Enhancing Membrane Permeability by Fatty Acylation of Oligoarginine Peptides

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The improved bioavailability of drugs in the treatment of diseases produces a prolonged therapeutic effect and, as a consequence, reduced toxicity and cost. Most oligonucleotides, peptides, or proteins are poorly taken up by cells due to their insufficient association with the lipid bilayer of the plasma membrane. In many cases, therapeutic agents need to possess lipophilic properties in order to achieve the desired pharmacokinetic profile. Thus, the lipophilicity of the molecules assists in the penetration of cytoplasmic and intracellular membranes.[1] Traditionally, the incorporation of homologous series of alkyl groups into a drug of interest produced increases in pharmacological effects. More recently, a short peptide (RKKRRQRRR), derived from HIV Tat-protein, as well as other arginine-containing peptides have attracted attention due to their high cellular uptake efficiency.[2,3] These membrane-penetrating peptides have been applied as delivery vectors for various biological and medical applications.[4–8] A systematic study of Tat-peptide indicated that the positively charged arginine residues are crucial to its membrane-penetrating ability, a property that has also been mimicked by a hepta-arginine peptide.[9] It has thus been concluded that the cationic guanidine moiety on the arginine side chain provides the exceptional translocation properties that are not observed in peptides containing similar amino acids such as ornithine, lysine, histidine or citrulline.[10] Although this membrane-translocalization phenomenon has been reported for more than a decade, the detailed mechanism still has to be determined.[4]

Here we described the systematic exploration of the changes in cell-localizing ability brought about by the modification of oligoarginine peptides with fatty acid analogues. We originally hypothesized that such modifications would lead to enhanced association with lipid membranes and, potentially, improved transmembrane delivery. The 7-mer oligoarginine Tat-peptide mimetic, originally reported by Wender et al.,[9] was selected as the peptide template on which to study the effect of the length of the acyl chain. A series of fatty acid groups, including hexanoyl, octanoyl, decanoyl, lauroyl, myristoyl, and palmitoyl, were attached to the N terminus of the amidated peptide via a β-alanine spacer on solid support by using a modified Schotten–Baumann reaction. Thereafter, the lipopeptides were labeled with fluorescein isothiocyanate

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(FITC) on their C-terminal lysine side chains. The synthesized compounds were purified by HPLC and characterized by MALDI-TOF Mass spectroscopy (Table 1). To quantify the amount of cellular association, we performed fluorescence-activated cell-sorting (FACS) analysis. HeLa cells were detached by trypsinization and incubated with 10 μM of lipopeptide at 37 °C for 5 min. After incubation, the cells were washed and underwent FACS analysis without fixation (Figure 1). Our finding was similar to the previously reported observation that R7 and Tat peptides had similar transmembrane properties.[9] Except for C6-R7 and C8-R7, in which the peptide was labeled with a six or eight-carbon moiety, significant fluorescence-signal increases were found with increasing lengths of the alkyl chains, with the trend reaching a maximum for the myristoyl lipid chain. C10-R7 and C12-R7, which contain ten and twelve carbons on their oligo-arginine backbones, experienced a two and threefold increase in cellular association, respectively. C14-R7 demonstrated the optimal association among the synthesized lipopeptides, with a sevenfold increase in fluorescence intensity as compared to R7. Further lengthening of the lipid chain to the C16-carbon palmitoyl group, however, decreased the efficiency of association with the cells.

Table 1. *Library of synthesized control and lipopeptides.*

Name	Sequence[a]	M_W $[M+H]^+$	MALDI-TOF
tat	BRKKRRQRRRK(*FITC*)-NH_2	1938.79	1938.59
R7	$B(R)_7K$(*FITC*)-NH_2	1699.91	1699.89
C6-R7	hexanoyl-$B(R)_7K$(*FITC*)-NH_2	1798.52	1798.91
C8-R7	octanoyl-$B(R)_7K$(*FITC*)-NH_2	1826.57	1826.56
C10-R7	decanoyl-$B(R)_7K$(*FITC*)-NH_2	1854.62	1854.94
C12-R7	lauroyl-$B(R)_7K$(*FITC*)-NH_2	1883.68	1883.13
C14-R7	myristoyl-$B(R)_7K$(*FITC*)-NH_2	1910.73	1910.58
C16-R7	palmitoyl-$B(R)_7K$(*FITC*)-NH_2	1938.79	1938.59
C14-C	myristoyl-BGFAGFAGK(*FITC*)-NH_2	1425.10	1425.11
C14-R4	myristoyl-$B(R)_4K$(*FITC*)-NH_2	1442.19	1442.26
C14-R11	myristoyl-$B(R)_{11}K$(*FITC*)-NH_2	2535.45	2535.73

