# Peptide Inhibitor of RED-SMU1 Interaction as a new anti-infectious agent

**Objective.** The aim of the project is to design new small bioactive peptides that target the human splicing factor complex RED-SMU1, for innovative anti-influenza therapies. For this purpose, a newly developed dynamic combinatorial chemistry (DCC) approach will be used as a tool to simultaneously build and screen a dynamic chemical library (DCL) of conformationally constrained protein mimetics, by grafting amino acid side-chains on a well-ordered cyclopeptide scaffold.

**Scientific Context.** The increasing incidence of drug-resistant pathogens calls for the development of novel therapeutic strategies. In recent years, the concept of host-directed therapies, which target host determinants essential for the infectious life cycle and/or pathogenesis rather than pathogen components, has been rapidly expanding.(1) This strategy is safer than traditional methods and provides broad-spectrum efficacy and reduced antiviral resistance. Following this approach, Partner 2 (**P2, Nadia Naffakh and coworkers, IP**) has recently characterized the structure of a human splicing factor, RED-SMU1, a protein-protein interaction (PPI) essential for the influenza A virus (IAV) life cycle, and investigated this cellular factor as a potential target for therapy.(2) They have shown that disrupting this protein-protein complex allows decreasing the level of endogenous RED-SMU1 level and inhibiting viral mRNA splicing and multiplication while preserving cell viability, representing thus a potential strategy for the development of new antiviral therapy. However, because of their large, shallow, and hydrophobic complementary interfaces, the design of compounds that specifically disrupt PPIs is quite challenging and intracellular PPIs have historically been described as undruggable.(3) Recent advances have shown that peptides can overpass these limits.(3) Thanks to their intermediate size between that of traditional small drug-like compounds and larger biologics, they combine advantages of the two drug's classes, with physicochemical properties resembling those of the former, together with the high potency and specificity of the later. Particularly, cyclic peptides in which either a head-to-tail or a side-chain-to-side-chain covalent bond is introduced, have received tremendous interest because they exhibit better drug-like pharmacological properties than their linear counterparts.(4) Indeed, their constrained 3-dimensional (3D) structure provides them with restricted conformational freedom allowing a preorganization of their backbone, that (i) reduces the entropic penalty upon binding, resulting in higher affinities to their partner (ii) limits the chances to fit to another partner, decreasing thus non-specific binding and off-target side-effect (iii) decreases their recognition by proteases, leading to the improvement of their *in vivo* half-life. Moreover, cyclic backbones exhibit most often a better membrane permeability, which makes them suitable candidates for challenging intracellular targets.(5) Not surprisingly, an increasing number of cyclic peptides has entered clinical trials over the last few years.(6)

The X-ray structure of the RED-SMU1 complex has been solved,(2) and reveals two interacting domains at the interface, one of them involving an α-helix of RED that project 4 hydrophobic side-chains residues located at one face of the helix into a hydrophobic groove of SMU1 (Figure 1). In the context of a collaboration between **Partner 1** (**P1, Roba Moumné & coworkers, CPCV, UMR8228, SU**) and **P2**, we have recently designed a conformationally constrained α-helical cyclic 15-mer peptide that recapitulates the native binding motif of the RED$^{[206–222]}$ sequence. For this purpose, helped by computer model we have introduced covalent constraints, between two pairs of side-chains that lies in close proximity upon α-helical folding, leading to a cyclic stapled peptide with a stabilized overall 3D structure and nM affinity for SMU1 (measured by Bio-layer interferometry, BLI). Moreover, the peptide present improved pharmacological properties favorable for its *in vivo* use, particularly cell membrane permeability and stability toward proteolytic degradation (*unpublished results*). The ability to inhibit the RED-SMU1 complex and IAV replication in a cell-based assay is currently evaluated. Although this compound represent the first peptide inhibitor for this interaction and has very promising feature, its poor solubility, moderate resistance to proteases and large size, makes it non-ideal as drug candidates and further optimisation would be requires for therapeutic development. In general, optimization of bioactive peptides is done through parallel or combinatorial synthesis and screening of libraries and is often the most challenging and time-consuming step of the process, particularly when considering peptides with non-canonical amino acids and main chain features.(7) In this context, **P1** has recently introduced an original approach based on DCC,(8) to graft amino acids-like side-chains on a small 3D peptide scaffold described in **Fig. 2**.(9)

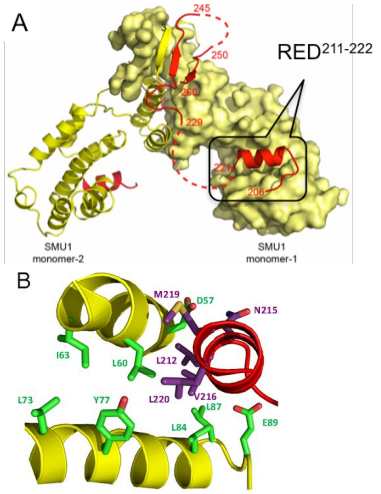


**Figure 1.** (A) X-Ray structure of the recombinant human RED (red)-SMU1 *N*-ter (yellow) complex (B) Zoom showing the side-chains involved in the interaction (green for SMU1 and purple for RED).

