Antigen-specific delivery of siRNA against Eucaryotic Elongation Factor 2 by rationally designed bivalent aptamer-siRNA transcripts

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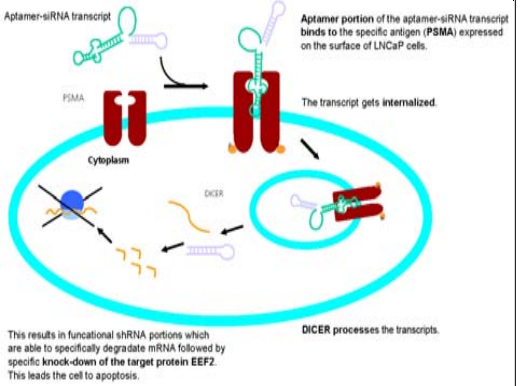
Introduction

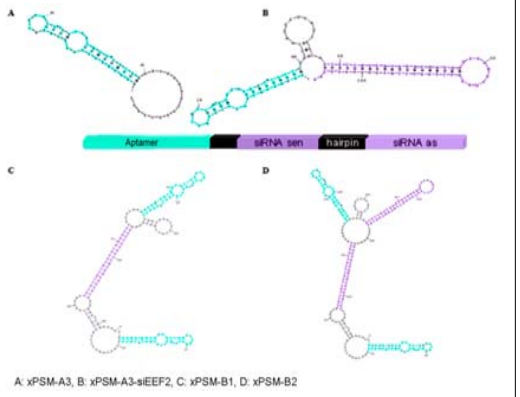
Eukaryotic Elongation Factor 2 (EEF2), a member of the GTPase superfamily, is an abundant cytoplasmic factor that is essential for protein synthesis. Inhibition of EEF2 by proteinous toxins, or by phosphorylation, is known to arrest protein synthesis and induce apoptosis, ultimately leading to cell death [Jorgensen, 2006, Biochem Soc Trans]. We chose *EEF2* as a candidate therapeutic target gene for selective inhibition by aptamer-targeted siRNA, a method analogous to the use of recombinant immunotoxins [Iglewski, 1977, Infect Immun]. Aptamers that bind selectively to tumor-associated antigens can be used as a nucleic acid-based method to deliver cytotoxic siRNAs to antigen-positive tumor cells [Hicke, 2001, J Biol Chem]. We used the anti-PSMA aptamer A10-3 for the construction of fused monocistronic xPSM-A3-siEEF2 transcripts [McNamara, 2006, Nat Biotechnol], [Chu, 2006, Nucleic Acids Res]. In order to enhance therapeutic efficacy of the monovalent anti-PSMA immuno-RNA transcript, we increased the valency of the construct by rational design. Two anti-PSMA aptamers were designed such that each binding sequence could fold independently into its active conformation. Fluorescently labeled monovalent and bivalent aptamer-siRNA transcripts bound selectively to PSMA-expressing tumor-cells and showed the same intensity of fluorescence. However, the bivalent transcripts showed significantly enhanced cell-cytotoxicity with IC50 values in the range of 80 to 150 nM assayed in a dose-dependent manner. We provide the first example of a tumor-targeted nucleic acid therapeutic approach based on the silencing of *EEF2*, whose efficacy and potency approaches that of protein-based drugs.

Idea/Concept

Knock down of EEF2 leads the cell to apoptosis



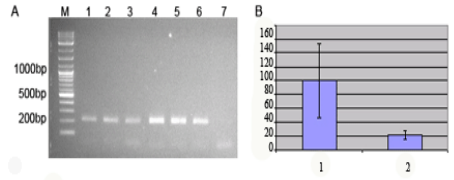
Design of all aptamer-siRNA transcripts



The RNA structure program MFOLD (http://bioweb.pasteur.fr/seganal/interfaces/mfold-simple.html) was used to predict the secondary structures of aptamer.
Ref.: PCT/EP05/112660.5 [EN] Immuno-RNA-Constructs Filed: Dec. 21 2005 (PCT)

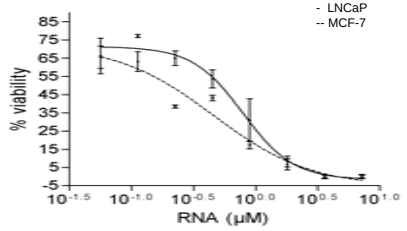
Results

1. Specific silencing with siRNA against target gene EEF2 via Real-time RT-PCR

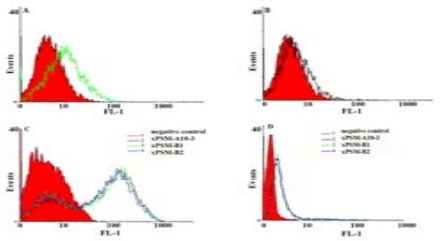


Cells were transfected with 200nmol siRNA and incubated for 8h by 37°C. Total RNA was isolated and cDNA synthesized using specific primers for EEF2 (Amplification product: 210bp). **A** RT-PCR was performed. Samples 1-3 show transfected cells. Sample 4-6 show untransfected cells. **B** A Real-time RT-PCR was performed using Sybr-Green. 80% knock-down of EEF2 mRNA expression could be determined.

