# Cell internalizing conjugates for MR/Optical Imaging: CPP cargo relationship affects uptake

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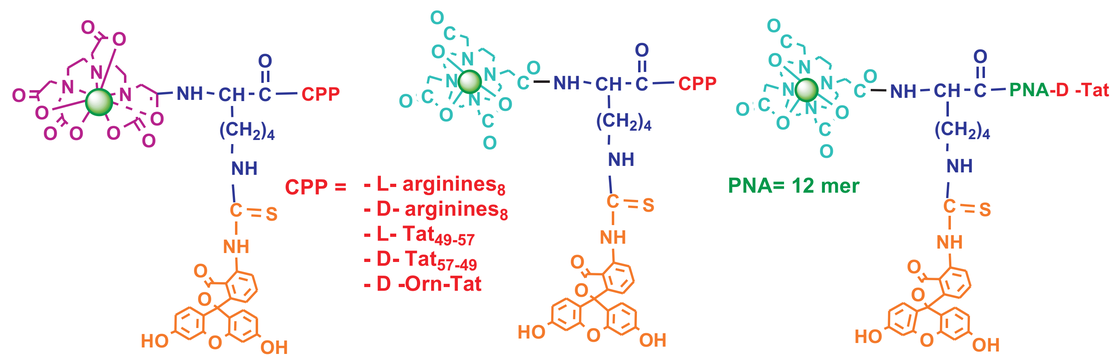
## INTRODUCTION:

The prerequisite of non-invasive cellular imaging is the ability of the contrast agent (CA) to cross the plasma membrane. In recent years, a class of peptides known as cell penetrating peptides (CPP) has been established as an efficient vector for intracellular delivery. CPP are being extensively employed for studies of biological processes in intact cells, but factors like uptake mechanism or toxicity are still under discussion.

Our aim is the development of 'targeted' intracellular CA using CPP as a delivery tool. Amongst CPP, polyarginines are known to be best internalizing but associated with high toxicity and Tat peptide is the most extensively studied CPP for trans-membrane delivery of diverse variety of agents (1). Here we describe the synthesis and cellular uptake of a series of CA based on Tat peptide and octa-arginines. Comparative analysis was performed on the nature of cargo and toxicity related to uptake.




General structure of the contrast agents
(Gd) DTPA, DOTA , FITC, Lysine, CPP-Cell Penetrating Peptide, PNA-Peptide Nucleic Acid

## SYNTHESIS:

Regular and modified CPP were synthesized on solid phase by Fmoc synthesis strategy on Wang resin. Coupling of fluorescein isothiocyanate (FITC) and gadolinium (Gd) chelators DTPA (bisanhydride) or DOTA (tris-t-Bu-ester) was done on resin as well (2). CA were cleaved from resin and loaded with Gd in water. Compounds were purified by RP-HPLC, freeze-dried and analyzed by ESI-MS and MALDI -TOF-MS.

Modifications include changing the natural L conformer of amino acids to D form (peptide D form is more resistant to enzymatic degradation), or substituting glutamine with ornithine in Tat peptide (3). All are discussed to enhance internalization.

## METHODS:

Cell experiments were performed with NIH-3T3 mouse fibroblast cells. Cells were cultured in 96 well microplates for 24 hrs. Additional incubation for 18 hrs was performed in the presence of various concentrations of CA.

After incubation, cells were stained with H33342 for nuclear labeling. External fluorescence was quenched by trypan blue for 3 mins followed by repeated HBSS washings. Cell related FITC fluorescence (CA, green) and nuclear fluorescence (H33342, blue) was evaluated in a multiplate reader. Subsequently, fluorescence microscopy was performed with the same cells to observe the cellular localization.

## REFERENCES:

(1) Jones S. W., *et al.*, 2005, *Br. J. Pharmacol., 145,* 1093-1102.
(2) Endres P. J., *et al.*, 2006, *Mol. Imaging,* 5, 485-497.
(3) Piwnica-Worms D. *et al.*, 2003, *Bioconj. Chem., 14*, 368-376.
(4) Su W. *et al.*, 2007, *Contrast Media Mol. Imaging, 2,* 42-49.

## RESULTS & DISCUSSION:

Uptake efficiency of the L-form of Tat and octa-arginine were compared using Gd loaded DTPA and DOTA as cargo (Fig. 1).

L-Tat was not affected by varying the cargo while in L-Arg8 uptake was Gd chelate dependent preferring DTPA to DOTA. This is in accordance with recently published literature (2).

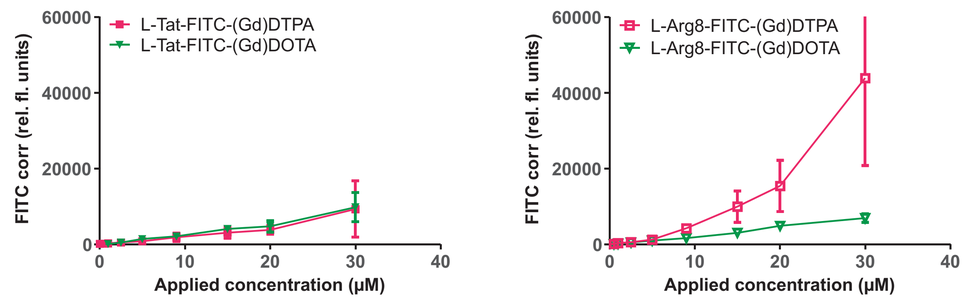



**Figure 1:** CA fluorescence of cells after incubation with different concentrations of L-Tat CA and L-Arg CA for 18 hrs. Values are means SEM (n=3).

