UNIVERSIDAD DE INVESTIGACIÓN DE TECNOLOGÍA EXPERIMENTAL YACHAY

Escuela de Ciencias Biológicas e Ingeniería

TÍTULO: Protein scaffolds for Phage Display and its Applications

Trabajo de integración curricular presentado como requisito para la obtención del título de Ingeniería Biomédica

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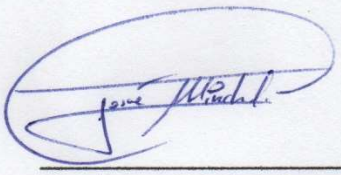

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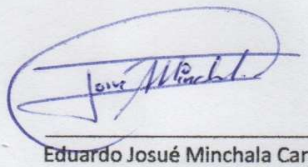

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Dedicatoria

A mis padres: *Luis y Martha*

A mis hermanos: *Carolina, Gabriela y Felipe*

Eduardo Josué Minchala Carangui

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A Urcuquí

Eduardo Josué Minchala Carangui

Resumen

Una comprensión clara del reconocimiento biomolecular, especialmente durante la interacción proteína-proteína o la interacción péptido-proteína, es de gran importancia durante los procesos que se desvían de lo normal y causan enfermedades. La exhibición sobre fagos ha surgido como una tecnología muy útil para crear bibliotecas que contienen ligandos exclusivos que pueden interferir en esas interacciones y servir como base para crear fármacos novedosos. El foco de investigación colocado en los péptidos ha sido desproporcionadamente menor que en las proteínas. Aquí, se describe una revisión de diversas bibliotecas basadas en péptidos, lineales, cíclicos y bicíclicos, con ejemplos que ilustran su utilidad. También se proporcionará una descripción de andamimajes de proteínas seleccionados (anticalinas, knottins, affibodies y DARPins) derivados de fuentes naturales que han servido como plataforma para constreñir estructuralmente a péptidos y crear bibliotecas que tienen potencial como alternativas a terapias basadas en anticuerpos. Finalmente, se dará una breve aplicación local al contexto ecuatoriano de librerías exhibidas en fagos para encontrar antivirales para enfermedades causadas por los virus del dengue y zika.

Palabras clave: Presentación sobre fagos, péptidos lineales, péptidos cíclicos, andamiajes proteicos, anticalinas, knottins, affibodies, DARPins.

Abstract

A clear understanding of biomolecular recognition, especially during protein-protein interaction or peptide-protein interaction, is an excellent deal during processes that deviate from average and cause disease. Phage display has emerged as a beneficial technology for creating libraries containing exclusive ligands that can interfere in those interactions and serve as the seat to create novel drugs. The research focus placed on peptides has been disproportionately less than on proteins. Here, a review of diverse peptide-based libraries such as linear, cyclic, and bicyclic are described with examples that illustrate their usefulness. A description will also be provided of selected protein scaffolds (Anticalins, knottins, affibodies and DARPins) derived from natural sources that have served as a platform for constraining peptides and creating libraries been found potential to find alternatives to antibodies based therapeutics. Finally, a brief local application to the Ecuadorian context of phage displayed-based libraries to find antivirals for diseases caused by dengue and zika viruses will be given.

Keywords: Phage display, linear peptides, cyclic peptides, protein scaffolds, anticalins, knottins, affibodies, DARPins.

Contenido

SECTION I

1. Introduction

Biomolecular recognition during intracellular and extracellular processes is essential in maintaining normal physiological functions and is one essential characteristic altered in diseases. As in many interactions, proteins play an essential role, and drug design aims to create or disrupt these connections. During the last 20 years, proteins have widely held the attention in research. A quick search in the PubMed database using the string "protein-based drugs" reveals a rise since 2000 (826 articles) compared to 2019 (>5500 articles). Although research on protein-based drugs have shown clear result as in the case of protein therapeutics and antibody (Ab) engineering, there are some limitations of proteins when it comes to crossing the cellular membrane and, therefore, tissue penetration, immunogenicity, engineering costs (Lee et al., 2019; Weil, 2014). In the face of Ab inconveniences, peptides have emerged as a promising alternative to overcome some drawbacks of proteins and at the same time offer the benefits of relatively small molecules in comparison to proteins. However, during the last decade, peptides have been relegated from focus due to several challenges accompanying their development.

