Intracellular MR Contrast Agents Based on Cationic Cell Penetrating Peptides: A Comparative Study

J. Engelmann[1], W. Su[1], D. Jha[1], R. Mishra[1], J. Pfeuffer[2,3], K. Ugurbil[1,4]

[1]High Field MR Centre, Max Planck Institute for Biological Cybernetics, Tuebingen, Baden-Wuerttemberg, Germany, [2]Physiology of Cognitive Processes, Max Planck Institute for Biological Cybernetics, Tuebingen, Baden-Wuerttemberg, Germany, [3]MREA Neuro, Siemens Medical Solutions, Erlangen, Bayern, Germany, [4]Radiology, Neurosciences, and Medicine, Center for Magnetic Resonance Research, Minneapolis, Minnesota, United States

Introduction The trans-membrane transport of exogenously administered molecular imaging agents to enable them to reach their molecular and cellular targets has been facilitated by the use of certain proteins or polypeptides known as cell penetrating peptides (CPPs). CPPs have the ability to traverse the formidable natural barrier of the lipid bilayer and convey cargo molecules attached to it across the cell membrane. CPPs are short peptides (generally less than 30 residues) with net positive charge and acting in a receptor- and energy-independent manner. Amongst the vast variety of natural and chimeric CPPs available, we have focused on the coupling of MR contrast agents (CAs) with cationic CPPs, namely Tat peptide and oligoarginines. Tat peptide (Tat_{49-57}) is derived from HIV-1 tat protein and received much attention mainly because of its high efficiency to deliver a large variety of cargo molecules across the membrane. Oligoarginines, constitute a class of simple synthetic peptides, that were demonstrated to be better internalized into cells compared to Tat [1]. Besides the native CPPs, modifications of peptides (e.g. use of d-form of amino acids, introducing fatty acid moieties) were used to study the change in internalization efficiency [2, 3]. Coupling these CPPs with fluorescence imaging agent fluorescein isothiocyanate (FITC) as well as with MR agent Gd-diethylenetriaminepenta-acetic acid (Gd-DTPA), we obtained various conjugates for testing their use as intracellular CAs. Based on optical imaging and relaxation time measurements we compared cellular internalization and contrast enhancement efficiencies of these bimodal cell internalizing contrast agents.

Methods The CPP fragments were synthesized by using standard protocols for fluorenylmethoxycarbonyl (Fmoc) solid-phase synthesis. Then Fmoc-lysine-FITC and DTPA dianhydride were coupled to CPPs, respectively. Finally the conjugates were chelated with Gd^{3+}. After purification by RP-HPLC, the products were lyophilized and characterized by ESI-MS and MALDI-TOF.

Cell experiments were performed with NIH-3T3 mouse fibroblasts and C6 rat glioma cells cultured in 96 well microplates for 24 h. Additional incubation for varying time points was performed in the presence of different concentrations of CAs. After repeated cell washing cell related FITC fluorescence was evaluated in multiplate reader. Subsequently, fluorescence microscopy was performed with the same cells to observe the cellular localization. Evaluation of cytotoxicity was done by addition of propidium iodide and a surfactant with subsequent fluorescent reading. For MR imaging, exponentially growing cells were labeled with various concentrations of CAs in 175 cm^2 tissue culture flasks for 18 h. Cells were repeatedly washed, trypsinized and re-suspended in 500 µL complete medium. MRI of the cell pellets was conducted at 300 MHz on vertical 7T/60 cm Bruker Biospec system using T_1- and T_2-weighted spin-echo sequences at room temperature. The axial slice of interest for the evaluation of relaxation rates R_1 and R_2 was positioned through the cell pellet.

Results and Discussion Fluorescence studies show the penetration ability of all the various synthesized CAs across the cell membrane. However, the efficiency of internalization was altered by introduction of modifications. CAs containing CPPs with d-form of the peptide exhibited better and more stable labeling compared to the natural l-form (Fig. 1). Internalization seems to take place via endocytotic pathway in both cell lines as observed by vesicular compartmentalization of fluorescence. The cellular uptake was sufficient to enhance contrast in T_1- as well as T_2-weighted images of labeled cells in comparison to unlabeled control cells (Fig. 2). The enhancement of MR relaxation rates R_1 was higher for cells loaded with 30 µM CA with d-form of peptide (180±23% of control) compared to the l-form of the CAs (156±1% of control). Interestingly, contrast in T_2-weighted images was increased compared to T_1-weighted images specifically for d-Tat CA (245±38% of control). The pronounced R_2-weighted increase is possibly caused by the accumulation of the compound in intracellular vesicles inducing local magnetic field inhomogeneities due to compartmentalization. The modifications of CPPs also enhanced the cytotoxicity as was observed especially with octaarginine containing stearoyl moiety which could be analyzed only up to a concentration of 5 µM. Nevertheless, this introduction of a stearoyl moiety increased many folds the labeling efficiency. In conclusion, our results demonstrated that modified CPPs conjugated to MR CAs can be used for stable labeling of cells for use in MR imaging studies.

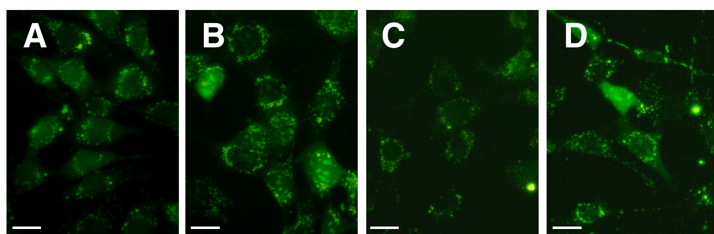

Fig. 1: Fluorescence microscopic images of NIH-3T3 cells after loading with contrast agents for 18 hours.
A: 20 µM l-Tat CA; B: 20 µM d-Tat CA; C: 20 µM l-Arg CA; D: 20 µM d-Arg CA;
Bars represent 20 µm

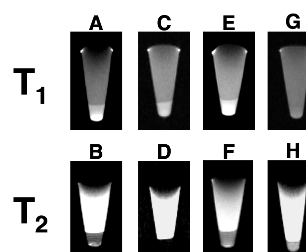

Figure 2: MR images of cell pellets labeled with 30 µM of l-Tat CA (A, B) and d-Tat CA (C, D), 5 µM of d-Arg-Stearyl CA (E, F), and unlabeled cells (G, H).

References [1] Rothbard J. B. *et. al.*, 2000, *PNAS*, *97*, 13003-13008. [2] Futaki S., *et. al.*, 2001, *Bioconj. Chem. 12*, 1005-1011. [3] Piwnica-Worms D. *et. al.*, 2003, *Bioconj. Chem.*, *14*, 368-376.