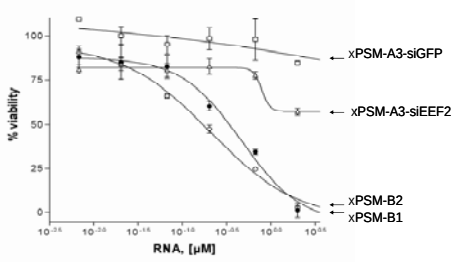
2. XTT-Viability Assay of LNCaP cells transfected with siRNA against EEF2



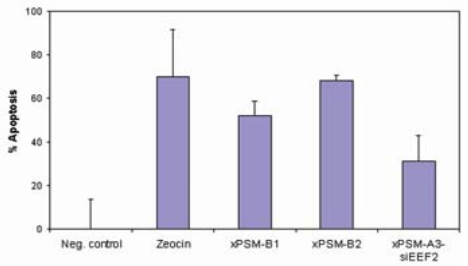
3. Binding analysis of xPSM-A10-3 and all aptamer -siRNA transcripts



4. XTT-Viability Assay on LNCaP incubated with xPSM-siEEF2,-siGFP,-B1 and B2



5. Apo-ONE Homogeneous Caspase-3/7: Induction of apoptosis on LNCaP cells



Specific binding of all aptamer-siRNA transcripts could be determine.

A The expression of PSMA was tested using the specific primary antibody against PSMA (anti-PSMA 3C6: Northwest Biotherapeutics) and secondary antibody GAM-FITC (Caltag Laboratories, Burlington, CA).
B To prove the specific binding the PSMA-negative cell line MCF-7 were used.
C RNA was directly FITC-labeled, purified and incubated with cells in 500µl 1xPBS for 30min. After 2 times washing cells were resuspended in 500µl 1xPBS and finally analysed by Flow Cytometry.
D All constructs show specific binding to LNCaP cells and only a slight unspecific background of FITC could be detected on MCF-7 cells.

Acknowledgements

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Conclusions

1. After 8h incubation of siEEF2 transfected LNCaP cells a **knock down of 80% mRNA expression** was determined.

2. Using the XTT-viability Assay we demonstrated **dose-dependent cytotoxicity on four transfected cell lines** (MCF-7, LNCaP, 293T and L540) and evaluated siEEF2 as a novel cell toxin.

3. All aptamer-siRNA **transcripts were successfully synthesized by *in vitro* transcription**, purified via a 8%Urea-SDS-Gel and resuspended in 1xPBS. After the refolding (2min, 94°C; 30min, 37°C) **all transcripts specifically bound to the antigen PSMA** expressed on LNCaP cells.

4. After 48h incubation with the **monovalent aptamer-siRNA transcript xPSM-A3-siEEF2 a maximal cytotoxicity of approx 40%** on LNCaP cells could be shown. The IC50 value obtained for xPSM-A3-siEEF2 (triangles) was 1.14 ± 0.2 µM. Both bivalent transcripts showed a dramatic increase in cytotoxic activity at the highest concentrations, e.g. **94% for xPSM-B1 and 96% for xPSM-B2**, respectively (p: 0.0012 ***). In addition, the IC50 values of both transcripts were less than 50% the value determined for xPSM-B1 and less than 20% of the value determined for xPSM-B2. No significant toxicity was induced in MCF-7 cells (data not shown).

5. **All aptamer-siRNA transcripts showed elevated caspase-3 activity** compared to the untreated cells. In addition, these data correlated well with the cytotoxicities observed in the proliferation assays discussed above.

6. Since a major safety concern surrounding the use of siRNA-based drugs is the induction of nonspecific inflammatory responses and subsequent cellular cytotoxicity, we performed a RT-PCR assay of five different genes known to be upregulated during the interferon response (*data not shown*). These results clearly indicate the **absence of a nonspecific interferon response in LNCaP cells following treatment with aptamer-siRNA transcripts** with different lengths and sequence compositions.

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