DEVELOPMENT OF A PLATFORM FOR IDENTIFICATION OF mRNA-TARGETING SMALL MOLECULES - PROOF OF CONCEPT

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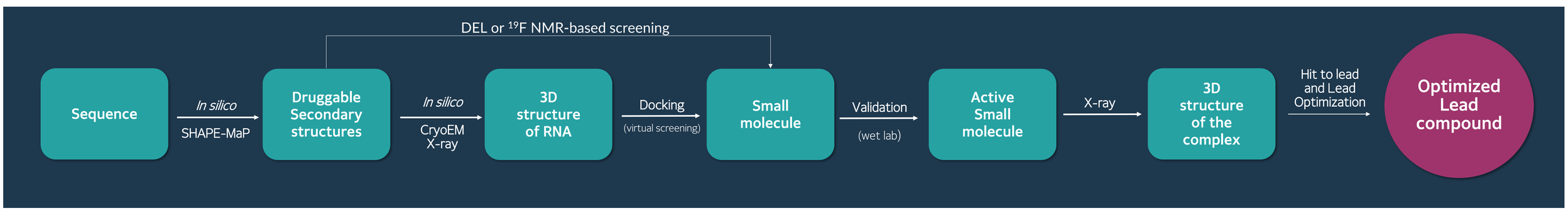
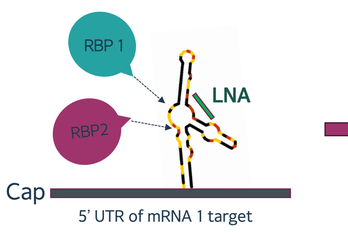
INTRODUCTION

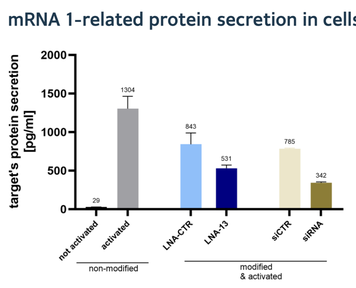
In recent years, the growing understanding of RNA's roles and functions has sparked increased interest in targeting RNA with small molecules as a therapeutic strategy. This challenging approach offers a promising alternative to attempts based on direct targeting of dysfunctional disease-related proteins, which were previously considered as undruggable. Consequently, it provides a new platform for development of therapeutics for many currently incurable diseases. In our mRNA-targeting small molecule (rSM) platform, we apply various methods to identify Hit molecules. Our goal is to discover and advance rSM for various mRNA targets related to unmet medical needs. The main steps of our approach include: **1. Prediction of Druggable RNA Region**: Assessing the stability, functionality, and druggability of selected mRNA regions using *in silico* methods, SHAPE-MaP, antisense oligonucleotides, or RNA-binding protein experiments; **2) High-Throughput Screening**: Utilizing methods such as DEL, ^{19}F NMR, or *in silico* techniques; **3. Interaction Verification**: Confirming small molecule interactions with mRNA using biophysical methods like MST, SPR, and AlphaScreen assay; **4. Activity Verification**: Testing activity in cell lines using ELISA, WB, and reporter assays. Hits identified through this process then enter the Med-Chem campaign to develop drug-like molecules.



DEL-BASED APPROACH – mRNA 1

Confirmation of Region Functionality

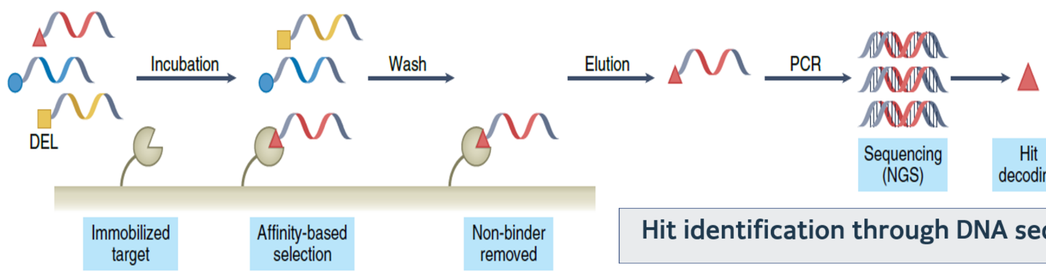




- The druggable region of mRNA 1 was selected based on:
  - *in silico* analysis and SHAPE-MaP results
  - ability to bind RBPs (confirmed by AlphaScreen)
- Locked nucleic acid (LNA) was designed for druggable region
  - 30-40% decrease in mRNA 1-related protein secretion was observed upon LNA13 treatment

In our mRNA 1 project, we used DNA-encoded library (DEL) screening. This technique involves a library of billions of compounds tagged with DNA fragments, which serve as structure decoding motifs. This library was screened against an immobilized target mRNA fragment. Compounds with no affinity to the RNA are washed away, while those showing interactions are eluted. The sequences of DNA-tags bearing interacting small molecules are then amplified using PCR, sequenced by next-generation sequencing (NGS) thereby revealing structures of potential Hit compounds. These Hits are resynthesized in off-DNA version and validated using experimental methods such as e.g. surface plasmon resonance (SPR), ultimately identifying Hit molecules for further development.