Delivery of such small and unconstrained molecules such as peptide drugs through the gastrointestinal tract would prematurely lead to undesirable interactions with proteolytic enzymes and the drug's consequent degradation before reaching the site of action. This limitation leaves injections as the preferable route. In addition to peptide degrading enzymes, the liver and kidneys also contribute to clear peptide drugs from the systemic circulation (Zorzi, Middendorp, et al., 2017). Another critical challenge for peptide drugs lays in its structure. Lacking of a defined folding and structure as in the case of proteins, peptides flexibility can cause an unwanted diversity of conformational structures adopted by the polymer that negatively affect its affinity to the intended target. Nonetheless, its lowered immune activation is one fundamental characteristic that has led researchers to overcome the afore-cited challenges.

On the bright side, peptides used in pharmaceuticals are mostly naturally occurring molecules that give off amino acids (AA) that do not elicit an immune response as other harmful metabolites do. In fact, in some cases, peptides' immunogenic response is low compared to that of antibodies and recombinant proteins (Gang et al., 2018; Weil, 2014). Another essential advantage of peptides is comparable compatibility and specificity. This opens a potential opportunity to alter protein-protein interactions (PPIs). PPIs are involved in various bioprocesses such as targeted protein degradation, signaling cascades, ion transportation, transcription of nucleic acids, and DNA repair (Akbulut & Olmez, 2016; Lee et al., 2019). Altering these communications by reaching the interaction site through a peptide can produce a more pragmatic way of dealing with a process of interest. A clear understanding of the main contributors for biomolecular recognition to take place pointed to peptides as coprotagonists, especially when it comes to bioprocess gone wrong as in several maladies such as gastrointestinal ailments caused by hormone peptide malfunction, immune responses such as inflammation, and even neurodegenerative disease and cancer (Browne, 1999; Picco et al., 2016; Woods et al., 2018).

This renewed interest has brought forth a research branch that focuses on peptide-protein Interactions (PepPI). As a result, the number of peptide-based drugs has increased since the year 2000. A search in the PubMed database using the string “peptide-based drugs” reveals a six-fold increase in the spam of 20 years since 2000. The development of peptide drugs initiated back in 1920 when insulin was isolated from pork and sheep and entered the market (Bozovicar & Bratkovi, 2020). Around thirty years later, synthetic peptides oxytocin and vasopressin also came into development. In the following years, biotechnological developments in genetic engineering, including solid phase peptide synthesis (SPPS), opened massive peptide production that lowered production cost.

Additionally, until 2016 more than 50 peptide drugs entered clinical trials (Akbulut & Olmez, 2016). According to a global analysis, future development for peptide therapeutics

predicted that in an eight-year span that ends in 2024, the sector will experience a 9.1% compound annual growth. (Lee et al., 2019).

Peptide interactions are not limited to proteins but also include “lipids, nucleotides or metabolites”(Akbulut & Olmez, 2016) as well as a variety of biomolecules present in physiological media that can potentially alter peptide structure. Therefore, peptides' flexibility poses a challenge that affects the affinity and selectivity of the peptide for the target. This particularity is highlighted if a screening process is involved in the investigation. These processes include phage display (Ph.D.), mRNA display, yeast display, bacterial cell surface display, ribosome display among others. In this circumstance, it is desirable to have the peptide constrained under a stabilizing structure. Consequently, biological scaffolds play an important role in peptide engineering development.

Ph.D., which can be performed *in vivo*, contrast with other display methods such as mRNA which is strictly an *in vitro* system. mRNA have also been used to discover novel peptides for biomedical applications, a similar endeavor as Ph.D. In mRNA display peptides are covalently linked to its corresponding mRNA tag through a artificial spacer whereas Ph.D. makes use of bacteriophages capsid proteins to display the library of peptides. Every display method presents its own advantages and challenges. mRNA display have the advantage of allowing the introduction of nonnatural aminoacids and being an *in vitro* method, does not require a transformation step. Thus, the variability of the library increases. Another important advantage is a notorious simplification of the PCR amplification procedure (Y. Huang et al., 2019). A disadvantage of mRNA is the potential for nonspecific binding of positively charged proteins which conform an important fraction of the protein genome (Lamboy et al., 2008). mRNA technique is not fully functional for proteins that require post translational modification and also the environment have to be RNAase free wich add to the work of providing clean spaces (Cotten et al., 2011).

In this graduation thesis, a comparative review between linear peptides, peptides constrained by one or two disulfide bonds, and peptides under protein scaffolds will be

provided. Particularly, how these different approaches have networked with the 2018 Nobel laureate technology of Ph.D. to create libraries used in several medical applications will also be addressed. As an additional local contribution, how these technologies have been applied to investigate different approaches to treat infectious diseases in Ecuador caused by Dengue and Zika viruses will be briefly discussed.

