Selective Substrate Identification by Chemically Modified Phage Display

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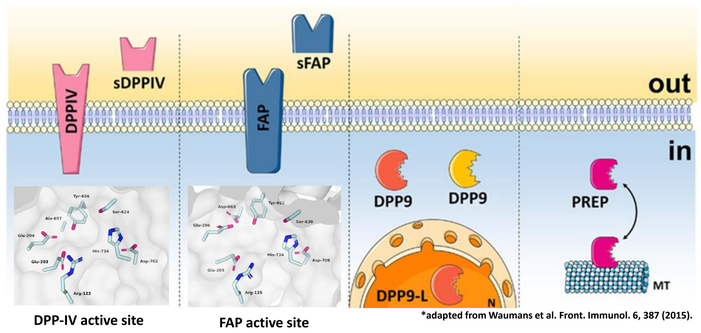
INTRODUCTION

Dipeptidyl Peptidase Family in Therapeutics

- Serine proteases with P1 specificity for Proline.
- FAP & DPPIV are type II membrane proteins but also the soluble form is present.
- Conserved catalytic triad: Ser, Asp, His -> **Cross reactivity of substrates**
- **CHALLENGE:**

  Achieve selectivity for 1 protease when developing substrates and inhibitors



*adapted from Waumans et al. Front. Immunol. 6, 387 (2015).

DPP-IV active site

- Glucose homeostasis
- Collagen metabolism

Validated target for type II diabetes

FAP active site

- Highly expressed in tissue remodeling
- Tumor growth and metastasis[1]

Promising target for inflammation & cancer[2]

- Largely unknown function
- Macrophage activation

- α-synuclein activation

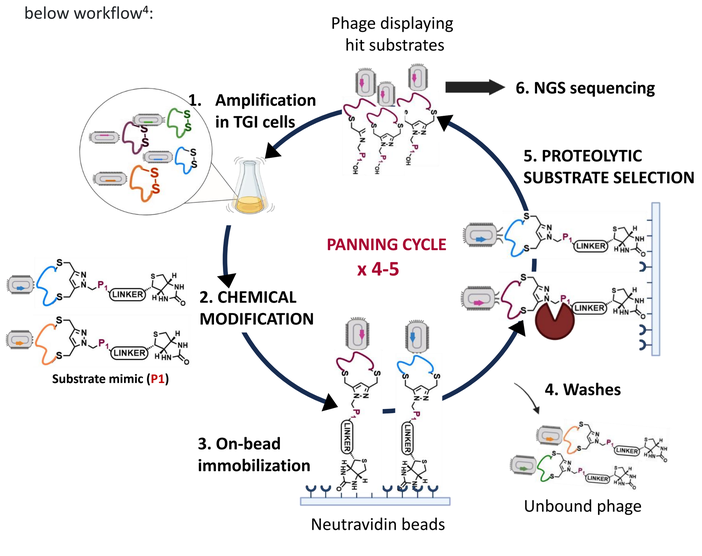
Targets of this work

GOAL

- Establish a phage display-based method to develop selective fluorogenic substrates in a fast unbiased way.
- Benchmark our method by identifying selective substrates for the therapeutically interesting serine proteases FAP and DPPIV.

Developing selective substrates[3] with phage display

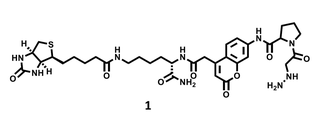
- Chemically modified phage display is a rapid and unbiased screening method to identify new classes of selective fluorogenic substrates from highly diverse pools of candidate molecules (up to 10^9 chemical diversity)
- We performed 4 rounds of panning for each target protease (FAP & DPPIV), following the below workflow[4]:

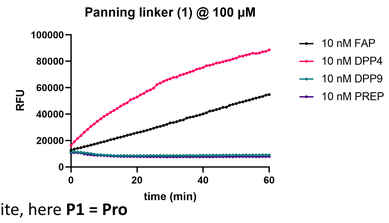


Thanks to the biotin incorporated via the linker addition, the phage library is immobilized on a solid support. This allows to perform stringent washes and, after incubation with the protease, to collect the selected substrate peptides in a straightforward way.

METHODS

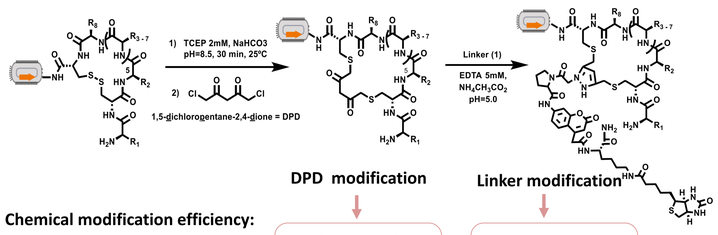
CHEMICAL MODIFICATION[5] STEP & LINKER DESIGN





