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COMMUNICATION

Cellular uptake of an α-AApeptide†

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Some short and cationic peptides such as the Tat peptide can cross the cell membrane and function as vectors for intracellular delivery. Here we show that an α-AApeptide is able to penetrate the membranes of living cells from an extracellular environment and enter the endosome and cytoplasm of cells. The efficiency of the cellular uptake is comparable to a Tat peptide (48–57) of the same length and is unexpectedly superior to an α-peptide with identical functional groups. The mechanism of uptake is similar to that of the Tat peptide and is through endocytosis by an energy-dependent pathway. Due to the easy synthesis of the α-AApeptides, their resistance to proteolytic hydrolysis, and their low cytotoxicity, α-AApeptides represent a new class of transporters for the delivery of drugs.

The cytoplasm of living cells is surrounded by a thin layer of plasma membrane which separates cells from their surrounding environment and protects them from outside changes.[1,2] The membrane further consists of a phospholipid bilayer which makes a hydrophobic interior that excludes the permeation of hydrophilic molecules.[1,2] Substances that are hydrophilic but necessary for cell survival can only be selectively transported into cells through channels or pumps associated with the cell membrane.[1,2] As a result, the inability of a large number of drugs to enter cellular compartments severely limits their therapeutic applications. Recently, a class of cell penetration peptides (CPPs), or alternatively called protein transduction domains (PTD), was discovered. These can be readily internalized by cells and thereby utilized to deliver hydrophilic macromolecules into the cytoplasm and nucleus.[3] One of the first identified CPPs was the Tat peptide which is currently the most commonly used transporting tool for cellular delivery. Comprised of 86–102 amino acids, Tat can bind to TAR RNA and then activate the transcription of the human immunodeficiency virus, type 1 (HIV-1).[3,4] Among the Tat's sequence, amino acids 49–57 (RKKRRQRRR) were identified to be responsible for Tat's cellular uptake and nuclear localization.[3–5] A detailed structure and activity analysis of this short sequence revealed that the cationic charges as well as the number of arginines are key to the peptide's cellular translocation.[5] Using fluorescein labeling followed by confocal microscopy, Tat_{47-57} was observed to enter cells through endocytosis, and later, exit the endosomes following the endosomal acidification.[6] The endosome-uptake process was ATP-dependent and can be blocked by the ATP inhibitor, sodium azide.[6] To date, truncated Tat has been used as a carrier for the intracellular delivery of a wide variety of cargoes including small molecules,[7] oligonucleotides,[8] small interference RNA,[9] peptides,[10] peptide nucleic acids,[11] proteins,[12–15] and nanoparticles.[16]

Despite their promising effects in cellular delivery, CPPs were, however, limited in clinical applications due to their peptide backbones that were vulnerable to biodegradation.[17] Although considered relatively more stable, Tat_{47-57} still only has a short half life (9–10 h) in most epithelial cells.[17] Though the metabolism of CPP is needed for a final release of chemically ligated cargos, a balance is required to avoid premature cleavage during the delivery. Hence, development of peptidomimetics that mimic Tat's ability of cellular transportation but have a higher stability represents a promising topic in chemical biology and attracts increasing attention in biomedical research. Up to now, several peptidomimetics of distinct frameworks have been reported. Both the β-peptide based β-heptalysine and β-heptaarginine were able to cross cell membranes and localize in the cytoplasm and nucleus, with the β-heptaarginine internalized to a greater extent.[18] In another example, β-peptide based β-(VRRVRRVRRVRR) entered cells by endocytosis and gradually translocated to the nucleus and the nucleolus.[6] For a direct comparison, a β-amino acid analogue of Tat_{47-57} was also synthesized which displayed comparable cellular translocation to the original Tat_{47-57}.[19] Other than β-peptide, Wender *et al.*[5] developed a 9-mer D-arginine as well as a class of polyguanidine peptoid derivatives which all exhibited greatly enhanced uptake compared to Tat_{49-57}. Nevertheless, in a very recent report, an oligomer of β-turn dipeptide mimics that was neither polycationic nor amphipathic turned out to be successfully internalized by cells to lysosomes.[20]