Next, cells were labeled with high (20 μM) and low (5 μM) CA concentrations as our previous results indicated that labeling concentration alters the uptake efficiency.

It was observed that the CA exhibiting significantly high uptake at 20 μM concentration also showed significantly increased toxicity (Fig. 2).

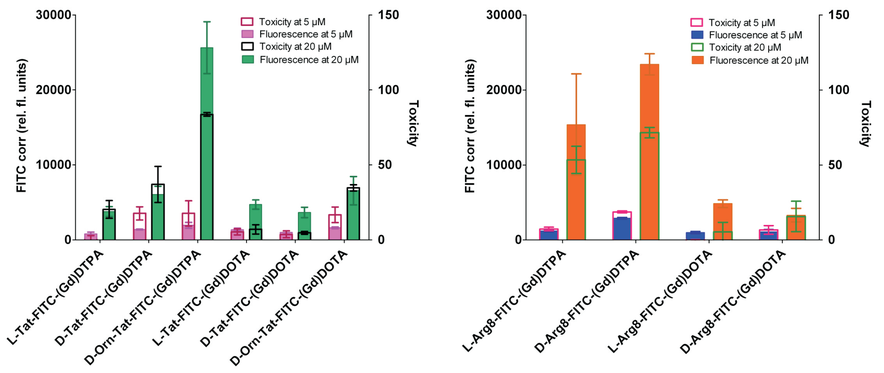



**Figure 2:** CA fluorescence and toxicity of cells after incubation with 5 and 20 μM of various Tat CA and Arg CA for 18 hrs. Values are means SEM (n=3).

Microscopy showed cells with diffused as well as vesicular distribution of CA at 20 μM while at 5 μM only vesicular uptake was observed (Fig. 3). These results indicate that the changed mechanism of uptake could be related to cell death.

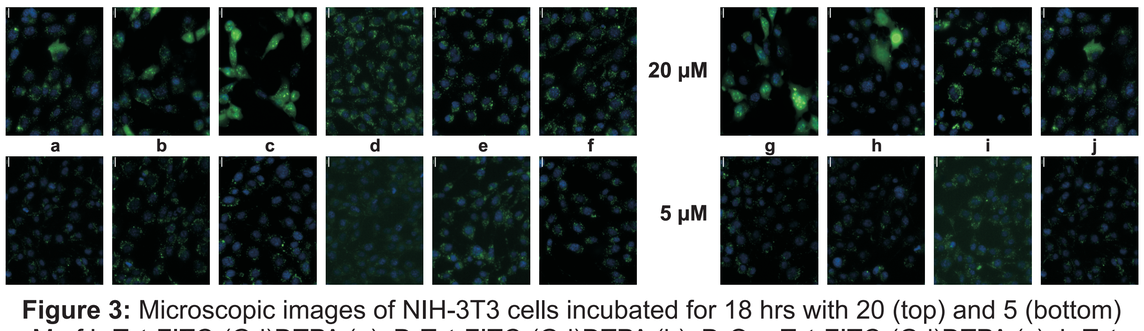



**Figure 3:** Microscopic images of NIH-3T3 cells incubated for 18 hrs with 20 (top) and 5 (bottom) μM of L-Tat-FITC-(Gd)DTPA (a), D-Tat-FITC-(Gd)DTPA (b), D-Orn-Tat-FITC-(Gd)DTPA (c), L-Tat-FITC-(Gd)DOTA (d), D-Tat-FITC-(Gd)DOTA (e), D-Orn-Tat-FITC-(Gd)DOTA (f), L-Arg-FITC-(Gd)DTPA (g), D-Arg-FITC-(Gd)DTPA (h), L-Arg-FITC-(Gd)DOTA (i), D-Arg-FITC-(Gd)DOTA (j).
Bars represent 16 μm. Green: CA, Blue: H33342 (nuclei)

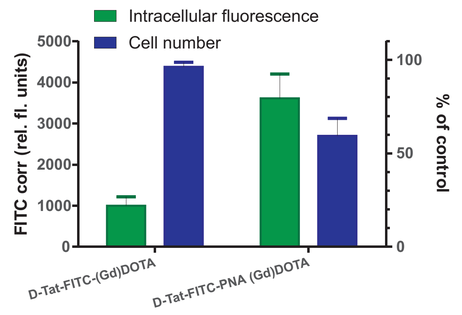



**Figure 4:** CA fluorescence and toxicity of cells after incubation with 5 μM of D-Tat-FITC-(Gd)DOTA and D-Tat-PNA-FITC-(Gd)DOTA for 18 hrs. Values are means SEM (n=3).

Increasing the size of the cargo from Gd loaded DOTA (~500 Da) to Gd loaded DOTA attached to a PNA sequence (~4000 Da) is accompanied by an improved delivery efficiency but also by enhanced cytotoxicity (Fig. 4). Thus, also cargo size plays an important role in uptake and biocompatibility of intracellular contrast agents.

## CONCLUSION:

Results indicate that for designing internalizing agents not only the CPP should be the governing criteria but also the nature of the cargo and the toxicity induced by the combination should be considered.