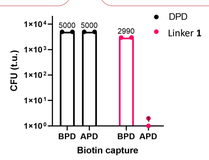
Panning linker (1) is designed to:

- enable the peptide cyclization
- feature a protease recognition site, P1 site, here **P1 = Pro**
- incorporate the selected fluorophore: ACC
- add a biotin to immobilize the library on affinity beads
- allow **bioorthogonal incorporation to the phage displayed peptide:**



Chemical modification efficiency:

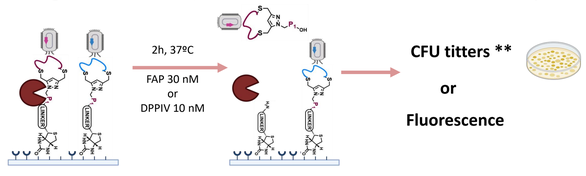
Titter supernatant before and after neutravidin pull down (**BPD** vs **APD**)



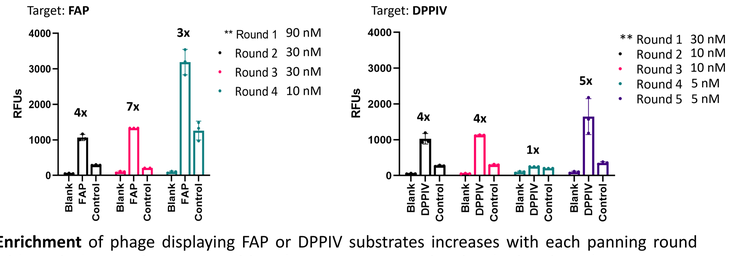
Linker **1** modification efficiency (%) = $\frac{\text{BPD-APD}}{\text{BPD}}$ = 99%

PROTEOLYTIC SUBSTRATE SELECTION

Traditionally the **enrichment** is measured by tittering TGI cells infected with released phage particles. Here we monitored it using ACC fluorescence, which is faster, less labor intensive and cheaper than the classic tittering.



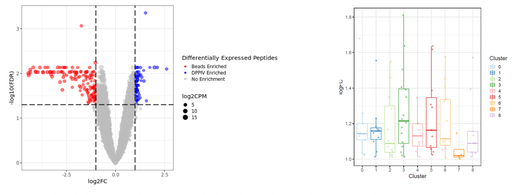
Library enrichment as monitored by ACC fluorescence:



Enrichment of phage displaying FAP or DPPIV substrates increases with each panning round with equivalent astringency conditions (enzyme concentration, incubation time...).

NEXT GENERATION SEQUENCING (NGS) & CYCLIC PEPTIDE HIT IDENTIFICATION

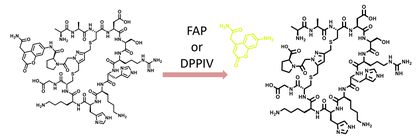
Peptide hits are identified via comparative data analysis (protease vs no protease) and a subsequent scoring system is used to locate the most promising substrates.



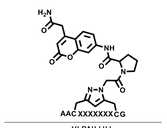
Data for DPPIV analysis. 254 peptides are 3-fold enriched comparing the protease vs no-protease libraries in the 5th panning round. Those can be classified in 9 clusters using the Gibbs clustering.

RESULTS

PEPTIDE HIT VALIDATION



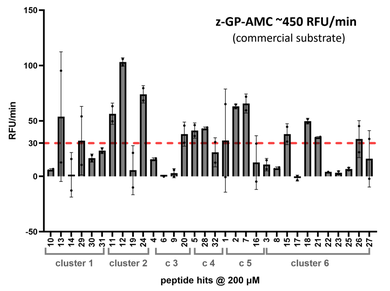
30 – 40 cyclic peptides were synthesized and tested, based on the panning output. **Left.** Activity assay scheme. **Right.** General structure of the peptide hits of this work & few of the FAP cluster 1 and 2 hits.



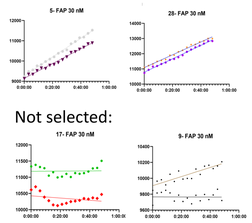
YLRNLHH
YLANLHH
YVPNLHH
YLPNVHH
YLPNLHD
YLPNLDH
SLRINSV
SLPINSL
.....

FAP CYCLIC PEPTIDES

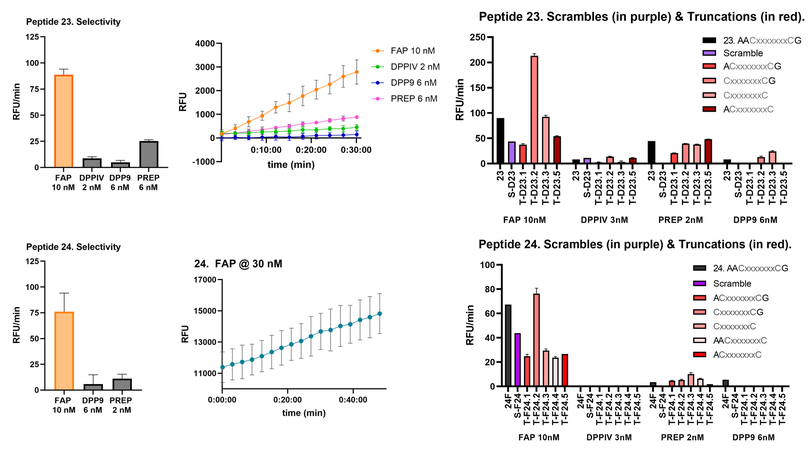
The 30 most promising FAP hits were synthesized and tested crude for their ability to be FAP substrates. Those with a slope over 30 RFU/min were purified and further tested for selectivity against a protease panel (data not shown).



