

Targeted MR agent based on Antisense Peptide Nucleic Acid - Cell Penetrating Peptide Conjugate: Endocytosis hinders targeting

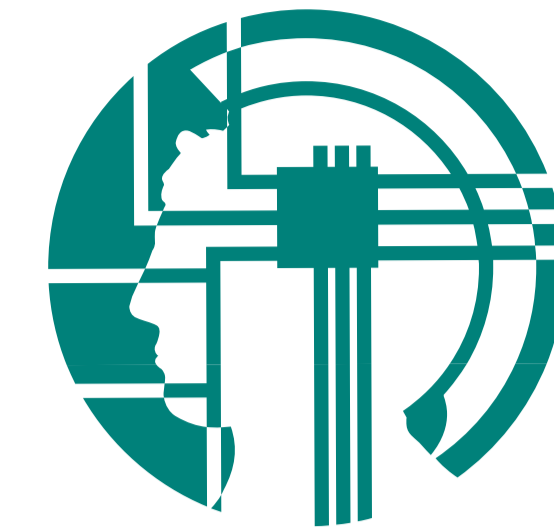


MAX-PLANCK-GESELLSCHAFT

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MPI FOR BIOLOGICAL CYBERNETICS

INTRODUCTION

Essential components of intracellular targeting for Molecular Imaging:

- targets** binding to sites present inside the cell e.g. DNA, mRNA, proteins/enzymes (Fig. 1)
- efficient **vectors** to provide cellular uptake e.g. cell penetrating peptides (CPP), liposomes, etc.
- reporters** to detect intracellular delivery and/or interaction with binding site of interest e.g. fluorophores, PET, SPECT, MR agents, etc.

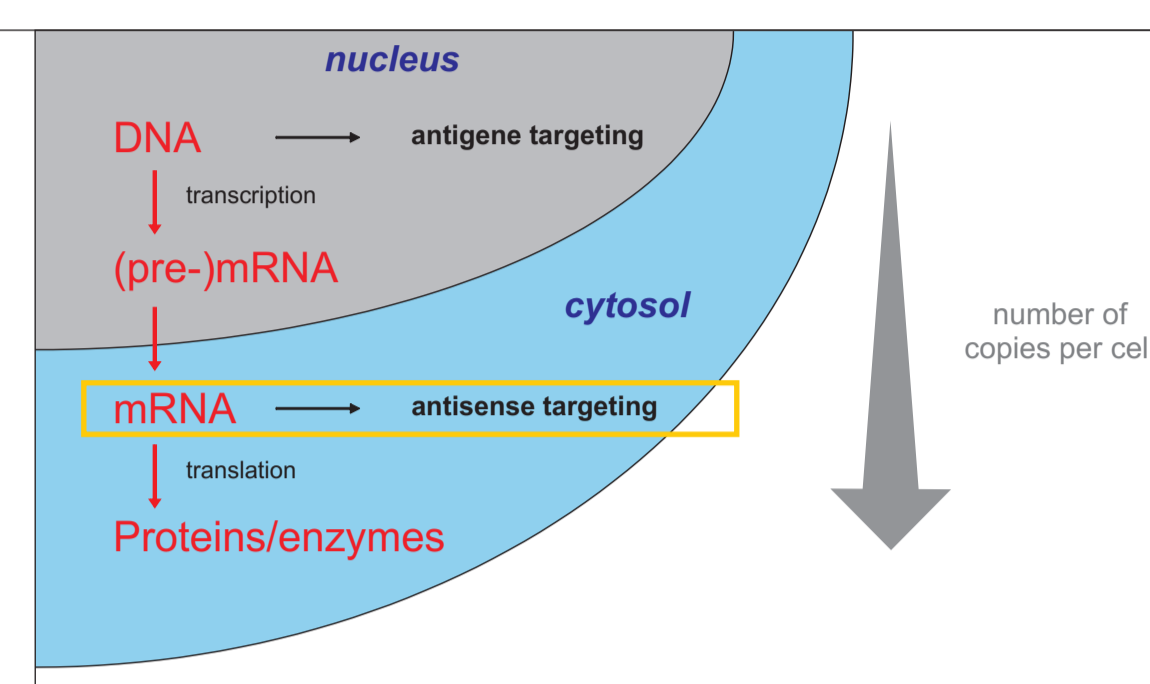


Figure 1: Potential binding sites for intracellular targeted contrast agents (CA)

