Target-Assisted Evolution of Foldameric Ligands Against the p300/HIF-1α Interaction

Final Report

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Introduction

The research programme focused on the development of peptidomimetic ligands targeting the p300/HIF-1α interaction, which plays a key role in the hypoxic response and a potential target in cancer therapy.[1,2] p300/HIF-1α is a challenging protein-protein interaction (PPI) to inhibit using conventional drug development strategies due to the large surface area of its interactions and its plasticity to bind different intrinsically disordered ligands.[3–5] Our goal was to design inhibitors using three different methodologies: i) sequence-based approach of peptidomimetic inhibitors based on native ligands; ii) screen and assembly of multivalent ligands from short helical foldamers using dynamic covalent chemistry and iii) develop an *in vitro* evolution method that can help the selection and identification of nonnatural ligands.

Results

1. *Sequence-based design of CITED2 analogues shed light on the molecular mechanism of allosteric regulation of p300/HIF-1α*

CITED2, the negative feedback regulator of the p300/HIF-1α interaction is an effective inhibitor and works as a unidirectional molecular switch, despite the fact that CITED2 and HIF-1α display similar affinities to p300.[6,7] Our goal was to better understand the inhibition mechanism and to take advantage of the functionality of CITED2 in the design of peptidomimetic inhibitors. First, we investigated the affinity and competition properties of different HIF-1α and CITED2 sequences using isothermal titration calorimetry (ITC). The ITC experiments revealed 2-4 fold higher affinity for the CITED2 constructs over HIF-1α, which in part explains their higher inhibitory potency. The thermodynamic signature of the interactions revealed favourable enthalpy with unfavourable entropic contributions, which is in line with a folding-upon-binding mechanism (Fig. 1a-b). Competition ITC experiments revealed that the ligands bind with negative cooperativity, with a mechanism depending on the sequence length of the competitor CITED2. For effective inhibition a ternary complex is required, which results in an allosteric change that facilitates the displacement of HIF-1α and the structure is locked into the CITED2-bound conformation. As a model for the ternary intermediate, we constructed a CITED2-HIF-1α hybrid and determined its structure in complex with p300 using X-ray crystallography (Fig. 1c). These results were published in *RSC Chemical Biology* (Hóbor, F., Hegedüs, Z., Ibarra, A.A., Petrovicz, V.L., Bartlett, G.J., Sessions, R.B., Wilson, A.J. and Edwards, T.A., 2022 *RSC Chem. Biol.*, *3*(5), 592.) and a crystal structure of the CITED2/HIF-1α hybrid peptide in complex with p300 deposited to the PDB (7QGS).

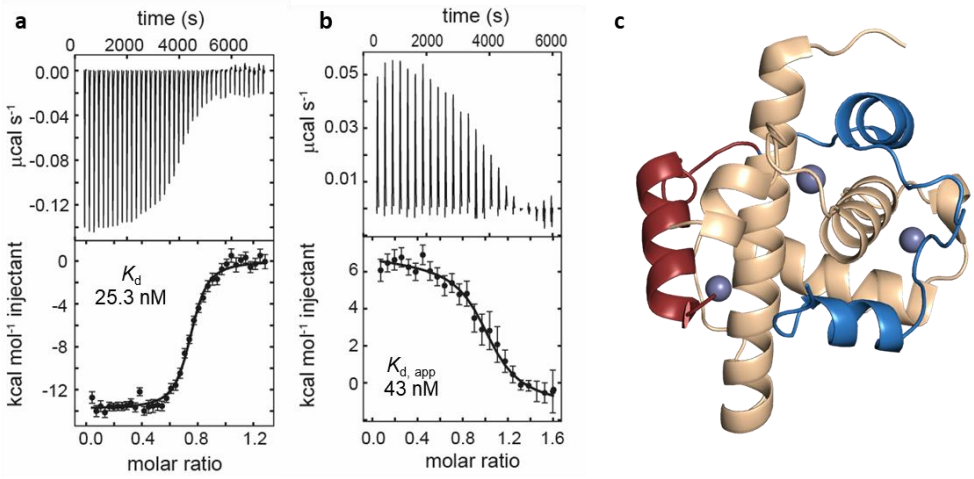

Figure 1. *Characterisation of CITED2 binding and competition using ITC. a) CITED2 titrated to p300. b) CITED2 titrated to the p300-HIF-1α complex. c) Crystal structure of the CITED2-HIF-1α hybrid bound to p300 (PDB: 1QGS, p300 in wheat, residues corresponding to CITED2 are coloured red and residues corresponding to HIF-1α are coloured blue, Speres indicated Zn atoms.)*

