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Liver Cell Specific Targeting of Peptide Nucleic Acid Oligomers

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Abstract—Chimeric molecules consisting of peptide nucleic acid (PNA) and lactose have been synthesized to test the hypothesis that lactose moieties can promote cell-specific uptake of PNAs. We find that lactose modified PNAs rapidly enter liver-derived HepG2 cells while unmodified PNAs do not and that lactose modified PNAs can inhibit cellular telomerase. © 2001 Elsevier Science Ltd. All rights reserved.

Peptide nucleic acids (PNAs) are DNA analogues in which the phosphate backbone has been replaced by (2-aminoethyl)glycine carbonyl units coupled to the nucleotide bases through the glycine amino nitrogen.[1] Because the PNA backbone is uncharged, PNAs are able to form exceptionally stable complexes with complementary DNA and RNA.[2]

This potential for high affinity hybridization has led to the hypothesis that PNAs have advantages for recognizing cellular targets. To test this hypothesis it is necessary to develop efficient methods for introducing PNAs into cells. Several methods for delivery of PNAs have been developed to date, but all have limitations. Electroporation is effective,[3] but causes substantial cell death, obscuring clear evaluation of PNA-induced phenotypes. Transfection of PNAs into cells using cationic lipid is a reliable approach,[4] but long term delivery requires repeated manipulation of cells, a process that complicates extended experiments. Attachment of import peptides, such as that derived from antennapedia homeodomain, allows uptake to be spontaneous.[5,6] The synthesis of conjugates, however, can be difficult and there is no evidence for preferential uptake by selected cell types.

Here we describe the modification of PNAs with lactose molecules. Lactose can be recognized by the hepatic asialoglycoprotein receptor (ASGP-R).[7] ASGP-R is localized to liver cells and provides an efficient entry point for lactose-modified macromolecules.[8–10] As with attachment of import peptides, modification with lactose may allow PNA uptake to be spontaneous. Another advantage from using lactose is that it may be possible to specifically target PNAs to liver cells, facilitating application of PNAs for the treatment of hepatitis B, hepatitis C, and liver cancer.

Synthesis of PNA–Lactose Conjugates

We pursued two strategies for the attachment of lactose to PNAs (Table 1 and Fig. 1). In our first approach we added several lysines to the N-terminus of PNA. The first lysine was attached to the PNA through a β-alanine linker. Three additional lysines were then added to both the α- and ε-amines of the first lysine. Since PNAs are synthesized by standard peptide synthesis protocols, this addition of amino acids to form a branched peptide is convenient. A total of eight lactose moieties were then coupled by reductive amination to afford **PNA I**, two on the α-amines, and the remaining six on the ε-amines.

As a second strategy, we synthesized the branched lysine rich peptide separately and included a C-terminal cysteine. We then labeled this peptide with lactose by reductive amination and coupled it to cysteine-containing PNA by disulfide exchange to afford **PNA II**. Disulfide coupling is less convenient than direct addition of lysines to the PNA because it requires a solution-phase crosslinking step. We reasoned, however, that cleavage of the disulfide upon exposure to the reducing environment inside the cell might improve subcellular localization and biological activity.

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For both syntheses, conjugates were purified by HPLC and analyzed by mass spectral analysis (Fig. 2).

Cellular Uptake of PNA–Lactose Conjugates

To determine whether the attachment of lactose affects the cellular uptake of PNAs we modified a PNA–lactose conjugate, in which the PNA was attached to the lactose peptide by an amide bond, with rhodamine (**PNA III**). We also synthesized the analogous rhodamine-labeled PNA (**PNA IV**) lacking lactose modification. We then examined uptake by HepG2 cells, a liver derived line that expresses ASGP-R. We observed that HepG2 cells take up lactose conjugated **PNA III** with high efficiency (Fig. 3a). No uptake of **PNA IV** was observed (Fig. 3b). **PNA III** did not enter DU145 prostate cancer-derived cells (Fig. 3c) which do not express ASGP-R. These experiments suggest that addition of lactose enables PNAs to selectively enter cells that express ASGP-R.