Our interest: **Antisense targeting of mRNA using peptide nucleic acids (PNA)**

- binding takes place by Watson-Crick base pairing (Fig. 2A)
- sequence of 12 or more bases enough for a specific binding

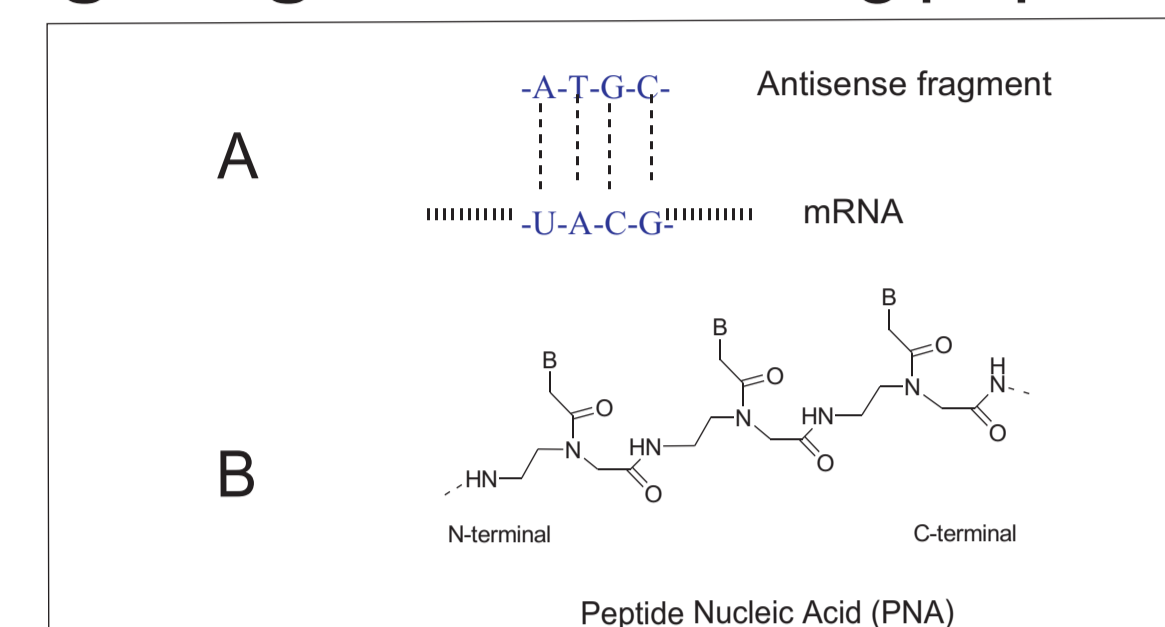


Figure 2: Principle of antisense targeting (A) and the schematic structure of PNA (B)

PNA are metabolically more stable compared to common DNA or RNA molecules (Fig 2B)

To image the presence of specific mRNAs by MRI, developed MR CA (Fig. 3) composed of:

- Gd-DOTA complex linked to a fluorophore (FITC)
- PNA sequence to bind to mRNA of DsRed (red fluorescent protein originating from *Discosoma coral*) or a non-sense sequence with no natural counterpart and a well studied CPP: Tat in its retro-inverso form