[a] B: β-alanine, *FITC*: fluorescein isothiocyanate

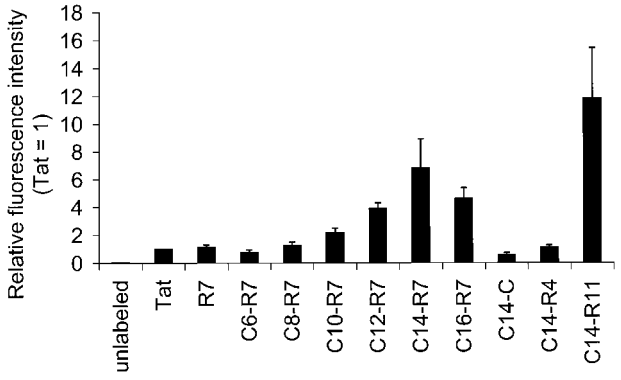

Figure 1. *Cellular association of the peptide library. The cell-associated fluorescence intensity of Tat was set as 1. Data are the average of two independent FACS experiments.*

Further exploration of the effect of arginine chain length on cellular association was accomplished by synthesizing a class of myristoyl lipopeptides containing varying numbers of arginine residues (Figure 1). The peptide chains were thus shortened to a 4-mer or extended to an 11-mer. We observed that the association efficiency of C14-R4 was similar to that of Tat-peptide, R7, and C8-R7. This implies that the myristate moiety makes a significant contribution to cellular association. On the other hand, increasing the number of arginine residues in the backbone from seven to eleven in C14-R11, demonstrated a remarkably higher cellular association (i.e. a 12-fold increase in fluorescence). When the positively charged peptide in C14-R7 was replaced by an uncharged control peptide, C14-C, the fluorescence signals were significantly reduced. These observations illustrate the combination effect of myristate and oligo-arginine groups.

While FACS analysis quantitates cell-associated fluorescence, we were also interested in knowing the specific intracellular distributions of the lipopeptide conjugates. To that end, a series of epifluoresence and confocal microscopy experiments in live cells were performed. Previously it had been reported that fixation could affect cellular distribution,[11] thus all experiments were performed by using live cells without fixation. A dynamic internalization of the probes into cells was observed at different time points, as shown in Figure 2. There was no specific intracellular localization within 5 min of incubation. The fatty acid moieties, such as myristate and palmitate, which are the driving force for membrane association, resulted in a significantly higher fluorescence intensity in C14-R7-, C16-R7-, and C14-R11-labeled cells as compared to the controls R7 and C14-C. After 90 min of incubation, the C14-R7 and C14-R11 lipopeptides demonstrated remarkable intracellular uptake. However, the distribution was different from the nuclear localization seen in R7 or Tat-peptide uptake profile. The modified peptide, C14-R7, appears to target the cytoplasm with minimal membrane staining, whereas the majority of the fluorescence signal for C16-R7 occurred in the cellular membrane. This suggests that the strong lipophilic interactions between the palmi-

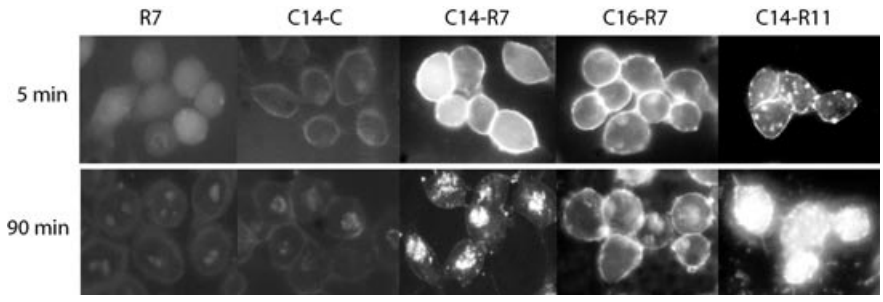

Figure 2. *Time course of cellular uptake. HeLa cells were incubated with a 10 μM solution of the respective peptide at 37 °C.*

toyl group and the cellular membrane overshadows the contribution of polyarginine peptide. FACS data correlate well with the fluorescence microscopy of C14-R11, for which the cells were labeled uniformly with high fluorescence intensity. The results also indicated that the entry of the modified probes into cells was time-dependent.

