Intracellular MR Contrast Agents based on *l*-Tat and *d*-Tat: A Comparative Study

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Introduction

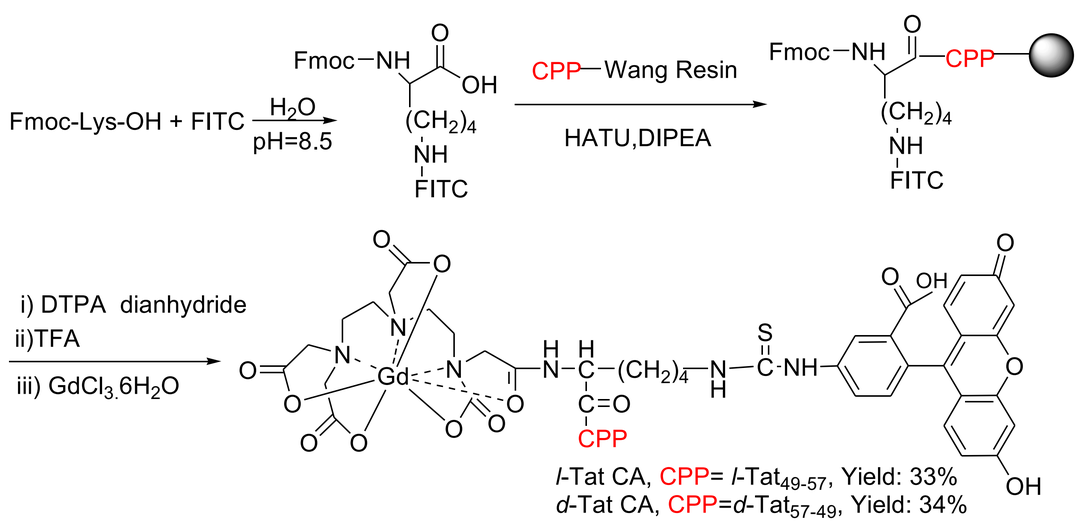
The lipid bilayer of the cell poses a formidable natural barrier for biomolecules. However, a unique class of peptides known as cell penetrating peptides (CPPs) has the ability to traverse this barrier and convey cargo molecules attached to it across the cell membrane [1]. CPPs are short peptides (generally less than 30 residues) with net positive charge and acting in a receptor- and energy-independent manner. Amongst a variety of natural and chimeric CPPs, HIV-1 tat protein derived Tat peptide (Tat_{49-57}) has received much attention mainly because of its high efficiency to deliver a large variety of cargo molecules across the membrane.

Noninvasive imaging techniques like MRI possess the prospective to observe molecular-genetic and cellular processes. The combination of these exogenously administered molecular imaging agents with CPPs may enhance their intracellular delivery, thus solving several queries at sub-cellular level.

Improved cellular uptake of the unnatural retro-inverso isomer of Tat, *d*-Tat_{57-49} (rrrqrrkkr), has been reported in comparison to *l*-Tat_{49-57} (RKKRRQRRR) [2]. Considering the potential of Tat as a molecular transporter, we coupled *l*-Tat_{49-57} and *d*-Tat_{57-49} with fluorescence imaging agent FITC as well as with MR agent Gd-DTPA, thus obtaining *l*-Tat-Lys(FITC)-(Gd)DTPA (*l*-Tat CA) and *d*-Tat-Lys(FITC)-(Gd)DTPA (*d*-Tat CA), respectively. Based on optical imaging and relaxation time measurements we compared cellular internalization and contrast enhancement efficiencies of these two bimodal cell internalizing contrast agents.

Synthesis

FITC (fluorescein isothiocyanate) was coupled to Fmoc-lysine in solution at first. The CPP fragments were synthesized by using standard protocols for fluorenylmethoxycarbonyl (Fmoc) solid-phase synthesis scheme with HATU as the peptide coupling reagent. Then FITC-Fmoc-lysine and diethylenetriaminepenta-acetic dianhydride (DTPA dianhydride) were coupled to CPPs, respectively. Finally the conjugates were chelated with Gd^{3+} to obtain two intracellular MR contrast agents (*l*-Tat CA & *d*-Tat CA). After purification by RP-HPLC, the products were isolated by lyophilization and characterized by ESI-MS (Scheme-1).



Scheme 1: Synthesis of CPP conjugates with Gd^{3+}-DTPA and FITC

Methods

Cellular uptake of compounds was confirmed by fluorescence microscopy and spectroscopy in NIH-3T3 mouse fibroblasts plated in 96 well plates as well as by MR analyses in Eppendorf tubes. Cells were treated with contrast agents at various concentrations in complete medium for 18 hours and washed three times. Internalized fluorescence was measured in a multiplate reader, and microscopic images were made. For MR studies cells were trypsinized after washing and resuspended in fresh medium without contrast agent at a cell density of around $1x10^7$ cells/500 µL. MRI of cell pellets was conducted at 300 MHz on a vertical Bruker 7T/60 cm MRI Biospec system using T_1- and T_2-weighted spin-echo sequences. Relaxation rates were obtained from axial slices as well as T_1- and T_2-weighted images of sagittal slices.

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Results & Discussion

Both the compounds showed concentration dependent enhancement in FITC fluorescence. At short incubation time points, *l*-Tat CA showed higher fluorescence while *d*-Tat CA transcended it after 14 hrs (Figure 1). This enhanced intracellular uptake of *d*-Tat CA is probably attributed by unnatural *d*-peptides that have increased stability against proteolysis.