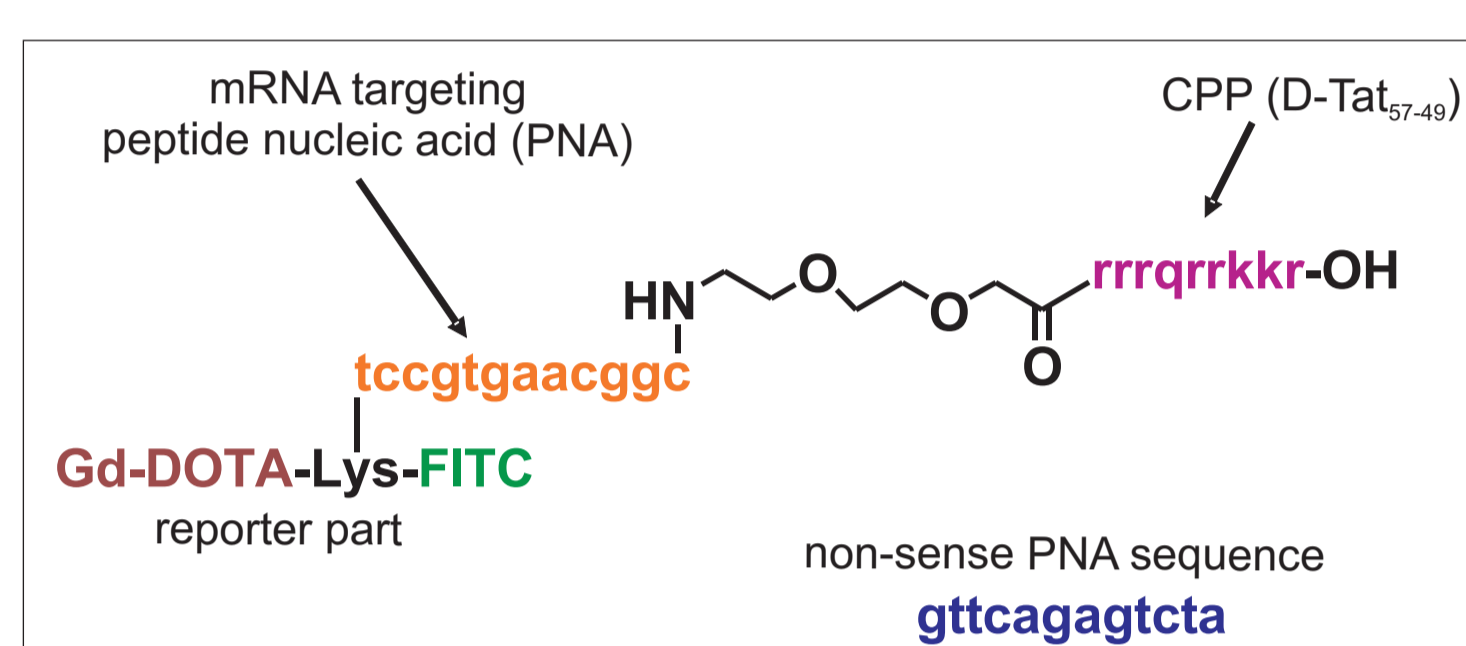


Figure 3: Schematic structure of antisense DsRed mRNA targeting contrast agent (DsRed CA).

Both MR contrast agents previously tested in NIH 3T3 fibroblasts [1].

Fluorescence studies:

- both CA entered efficiently into cells showing concentration dependent uptake
- no significant difference detected between both CA as expected for these cells
- CA located in vesicles around nucleus, indicating internalization by endocytosis

In vitro MR studies:

- contrast enhancement also detectable in MR images
- statistically significant increase of the intracellular relaxation rate $R_{1,cell}$ already at a labeling concentration of 0.5 μ M

RESULTS & DISCUSSION

In vitro binding assay:

- 45mer synthetic deoxynucleotide sequence containing complementary bases to target site of DsRed CA commercially procured
- target oligodeoxynucleotide sequence immobilized on DNA binding plates
- significantly higher specificity of the antisense CA in comparison to its non-sense counterpart observed (Fig. 4B)

