GENERATION OF RESPIRATORY SYNCYTIAL VIRUS SPECIFIC APTAMERS

PhD thesis

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1. Introduction

Aptamers are short, single stranded oligonucleotides of unique spatial conformations that bind their target molecules and rival antibodies in terms of selectivity and affinity. Moreover, they offer a variety of benefits over antibodies, e.g., their ease of production using *in vitro* selection (SELEX, Systematic Evolution of Ligands by EXponential enrichment) which can theoretically target any molecule, even toxic or non-immunogenic compounds. SELEX can be engineered so that aptamers recognize specific regions of the target molecule under a range of conditions tailored to the future application of the aptamer. Owing to their cost-efficient production by phosphoramidite chemistry, there is low batch-to-batch variation and their high stability in various environmental conditions. Their low immunogenicity makes them noteworthy drug candidates.

Even though there is a myriad of advantages of aptamers in comparison to antibodies, aptamers have yet to reach their translational potential for therapeutics and diagnostics. Essentially, two main factors hinder the practical application of aptamers. On one hand, practical applicability of the published aptamers is often not thoroughly assessed in a clinically relevant setting. On the other hand, challenging the well-established antibody-based methods requires a great capital investment and novel know-how that delays the commercialization of aptamer-based products.

Traditionally, SELEX requires the immobilization of the target molecule; thus, the partition of bound and unbound sequences can be accomplished. The use of magnetic beads for immobilization (FluMag-SELEX) ensures rapid and efficient separation of the target molecule binding oligonucleotides. The protein demand of SELEX usually could be met using protein overexpressing systems; however, most of the diagnostically significant virus proteins are posttranslationally modified which might not be accurately represented when the protein is expressed by cell systems. The so-called cell-SELEX, in which the selection is performed on whole cells, is also a widespread method. A variation of cell-SELEX may also be applied, in which the selection target is either a whole virus or a virus-like particle (VLP) that is immobilized either by affinity interaction or by covalently linking to a solid support. The most advantageous feature of this approach is that it’s possible to perform cell-SELEX without prior knowledge of the structures of the viral proteins.

The interactions between proteins and natural oligonucleotides are constrained due the limited chemical diversity of the nucleobases as opposed to the diversity of amino acids. Therefore, a series of unnatural modifications have been incorporated into aptamers in order to improve the efficiency of SELEX and increase the durability of aptamers in the prevailing environmental conditions. Mod-SELEX, that is, the selection of unnatural oligonucleotides, can be implemented via directly introducing the modified nucleotide during the synthesis of oligonucleotides or by the addition of "clickable" nucleotides and the post-synthetic functionalization by click-chemistry.

Introduction of hydrophobic and aromatic functional groups at positions oriented away from the hydrogen bonding sides of the nucleobases seems to have the most substantial effect on the outcome of aptamer selection. While the initial modified oligonucleotide library could be generated by solid-phase synthesis and by click chemistry, the amplification of target selective oligonucleotides in SELEX requires specific polymerases that are capable of incorporating the applied unnatural nucleotide. Several different commercial DNA polymerases have been studied to evaluate their ability to accept modified nucleotides as substrates of DNA polymerisation. These studies proved that family B polymerases are superior both in terms of effectivity and accepting a more extended repertoire of unnatural nucleotides to the members of family A. Although some of these enzymes were successfully applied to amplify base-modified aptamers, very few publications have illustrated the level of distortion of oligonucleotide libraries upon incorporation of non-natural nucleotides by PCR yet.

Even a successful SELEX results in a panel of aptamer candidates; therefore, pinpointing the most promising oligonucleotide with diverse approaches is crucial. Due to PCR bias, the most abundant sequence or motif holding aptamer in the final oligonucleotide pool is not necessarily the best binder; therefore, a combination of *in silico* and *in vitro* analysis of aptamers, preferably in clinically relevant matrices, is a superior approach to identify the lead aptamer.