Table 1. IC_{50} values and mass spectral characterization of PNAs used in these studies[a,b]

PNA[a]		MS	T_m (°C)	IC_{50} (μM)
I	Lac-pep-PNA-Gly	7252.18 (7241.78)	74	20
II	Lac-pep-Cys-s-s-Cys-PNA	7534.48 (7536.02)	71	6
III	Lac-pep-PNA-Cys-Rho	7764.15 (7767.28)	—	—
IV	Rho-Cys-PNA-Lys	4249.17 (4251.60)	—	—
V	PNA-Lys	3735.37 (3736.60)	72	ND[c]

[a]PNA sequence: TAG GGT TAG ACA A (C terminus); Lac-pep: $Lac_8(Lys_3)_2Lys\beta Ala$.
[b]Predicted mass values are in parentheses.
[c]ND, inhibition not detectable at PNA concentrations >40 μM.

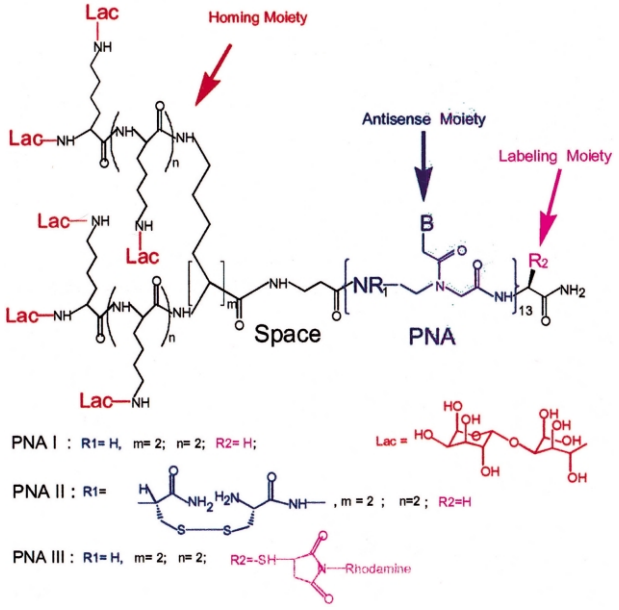

Figure 1. Structures of lactose–PNA conjugates **I**, **II** and **III**.

Inhibition of a Cellular Enzyme by PNA–Lactose Conjugates

When developing new methods for intracellular delivery of PNAs it is helpful to have a cellular target that is well characterized and whose activity is easy to measure. Recognizing this, we chose to synthesize PNAs to target human telomerase. Telomerase is a ribonucleoprotein that is required for maintaining telomere length. It contains an essential RNA component that is highly accessible to Watson–Crick pairing by oligomers because it functions by binding to single-stranded telomere ends. Because of this accessibility, telomerase is an ideal target for PNAs and we have already shown that cellular telomerase can be inhibited by PNAs delivered by complexes of DNA and cationic lipid.[4] Telomerase activity can be readily measured using the PCR based Telomere Repeat Amplification Protocol (TRAP).

We introduced PNA–lactose conjugates **I** and **II** at final concentrations in cell growth media ranging from 500 nM to 40 μM into cells and assayed telomerase activity. Incubation times varied from 24 to 72 h. No toxicity was observed, and telomerase activity was inhibited by **PNAs I** and **II** with IC_{50} values of 20 and 6 μM, respectively (Fig. 4a and b). The lower IC_{50} value of PNA **II** may be due to improved nuclear and cytoplasmic localization upon reduction of the disulfide.

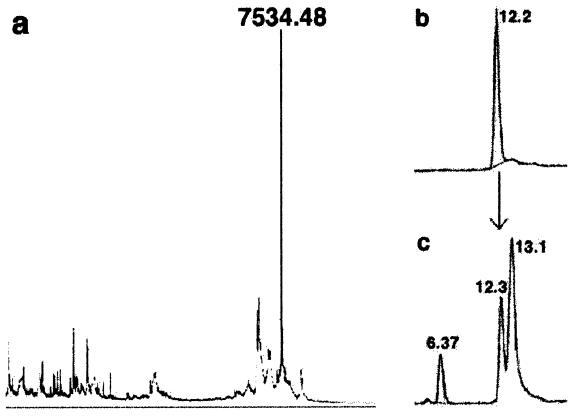

Figure 2. Mass spectral and HPLC analysis of disulfide-linked lactose–PNA conjugate (**PNA II**): (a) mass spectral analysis of **PNA II**; (b) HPLC analysis of the **PNA II**; (c) effect on retention time of adding 10 μL 1 M dithiothreitol (DTT) and incubation at room temperature for 30 min. The peak with a retention time of 6.4 min is DTT, the peak with a retention time of 12.3 min is **PNA II**, and the peak with a retention time of 13.1 min. is reduced cysteine-containing PNA.