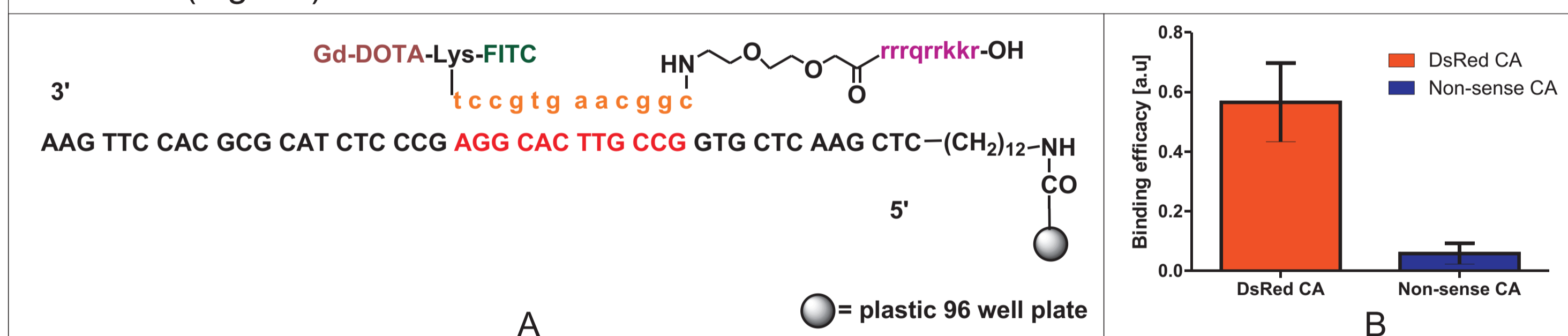


Figure 4: Schematic representation of interaction between synthetic deoxynucleotide sequence and DsRed CA (A) and binding efficacy of DsRed CA and non-sense CA in *in vitro* binding assay (B). Synthetic DNA immobilized on DNA binding plates and unspecific binding sites blocked with BSA/FCS. Hybridization was performed with DsRed and non-sense CA followed by extensive washing to get rid of non-specific binding. Detection by anti-FITC HRP conjugated antibody; values are means \pm SEM, n=3.

In vitro MR study:

- DsRed cells containing target mRNA and parent cell line were incubated with both CA. DsRed as well as CCL-11 cells internalized efficiently the CA.
- a significantly higher contrast enhancement could be observed at labeling concentrations more than 1 μ M (Fig. 6)
- slightly increased uptake in DsRed cells compared to CCL-11 cells was visible.
- no specific accumulation of the antisense DsRed CA in comparison to the non-sense CA could be detected in the target containing DsRed cells.

