly suggesting the existence of caveolae and clathrin-mediated endocytic pathways during Tat peptide **1** cellular uptake. Interestingly, the same simultaneous caveolae and clathrin-mediated endocytic uptake mechanisms were also identified for the uptake of $\gamma$-AApeptide **2**, since its uptake was also significantly inhibited upon pretreatment of filipin (Figure 5C) and sucrose (Figure 5D). The results further support that the cellular uptake mechanisms of $\gamma$-AApeptide **2** are similar to those of Tat peptide **1**.

The toxicity of a molecular transporter has to be considered before its further development for *in vivo* applications. To

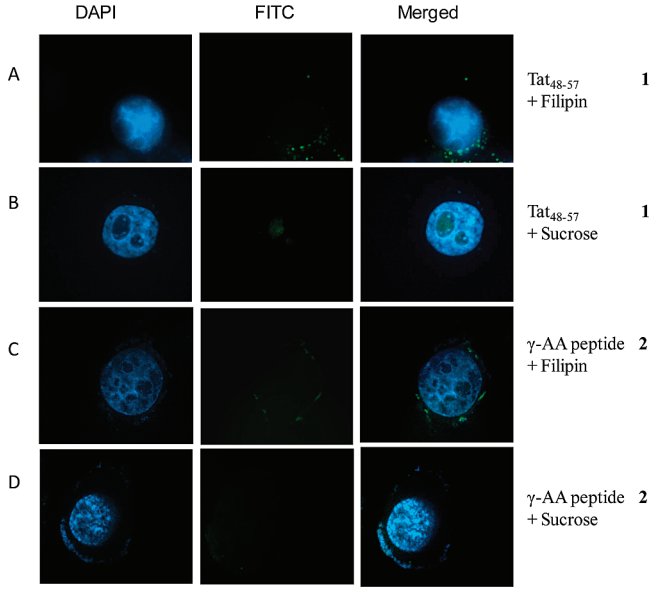


**Figure 5.** Confocal images of HeLa cells pretreated with filipin or sucrose and then with oligomers **1** and **2**. HeLa cells were pretreated with 3 $\mu$g/mL of filipin or 200 mM sucrose for 10 min, and then incubated with 5 $\mu$M $Tat_{48-57}$(FI-GRKKRRQRRR) **1** (A and B) or $\gamma$-AApeptide **2** (C and D).

assess the potential application of $\gamma$-AApeptide **2** as a molecular transporter, its toxicity against mammalian cells was evaluated and compared to that of $Tat_{48-57}$ peptide **1** (Figure 6). Under current experimental conditions, peptide **1** did not show significant toxicity against both HeLa and Jurkat cells, consistent with the fact that it has been frequently used for molecular delivery. Surprisingly, although the toxicity of **2** is comparable to that of **1** against HeLa cells at higher concentrations, **2** is less toxic than **1** at lower concentrations (0.39−1.56 $\mu$M). Moreover, **2** is generally less toxic than **1** in Jurkat cells; especially at high concentrations (6.25 and 12.5 $\mu$M), it shows at least 10% lower cytotoxicity than Tat peptide **1**. The overall less cytotoxicity shown by **2** may be due to its $\gamma$-AApeptide framework, which represents another advantage over natural peptides besides the already known superior stability. The results demonstrate that $\gamma$-AApeptide **2** has a strong potential for the further development of novel molecular transporters for intracellular delivery.

## ■ CONCLUSION

In conclusion, our work demonstrates that, as a novel type of peptidomimetics, $\gamma$-AApeptide not only can mimic Tat peptide's binding with RNA[17] but also possesses comparable cellular translocation ability to Tat peptide. The translocation activity of $\gamma$-AApeptide is somewhat even better at low concentrations, presumably due to its unnatural backbone where most of the guanidine side chains are appended to the nitrogens. Accordingly, an enhanced cellular uptake of polyguanidine peptoid derivative has been previously observed.[3] Our findings in confocal microscopy are complementary to Potocky et al.,[4] that peptidomimetics' cellular uptake is through endocytosis, which is the same as Tat itself. In addition to the findings that **2** is comparable to or even less toxic than Tat peptide **1** against mammalian cells, $\gamma$-AApeptide based unnatural peptidomimetics are also extremely stable toward biodegradation, which, taken together, suggest that the