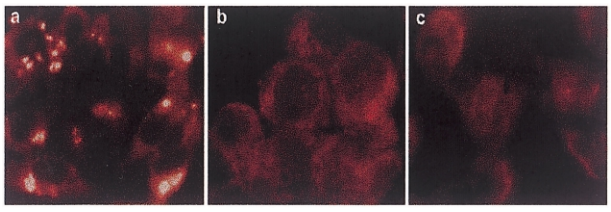

Figure 3. Incubation of 1 μM rhodamine-labeled PNA–lactose **IV** and analogous **PNA V** with different cell lines at 37 °C for 30 min: (a) uptake of rhodamine labeled PNA–lactose conjugate **IV** by Hep G2 cells (ASGP-R +); (b) uptake of rhodamine labeled **PNA V** by Hep G2 cells; (c) uptake of **IV** by DU145 cells (ASGP-R−).

PNA V, the analogous unmodified oligomer, did not inhibit telomerase. A PNA–lactose conjugate containing mismatch bases, and a PNA–maltose conjugate also did not inhibit telomerase activity when present at 20 μM, suggesting that inhibition is sequence specific and depends on modification of the PNA with lactose.

Inhibition of telomerase by lactose conjugates was 50-fold less efficient than the inhibition we had previously observed by PNAs delivered by cationic lipid.[4] One explanation for this discrepancy is that the lactose-modified peptide may prevent recognition of the telomerase RNA template. This is unlikely for three reasons. The first is that the lactose-modified PNA binds to a complementary DNA oligomer with approximately the same melting temperature (T_m) value as the corresponding PNA (Table 1). The second is that the lactose

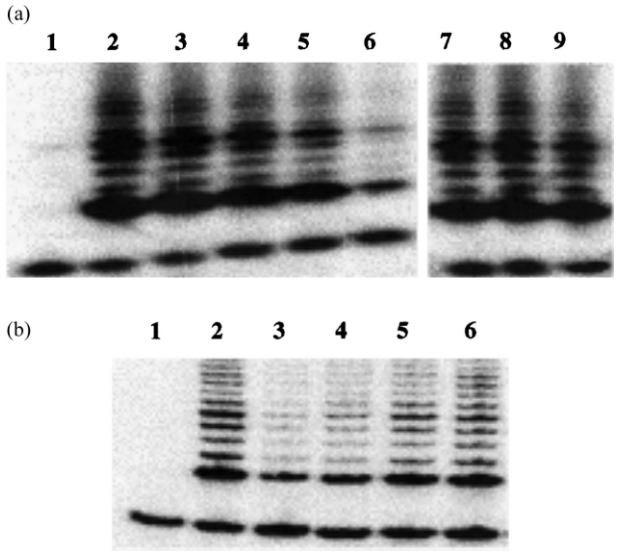

Figure 4. Inhibition of telomerase in cultured HepG2 cells by (a) disulfide linked PNA lactose conjugate **PNA II** and (b) amide linked conjugate **PNA I**. (a) Lane 1, no extract was added. Lane 2, cell extract alone. Lanes 3–6 show the effect on telomerase activity of adding **PNA II** at 10, 5, 1, and 0.5 μM respectively. Lanes 7–9 show the effect of adding 20 μM unmodified PNA, mismatch containing lactose PNA, or an amide linked PNA–maltose conjugate respectively. (b) Lane 1, no extract added. Lane 2, cell extract alone. Lanes 3–6 show the effect on telomerase activity of adding 5, 10, 20, and 40 μM **PNA II**. The lower band is an internal amplification standard.