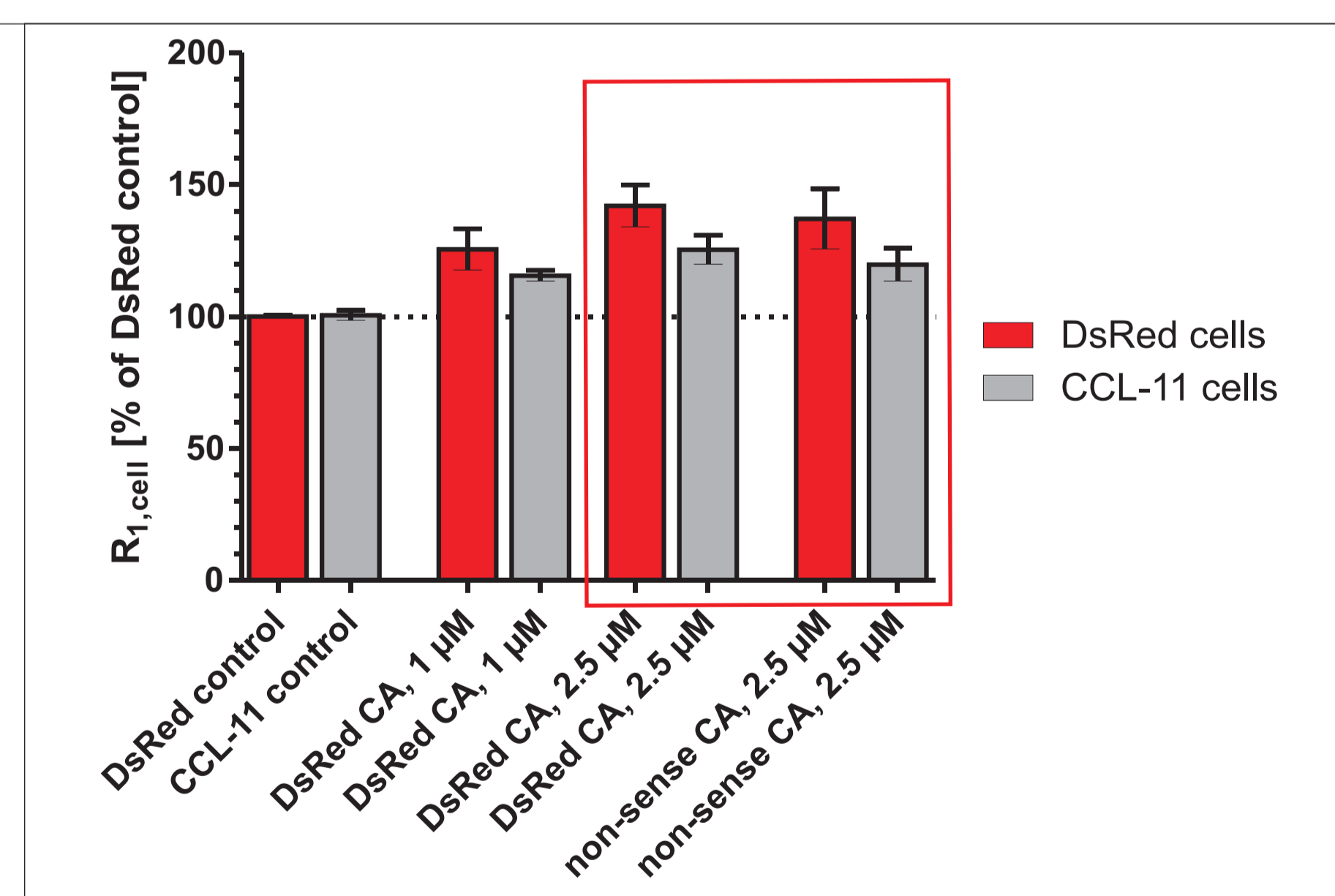


Figure 6: Cellular relaxation rate $R_{1,cell}$ in DsRed and CCL-11 cells after labeling with DsRed or non-sense CA. After treatment with DsRed CA for 18 h, cells were trypsinized, centrifuged and re-suspended in 1.5ml Eppendorf tubes at 2×10^7 cells/500 μ l in complete Medium for MR studies. The measured relaxation rates were plotted vs. the extracellularly applied labeling concentration. Control: cells incubated with culture medium without CA.

Fluorescence spectroscopy:

- uptake studies were performed in a mouse fibrosarcoma cell line expressing DsRed (DsRed) and their parent cell line deficient of the target sequence (CCL-11)
- concentration dependent uptake was observed in both cell types without observable toxicity upto a concentration of 5 μ M (Fig. 5)
- no significant differences were detected between both CA irrespective of the presence or absence of target site in cells