CITED2 consists of different binding motifs that act cooperatively to exert its allosteric function and render the competition irreversible.[8] To better understand which binding motifs of CITED2 are responsible for effective inhibition our goal was to modify the CITED2 sequence in a way that maintains its native properties but induces different structural changes upon binding to p300. We achieved this through incorporation of motif-by-motif β-amino acids and synthesized six 40mer CITED2 analogues. We used ITC and fluorescence anisotropy to characterise the modification effect on direct binding to p300 and competition efficiency with HIF-1α. These replacements enabled us to produce CITED2 analogues that have native-like properties (maintained intrinsic disorder, original side-chains and charge), and most of the modified sequences retained an enthalpy-driven high affinity binding to p300. However, their efficiency in competing with HIF-1α/p300 was strongly dependent on the modification site. To elucidate the effect of the β-amino acid modifications on the structure of p300, we recorded ^{1}H-^{15}N HSQC spectra using ^{15}N-^{13}C labelled $p300_{330\text{-}424}$ in complex with all peptides. The weighted average chemical shift differences were calculated relative to the native p300–CITED2 complex for amide and methyl resonances. These data revealed which binding motifs induce local or more distributed changes in the structure of p300. The structural changes could be related to the efficiency of the competition of a CITED2 variant, which clarified the role of CITED2 binding motifs in rendering the interaction unidirectional.

In conjunction with previous studies, our data provide a more detailed picture of molecular mechanism of the competition between CITED2 and HIF-1α. We have shown that $CITED2_{216\text{-}256}$ is the shortest sequence reported so far that contains all the necessary binding motifs that render the competition irreversible. The key in our strategy was to maintain native-like properties of CITED2, for which β-amino acid replacements proved invaluable. The combination of modifications that do not abrogate the allosteric function of CITED2 can lead to stable and biologically active variants of CITED2. We believe that this approach can generally be applied to the investigation of interactions mediated by intrinsically disordered proteins with complex mechanisms. A manuscript discussing these results is under submission.

2. Short foldameric sequences recognize the p300 TAZ1 surface with low affinity

Short, helical foldamers can act as local protein surface mimetics[9], thus can be used as building blocks of a multivalent ligand targeting solvent-exposed protein surfaces. The first step was to identify foldameric fragments that recognise the surface of the p300 TAZ1 domain. Libraries of foldamers that have the propensity to fold into a helical structure were synthesised. L1 and L2 fold into a H14 helix projecting the variable side-chains on the same face of the helix; L3 synthesized with an $\alpha\alpha\beta$ pattern and L4 with a $\alpha\alpha\alpha\beta\alpha\alpha\beta$ pattern (Fig. 2). The binding of the library members was tested using a pull-down assay and the bound fragments were quantitated using LC-MS. We detected low affinity binding (expected in the low mM range) with the most successful fragments containing phenylalanine, tryptophane and leucine side-chains, which are common hot-spot residues in protein-protein interactions. Differences between library structures indicated that the secondary structure has a major influence on the correct projection of the proteinogenic side-chains.