SECTION II

2.1.Problem Statement

Although antibody engineering has produced consistent results as evidenced by many FDA-approved monoclonal antibodies to treat diseases that include cancer, there are some inherent limitations of Abs regarding size, immunogenicity, and consequent side effects. Therefore, there is a need to develop alternative molecules with similar affinity and selectivity that at the same time can induce the desired outcome. In this endeavor, Ph.D. technology makes possible the creation of libraries with diverse conformations of peptides and proteins. The structure in which a peptide is presented to the target is an essential factor to create a drug with an excellent pharmacological profile. Linear peptides have an inherent instability and high entropy that limits their applicability.

Conversely, peptides constrained by themselves (e.g., cyclic/bicyclic peptides) or peptides that use an underlying protein scaffold show more potential to become alternatives to Abs-based molecules. Thus, diverse structures that exist in nature have been mimicked to engineer scaffolds. Its field of application can include diseases present in Ecuador, such as the ones caused by dengue and zika viruses.

2.2. Research Hypothesis

A contemporary and relevant body of research and literature contains information regarding creating protein scaffolds for creating peptides displaying libraries that show potential for translation in drug development and offer an alternative to antibody-based therapeutics.

2.3.General objective

To provide a review document that highlights constrained peptides, focusing in protein scaffolds, used for elaborating Ph.D.-based libraries along with significant evidence in the literature of some of the results achieved.

2.4. Specific objectives

- To analyze the superiority of constrained structures in peptides based on its respective thermodynamic profiles.
- To describe the structure and synthesis of cyclic peptides and how they are used in combination with Ph.D. technology in medicine.
- To describe the structure and examples of two categories of protein scaffolds and how they are combined with Ph.D. technology to be used as alternatives to Abs-based drugs.
- To suggest an application of Ph.D. libraries in the Ecuadorian context to create antivirals against diseases caused by dengue and zika viruses.

SECTION III

3. Linear and cyclic peptides

3.1. Linear peptides

When it comes to peptides, especially short sequences, it is not thermodynamically favorable to fold into 3D shape as proteins do. This happens due to intra-molecular level interaction that does not favor the formation of secondary strucutres. So peptides tend to maintain a linear structure. But this conformation renders the peptide chain with undesirable flexibility that can lead to off-target interaction that subsequently could cause side effects. This lack of rigidity makes the peptide chain a suitable target for peptidases that lead to the molecule's degradation (Figure 1) (Rubin & Qvit, 2016). This flexibility is accentuated for sequences 2-10 AAs long; beyond this point, from 10 to 20 AAs long, secondary structures that resemble α-helices or β-strands start to emerge (Fischer et al., 2000).

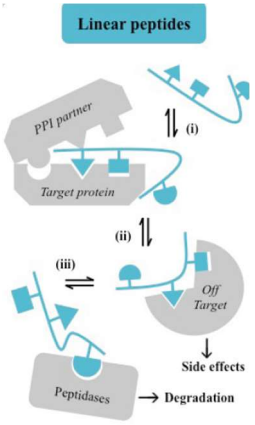

Figure 1. Possible paths of linear peptides.Taken from (Rubin & Qvit, 2016)

The lack of constraints in the peptidic bonds facilitates the formation of various geometries. For shorter sequences, the peptide bond geometry makes it difficult for the backbone to assume a conformation that favors macro cyclization. For larger peptides, peptide bond geometry does not represent a challenge, but side-to-side interactions are evaded. (White

& Yudin, 2011). To illustrate one of the reasons some peptides abide by a linear structure, analogously when a person tries to coil the ending of a chain, the shorter the lead portion of the chain, the more complex it is to get it coiled, and as a consequence, the peptide will stick to a linear structure.

The great majority of geometries the short linear peptide adopts are inactive, and as a consequence, the entropy is expected to be higher than a constrained peptide. Furthermore, it is challenging to assess precisely the complete thermodynamic profile of a linear peptide because a referential stable state of the peptide is lacking. This means the peptide adopts numerous conformations and stabilities (Camacho et al., 2008). The ambiguity regarding its structure, particularly for shorter linear peptides, is responsible in part for the vague role that they play when the target requires a more stable ligand (Empting, 2017).