DEL-Based Screening Principle

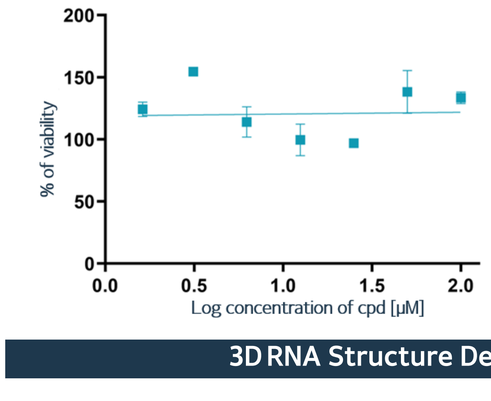


Hit identification through DNA sequencing

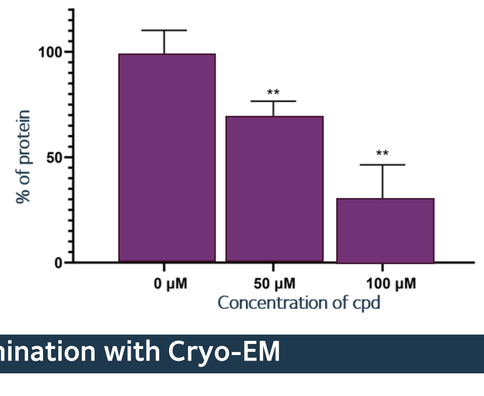
Analysis of on-DNA Hits from the standard DEL screening revealed that many potential binders might result from non-specific interactions between the DNA-tag sequence and the target mRNA 1 sequence (up to 9 complementary bases). To eliminate non-specific interactions, a subsequent screening campaign was conducted using RNA oligonucleotides specifically designed to block interactions between DNA tags and target mRNA. This approach led to the identification of 100 potential on-DNA Hits. From these, 20 structures were selected for off-DNA synthesis and SPR-based binding validation. Five compounds were identified as Hits and tested in cell-based assays, with at least one showing an influence on the mRNA 1-related protein level.

Decrease of mRNA 1-Related Protein Level Without Cytotoxic Effects

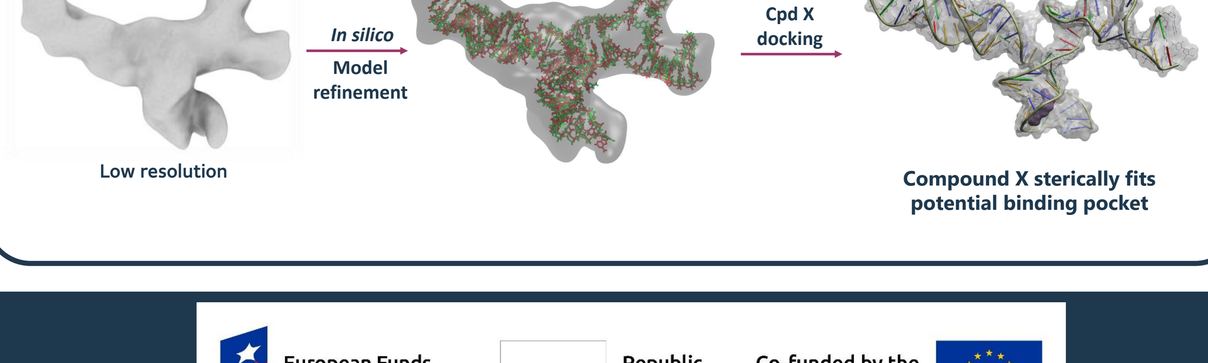
Viability of activated cells upon treatment with compound