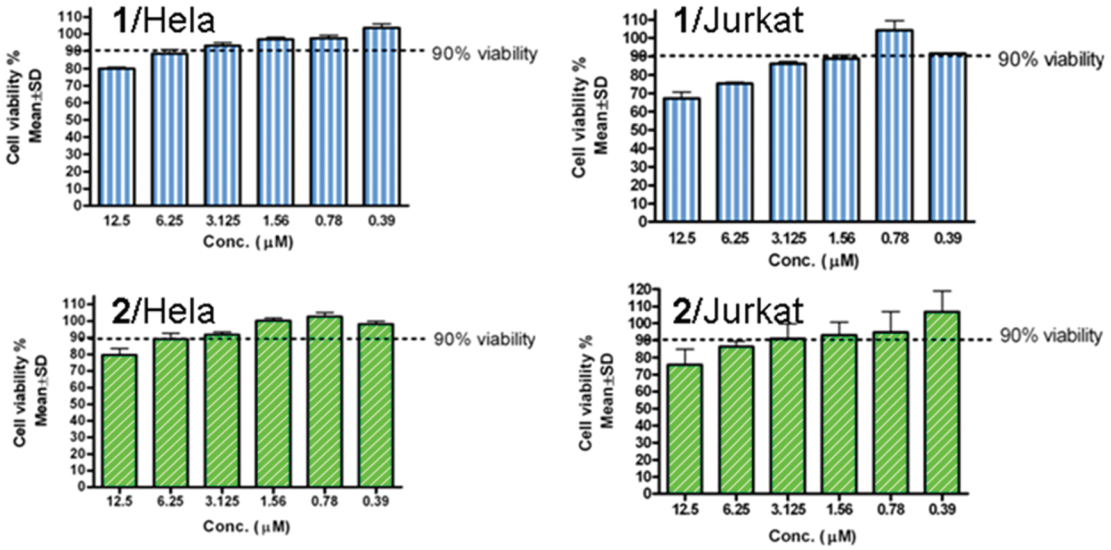


**Figure 6.** MTT assay for $Tat_{48-57}$ (Fl-GRKKRRQRRR) peptide **1** and γ-AApeptide **2** against HeLa and Jurkat cells.

new cell penetration peptide mimic **2** can be an efficient and robust agent in cellular delivery. The specific targeting to the nucleolus by **2** is also expected to be exploited in the future to selectively deliver cargo to certain domains of the nucleus. For future work, the mechanism of nucleolus targeting needs to be further elucidated. Moreover, in the search for CPPs of enhanced cellular translocation, more derivatives of γ-AApeptide **2** can be constructed, for example, by increasing the length of backbone, by introducing more functional groups to side chains, or by optimizing the distance between backbone and functional groups, which can be easily achieved by the limitless potential of derivatization of γ-AApeptides.[16,17] Delivery of different types of cargo by **2** is currently under investigation.

## ■ ASSOCIATED CONTENT

### Supporting Information

Synthesis and characterization of γ-AApeptides; experimental procedures for confocal, flow cytometry, and MTT assay; time-resolved fluorescence spectroscopy of γ-AApeptide 2 (mpeg). Snapshots were taken every 6 s for 90 min, and the video file was produced by compressing 20 snapshots per second. This material is available free of charge via the Internet at http://pubs.acs.org.

## ■ AUTHOR INFORMATION

### Corresponding Author

*J.C.: Department of Chemistry, University of South Florida, 4202 E. Fowler Ave, Tampa, FL 33620; tel, 813-974-9506; fax, 813-974-3203; e-mail, jianfengcai@usf.edu. C.C.: USF College of Pharmacy, 4001 E. Fletcher Ave., Tampa, FL 33613; tel, 813-396-0742; fax, 813-974-3081; e-mail: ccao@health.usf.edu.

### Author Contributions

‡These authors contributed to the work equally.

### Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

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