

CYTOPLASMIC DELIVERY AND NUCLEAR TARGETING OF HPMA COPOLYMER-TAT CONJUGATES TO HUMAN OVARIAN CARCINOMA CELLS

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ABSTRACT SUMMARY

The study was designed to verify the hypothesis that the attachment of protein transduction domains (PTDs) to water-soluble macromolecules will result in cytoplasmic delivery. To this end, N-(2-hydroxypropyl)methacrylamide (HPMA) copolymer – Tat (GRKKRRQRRRGYK(FITC)C) conjugate was synthesized and its subcellular fate after incubation with A2780 human ovarian carcinoma cells evaluated by confocal fluorescence microscopy. Preliminary data show cytoplasmic and nuclear accumulation of the conjugate by a non-endocytic pathway in a time dependent fashion.

Keywords: HPMA copolymer, cytoplasmic delivery, nuclear targeting, Tat peptide, ovarian carcinoma.

INTRODUCTION

The rationale for the use of water-soluble polymers as carriers of anticancer drugs is based on the mechanism of cell entry. Binding a drug to a carrier, such as HPMA copolymer, renders the conjugate lysosomotropic¹. Since, the lysosomal membrane is not permeable to macromolecules, drug – polymer linkages have to be designed to be stable in the bloodstream and interstitial space but susceptible to enzymatically catalyzed hydrolysis in the lysosomal compartment resulting in drug release from the carrier.

However, the endocytic route is not suitable for therapeutic molecules such as genes, antisense oligonucleotides, peptides and proteins, which are susceptible to degradation by lysosomal enzymes. It would be therapeutically more effective to deliver them into the cytoplasm.

Numerous attempts have been made to deliver macromolecules directly into the cytoplasm. It was shown that protein transduction domains (PTDs) composed of ~10-16 amino acid residues, are capable of crossing the lipid bilayer and bypassing the endocytic pathway. Three protein domains with high transducing efficiency have been identified, namely the Tat protein from the HIV virus, the *Drosophila* homeotic transcription factor ANTP, and the herpes simplex virus type 1 (HSV-1) VP22 transcription factor. The common feature of these PTDs appears to be the presence of basic amino acids, such as arginine and lysine. Though the mechanism of cellular entry is yet unknown, it may be possible that these basic amino acids are responsible for lipid interactions and membrane penetration². The Tat PTD has enabled the translocation of a 120kDa

β -galactosidase fusion protein across plasma membranes in different tissues including the brain, in mice. Interestingly, it was also suggested that the fusion protein entered the nucleus due to the nuclear localization signal embedded in the Tat PTD³. In addition, the Tat PTD has successfully been employed to ferry derivatized nanoparticles into progenitor cells, thus allowing their facile detection by magnetic resonance imaging⁴.

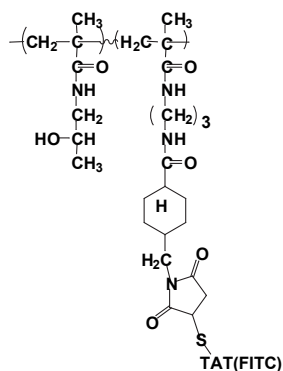
We hypothesize that attachment of PTDs to water soluble synthetic macromolecules will result in cytoplasmic delivery of the conjugate. To test the hypothesis we have synthesized a fluorescein labeled HPMA copolymer - Tat conjugate and evaluated its internalization and subcellular trafficking in A2780 human ovarian carcinoma cells by confocal fluorescence microscopy. The time and concentration dependence of these processes was also evaluated.

EXPERIMENTAL METHODS

Cell lines. The A2780 human ovarian carcinoma cell line was obtained from Dr.T.C. Hamilton (Fox Chase Cancer Center, PA).

HPMA Copolymer-Tat Conjugate. The Tat containing HPMA copolymer was prepared in a three-step procedure. First, a polymer precursor containing amino groups at the side-chain termini (0.44 mmol/g) was synthesized by copolymerization of HPMA and N-(3-aminopropyl)methacrylamide in the presence of AIBN (initiator) and 3-mercaptopropionic acid (chain transfer agent) in methanol. The molecular weight of the copolymer was 26 kDa and Mw/Mn = 1.5 as estimated by size exclusion chromatography. In the second step, maleimido groups were incorporated by the reaction of the amino groups with SMCC (succinimidyl 4-[N-maleimido-methyl]cyclohexane-1-carboxylate) in DMF. The last step involved the binding of the Tat peptide (Fig. 1) via -SH groups of Cys to maleimido groups on the polymer (PBS buffer pH 6.5), thus forming stable thioether bonds. The HPMA copolymer-Tat conjugate was purified on a Sephadex G-25 PD-10 column. The conjugate contained approximately one Tat moiety per chain as determined by UV spectroscopy (0.037 mmol/g; 8.1 wt.-%).

