Phage display validation of lysosomal targeting receptors for the treatment of lung cancer

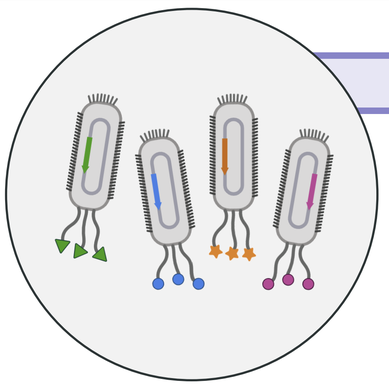
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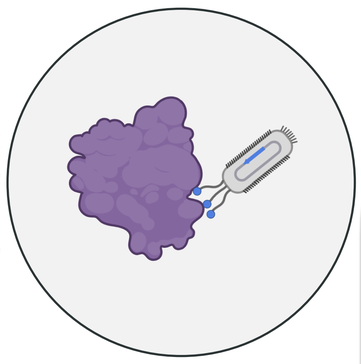


INTRODUCTION

Phage display libraries have been used to identify new peptide-binders for the treatment of non-small cell lung cancer (NSCLC). This type of cancer is one of the leading causes of cancer-related mortality and the second most frequently diagnosed cancer worldwide. With the current frontline therapies relying on tyrosine kinase inhibitors emergence of resistance after 12 to 24 months alternative therapeutic strategies such as targeted protein degradation offer a promising approach[1]. In this work, we are developing new lysosomal targeting chimeras (LYTACS)[2,3] exploiting the well-characterized anti VEGF antibody bevacizumab combined with a lysosome-targeting moiety which will be a novel peptide. Two different workflows have been applied to maximise the success in the identification of novel peptides with the potential to direct our protein of interest to the lysosome.

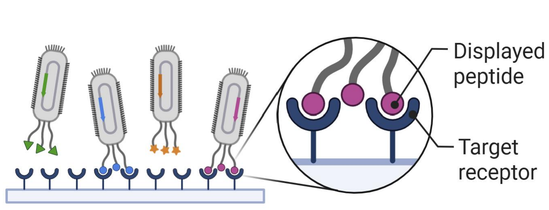
TARGET DIRECTED

With the aim to treat NSCLC we have identified two relevant receptors with the potential to direct the target to the lysosome. These two receptors have been immobilised and presented to a peptide phage display library with 12 amino acids in the protein III of the M13 phage:

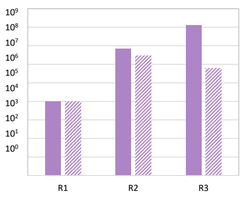


PHAGE LIBRARY

- M-13 phage
- 12-mer linear peptide library
- Diversity 10^{12}
- Fused to pIII: 3-5 copies per phage
- Leucine-Arginine sequence: trypsin sensitive for elution
- Amp resistance



1 PANNING



3 rounds of panning with increasing stringency and decreasing concentrations of immobilised protein were performed to obtain promising peptide candidates.

Figure 1. Library enrichment after each panning cycle for Receptor 1 (plain purple) and Receptor 2 (striped purple).

2 PHAGE ELISA

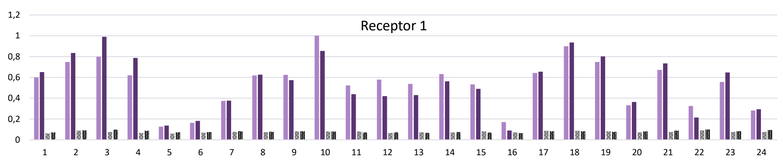


Figure 2. Screening of positive clones by phage ELISA from competitive elution at two concentrations (purple bars) for Receptor 1, non receptor coated wells (dotted bars) were used as negative control.

3 SANGER SEQUENCING AND PEPTIDE SYNTHESIS

Two new peptides were identified for receptor 1 and four for receptor 2. These peptides were synthesized including a GGG-motif for sortA conjugation to the mAb. Their biotinylated versions were also synthesized in order to determine their Kd.

RECEPTOR 1 — 2 PEPTIDES

RECEPTOR 2 — 4 PEPTIDES

Kd peptide B: 1.2 µM Kd peptide D: 2.6 µM

4 CELLULAR EXPERIMENTS

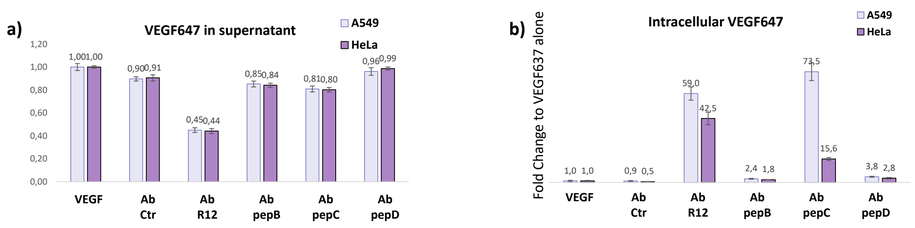


Figure 3. a) Quantification of VEGF647 supernatant and b) intracellular levels in A549 and HeLa treated for 6h with 50nM VEGF647 and 100nM Beva Enzymatic Sortase Conjugates.