In spite of the progress made so far, the development of peptidomimetics is still necessary[21,22] and the search for peptidomimetics of new frames becomes crucial to facilitate the development of cellular transductible agents. In our case, we have recently proposed a new class of oligomers termed "α-AApeptides"

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since they are based on *N*-acylated-*N*-aminoethyl amino acids. The α-AApeptides, which, compared to traditional α-peptides, are resistant to protease degradation and can be limitlessly derivatized at their secondary amines.[23,24] Herein, we show that in our exploration of potential applications of α-AApeptides, a α-AA-poly (KR mimic) repeat was found to result in an unexpectedly higher cellular-uptake than the natural Tat_{48-57} peptide. Coupled with their facile solid phase synthesis and enhanced stability, α-AApeptides may represent a new type of transporter for the delivery of therapeutic agents.

A cellular permeable α-AApeptide was designed based on the notion that cationic residues such as lysine and arginine play substantial roles in an oligomers' uptake.[5,18] Given that polylysine or polyarginine based peptidomimetics have been well studied,[5,6,18] we prepared a α-AApeptide **2** (Fig. 1) with side chains composed of functional groups identical to alternated lysine and arginine instead. Oligomer **2** was the same length as natural Tat_{48-57} peptide, and can be readily synthesized following the standard Fmoc solid phase synthesis, with a α-AApeptide building block **5** which bore an extra *N*-acetylated lysine-like side chain in addition to a regular arginine. The preparation of **5** was carried out based on previously published procedures (Fig. 2).[23,24] Briefly, Pbf protected arginine amino acid benzyl (Bzl) ester was reacted with Fmoc–amino ethyl aldehyde to form an intermediate **4**, which possessed a secondary amino group that can be further acetylated with Boc–$NH(CH_2)_3COOH$, followed by hydrogenation to give the desired building block **5**. As controls, α-peptide Tat_{48-57} **1** was also synthesized, together with α-peptide **3** that has identical functional groups as α-AApeptide **2** (Fig. 1). All three oligomers were modified with fluorescein at their N-terminals to allow for the fluorescence-based detection of their cellular-uptake activities.

To evaluate their ability to enter Jurkat cells, oligomers **1–3** at a series of concentrations were incubated with Jurkat cells for 10 min at 37 °C in complete DMEM supplemented with 10% FBS, after which the cellular fluorescence was measured by fluorescent flow cytometry. As shown in Fig. 3, α-AApeptide **2** unexpectedly displayed better cell binding ability than the natural peptide Tat_{48-57} **1** at all of the tested concentrations, even when both oligomers were administered at 12.5 μM. The difference between **1** and **2** became more significant when both were used at a low concentration range, *e.g.* from 0.39 μM to 3.13 μM. The control α-peptide **3** had the same functional groups as α-AApeptide **2**, yet its incubation with cells resulted in the lowest fluorescence, indicating its poor cellular translocation. Taken together, the lysine–arginine alternated α-AApeptide oligomer **2** had comparable cellular-binding to Tat_{48-57} itself, which is also unexpectedly superior to its natural peptide counterpart **3**. Early studies suggested the importance of the number of arginines to an oligomer's cross-membrane ability. For example, a detailed structure–activity study revealed that arginine homo-oligomers containing more than seven arginines can exhibit enhanced cellular translocation compared to Tat_{47-57}.[5] The theory was further supported by an independent report that an eight arginine containing β-peptide resulted in better cellular-uptake than Tat_{47-57}.[6] In our case, α-AApeptide oligomer