Protein level in activated cells upon treatment with compound

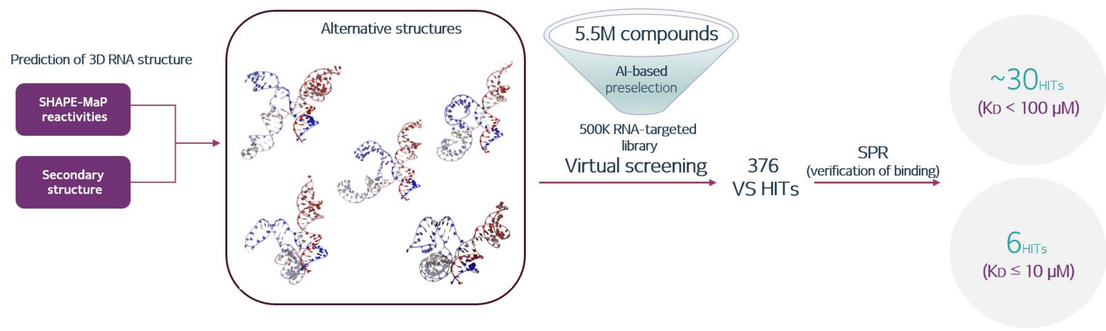


3D RNA Structure Determination with Cryo-EM



VS-BASED APPROACH – mRNA 2

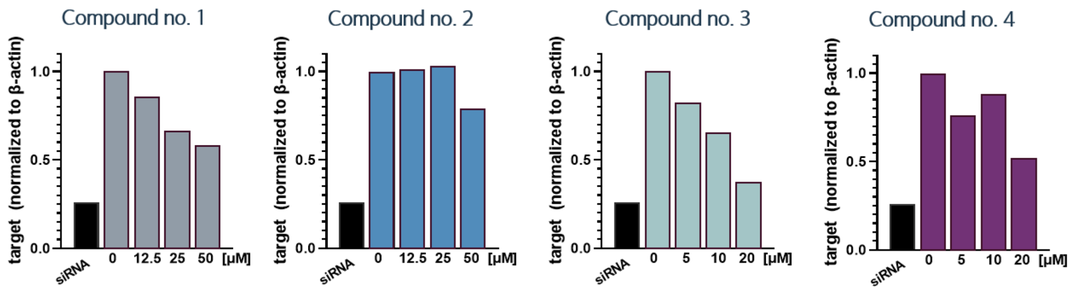
Our virtual screening-based approach involves utilisation of a 5.5M compound library as a starting point. The number of compounds is reduced through filtration using several AI-based filters, which eliminate compounds with unfavorable features while retaining those with known so-called RNA-liking motifs. In a parallel process, 3D structure of target mRNA was predicted with the aid of experimental data (SHAPE-MaP, Cryo-EM). Five stable alternative RNA structures were selected as virtual screening input. Then virtual screening was performed applying innovative SimRNA-L-aided technique, which resulted in 376 potential Hits that were purchased and screened using SPR. We identified ~30 Hits with Kd < 100 μM, including 6 compounds with Kd < 10 μM.



Preliminary Med-Chem analysis VS Hits' properties

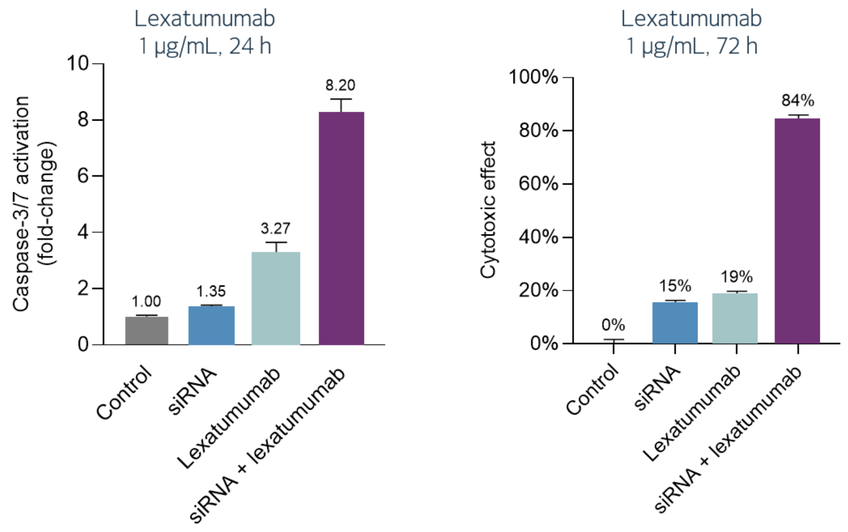
- No PAINs
- No aggregation
- No redox
- MW < 500 g/mol
- LogP < 5
- PSA < 100 Å^2
- No solubility issues in the assay
- Reasonable IP space
- No safety concerns (no hERG, no CYP inhibition, no genotoxicity) – *In silico* predictions
- No reactive functional groups or tricky moieties in the chemical structure

Cellular Tests (target protein level)



Treatment of cancer cells with some of the tested compounds leads to significant decrease in the mRNA 2-related protein. Cellular activities of compounds are consistent with the trend of determined Kd values.

mRNA 2 Knockdown Enhances Drug-Induced Apoptosis in Cancer Cells



Silencing of the mRNA 2-related gene expression via siRNA resulted in sensitization of cancer cells to apoptosis induced by lexatumumab (TRAIL agonist) which led to enhanced cytotoxic anticancer effect. Experiments with Hits are ongoing.

Conclusions & Perspectives

- Two different approaches have been developed to increase the success rate of our rSM platform
- HTS by DEL for mRNA 1 target and VS approach for mRNA 2 target generated Hits that decreased level of target proteins in cells
- Confirmation of the specificity of interactions of Hits with target mRNA is in progress (reporter assay, qPCR)
- Optimization of Hits, guided by Structure-Activity Relationship (SAR) studies, is ongoing

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FINANCIAL SUPPORT:
"Breakthrough discovery services for new small molecule medicines targeting mRNA for incurable diseases"

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