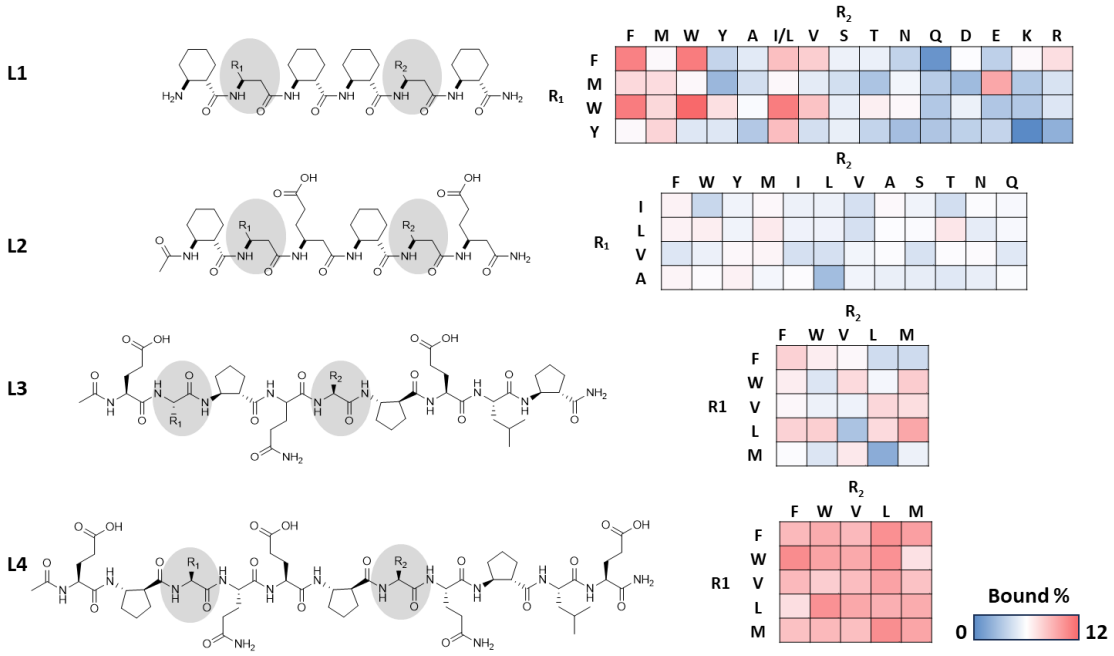

Figure 2. *Structures of the different foldamer libraries with the results of the pull-down experiments (right). The heatmap shows the percent bound calculated based on LCMS peak integration relative to a control sample. The amino acid side chains used in the variable positions (R_1 and R_2, highlighted grey) are indicated with single letter codes on the heatmaps.*

Since the identified fragments displayed low affinity to the protein, we modified our approach to combine these fragments with the native CITED2 sequence, by replacing sequence parts with foldamers, creating hybrid structures. For this, we set out to use dynamic covalent libraries (DCL)[10] in the presence of p300 as a template to amplify the best binders. To construct a DCL, a reversible chemical reaction is needed that is compatible with the target protein and reaches equilibrium in a short time. Disulphide exchange can be used with foldamers as building blocks in protein templated reactions[11], however, in case of p300 reductive media is needed to maintain its activity, which is incompatible with disulphide formation. As an alternative, we optimized conditions for thioester exchange[12] using fragments of CITED2. The peptides were prepared with thiol and thioester functionalities[13] in order to construct a full-length thioester analogue of CITED2 in which the sequence parts can be replaced by thiol-functionalised foldamers. To optimize conditions for the exchange, time-

course experiments were performed to follow the reaction between the peptide-mercaptopropionic acid thioesters and glutathione under reductive conditions. These experiments showed that the exchange is fast and reaches equilibrium in a couple of hours, and that thioester hydrolysis is negligible, which makes it suitable for use in our DCL experiments. However, when constructing the thioester analogue of CITED2, we observed an unexpected ring-closed thioester product, which we could not eliminate so far, which prevented us to perform the DCL experiments in the presence of the protein template.

3. *Ligatable foldamer-oligonucleotide conjugates as building blocks of an in vitro evolution method*

DNA templated synthesis is a powerful tool to prepare nonnatural ligands capable of *in vitro* evolution.[14] Our goal was to develop the steps required for an *in vitro* evolution method for non-natural foldamers which involved the following: i) preparation of a library of DNA-foldamer hybrids, ii) affinity-based selection in the presence of the target protein, iii) amplification of DNA code using PCR and ligand identification using sequencing, iv) reuse DNA to prepare ligands for the next selection round, allowing the *in vitro* evolution cycle.

To prepare foldamer-DNA conjugates, we employed thiol-maleimide ligation, using 5'thiol-modified oligonucleotides (synthesis by Györgyi Ferenc, BRC) and functionalised maleimide foldamers in solution. Protocols for solution phase conjugation, purification, and LC-MS analysis of the conjugates have been established. We showed that conjugate synthesis can be performed on solid phase, the conjugated foldamer withstands oligonucleotide cleavage using concentrated ammonia. Using native acrylamide gel electrophoresis, we were able to show that the foldamer-oligonucleotide conjugates hybridise to a corresponding DNA template.

To increase the stability and flexibility of the foldamer bearing DNA, our next goal was to prepare ligatable conjugates, therefore we modified our system and functionalised the oligonucleotides in the middle of the sequence. For this, a thiol-containing phosphoramidite synthesis was a prerequisite (synthesis by Zoltán Kupihár, SZTE). This allowed us to perform the ligation of these conjugates in the presence of a DNA template. After strand separation, a single-stranded DNA bearing multiple functionalities could be isolated, and the DNA code could be amplified by PCR. The methodology of nucleotide synthesis, peptide-conjugation on solid or in solution phase, and the templated ligation procedure has been published in *Pharmaceutics*, 15 (1) 248., **2023.**

Next, we tried to optimise the affinity-based selection step using a well-known model protein, Calmodulin. Our results indicated however, that the DNA interferes with protein binding, probably due to long range electrostatic repulsion, which is not uncommon using DNA encoded systems and may require further optimization.