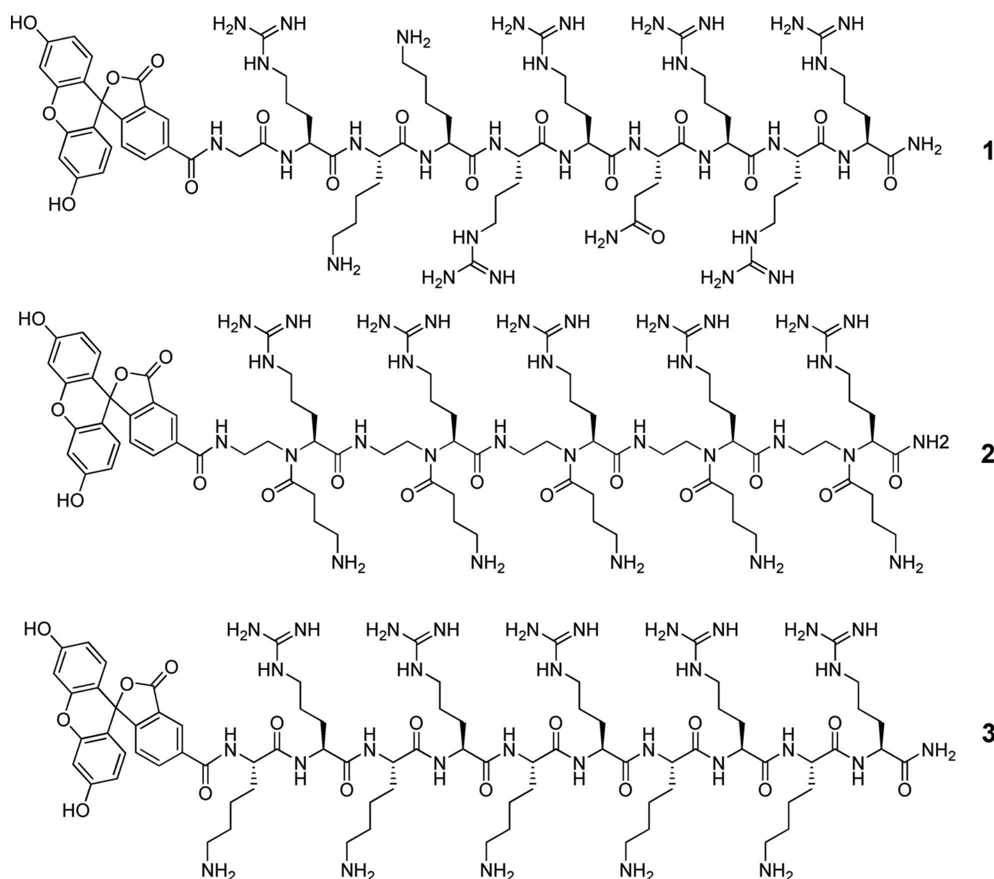

Fig. 1 Chemical structures of fluorescein-labeled oligomers **1–3**.

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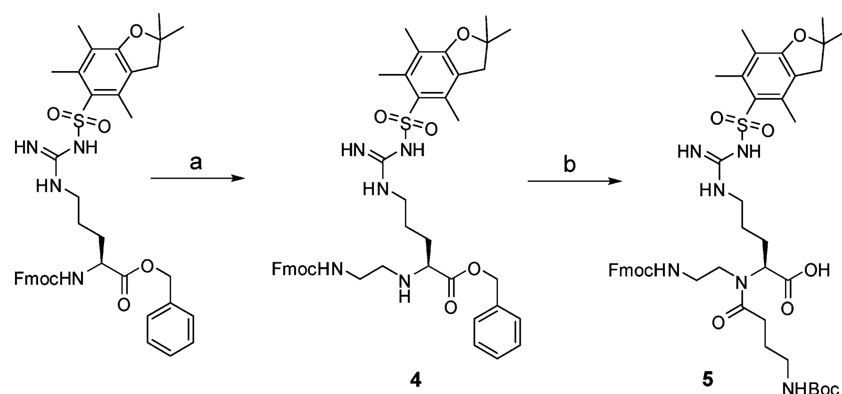

Fig. 2 Synthesis of the AApeptide building block **5**. Pbf = 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl. (a) 1. Et_2NH/CH_3CN, 2 h; 2. Fmoc–amino ethyl aldehyde, $NaBH_3CN/CH_3OH$, overnight. (b) 1. Boc–$NH(CH_2)_3COOH$, DhBtOH/DIC, overnight; 2. Pd/C, H_2, MeOH.

