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CPP or cholesterol conjugation to antisense PNA for cellular delivery

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Introduction

Peptide nucleic acids (*Proc Nat Acad Sci USA*) are DNA mimics consisting of the four common bases as in DNA on a pseudopeptide backbone that makes them extremely stable in biological fluids. Antisense PNA can be targeted to mRNA in the cytoplasm in a complementary base pairing manner. In order to achieve efficient mRNA based targeting, endosomal release or direct uptake of *Proc Nat Acad Sci USA* into the cytosol is mandatory. However, relatively poor internalization of these agents is reported for most cells.[1] Several reports suggested cell penetrating peptide (CPP) based delivery systems for PNA delivery into cells.[2] Unfortunately, endosomal capture seems to be a major challenge in these approaches restricting the ability of PNA to bind with mRNA. Recent studies have explored the use of cholesterol-siRNA conjugates to enhance cellular import.[3] In order to attain efficient PNA internalization into cells, we synthesized two different sequences of cholesterol coupled antisense PNA and evaluated their uptake efficacy in comparison to a PNA-CPP conjugate previously reported by our group.[4]

Methods

The synthesis of PNA (anti-dsRed, specifically targeted to mRNA of red fluorescent protein dsRed) conjugated to CPP (D-Tat_{57-49} i.e. rrrqrrkkr) or cholesterol (Fig. 1) was performed in a fully automated synthesizer (peptides&elephants, Germany) using Fmoc continuous solid phase chemistry. All compounds were labeled with fluorescein isothiocyanate (FITC) to confirm the cellular uptake and were characterized by ESI-MS. Cellular uptake was estimated by fluorescence microscopy and spectroscopy in NIH-3T3 mouse fibroblasts plated in 96well plates. Briefly, cells were labelled with conjugates in complete, serum-containing medium for 18h. Afterwards, cell nuclei were counterstained with H33342, a DNA dye, and external fluorescence was quenched with trypan blue followed by repeated washes to ensure that only intracellular fluorescence was detected.

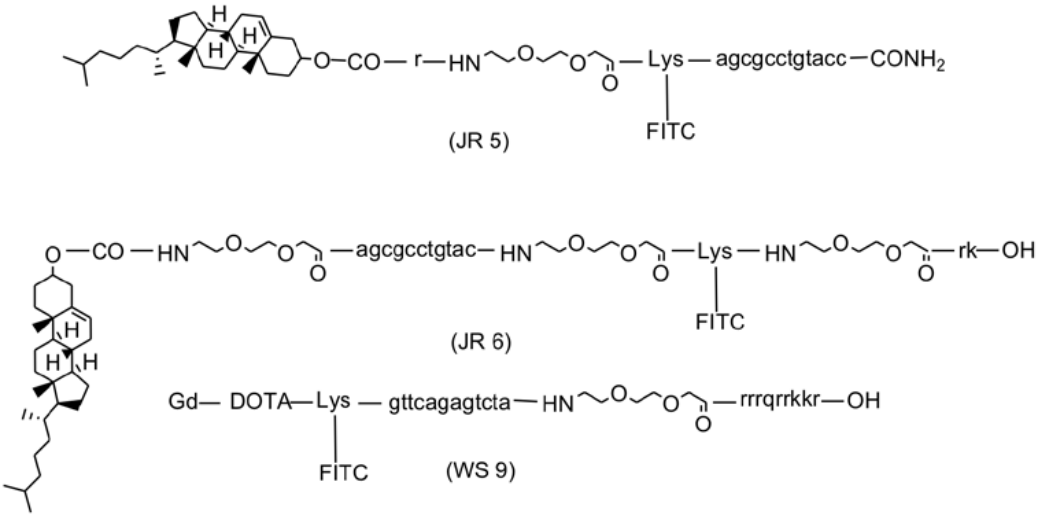

Figure 1. Schematic structure of PNA conjugated to cholesterol (Jr 5 & Jr 6) or CPP (WS 9). Antisense PNA sequence (a: adenine, c: cytosine, g: guanidine, t: thymine); CPP sequence (r: D-arginine, q: D-glutamine, k: D-lysine); Gd: gadolinium; DOTA: 1,4,7,10-tetraazacyclododecane-*N,N',N'',N'''*-tetraacetic acid; FITC: fluorescein isothiocyanate.