A cyclic peptide scaffold (**Sca**) of defined 3D structure is covered on one face by chemical functional groups prone to react in reversible processes, in physiological conditions. A series of building blocks (**BBs**) bearing amino acids side-chains are mixed, allowing the side-chains exchange between **Sca** and **BBs**, leading to a dynamic combinatorial library of peptides (**DCL**) presenting multiple side-chain combinations. Such a library is *stimuli*-responsive according to Le Chatelier's principle: upon the addition of a biological target, compounds able to bind this target shift the equilibrium to favor their own formation at the expense of the others, in a target-directed DCC approach (td-DCC).[10] After freezing the reaction mixture and target's dissociation, comparison of the mixture's composition at equilibrium, for a **templated-DCL** to **a blank-DCL**, allows the identification of the binders. The proof of concept is currently established for the identification of mimetics of the tumor suppressor p53 that would inhibit its interaction with the oncoprotein HDM2, a protein-protein interaction involved in apoptosis relevant for cancer therapy that has been repetitively used as a model for the development of new strategies for PPIs inhibition (*manuscript in preparation*). Application of the method to the discovery of mimetics for less known PPIs would significantly expand the potential of the method for bioactive cyclopeptide's discovery.

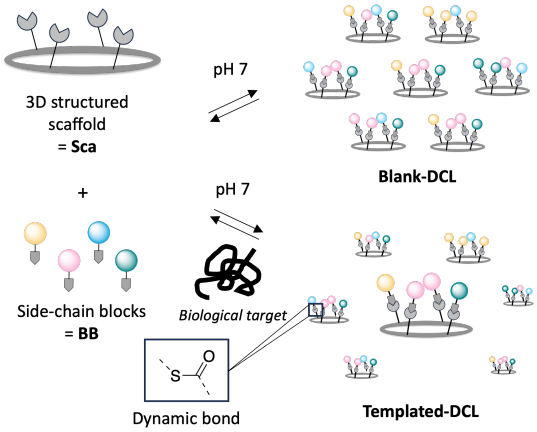


**Figure 2.** Dynamic side-chains exchange between 3D-folded cyclopeptides (**Sca**) and thioester (**BB**), leading to a dynamic combinatorial library of cyclopeptides and amplification of the best binders by a biological template.

**Methodology.** The aim of the project is to downsize the RED protein to a short helical peptide that retains the target affinity and activity of the whole protein and embedded with favorable pharmacological properties.

**WP1: Rational Design of a scaffold for DCC grafting. (P1)** Based on the existing stapled RED mimetic, an α-helical scaffold will be designed that projects four thiol groups prone to react in thioester to thiol exchange on one face. The peptide will be prepared by solid phase peptide synthesis (SPPS), its structure confirmed (circular dichroism, CD and nuclear magnetic resonance, NMR) and its reactivity toward thioester BBs evaluated. DCLs will be generated by mixing this **Sca** with a series of **BB** bearing hydrophobic natural and non-natural amino acids side-chains, in physiological conditions. The libraries will be characterized and quantified by HPLC and mass spectrometry (MS).[11]

**WP2: Screening of SMU1 peptide ligand by DCC. (P1&P2)** The SMU1 *N*-ter domain will be prepared by recombinant protein production in *E. Coli*, at a concentration compatible with the DCC assay, *i.e.* 200-300 µM.[2] Amplification of the DCLs will be studied by comparative DCC, between a **blank-DCL** and a **templated-DLC**, allowing to identify those combinations that are amplified in the presence of the SMU1 protein and represent thus potential ligands. After sequence(s) identification (MS-MS),[11] thioester peptides will be transposed into a robust sequence by exchanging the labile thioester function with its isosteric amide groups and their binding affinity to SMU1 will be confirmed by *in vitro* binding assay (BLI, surface plasmon resonance SPR, isothermal titration calorimetry ITC and fluorescent polarisation). The stability against proteolytic enzymes will be assessed. Their intrinsic cell-permeability will be evaluated, using flow cytometry and MALDI-based assay.

**WP3: Biological evaluation. (P2)** Finally, the ability of the optimized peptides to disrupt the RED-SMU1 complex and inhibit IAV replication will be evaluated, using well-established cell-based assays.[2] Disruption of the RED-SMU1 complex will be assessed using a split-luciferase based complementation assay (overexpressed RED-SMU1) or western-blot analysis of the endogenous RED and SMU1 steady-stated levels (formation of the RED-SMU1 complex protects each subunit from degradation). Inhibition of IAV replication will be initially assessed using a recombinant Nanoluc-expressing reporter virus. Peptides showing the best antiviral activities will then be tested against various subtypes of circulating IAV, using a plaque assay to monitor the production of infectious particles and RTqPCR to monitor the splicing of viral mRNAs.


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