The well-known Lipinski rule of five suggests that for a drug to be orally active, the number of hydrogen bond donors should not exceed 5, and hydrogen bond acceptors should be 10 at most (Mullard, 2018). Even a peptide chain as short as 10 residues can surpass those requirements of the Lipinski rule. Additionally, in a linear peptide lacking a defined secondary structure, its unfavorable hydrogen bonds are exposed to the medium's interaction. This hurts the peptide permeability, which in turn is associated with oral bioavailability (Wang & Craik, 2016).

Although there are traditional and contemporary chemical methodologies to obtain a cyclic peptide (CP) from the linear precursor, it does not imply that linear peptides should by any means be considered as of little to no use biomolecules that cannot exert biological activities. Approximations point to endogenous linear peptides to be modulators of 15% to 40% of PPI described (Rubin & Qvit, 2016). A comparison of linear peptides and their cyclized equivalents regarding cancer-targeting properties revealed that the linear peptides outperform cyclic ones under some circumstances.

For example, integrins are a transmembrane family that acts as receptors that anchor ligands from the extracellular matrix to the cell's surface. Eight members of this protein family are directly linked to tumor progression (Nieberler et al., 2017). A structure activity study evaluated the binding affinity of (arginine-glycine-aspartic acid) RGD containing linear and CP to RGD integrin's binding motif. Researchers found that the linear RGD bound to a specific integrin subtype ($\alpha_5\beta_1$) showed a 4 times greater affinity than specific CP. But cyclization in other cases did improve binding affinity. Additionally, recognition by the intended receptor does not require cyclic conformation due to a stable enough linear structure (Roxin & Zheng, 2012). Although this shows the utility of linear peptides, CP has found a broader application field, especially concerning Ph.D.

3.2. Cyclic peptides

The importance of correct folding for proteins to exert the intended function is textbook knowledge well established and widely accepted. By adopting a tertiary structure and folding into the 3D entity, proteins become stable enough to play their determined function. Evidence shows that CP withstands hydrolytic degradation better than linear ones (Díaz-Eufracio et al., 2018; Tapeinou et al., 2015). To expand its pharmacokinetic profile, cell permeability is also improved in several cases (Bockus et al., 2013). A constrained peptide does not conform to the required structure that allows recognition by catalytic endo and exopeptidases to off-target proteins that can potentially lead to side effects. (Figure 2) (Bhardwaj et al., 2016; Prabu-Jeyabalan et al., 2002; Rubin & Qvit, 2016). Besides, as it will be further explained, specific paths for cyclization lead to reduced polarity and hydrogen acceptors and donors (Empting, 2017). All the previously mentioned progress using different approaches to perform the cyclization process has contributed to upgrading this compound classically regarded as "undruggable".

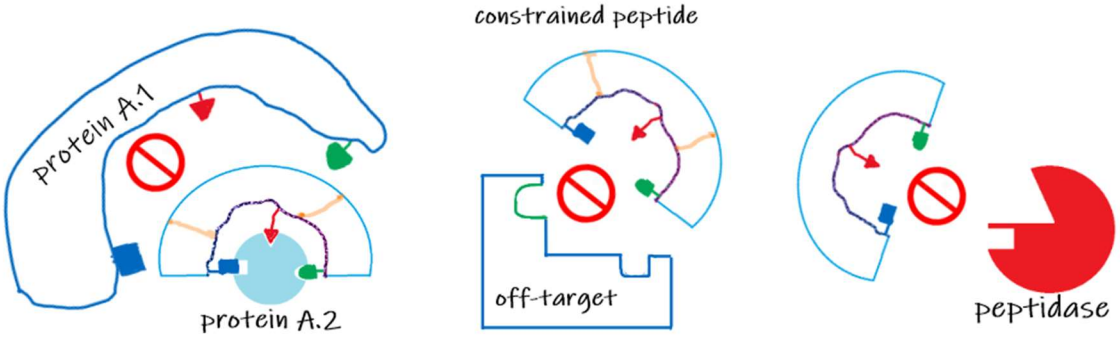

Figure 2. Advantages of cyclic peptides. Protein A.2 is the target

3.2.1. Thermodynamic characteristics

While in the case of CP derived from a linear precursor, presumably, it would be more feasible to obtain information about the thermodynamic profile, a clear understanding at the molecular level of concepts such as configurational entropy has not been reached yet (H. Huang et al., 2021). Nevertheless, some efforts have been made. In contrast to linear peptides, which lack a reference point, the constrained structure of CP has enabled the assessment of entropy and enthalpy values that are related to the free Gibbs energy of binding by the formula:

$$\Delta G = \Delta H - T\Delta S \qquad (1)$$

Where: ΔG: $Gibbs\ free\ energy$; ΔH: $Enthalpy$; $T\Delta S$: $Entropy$; R: $Universal\ gas\ constant$