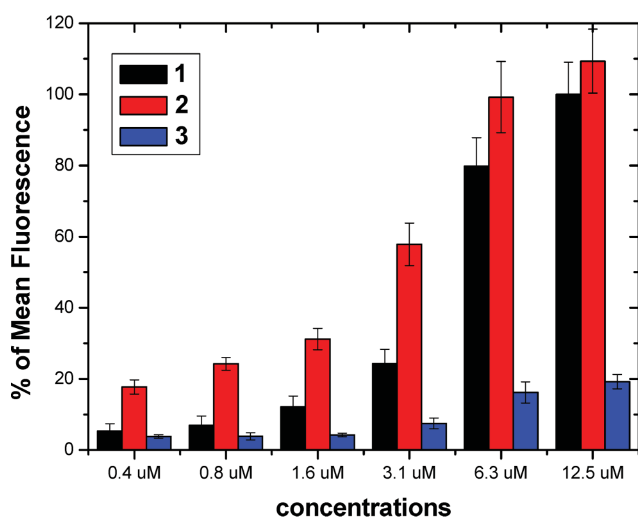

Fig. 3 Fluorescent flow cytometry of oligomers **1–3**. Oligomers **1–3** at a series of concentrations from 0.4 μM to 12.5 μM were incubated with Jurkat cells at 37 °C for 10 min, and their cellular uptake was measured by fluorescent flow cytometry. The relative readings were normalized according to the highest mean fluorescence of oligomer **1** (at 12.5 μM), and were represented by bars. (black colored: oligomer **1**, red colored: oligomer **2**, blue colored: oligomer **3**).

2 comprised of only five arginines, yet it has already shown a comparable translocation ability from extracellular milieu to cytoplasm, thereby suggesting the possible contribution from other cationic groups such as lysines. Moreover, given that the control oligomer **3** bears all cationic groups identical to **2** but failed to have significant cellular permeation, the exceptional cellular transduction ability of **2** should be not only due to the cationic charges, but also because of its peptidomimetic framework. In this regard, improved cellular translocation has been previously observed for oligomers of a diverse set of frameworks, such as the d-isomer and retro-inverso isomer of Tat_{49-57},[5] or a polyguanidine peptoid.[5] The α-AApeptide framework reported here may thereby serve as a promising supplement to those existing ones.

To gain more insight in to their internalization and intracellular distribution, the cellular uptake of oligomers **1–3** was additionally monitored by confocal microscopy. Similar to literature reports,[6,19] Tat_{48-57} **1** was found in both the cytoplasm and nucleus of HeLa cells, and in a diffused pattern out of nucleus (Fig. 4A). Some sporadic punctation is shown that presumably represents an endocytic vesicle's remnants,[6] which is in agreement with the reported observation that the Tat peptide enters cells through the endocytic uptake pathway.[6] In the case of α-AApeptide oligomer **2**, however, although some of it was found localized in the cytoplasm and nucleus, most oligomers still remained in endosomes, showing a characteristic punctate fluorescence (Fig. 4B). As a negative control, the α-peptide **3** expectedly failed to display any cellular uptake (Fig. 4C) and this negative result is consistent with the aforementioned observations in the flow cytometry studies. To further confirm that the ATP-dependent endosomal uptake mechanism was adopted by oligomers **1–2** for their cellular translocation, HeLa cells were pretreated with the energy poison—sodium azide,[6] before the incubation with oligomers. As shown in the b column of Fig. 5, neither Tat_{48-57} **1** nor α-AApeptide **2** were detectable inside the cells treated with sodium azide, and their corresponding confocal images appeared similar to that of the negative control **3**. These results firmly indicate that endocytosis is the cellular path for oligomers **1–2**.