Streptococcus pneumoniae, Haemophilus influenzae and Respiratory syncytial virus (RSV) are the major infectious agents responsible for severe lower respiratory tract infections (LRTI), which lead to significant morbidity and mortality, especially in childhood. Almost all children in their first couple of years of life undergo RSV

infection and in the majority of cases the disease resembles the symptoms of the common cold. Infection of both the elderly and immunosuppressed patients are also of great concern as RSV is most common viral pathogen that is transmitted nosocomially in both paediatric wards and nursing homes. Secondary bacterial infections occur less than 1% of RSV cases; on the other hand, co-infections with other respiratory viruses are more frequent and increase the risk of severe LRTIs leading to hospitalization, e.g. co-infection with Rhinovirus.

RSV has three envelope proteins, namely the fusion (F) glycoprotein, the attachment (G) glycoprotein and the small hydrophobic (SH) protein. Neutralizing antibodies target both the F and the G protein upon natural infection by RSV. Antigenic drift of F protein is less significant compared to those of G protein, and F protein induces higher levels of neutralizing antibodies *in vivo*. Consequently, F protein is a more favourable target for antiviral development.

The development of a safe and clinically effective vaccine against RSV is hampered by several factors including the early age of infection and the lack of adequate animal models for the pathogenesis of human RSV infection. Management of RSV infection is mainly supportive; currently, there is only one commercially available humanized monoclonal antibody treatment (palivizumab) for the prophylactic therapy of high-risk infants. Other human antibodies are in the pipeline, e.g., D25 that renders stronger neutralizing effect by stabilising the prefusion conformation of F protein. The prefusion form of F protein is a reasonable choice for a potent vaccine candidate. McLellan *et al.* provided one of the most stable mutants of F protein (RSV-A) using structure-guided stabilization, the so-called the DS-Cav1.

Rapid diagnosis of viruses is crucial for creating the most appropriate treatment plan. The traditionally applied methods, such as viral culturing, require not only highly skilled staff but are also time-consuming. Therefore, qPCR of the viral genome has become the golden standard for detection of viral infections, alongside with the use of surface protein selective antibodies for immunoassays, e.g., in rapid antigen-detection tests (RADT). Of note, antibody-based antigen detection tests are less suitable due to loss of reliability caused by, e.g., thermal inactivation in resource limited settings.

2. Objectives

The respiratory syncytial virus (RSV) is the most common cause of acute lower respiratory tract infection during infancy and late adulthood with a disease burden comparable to that of influenza. Appropriate clinical management and diagnostics of RSV is hindered by the fragility, immunogenicity and high cost of antibodies. The aim of the thesis work is to exploit the unique attributes of aptamers to provide RSV specific oligonucleotides of diagnostic and therapeutic potential.

In order to realize our goals, the following tasks were carried out:

1. Design of a SELEX procedure that circumvents the time-consuming target protein purification and may be generally applied for selection of virus specific aptamers;
2. Selection of highly specific and selective aptamers that are suitable for the determination and distinction of RSV from its most common co-infecting agent, HRV;
3. Characterization of aptamers using several approaches to determine target specificity and selectivity in complex biological matrices at clinically relevant virus titers;
4. Development of a suitable method for the generation of oligonucleotide libraries of modified nucleotides by PCR;
5. Devising a SELEX protocol for exploiting the modified oligonucleotide library to provide aptamers of therapeutic potential for RSV infection;
6. Identification of a lead aptamer candidate for virus infection inhibition assay.

3. Methods

3.1. Selection of aptamers for RSV and F protein of RSV

In order to obtain RSV selective oligonucleotides, SELEX was performed in five iterative cycles with gradually increasing selection pressure using inactivated whole RSV and HRV, as selection targets and counter targets, respectively. The selection buffer was complemented with BSA, Tween 20, and poly(dI-dC) acid in PBS. Prior to each selection step, RSV was *in situ* captured by palivizumab-coated paramagnetic beads to ensure proper separation of the RSV-bound and unbound aptamer fractions. Negative selection steps were also performed to ensure the exclusion of aptamers binding to either the protein A paramagnetic beads or the antibody used during the selection.