T: $Temperature$; K_d: dissociation constant

From (*1*) it can be noted that for a process, cyclization of a linear peptide, to be spontaneous, has to be favorable either entropically or enthalpically. It has been found that there is an interplay of both factors in the process of cyclization, as it will be shown in the next paragraphs. A study evaluated the thermodynamics of the bonds between PDZ10 and

serotonin receptor type 2c ($5HT_{2c}$). PDZ10 is the tenth PDZ domain of 13 that comprises MUPP1 (Multiple PDZ-containing Protein). MUPP1 plays an essential role in synaptic organization and plasticity (Donaldson et al., 2016). Sharma and collaborators obtained three linear peptides (controls) from the PDZ domain partner protein and two cyclic analogs; one CP had two controls. The linear peptides were in some manner a "precyclized" version. Through isothermal titration calorimetry (ITC), they obtained thermodynamic characteristics. CPs were obtained from replacing one residue in each peptide (Glu and Leu, respectively) and a subsequent coupling through a β-alanine linker that prompted the bridging. They concluded that for "Cyclic-1 and Cyclic-2, nearly total enthalpy-entropy compensation was observed, and in both cases, entropy is the dominant factor" (S. C. Sharma et al., 2007). Nevertheless, in comparison to the linear peptides, the free energy of binding of linear and cyclic did not show significant differences in the results as they were very similar.

Its internal dynamic changes have to be noted through a thorough analysis of a cyclized peptide's thermodynamics. These changes impact the enthalpy-entropy compensations and therefore impact the free energy of binding (Wang et al., 2015). Wang and colleagues used Nuclear Magnetic Resonance techniques to characterize precisely molecules in motion and assess their internal dynamics. This technology has proved beneficial to determine, for example, that head-to-tail cyclization confers the peptide backbone a rigid assembly and, as a consequence, shows a decrease in amide temperature coefficient. Data obtained through NMR relaxation, a sophisticated technique to study protein flexibility, used in conjunction with molecular dynamics (MD), enabled a group of researchers to determine the internal dynamics of mono and poly cycle peptides. The data showed that switching the disulfide bond, which plays an essential role in the internal peptide dynamics, reduces rigidity (Wang et al., 2015). Although this result seems obvious, it will be detailed in sections 3.2 and 3.3 how inserting disulfide bonds has been very useful in cancer therapy and research.

3.2.2. Classification

CPs first appeared in nature. In the light of the benefits and all the potential they harbor, we have witnessed the emergence of techniques to simulate nature and synthetically generate CP. A brief overview of the synthesis of natural peptides that have shown clinical potential will be given in the following sections. Afterward, a summary of the synthetic approaches to obtain CP will be mentioned, with a particular focus on those that are compatible with Ph.D. screening technology.

Nature has evolved bringing multiple CP of many organisms, from bacteria, marine organisms to plants and mushrooms. They have been isolated and classified over the last decades (Morewood & Nitsche, 2020). Although a thorough understanding of some cyclization processes has been reached for some peptides, there are still 'unanswered questions' that ongoing research is trying to address. The knowledge reached has enabled scientists to combine this knowledge with molecular engineering tools to develop synthetic peptides. CP can be either biosynthesized by ribosomes and go through enzymatic post-translational modification (RiPPs) or have a non-ribosomal origin (NRP).

3.2.2.1. Ribosomally synthesized and post-translationally modified peptides (RiPPs)

Many RiPPs have entered clinical trials to treat maladies such as cancer or vasoconstriction and are also used as antimicrobial agents (Singh & Abraham, 2014; Takase et al., 2019). It could directly serve to develop new drugs or unveil novel targets (Ting et al., 2019). They are present in Archaea, Bacteria, and Eukarya, and the genomes sequenced so far from these domains give evidence of the abundance of the biosynthetic genes (Arnison et al., 2013). The post-translational modifications experienced by the peptide bestow it with an extensive display of possible chemical conformation. Macro cyclization being one of them.