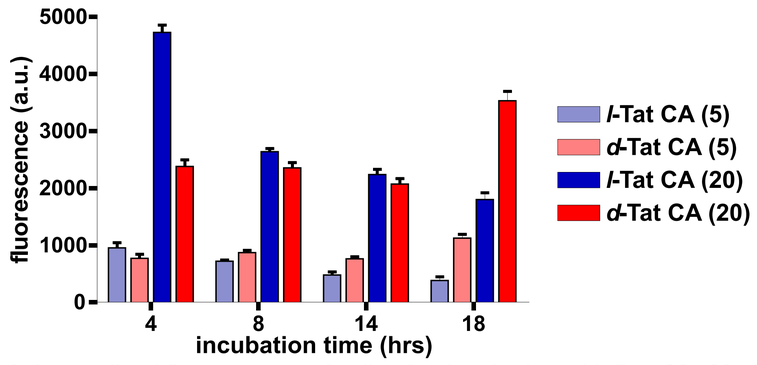

Figure 1: Internalized fluorescence of cells after incubation with 5 or 20 µM of *l*-Tat CA or *d*-Tat CA at varying time points. Values are means SEM. n=4-6

l-Tat CA and *d*-Tat CA were localized inside the cells after incubation for 18 hrs (Figure 2). Higher fluorescence was observed in cells labeled with *d*-Tat CA compared to *l*-Tat CA at the same concentration, correlating with spectroscopic results. Both the CAs were taken up by cells as vesicles and *d*-Tat CA also exhibited some localization to specific regions in the nucleus.

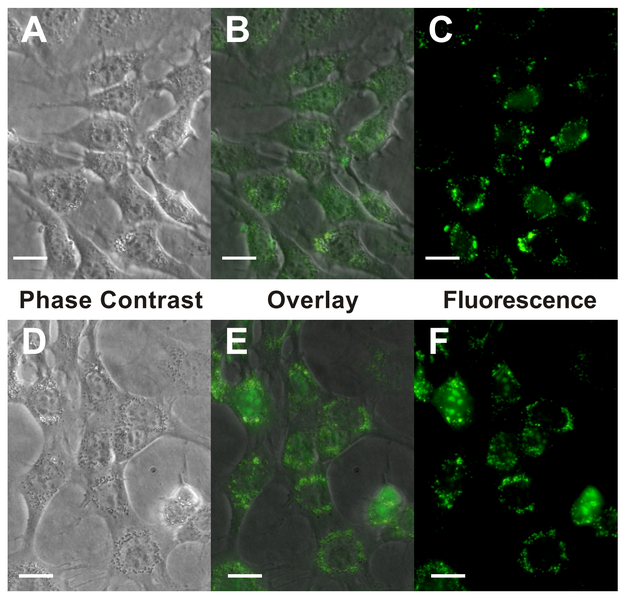

Figure 2: Microscopic images of NIH-3T3 cells incubated with 20 µM of *l*-Tat CA (**A**-**C**) and *d*-Tat CA (**D**-**F**) for 18 hrs. Bars represent 20 µm.

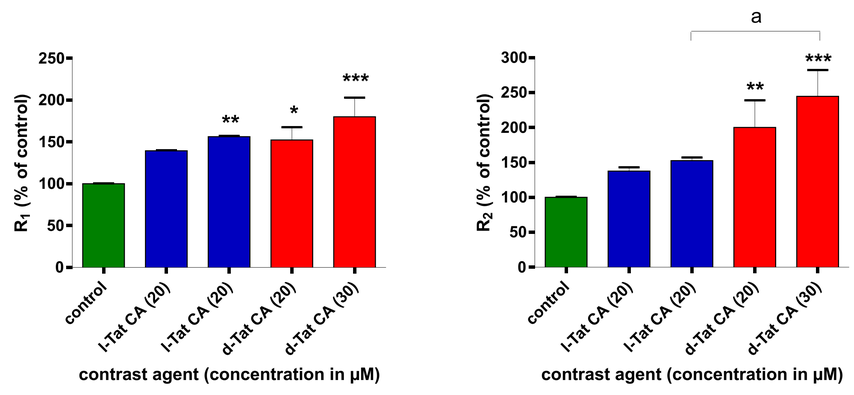

Figure 3. Changes in relaxation rates of R_1 and R_2 in the cells after internalization of CAs. Values are means ± SEM, n=2-5; *p<0.05, **p<0.01, ***p<0.001, statistically significant different compared to control; [a]p<0.05 statistical significant difference.

Enhancement of MR relaxation rates, both R_1 and R_2, was higher for *d*-Tat CA loaded cells than *l*-Tat CA (Figure 3). Interestingly, also a large change in R_2, thus in T_2 contrast, was observed for *d*-Tat CA, not expected from a Gadolinium loaded T_1-based CA.

Conclusion

Both *l*-Tat CA and *d*-Tat CA were delivered inside the cells at concentrations detectable not only by fluorescence imaging but also by MR measurements. *d*-Tat CA was internalized more efficiently but exhibited moderate cytotoxicity. Our results suggest that both these CAs can be useful for developing new intracellular MR contrast agents.

References

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[2.] Rothbard J. B. *et. al.*, 2000, PNAS, 97, 13003-13008.