To generate F protein selective modified aptamers, seven selection steps with increasing selection pressure were applied to ensure the specificity of prefusion F (Ds-Cav1) selective aptamers. Target and counter-target molecules were immobilized on paramagnetic beads. As a counter-selection target, the prefusion form of F was utilized. The selection pressure was further increased by applying competitor molecules, e.g. L-tryptophan, mucin, BSA, Salmon DNA.

In both cases, PCR products from the last selection cycle were inserted into a cloning vector and the sequences of 96 colonies were determined by Sanger sequencing. *In silico* analysis was carried out using BioEdit, Microsoft Excel and MEME motif search.

3.2. Modified oligonucleotide library generation and high-throughput sequencing

Several family B DNA polymerases were tested for the ability to incorporate a modified nucleotide, TAdUTP, into oligonucleotides by PCR. The error rates of DNA polymerases were assessed using high-throughput sequencing (HTS) by Xenovea Kft. The modified aptamer library was produced by water-in-oil emulsion PCR using KOD XL DNA polymerase according to the manufacturers' protocols.

3.3. Fluorescence polarization (FP)

FP was leveraged to determine interactions of aptamers and their targets in free solution: first, characterization of consensus-motif holding aptamers that target RSV

were accomplished using FP. Then, to determine if palivizumab and aptamer H8 target the same epitope, a competition assay was set up. Finally, K_D of the most promising aptamer, H8, was determined by FP using its target molecule, the G protein of RSV. Aptamers labelled with fluorescent dyes were mixed with inactivated RSV or G protein and their fluorescence polarization was measured.

3.4. DotBlot

To assess the selectivity of aptamers, DotBlot was employed. A constant amount of FAM-labelled aptamers were mixed with decreasing amount of inactivated RSV or HRV particles, either in selection buffer or throat swab matrix. Throat swab was obtained from the *nasopharynx* and *oropharynx* of healthy volunteers. Samples were spiked with RSV and HRV at clinically relevant concentrations. After incubation, the mixtures were blotted onto nitrocellulose membrane using vacuum, and stringent washing steps were carried out before fluorescence signal detection.

3.5. AlphaScreen

Protein-aptamer interactions were analysed using AlphaScreen. RSV or protein F and biotinylated aptamers were immobilized onto Protein A Acceptor beads using palivizumab and Streptavidin Donor beads, respectively. The mixture was complemented with competitors, e.g., BSA, Salmon sperm DNA, or mucin. Following appropriate incubation, the specific interactions were determined by chemiluminescence measurement.

3.6. Quantitative PCR (qPCR)

To determine the stability of H8 in throat-swab matrix, the concentration change of H8 upon infusion with throat-swab matrix was measured by qPCR. Samples were taken from the throat-swab mixture containing 100 pM H8 aptamer at various time points up to 24 hours. The effect of TAdUTP incorporation on the T_m of oligonucleotides was also assessed by qPCR.

3.7. Microscale thermophoresis (MST)

The K_D of aptamers was determined by MST. A 16-fold serial 1:1 dilution was made of F proteins and mixed with a constant concentration of Cy5-labelled modified aptamers, then the IR induced thermophoresis was measured. Affinity MO program was used to fit a model to the obtained data points using Michaelis-Menten kinetics.

4. Results

4.1. Selection of aptamers for RSV

Inactivated, partially purified whole virus for isolation of RSV discriminating aptamers was applied in SELEX. Five selection steps with increasing selection pressure were applied to ensure the specificity aptamers. The combined use of high purity target and counter-selection ensured the enrichment of specific aptamers. *In silico* analysis was carried out using Microsoft Excel and MEME motif search. The former revealed two aptamers in duplicated copies (B5, F6 and D10, D12), the latter showed three pairs holding consensus sequence motifs (H8, E6; E11, E10; B10, F10).