To further determine the uptake profile, laser scanning confocal microscopy images of the modified peptides were taken. HeLa cells were incubated with peptides at 37 °C for 90 min. The live-cell images in the FITC channel clearly showed that C14-R7 localizes within the cytoplasm with very minor membrane staining (Figure 3). This observation is consistent with

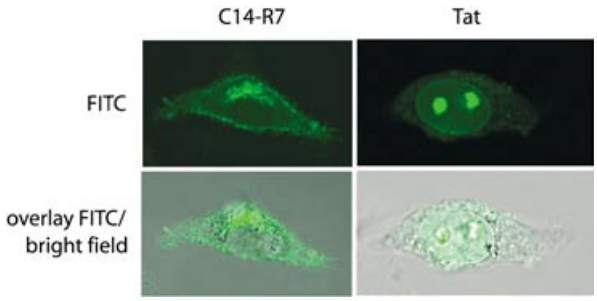

Figure 3. *Visualization of intracellular distribution by confocal microscopy. HeLa cells were incubated with peptides (10 μM) for 90 min.*

the fact that, at first, the hydrophobic interaction caused by the myristate group promotes membrane associations, yet this interaction provides barely sufficient energy to anchor a peptide to a biological membrane.[12] Intracellular distribution of the fatty acylated polyarginine, C14-R7, was further compared to R6 dye (0.5 $\mu g\,mL^{-1}$), which has been reported as a staining reagent for cytoplasmic membrane-containing organelles such as ER and Golgi apparatus.[13,14] Similarly to the previous observation, the fluorescence signal of C14-R7 was peripheral to the cellular nuclear envelope and profoundly overlapped with that of R6 dye (Figure 4).

We further extended our work to determine the potential cytotoxic effects of C14-R7, given its highly increased membrane permeability and intracellular accumulation. Cell death was determined by the conventional colorimetric method, in which the released lactate dehydrogenase (LDH) in culture medium, after exposure to various concentrations of C14-R7, converts the substrate tetrazolium salt into a red product with

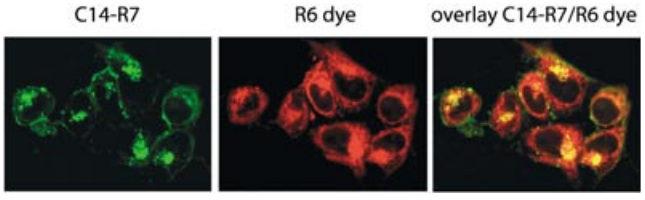

Figure 4. *Comparison of intracellular distribution of C14-R7 to that of R6 dye (staining endoplasmic reticulum/Golgi-apparatus) in HeLa cells. FITC channel for C14-R7 (left), rhodamine channel for R6 dye (middle), and overlay of FITC and rhodamine channel (right).*

an affordable quantitative absorbance at 490 nm.[15–17] The results show that treatment of the cells with 0.1 to 30 μM of C14-R7 followed by 30 min of incubation at 37 °C, did not induce significant LDH leakage (Figure 5). The percentage of LDH released for the tested concentrations was similar to that of the spontaneous LDH release.

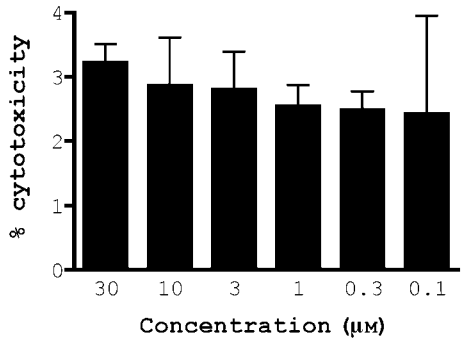

Figure 5. *Effects of C14-R7 on cell toxicity. HeLa cells were incubated with different concentrations of C14-R7 at 37 °C for 30 min. Data were normalized to the amount of maximum LDH released from the cultures treated with lysis solution alone and corrected for baseline LDH release from cultures exposed to buffer only. Experiments were performed in triplicate, mean ± SE.*

In summary, we have synthesized a library of fatty acylated oligoarginine peptides and tested their roles as potential alternative delivery vectors. With an optimized combination of oligoarginine and fatty acid chain length, as in C14-R7, we observed seven- to eightfold higher uptake into the cells than the previously reported R7 or Tat peptides. These results indicate that the tested lipopeptides can improve the efficiency of intracellular delivery.

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