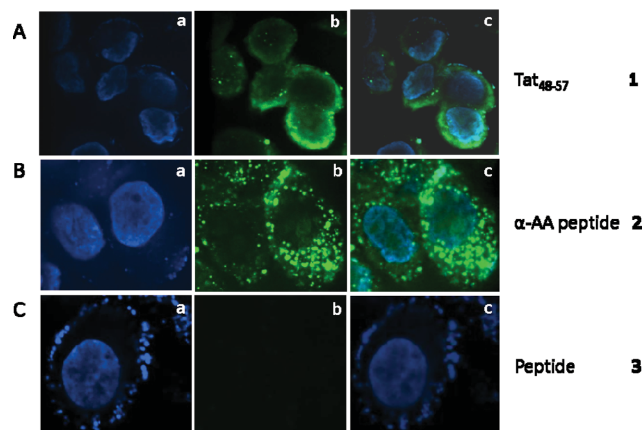

Fig. 4 Confocal images of HeLa cells treated with oligomers **1–3**. HeLa cells were incubated with 5 μM Tat_{48-57} (FI-GRKKRRQRRR) **1** (A), α-AA peptide **2** (B), peptide **3** (C) at 37 °C for 25 min, and then further processed for confocal microscopy. For each subsection: a, DAPI channel (stains DNA in nucleus); b, FITS channel (fluorescence from oligomers **1–3**); c, merged view.

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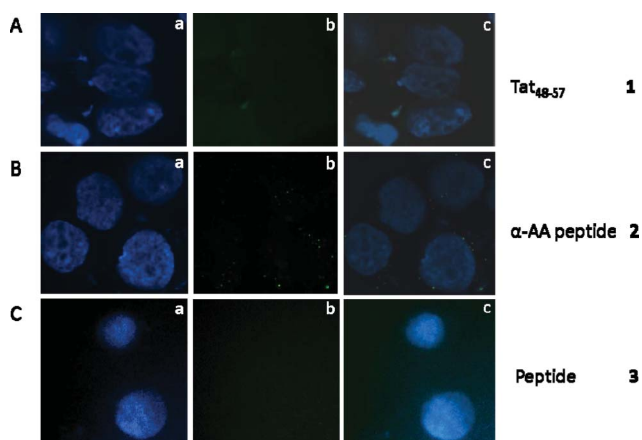

Fig. 5 Confocal images of HeLa cells pretreated with sodium azide and then with oligomers **1–3**. HeLa cells were pretreated with 0.5% sodium azide for 30 min, and then incubated with 5 μM Tat_{48-57} (FI-GRKKRRQRRR) **1** (A), α-AA peptide **2** (B), peptide **3** (C) at 37 °C for 25 min before confocal microscopy. For each subsection: a, DAPI channel (stains DNA in nucleus); b, FITS channel (fluorescence from oligomers **1–3**); c, merged view.

To gain an insight on the endocytic uptake mechanisms of α-AApeptide **2**, HeLa cells were pretreated with filipin or sucrose and then incubated with oligomer **1** and **2**, respectively (Fig. 6). Both filipin and sucrose are endocytosis inhibitors;[25] filipin is known to inhibit caveolae-mediated endocytosis by binding to cholesterol, while high sucrose concentration generates hyper-osmolarity conditions and blocks internalization and clathrin recycling *via* clathrin-coated pit mechanisms. Tat peptide **1** shows significantly decreased cellular uptake upon treatment with both endocytosis inhibitors (Fig. 6A and 6B), indicating that Tat peptide **1** simultaneously uses both caveolae and clathrin-mediated endocytic pathways, which is consistent to the previous report.[26]