Figure 1 Chemical structure of HPMA copolymer-Tat conjugate and structure of Tat peptide
H-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Gly-Tyr-Lys(FITC)-Cys-OH



Confocal microscopy. A2780 cells were plated onto glass coverslips before incubation with the fluorescein labeled polymer-Tat conjugate. The cells were incubated with 100, 300, 500, 625 $\mu\text{g}/\text{mL}$ of the conjugate for 1 h at 37°C and with 150 $\mu\text{g}/\text{mL}$ of the conjugate for 1 h at 4°C. For comparison, the cells were incubated with an FITC labeled HPMA copolymer lacking the Tat peptide (control polymer) or with no polymer at all for 1 h at 37°C. Nuclear localization was determined using nuclear marker propidium iodide (Molecular Probes). For the time point experiment, the cells were incubated with 200 μg of the conjugate for 5, 10, 20, 30 min, and 1, 2, 4, and 24 h. The cells were fixed with 3% paraformaldehyde. The cells were imaged on a Zeiss (Thornwood, NY) LSM 510 confocal imaging system with an Axioplan 2 microscope with a 100x plan-apo objective (NA=1.4, oil) and an argon laser (excitation at 488 nm, emission collected with a 505 nm long-pass filter). The untreated cells were dark at the settings used for imaging.

RESULTS AND DISCUSSION

Laser scanning confocal microscopy analysis of cells, to which no polymer was added, showed no fluorescence. Cells incubated with the control polymer exhibited localization to endocytotic vesicles, indicating the endocytic uptake pathway (Figure 2a), consistent for polymers and other macromolecules¹. In contrast, cells incubated with HPMA copolymer-Tat conjugates revealed distinct intense membrane staining accompanied with diffuse cytoplasmic and nuclear staining and presumable nucleolar staining (Figures 2b, 2c). Nuclear staining was confirmed by the co-localization of the nuclear marker propidium iodide (red) and the conjugate (green) (Fig. 2c). This was observed at both 37 °C and 4 °C. In addition, traces of vesicular compartments visible at 37 °C were abolished at 4 °C (Fig. 2d). These data seem to indicate a non-endocytic uptake mechanism of the HPMA copolymer-Tat conjugates.

Preliminary fluorescence microscopy data suggested that the distribution of the conjugates within the cytoplasm and nucleus was similar for all concentrations, indicating concentration independent subcellular fate. Internalization into the cytoplasm and nuclear localization were apparent

with amounts of conjugate as low as 100 $\mu\text{g}/\text{mL}$. Confocal imaging also demonstrated that the internalization of the HPMA copolymer-Tat conjugates was time dependent. Longer incubation periods produced both persistent membrane staining as well as a proportional increase in the fluorescence intensity within the cytoplasm, the nucleus and possibly the nucleolus (data not shown). This appears to imply an augmented subcellular distribution of the conjugates with increased duration. Endocytotic vesicles (which are seen typically with macromolecules) were more prominent at time points greater than 1 h as expected. Interestingly, accumulation of the conjugate in the cytoplasm was observed as quickly as ~5 min.

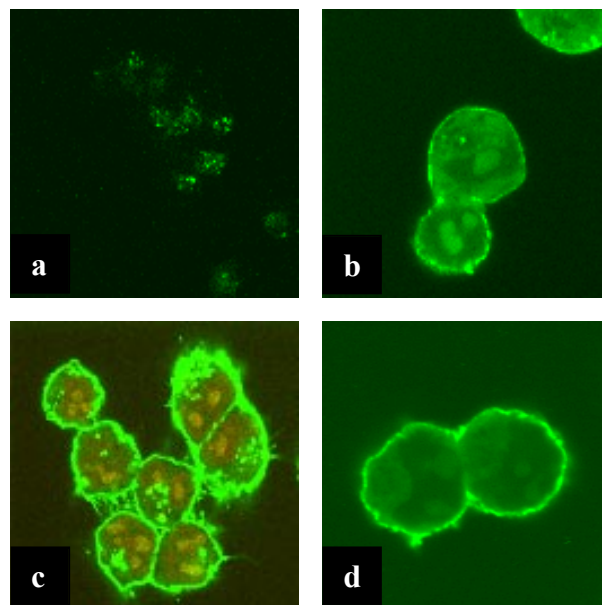


Figure 2 Confocal Image of A2780 cells incubated with a) control polymer, 1 h, 37°C, b) HPMA copolymer-Tat, 15 min, 37°C, c) HPMA copolymer-Tat and propidium iodide, 1 h, 37°C, d) HPMA copolymer-Tat, 1 h, 4°C.

CONCLUSIONS

The microscopic analysis of the incubation of HPMA copolymer-Tat conjugate with A2780 human ovarian carcinoma cells revealed powerful membrane staining associated with cytoplasmic, nuclear and presumable nucleolar staining indicating a non-endocytic pathway of internalization. Though further investigations are warranted, this preliminary study seems to indicate that the development of HPMA copolymer-Tat conjugates as cytoplasmic and nuclear delivery systems holds promise.

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