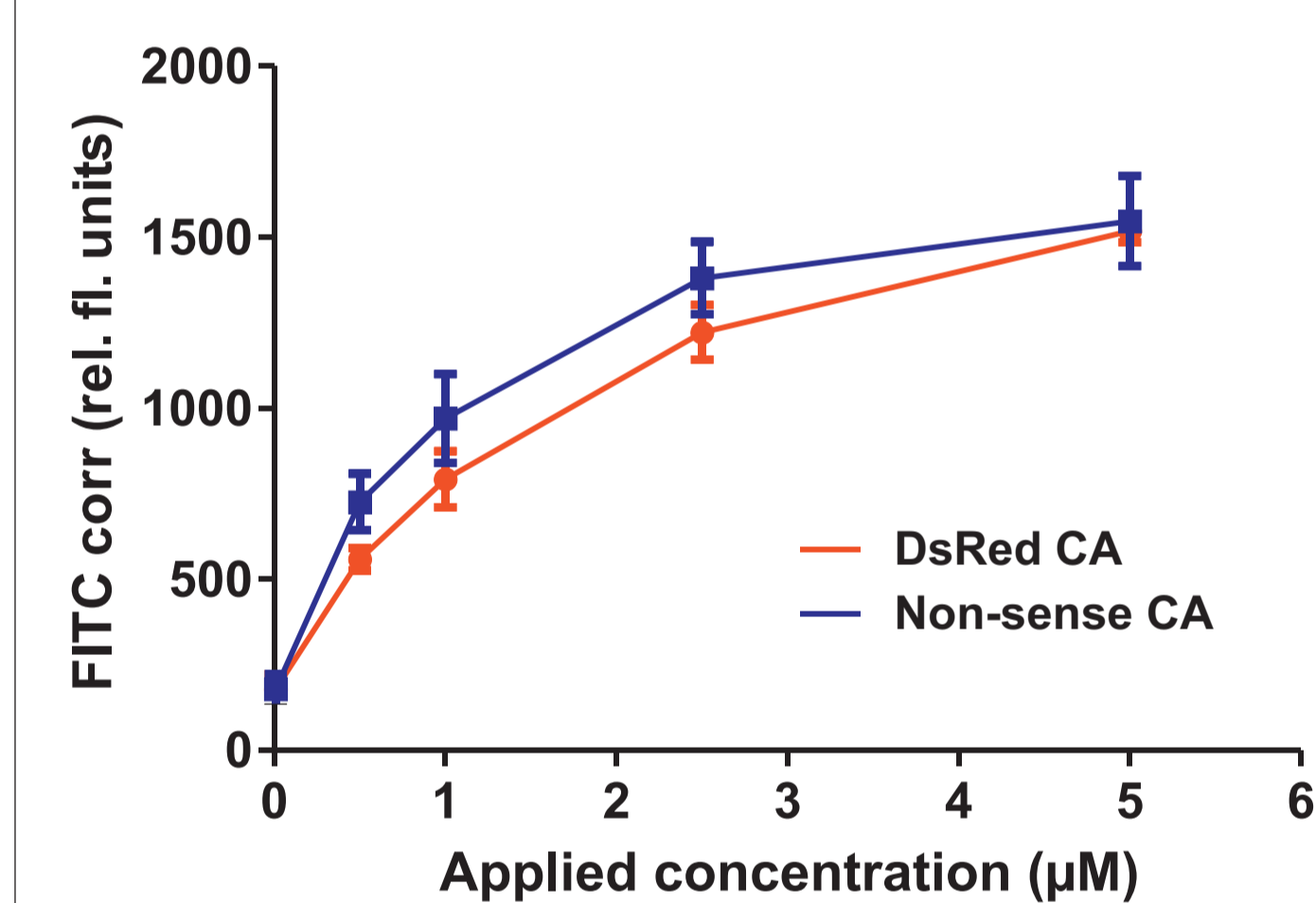


Figure 5: Cell internalization of DsRed CA and non-sense CA into DsRed cells measured by fluorescence spectroscopy. Cells were incubated with contrast agent at various concentrations in complete medium for 18h. External fluorescence was quenched by trypan blue and subsequently washed with HBSS; values are means \pm SEM, n=4 with six replicates each.

Fluorescence microscopy:

- exclusive endosomal localization of the CA was observed by fluorescence microscopy of labeled DsRed cells (Fig. 7A).
- since target mRNA is located in cytosol, the vesicular entrapment prevents a specific interaction between the CA and the target. Thus, further modifications of the CA are required to achieve the release from endosomes or a direct uptake into the cytosol.
- similar vesicular localization of CA was observed in non-target containing parent cell line (Fig. 7B) as well as 3T3 cells (Fig. 7C)