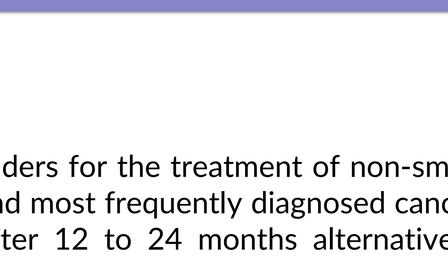
Further experiments did not conclusively confirm the receptor-dependent uptake of the Ab-peptide conjugates. The observed decrease in VEGF levels may be related to internalization processes, potentially involving non-specific precipitate engulfment, and additional studies will be required to clarify this mechanism.



TARGET AGNOSTIC

We tested lung carcinoma cells against the MIM-12 library in order to identify peptides which would bind selectively to the disease. We also introduced two rounds of negative selection to increase the selectivity of the process.

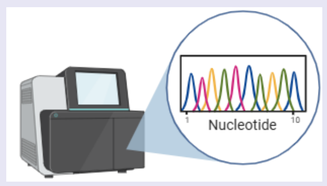
1 PANNING



Four rounds of panning using the MIM-12 library have been performed. Two negative selection steps using BEAS-2B bronquial epitelial cell lines were performed in R1 and R3 to guide the panning towards increased cellular selectivity. A round of degradation was also performed, using degradation inhibitors.

Figure 4. Library enrichment after each panning cycle for whole cell panning.

2 NGS SEQUENCING



We analysed R3 and R4 and observed an enrichment of two different peptide sequences. When the library was tested for cellular degradation, the two lead peptides were not among the most abundant examples, suggesting a that those peptides get degraded via lysosomal pathway.

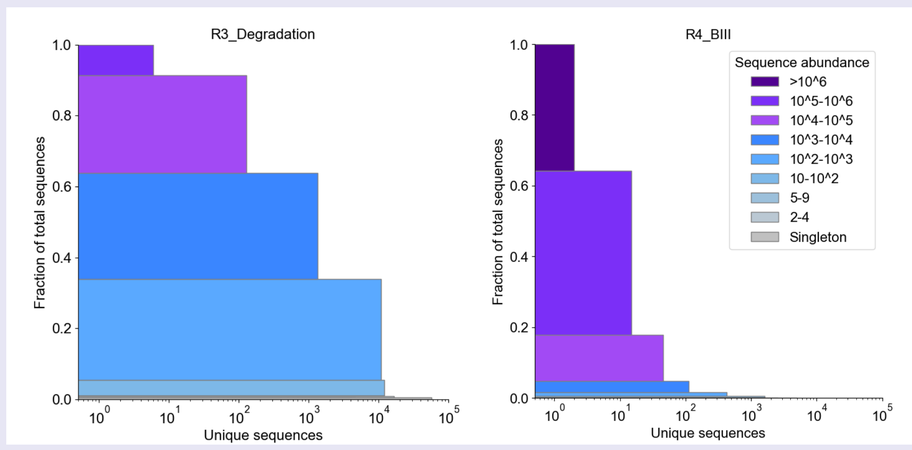


Figure 5. Diversity plots to visualize selection effects on sequence abundances per panning round as revealed by complete library sequencing by NGS. Each colored segment contains all sequences with the specific abundance. The segment height is equal to the fraction of total library, and the width corresponds to the number of unique sequences per segment.

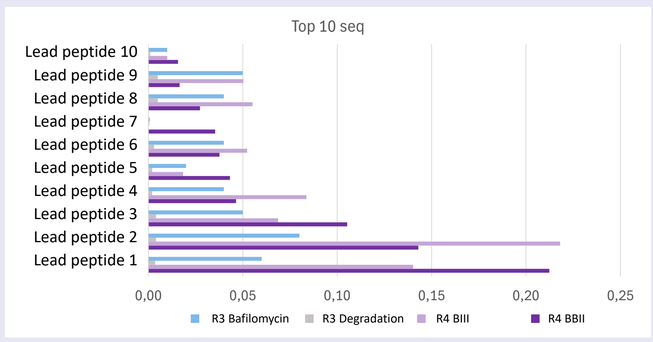


Figure 6. Relative abundance of the top 10 lead peptides in the four NGS-analysed rounds. R3 bafilomycin cells were evaluated under lysosomal inhibition, while R3 degradation assays were conducted in the absence of the inhibitor.

3 PEPTIDE SYNTHESIS AND CELLULAR VALIDATION

The next steps include the synthesis of a peptide series and their conjugation to fluorescent markers to evaluate lysosomal colocalization. Follow-up antibody conjugation and detailed cellular experiments will then assess the potential of these peptides to promote degradation of the protein of interest.

CONCLUSION

New peptide-binders have been identified for the selected lysosomal targeting receptors, the affinity of two of them has been successfully reported. However, their ligation to therapeutic antibodies suggests that the protein of interest may undergo non-specific internalization, and additional experiments will be required to confirm this possibility. Whole cell-panning experiments have highlighted two lead peptides which could potentially selectively degrade the target of interest in the desired cell lines. Subsequent chemical synthesis and fluorescent labelling will confirm the potential of the newly discovered peptides.

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