Covalent Pyrenebutyrate-Cell Penetrating Peptide Conjugates: Enhanced Direct Membrane Translocation of Coupled Molecular Imaging Agents

Ritu Mishra[1], Wu Su[1], Josef Pfeuffer[2], Kamil Ugurbil[1], Joern Engelmann[1]
[1]Max Planck Institute for Biological Cybernetics, Spemannstrasse 41, Tübingen, Germany 72076
[2]Siemens AG Medical Solutions USA, MR Research & Development, Boston, USA
ritu.mishra@tuebingen.mpg.de

ABSTRACT SUMMARY:

Intracellular targeting with cell penetrating peptides (CPPs) is limited by endocytotic mechanism of uptake. Here, we report that the well known CPP, dTat, chemically coupled with pyrenebutyrate (PB) can efficiently deliver directly into the cytosol. Results indicate that this combination could successfully translocate magnetic resonance imaging (MRI) agents into cytosol.

INTRODUCTION:

Arginine-rich peptides have extensively been used in the last years for delivery of a wide variety of exogenous cargoes into cells. Tat, in particular, is a CPP of this category that has been comprehensively studied and utilized for delivering biomolecules that can modulate cellular functions or for imaging of cellular processes.

In order to observe specific biological processes, membrane permeating targeting agents can be developed for labeling cells of interest. Potential intracellular targets would be DNA, mRNA or proteins/enzymes. In an attempt to image mRNA transcription, we developed a MRI agent targeting mRNA using peptide nucleic acids (PNA), with dTat (Tat in retro-inverso form) to translocate via the cell membrane (1). The agent could very efficiently permeate cells and microscopically vesicular distribution of agent was observed. Ironically, the targeted mRNA residues reside mainly in the cytosolic compartment of the cell. Due to an endosomal trapping of the anti-sense PNA containing agent, our attempts to target specifically the mRNA localized in the cytosol were not satisfying.

As better understanding of the mechanism of uptake for CPPs was obtained by several groups (2), it revealed that endocytosis is the main driving force. Thus, the confinement of biomolecules into endosomes becomes evident. Several interesting studies on endosomolytic peptides or non-peptide carriers are being undertaken to overcome this bottleneck (3-5).

In 2006, Takeuchi *et al*. came up with an interesting approach of co-incubating CPP with the counteranion PB in order to obtain rapid cytosolic delivery (6). But this strategy, like most noncovalent co-incubation methods, works best with molar excess of PB. In addition this approach is reported to be not applicable in the presence of medium or serum. Thus, *in vivo* application of the same would always be a limiting factor.

We attempt, in this study, to present an improvement over the above approach by covalently coupling PB with the CPP attached to (Gd)DOTA (a MRI agent) and fluoroscein isothiocyanate (FITC) for MR and optical imaging as well. In such a way the cytosolic delivery is retained along with possibility for a use not just limited to application in cells but also *in vivo*.

EXPERIMENTAL METHODS:

Cell experiments were performed with NIH-3T3 mouse fibroblast cells. Cells were cultured in 96 well microplates for 48 hrs. Incubation was performed for different time points in the presence of 5µM of chemically coupled dTat-FITC-PB, dTat-(Gd)DOTA-FITC-PB or a 1:1 mixture of free PB with free dTat-(Gd)DOTA-FITC.

After incubation, cells were incubated with Bisbenzimid 33342 (Hoechst 33342), a nuclear stain, in order to estimate the cell number. External fluorescence was quenched by incubating with trypan blue for 3 mins followed by repeated buffer washings. Cell-related FITC fluorescence (Ex 485 nm/Em 530 nm) and cell number (Ex 346 nm/ Em 460 nm) were evaluated in a multiplate reader. Subsequently, fluorescence

microscopy was performed with the same cells to observe the cellular localization.

RESULTS AND DISCUSSION:

We synthesized dTat-FITC-PB by chemically coupling the two units together and observed a direct cytosolic uptake of compound into cells within a few minutes (Fig. 1 left panel). Also a low micromolar concentration of the applied compound was enough, which is a factor 4-5 less than what is needed for internalization with dTat alone. However, endocytotic mechanism takes over when the time of incubation with compound is increased to 18 hrs (Fig. 1 right panel). Our results indicate that PB only transiently delivers into cytosol while on longer incubations, endocytosis dominates. No cytotoxic effect was observed on cells at this concentration even with the long term incubation.

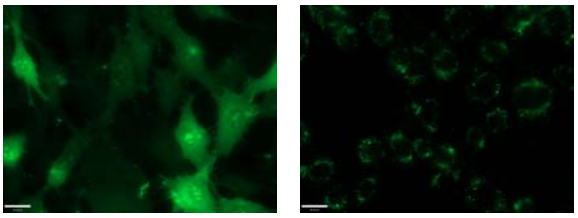

Fig. 1: dTat-FITC-PB (5 µM in HBSS) incubated in 3T3 cells for 10 mins (left panel) and 18 hrs (right panel).

The covalently coupled dTat-(Gd)DOTA-FITC-PB when incubated in medium showed almost the same uptake as observed when dTat-(Gd)DOTA-FITC was co-incubated in HBSS with PB in a 1:1 ratio (Fig. 2). However, a complete loss of uptake was observed when the co-incubation was performed in culture medium. This highlights the applicability of covalently coupled conjugates in presence of serum or medium and possibly also *in vivo*. Since PB is a fluorophore itself, it might be used directly for optical imaging, avoiding the additional coupling of groups such as FITC.

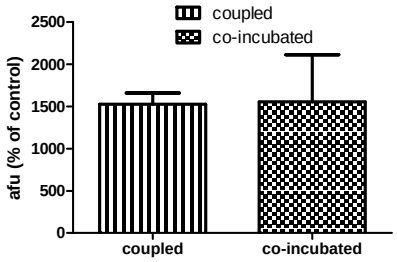

Fig. 2: Fluorescence spectroscopy results of dTat-(Gd)DOTA-FITC chemically coupled to PB (5 µM in medium) compared to the co-incubated mixture (1:1 ratio, 5 µM in HBSS).

We observed that shortcomings of the co-incubation concept can be overcome by chemical coupling of the two moieties, PB and CPP. This strategy can be used for escaping endosomal trapping and successfully delivering agents directly into the cytosol.

CONCLUSION:

The results show that covalently bound PB can be helpful to achieve efficient colocalization of targeted molecular imaging agents with targets like mRNA or enzymes located in cytosol, a pre-requisite for specific interaction. However, further experiments are needed to prove the full capacity of this approach and regarding the specificity of targeting.

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