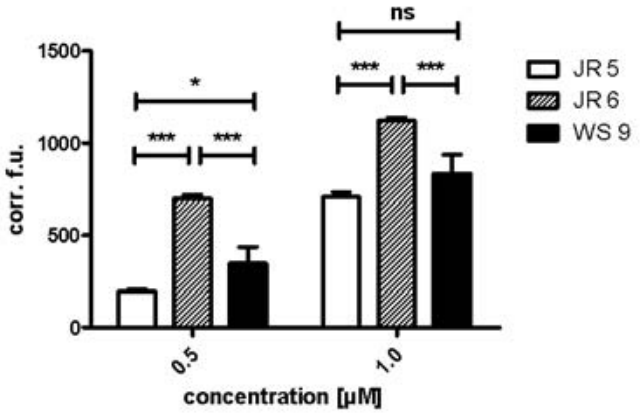

Figure 2. Comparison of cell internalization among Jr 5, Jr 6 and WS 9. ns, not significantly, *p<0.1,***p<0.001, significantly different (Tukey's Multiple Comparison Test).

Results and discussion

The synthesis of PNA, often laborious and lengthy, was made facile by developing and optimizing the Fmoc synthetic scheme for an automated peptide synthesizer. The advantage of this scheme was that all reactions were performed under mild conditions preventing the formation of side products, such as excess coupling on the phenol group of FITC.

Two different conjugates (Jr 5 and Jr 6) were synthesized altering the position of cholesterol, PNA and linkers in order to achieve better aqueous solubility as well as efficient internalization into cells. However, limitation in these syntheses was the removal of cholesterol during the cleavage of the product from the resin. This side reaction was dependent upon incubation time of resin in the cleavage cocktail and could be suppressed to a great extent by choosing the proper reaction time.

The results of fluorescence spectroscopy showed that cholesterol conjugates (Jr 5 & Jr 6) could enter efficiently into 3T3 cells in a concentration dependent manner from 0.5 µM to 2.5 µM (data not shown). However, at concentrations ≥ 2.0 µM, precipitation was observed after 18 h indicating a solubility problem of these conjugates under physiological conditions. Thus, further comparisons with CPP-PNA conjugates were made solely at 0.5 µM and 1 µM. Jr 6 was most efficiently internalized among all three (Fig. 2). This might be either governed by the molecular size or the interaction of cholesterol specifically with cell membrane may play a role in enhancing cellular uptake.

Fluorescence microscopy demonstrated that all the three conjugates were located in vesicles around the cell nucleus indicating a predominantly endosomal uptake mechanism (data not shown).

Conjugate Jr 6 was internalized most efficiently into cells indicating that by the conjugation of cholesterol not only the number of synthetic steps was decreased significantly but also a better internalization was achieved as compared to PNA-CPP. However, cholesterol-PNA conjugates were poorly soluble in aqueous solution at higher concentrations. Nevertheless, it can be expected from the above results that Jr 6 could be a promising delivery agent after further improvements like adding more linkers or residues which enhance solubility in water at physiological pH. In addition, Streptolysin-O, a bacterial protein able to reversibly permeabilize cell membranes, can be used to enhance direct cytosolic uptake into cells as reported elsewhere.[5]

Conclusion

Cholesterol-PNA conjugates need fewer synthetic steps, have a modest molecular size and better or comparable internalization properties as compared to PNA-CPP. However, its applicability on targeting mRNA is still restricted due to endosomal trapping of these conjugates. Nevertheless, the efficient uptake might make it a promising cellular delivery agent after further improvements.

References

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