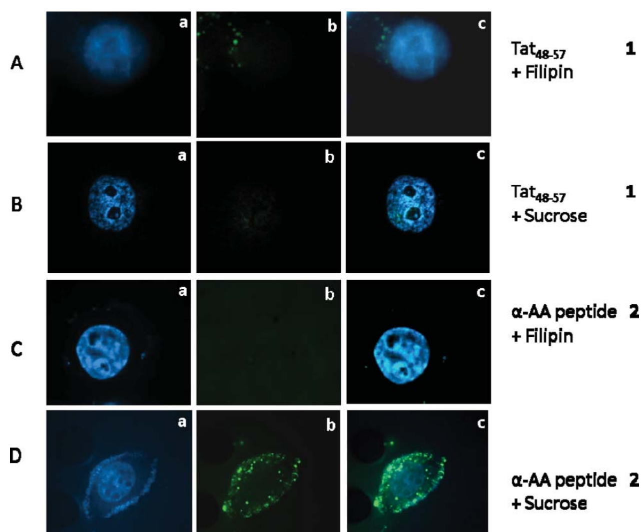

Fig. 6 Confocal images of HeLa cells pretreated with filipin or sucrose and then with oligomers **1–2**. HeLa cells were pretreated with 3 μg mL^{-1} of filipin or 200 mM sucrose for 10 min, and then incubated with 5 μM Tat_{48-57} (FI-GRKKRRQRRR) **1** (A and B) or α-AApeptide **2** (C and D). For each subsection: a, DAPI channel (stains DNA in nucleus); b, FITS channel (fluorescence from oligomers **1–2**); c, merged view.

Interestingly, the significant inhibition of cellular uptake for α-AApeptide **2** was only observed with the treatment of filipin (Fig. 6C). The treatment of sucrose appears to have no or minimal affect on the cellular uptake of α-AApeptide **2**. The results suggests that although the clathrin-mediated endocytic pathway possibly exists in the cellular uptake of α-AApeptide **2**, α-AApeptide **2** mainly uses the caveolae-mediated endocytic pathway for cellular translocation.

Endosome acidification was believed to be an important factor for a cationic peptide's emission from endosomes, and a cationic β-peptide was previously shown successfully translocating from endosome to cytoplasm, under the mediation of endosome acidification.[6] Given that a considerable amount of **2** is also found in the cytoplasm (columns b–c of Fig. 4B), it is conceivable that instead of being completely trapped inside endosomes, α-AApeptide **2** has a relatively slow kinetic escape. This behavior may be again attributed to its unique backbone structure, which is significantly different from traditional α- or β-peptides and could thereby compromise the effect of endosome acidification. The detailed mechanism, however, remains elusive. In another aspect, the long-term accumulation of **2** in endosomes may make it a potential molecular transporter for endosome-targeted drug delivery. Despite the general accepted rule that the endosomal escape is desired for drug delivery, the endosomal system has been long regarded as an essential site of signal transduction.[27] Numerous activated receptors and signaling components stored in endosomes have been regarded as emerging targets for drugs in the treatment of psychiatric disorders,[27] inflammation,[27] pain,[27] Alzheimer's disease,[27–29] and cancer.[30,31] Most recently, novel cellular translocating agents, such as pH-sensitive nanoparticles[32] and noncationic oligomers,[20] were developed to specifically target the endocytic organelles.

The toxicity of α-AApeptide **2** against mammalian cells was also evaluated, in order to demonstrate their potential for the development of novel molecular transporters for *in vivo* applications. For comparison, the control Tat_{48-57} peptide **1** was also tested (Fig. 7). Consistent with previous findings, peptide **1** did not show significant toxicity against both HeLa (a1) and Jurkat cells (b1). Interestingly, although **2** displayed similar toxicity (a2) to **1** against HeLa cells, **2** is generally less toxic than **1** at all concentrations when incubated with Jurkat cells (b2). These results suggest that α-AApeptide **2** has great potential for molecular transportation development.