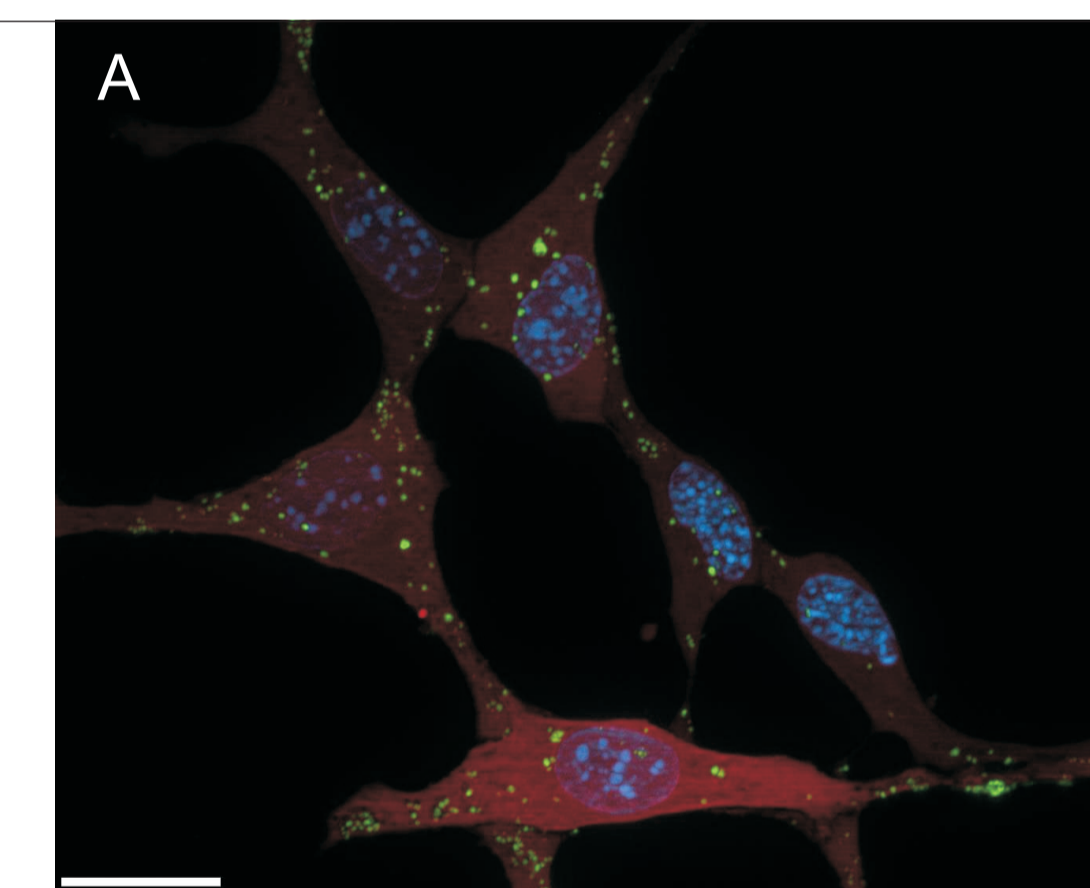


Figure 7: Endosomal localization of DsRed CA in DsRed expressing cells (A), non target containing CCL-11 cells (B) and 3T3 cells (C). CA is trapped inside the endosomes and cannot interact with target. Cells were incubated with DsRed CA at 5 μ M in complete medium for 18 h, cell nuclei were counterstained by Hoechst 33342 and external fluorescence was quenched by trypan blue and subsequent washes with HBSS; DsRed CA: green (FITC fluorescence); nuclei: blue (Hoechst 33342); bar represents 20 μ m.

REFERENCES

- [1] Su W et al. Contrast Med Mol Imaging 2007;2,42-49.

ACKNOWLEDGEMENT

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CONCLUSION

synthesized contrast agents showed excellent ability for intracellular delivery presented contrast agent DsRed CA needs further modifications to be used as a specific targeted contrast agent for MR imaging in cells expressing the DsRed gene exclusive endosomal uptake prevents a specific interaction between CA and target endosomal release or the direct uptake into the cytosol is an important prerequisite to obtain mRNA-based targeting and specific accumulation