Right panel shows examples of time vs fluorescence curves of tested peptides.

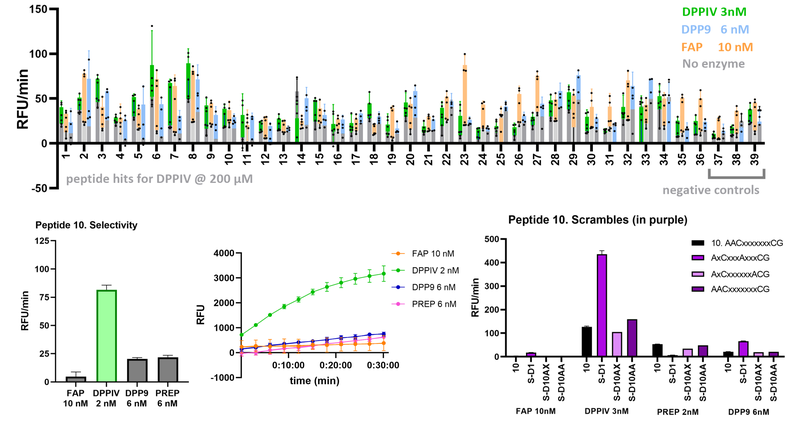


The peptides that showed selectivity for FAP over DPPIV, DPP9 and PREP were selected, total of 4 peptides, and the sequence specificity of their activity was tested by preparing a scrambled version (data partially shown). Two peptide hits **23** and **24** showed sequence specificity and we initiated the optimization of their activity by preparing structurally truncated versions. Data shown below:



DPPIV CYCLIC PEPTIDES

We prepared 36 hits + 3 control cyclic peptides and screened them for selectivity (see below), with 9 selected to further test (data not shown). Peptide 10 resulted in the best hit (see bottom panels).



CONCLUSIONS & OUTLOOK

- We have developed a chemically modified phage display methodology to prepare substrates that are selective even for closely related proteases.
- Currently we are working on optimizing the activity and selectivity of our best peptide hits for FAP (peptides **23** and **24**) and DPPIV (peptide **10**).

REFERENCES & ACKNOWLEDGEMENTS

1. Šimková A, Busek P, Sedo A, Konvalinka J. Molecular recognition of fibroblast activation protein for diagnostic and therapeutic applications. Biochim. Biophys. Acta Proteins Proteomics. 2020;1868:14040929. doi: 10.1016/j.bbapap.2020.140409.
2. Hamson, E. J., Keane, F. M., Tholen, S., Schilling, O. & Gorrell, M. D. Understanding fibroblast activation protein (FAP): Substrates, activities, expression and targeting for cancer therapy. Proteom - Clin Appl 8, 454–463 (2014).
3. Kasperkiewicz, P.; Poreba, M.; Groborz, K.; Drag, M. Emerging challenges in the design of selective substrates, inhibitors and activity-based probes for indistinguishable proteases. FEBS J. 2017, 284, 1518– 1539, DOI: 10.1111/febs.14001
4. Chen, S.; Lovell, S.; Lee, S.; Fellner, M.; Mace, P. D.; Bogyo, M. Identification of highly selective covalent inhibitors by phage display. *Nat. Biotechnol.* 2020, DOI: 10.1038/s41587-020-0733-7
5. Ekanayake, A. I.; Sobze, L.; Kelich, P.; Youk, J.; Bennett, N. J.; Mukherjee, R.; Bhardwaj, A.; Wuest, F.; Vukovic, L.; Derda, R. Genetically Encoded Fragment-Based Discovery from Phage-Displayed Macrocyclic. J. Am. Chem. Soc. 2021, 143, 5497– 5507, DOI: 10.1021/jacs.1c01186

*** We thank Jef Callebaut & Matilde Bertolini for providing assistance in this work*

M.B.-X. thanks the support from the Research Foundation Flanders (Fonds Wetenschappelijk Onderzoek) for the following financial support: postdoctoral fellowship **12Y0720N** *(2019-2022) and travel grant* **165772**

The project that gave rise to these results received the support of a fellowship from "la Caixa" Foundation (ID 100010434) to M.B.-X. The fellowship code is ***LCF/BQ/PI22/11910012****.*