With the recently developed α-AApeptide frame, we unexpectedly found that a poly(lysine–arginine) oligomer has a cellular penetration efficiency comparable to the natural Tat peptide, but much better than its own α-peptide counterpart, thereby making itself a novel carrier for molecular transportation. Whereas this oligomer crosses membranes *via* endocytosis, in a way similar to that of the Tat peptide, it is mainly sequestered in endosomes, suggesting the potential of α-AApeptide based agents in endosome-targeted drug delivery. Given that previously reported α- or β-peptides are either cytoplasm or nucleus targeted, and that only noncationic oligomers and nanoparticles were shown to remain in endosome, to our best knowledge, the α-AApeptide based oligomer introduced here is the first case of an endosome-targeted cationic peptidomimetic. The low cytotoxicity of the α-AApeptides further supports their potential for molecular transporter development. For future studies, more detailed structure/function studies are

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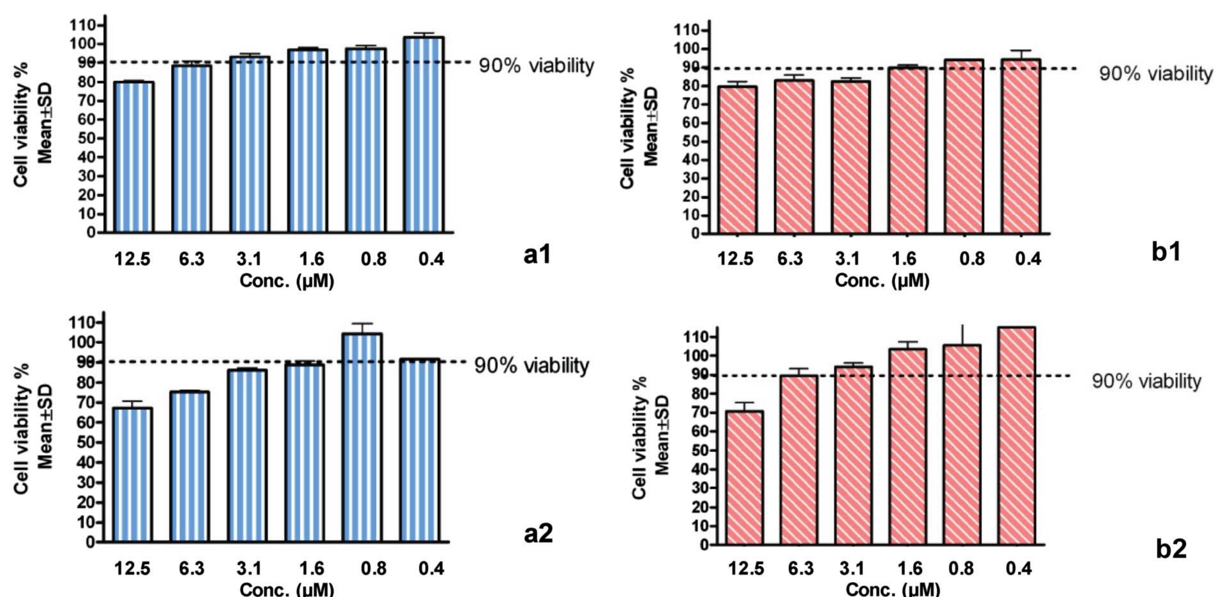

Fig. 7 MTT assay for the Tat_{48-57} (Fl-GRKKRRQRRR) peptide **1** and α-AApeptide **2** against HeLa and Jurkat cells. a1, HeLa cells treated with **1**; a2, HeLa cells treated with **2**; b1, Jurkat cells treated with **1**; b2, Jurkat cells treated with **2**.

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needed, especially to identify the optimal backbone length and the optimal length of the guanidine/primary amino ended side-chains that may be crucial to further improve the oligomer's cellular uptake. Studies to deliver chemotherapeutic drugs to tumor cells by this oligomer are currently in progress.

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