FP analysis determined that all analysed aptamers bound to RSV and HRV in a concentration dependent manner. The changes in FP value are influenced by two factors, i.e., the binding of the aptamer to the target molecule and the conformation of the aptamer that could constrain the free rotation of the FAM label. As these factors vary from aptamer to aptamer, classification of aptamers solely based on the FP measurements must be handled cautiously. Notwithstanding, three aptamers, E10, B10 and H8 seem to possess higher RSV binding capacity and H8 is clearly superior in discriminating between RSV and HRV. B5, D10, H5 and H8 were chosen for further studies. These aptamers were assessed using DotBlot interaction assay that demonstrated that all of them are selective to RSV in selection buffer and even in throat swab matrix, since they produced a higher fluorescence signal with the RSV than HRV containing mixtures. The consensus motif holding aptamer, H8, provided the highest signal, while the lowest signals were produced by the sequences present in duplicates (B5 and B10).

Another approach, Amplified Luminescent Proximity Homogenous Assay (ALPHA) was also implemented to further assess the binding of aptamers to RSV. The measured fluorescence indicated the RSV binding of aptamers. The results of ALPHA measurement confirmed that all aptamers recognized RSV and the most promising aptamer candidate is the consensus motif holding oligonucleotide, H8. Additionally, an antibody-aptamer and an aptamer-aptamer sandwich assay was carried out by using the highest affinity aptamer, H8 in throat swab matrix. In theory, aptamer-based sandwich assays for virus detection can be implemented by applying the same aptamer as capture and recognition element of the sandwich assay since many copies of

the envelope proteins are present on the virus surface. Both experimental setups succeeded in detecting clinically relevant titers of RSV, producing high fluorescence signals and showed no signal increase when combined with HRV. Notably, the aptamer-based sandwich detection showed a greater sensitivity than that of antibody-aptamer assay for RSV detection.

In the next experiment, a FP-based competition assay was set up to determine if H8 and palivizumab recognize the same epitope. The fluorescently labelled H8 aptamer was mixed with two orders of magnitude higher concentration of anti-RSV antibody (palivizumab) and incubated with inactivated RSV. There was no evident difference in the fluorescence polarization between the H8 aptamer only samples and samples containing both antibody and aptamer. These data prove that the H8 aptamer and the palivizumab antibody did not compete for the same recognition site. In search for the target protein of H8, G protein of RSV was tested as a putative interacting partner by FP. There was clear FP change upon addition of the protein and a 30 nM K_D value was calculated for H8 aptamer and G protein complex by using a 1:1 binding model.

To assess the stability of H8, the concentration change of H8 upon infusion with throat-swab matrix was measured by qPCR. A slight change in concentration was detected during the first 2 hours of incubation, that is, the concentration of the H8 aptamers dropped to 90 from 100 pM. Following one day incubation, the concentration of H8 aptamer was calculated to be ~30 pM.

4.2. Selection of modified aptamers for F protein

A base-modified nucleotide, 5-indolyl-AA-dUTP (TAdUTP) was chosen to be incorporated into an oligonucleotide library by PCR. First, we set out to analyse eight family B thermostable DNA polymerases that were previously reported to accept modified nucleotides as substrates to see if they could incorporate TAdUTP instead of thymine into the amplicons. Amplification reactions were only successful in generating PCR products of expected size in two cases, i.e., with application of Vent(exo-) and KOD XL polymerases, which both lack 3’-5’ exonuclease activity. The effect of TAdUTP incorporation was demonstrated by melting temperature analysis (T_m) which has shown that the insertion of TAdUTP into oligonucleotides results in about 5–10 °C lower melting temperature compared to that of dTTP containing amplicons.

Generation of a random oligonucleotide library poses challenged due to its large sequence space. Water-in-oil emulsion PCR was exploited to amplify an aptamer library containing up to 10^{14} different sequences. PAGE analysis revealed that by-product formation greatly decreased when the micro-reactor forming emulsion PCR was applied to generate oligonucleotide libraries in reactions catalysed by Vent(exo-) or KOD XL.

High throughput sequencing analysis of the obtained PCR products indicated that the substitution of dTTP with TAdUTP had a minor effect on the error rate of DNA polymerisation, the fidelity of both enzymes was in concert with the previously published values and the sequence space of the modified oligonucleotide library was not distorted.