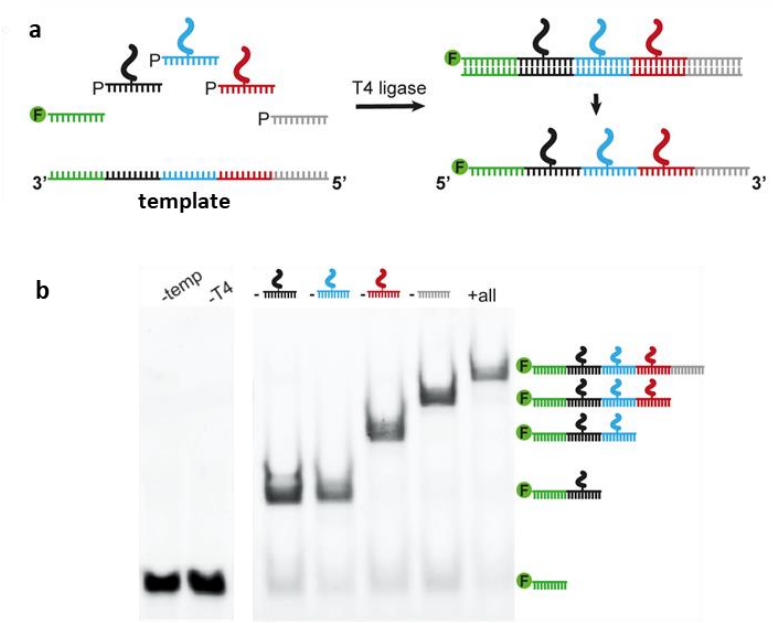

Figure 3. *a) Scheme showing the templated ligation process: peptide-oligonucleotide conjugates are mixed with a DNA template followed by ligation using T4 ligase. b) Denaturing acrylamide gel showing the ligation products excluding one reagent at a time.*

Summary

We have explored different methodologies to target the p300/HIF-1α interaction using nonnatural ligands. Our most successful strategy was the backbone modification of the competitor peptide CITED2, which allowed us to better understand the underlying molecular mechanism of the competition and resulted in potentially active inhibitors. Using short foldameric sequences we selected fragments that bind to p300 with low affinity and optimized a thioester exchange-based method that can be used to incorporate these fragments into native ligands. We successfully made steps toward an *in vitro* evolution method that can help the selection and identification of non-natural protein-binding ligands. We established steps for foldamer-oligonucleotide synthesis and ligation in presence of a DNA template and PCR amplification. Although the methodologies to create multivalent ligands require further improvement, our results provide key steps toward these methods. Overall, these findings improve our understanding of the p300/HIF-1α interaction and provide potential inhibitors.

During the time period of the programme, 3 poster and 4 oral presentations were presented (by Zsófia Hegedüs and Vencel Petrovicz, Ph.D. student) at different national and international conferences (EMBO Chemical Biology Symposium 2022; Peptidkémiai Munkabizottsági ülés 2022, 2023.; László Zechmeister lecture competition 2022, 2023, Tavaszi Szél Konferencia 2023).

During the time period of the programme, 2 articles have been published, with a third manuscript currently under submission.

Hobor, F.; **Hegedüs, Z.*;** Ibarra, A. A.; Petrovicz, V.; Bartlett, G.; Sessions, R. B.; Wilson, A.*; Edwards, T. A.* Understanding P300-Transcription Factor Interactions Using Sequence Variation and Hybridization. *RSC Chem. Biol.* **2022**, 3, 546–550. https://doi.org/10.1039/d2cb00026a.

Kupihár, Z.; Ferenc, G.; Petrovicz, V. L.; Fáy, V. R.; Kovács, L.; Martinek, T. A.*; **Hegedüs, Z***. Improved Metal-Free Approach for the Synthesis of Protected Thiol Containing Thymidine Nucleoside Phosphoramidite and Its Application for the Synthesis of Ligatable Oligonucleotide Conjugates. *Pharmaceutics* **2023**, 15 (1), 248. https://doi.org/10.3390/pharmaceutics15010248.

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