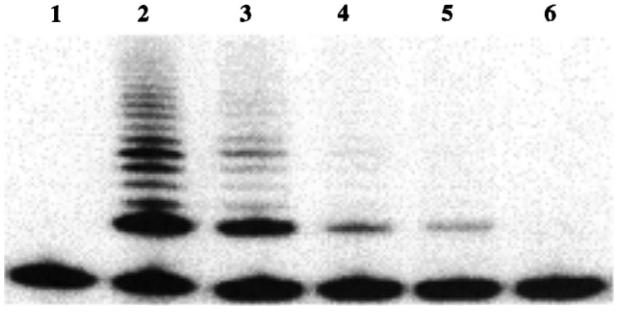

Figure 5. Inhibition of telomerase by PNA–lactose conjugate (**PNA I**) in HepG2 cell extract. Lane 1, no extract was added. Lane 2 cell extract alone. Lanes 3–6 show the effect on telomerase activity of addition of 0.2, 2, 20, and 200 nM **PNA I**. The lower band is an internal amplification standard.

modified **PNA I** was able to inhibit telomerase in cell extract with an IC_{50} value of 1 nM (Fig. 5), similar to the IC_{50} value of the unmodified PNA. Most convincingly, we found that we could deliver the PNA–lactose conjugate **PNA I** into HepG2 cells in complex with DNA and cationic lipid. **PNA I** was able to inhibit cellular telomerase when introduced by this method, and inhibition was as efficient as inhibition by unmodified PNA delivered similarly. It is likely, therefore, that the lactose–PNA conjugate is compartmentalized upon uptake and that this reduces its availability. This hypothesis is supported by the punctate localization of rhodamine-labeled PNA–peptide observed by microscopy (Fig. 3a).

Conclusion

We have developed two simple syntheses for lactose–PNA conjugates. Conjugates enter cells that express ASGP-R, but not cells that lack the receptor. A lactose–PNA conjugate targeted to the RNA template of telomerase inhibits telomerase, but only at high concentrations. These results suggest that lactose–PNA conjugates can specifically enter liver cells, but that they are compartmentalized and their efficient release into the cytoplasm is hindered. It is important to note, however, that oligonucleotides have been noted to possess much more favorable cellular uptake properties in animals relative to cell culture. Animal testing, therefore, will provide the best system for conclusively evaluating the potential of lactose–PNA conjugates.

Synthesis of PNAs and PNA–lactose conjugates

All PNAs and amide-linked PNA–peptide conjugates were synthesized using an Applied Biosystems (Foster City, CA) Expedite 8909 synthesizer.[11,12] PNA–lactose conjugates were synthesized by two different methods. In the first method lactose was added directly to an amide linked PNA–peptide conjugate by reductive amination.[13] β-D-Lactose and PNA–peptides were separately dissolved in 10 mM Na–phosphate buffer (pH 7.5). A 50 μL solution of 1 M lactose was then mixed with 1 mM PNA–peptide. The mixture was vortexed and reacted at room temperature for 6 h, followed by the addition of 10 μL 1 M sodium cyanoborohydride and an additional 10 h of mixing.

In the second method, disulfide exchange was used to link PNAs to a lactose derivatized peptide. Both PNA and peptide were synthesized to contain cysteine. Lysine containing peptides were modified with lactose by reductive amination as described above. To promote crosslinking, the thiol group of cysteine at the C-terminus of the PNA was activated by 2,2′-dithiodipyridine as described. The lactose-modified peptide was then added dropwise to the solution of S-thiopyridyl PNA and allowed to sit at room temperature for 10 h. For both methods, PNA–lactose conjugates were purified by C-18 HPLC and were analyzed by mass spectral analysis.

Cell Culture

The human hepatoblastoma cell line HepG2 was obtained from American Type Cell Culture Collection (ATCC, HB8065). DU145 prostate tumor-derived cells were also obtained from ATCC (HTD-81). HepG2 cells were grown in Eagle's Minimal Essential Medium (EMEM) with 2 mM L-glutamine and 10% FBS. DU145 cells were grown in Dulbecco's Modified Eagle's Media (DMEM) supplemented with 10% fetal bovine serum, 100 units/mL of penicillin G and 100 μg/mL of streptomycin.

Uptake and Visualization of PNA–Lactose Conjugates by Cells

25,000 HepG2 and DU145 cells were grown on the cover slide in a 24-well plate for 12 h in EMEM with 2 mM L-glutamine for HepG2 or in DMEM for DU145 containing 10% fetal bovine serum. The media was removed and the cells were washed three times with 1× PBS. Rhodamine-labeled PNA was added (1 μM final concentration) to cells and incubated for 30 min. Cells attached to the cover slide were then rinsed six times with PBS and fixed with 3% paraformaldehyde for 30 min. Cells were then rinsed once more with PBS prior to visualization.

Assay for Telomerase Activity

Telomerase activity was determined with the telomere repeat-amplification protocol (TRAP) and analyzed as described.[4]

Acknowledgements

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