Mod-SELEX was carried out using both the immobilized prefusion and postfusion form of RSV F protein by applying the former as the target for the selection procedure and the latter as a counter-target molecule. In the 96 sequenced aptamers, the primary sequence analysis revealed that 10 aptamers were present in multiple copies, showing a definite enrichment of potential RSV selective modified aptamers. All aptamers were screened for their affinity towards the prefusion F protein using AlphaScreen. The majority of aptamers showed binding to the target molecule and 14 aptamers produced 10-50 times higher fluorescence signal upon binding the prefusion form of F protein in comparison to the no F protein and no antibody controls. Next, these aptamers were mixed with either the prefusion or the postfusion form at different concentrations to determine the F protein discriminating capacity of aptamers. All analysed aptamers produced an approx. 2 times higher relative fluorescence signal when mixed with the prefusion form than upon mixing with the postfusion form.

MST was applied to determine the K_D of the most promising aptamer candidates. The lowest K_D values were acquired in case of A2, H9 and D4 when complexed with the prefusion F protein. Significantly higher K_D values were obtained for the postfusion form using these aptamers. Aptamers producing the highest fluorescence signals in AlphaScreen measurements did not necessarily possess high affinity towards the prefusion F according to MST. This may be due to the inherent technical differences between the two methods; nevertheless, both methods provide valuable insight in terms of the practical applicability of aptamers. These aptamers may have an inhibitory effect on RSV infection that will be determined by cell-based virus neutralization assays.

5. Conclusions

The rationally designed SELEX method that applied monoclonal antibody immobilized whole inactivated RSV particles as targets of selection and counter-selection steps using a common co-infecting virus, HRV, resulted in selective aptamers with potentially diagnostic value.

Our approach generated an aptamer, which remains to be capable of detecting RSV and distinguishing it from HRV at clinically relevant concentrations even when challenged with complex biological matrices.

Our variation of cell-SELEX may be generally applied for other viruses thus accelerate the process of aptamer selection since there is no need to use purified viral protein since the target virus could be directly immobilized using the antibody.

To generate chemically modified oligonucleotide libraries containing 5-indolyl-AA-dUTP (TAdUTP), two DNA polymerases, Vent(exo-) and KOD XL can be applied. Substitution of dTTP with TAdUTP has no significant effect on their error rate of these polymerase and do not distort the sequence space of the aptamer library.

Amalgamation of water-in-oil emulsion PCR with the application of KOD XL thermostable polymerase could be the method of choice for the selection of chemically modified aptamers with amino acid-like properties since it evades excessive by-product formation during SELEX.

The presented data also highlights that the most abundant sequences do not necessarily possess high affinity towards the target molecule; therefore, the screening of aptamers is inevitable and further functional analysis of aptamer candidates by diverse approaches is required to pinpoint the most appropriate aptamers.

Devising and implementing a mod-SELEX protocol using a modified library possessing tryptophan-like side chains to generate F protein selective oligonucleotides results in high-affinity aptamers that may stabilize the F protein in its prefusion form, thereby inhibiting RSV infection.

Abbreviations: **ALPHA**: Amplified Luminescent Proximity Homogenous Assay; **BSA**: bovine serum albumin; **DNA**: deoxyribonucleic acid; **F protein**: fusion glycoprotein; **FP**: fluorescence polarization; **G protein**: attachment glycoprotein; **HRV**: human Rhinovirus; **HTS**: high-throughput sequencing; $\mathbf{K_D}$: dissociation constant; **kDa**: kilodalton; **LRTI**: lower respiratory tract infection; **MST**: Microscale Thermophoresis; **PCR**: polymerase chain reaction; **Poly(dI-dC)**: poly(deoxyinosinic-deoxycytidylic) acid; **qPCR**: quantitative polymerase chain reaction; **RADT**: rapid antigen detection test; **RSV**: Respiratory syncytial virus; **SELEX**: Systematic Evolution of Ligands by Exponential enrichment; **SOMAmer**: slow off-rate modified aptamer; **ssDNA**: single stranded deoxyribonucleic acid; **TAdUTP**: 5-indolyl-AA-dUTP; $\mathbf{T_m}$: melting temperature; **VLP**: virus-like particle

6. Bibliography of the candidate's publications

Publications related to the thesis:

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