The enzymes in charge of post-translational cyclization of the peptide have to surmount some challenges. One of them is the length of the precursor peptide. Each class of natural CP undergoes its particular process at a certain level, accompanying a specific set of enzyme cyclization that ultimately gives its particular properties, but they all share some typical phases as RiPP's. As a general description of the process, a precursor peptide is expressed by the known ribosomal path. As in all peptides, it poses an N-terminus, the head, also called the leader sequence that is usually recognized by the biosynthetic apparatus to start the pronation and cyclization of the core peptides, which commonly have more than one copy. After recognizing and binding the leader sequence or, less often, the C-terminus, the core peptide is enzymatically modified to give off not just several macrocyclic peptides but heterocycles, D-amino acids, lanthionine, or sactionine cross-links (Sikandar & Koehnke, 2019). The best representative classes of RiPP's are Cyanobactins, Lanthipeptides, Thiopeptides, and CPs from mushrooms and plants. Detailing each one of these peptides is beyond the scope of this graduation project.

3.2.2.2. Non-Ribosomal Peptides (NRP)

This class of biosynthesized peptides has proven beneficial in affecting many biological activities. Notable examples are vancomycin, which acts as an antibiotic; cyclosporine A, which acts as an immunosuppressant, bleomycin which acts as an anticancer agent (Schwarzer et al., 2003). A significant number of these natural peptide products underwent macro cyclization of their peptidic backbone. As mentioned earlier, a macrocyclic structure provides the molecule properties such as a more rigid conformation, improved membrane permeability, and proteolytic resistance.

The biosynthesis is carried out by mega-enzymes called Non-Ribosomal Peptide Synthetases (NRPSs). These enzymes are subdivided into modules that are in control of the addition of residues into the NRP. In turn, each module contains at minimum three primary domains, adenylation, peptidyl carrier protein, and condensation. These submodules carry out selection, activation, loading, and condensation to obtain a NRP. The

thioesterase (TE) domain carries out the termination step (Konno et al., 2017). This domain is in charge of liberating the precursor as a macrocyclic-containing peptide (Sieber & Marahiel, 2003). The role TE domain plays has motivated research to engineer TE to produce non-natural macrocyclic NRP.

3.2.3. Synthesis techniques

One of the earliest techniques chemist devised to engineer peptides was solution-phase peptide synthesis (SPS), a technique started in the last century (Du Vigneaud et al., 1953). When the peptide could not adopt a secondary structure (like α-helices or β-sheets) spontaneously, they aimed to convert peptides into "druggable" entities by focusing on backbone constrain for which several protocols were designed. However, SPS allowed the synthesis of constrained peptides with a certain ease, the exhausting process of purification that included many repetitions, caused in some cases a toll in the yield of the peptide product (Tapeinou et al., 2015).

Solid-phase peptide synthesis (SPPS) overcome these limitations. SPPS was started after ten years since SPS (Merrifield, 1963). According to the nature website, SPPS is the "synthesis of chemical compounds whereby the reactant molecule is chemically bound to an insoluble material and reagents are added in the solution-phase" (NatureISSN, 2021). Besides peptide synthesis, SPPS has also used for nucleic acids and oligosaccharides synthesis. The purification is performed with less effort than SPS. SPPS also have a versatility that tolerates the chemical variety of AA structures. CPs obtained using this technology are used for screening proteins (Gang et al., 2018).

3.2.4. Approaches to constrain a peptide

Peptidomimetics, a group of peptides that undergoes rational chemical modification (Bock et al., 2013), comprises multiple techniques to obtain a looped structure in a process that usually takes several steps. Among the modifications are residues substitution, L to D isomerization, cyclization, N-methylation, PEGylation, and incorporation of structural

restraints such as disulfide bonds (Qvit et al., 2017; Tapeinou et al., 2015). Cyclization by artificial means using a combination of peptidomimetics techniques.

Depending on the nature of the bonds attaching one AA to the other, CPs are classified as 'homodetic' if only peptidic bonds are involved; or 'heterodetic' if AAs are connected through other functional groups (Tapeinou et al., 2015). The following strategies are currently designated to describe the constraints placed in a macrocycle. Having a linear peptide as a substrate, the standard cyclization methods commonly used are described next and illustrated in Figure 3.

- Head to tail cyclization. When both *termini* of the chain are joined together, the C-terminus to the N-terminus, it's called head-to-tail cyclization.
- Cyclotide. Additional disulfide bonds reinforce a head-to-tail cyclization. Side chain to either of the terminals' cyclization. Whichever terminal, N or C, of the peptide attaches to the side chain.
- Side chain to side-chain cyclization. Two side-chain AAs are attached through homodetic and heterodetic AAs.
- Disulfide bond. A bond formed between two cysteines attached by the thiol groups.
- Thioether. When a cysteine residue of a side chain is attached through its thiol group to the α-carbon of a residue.

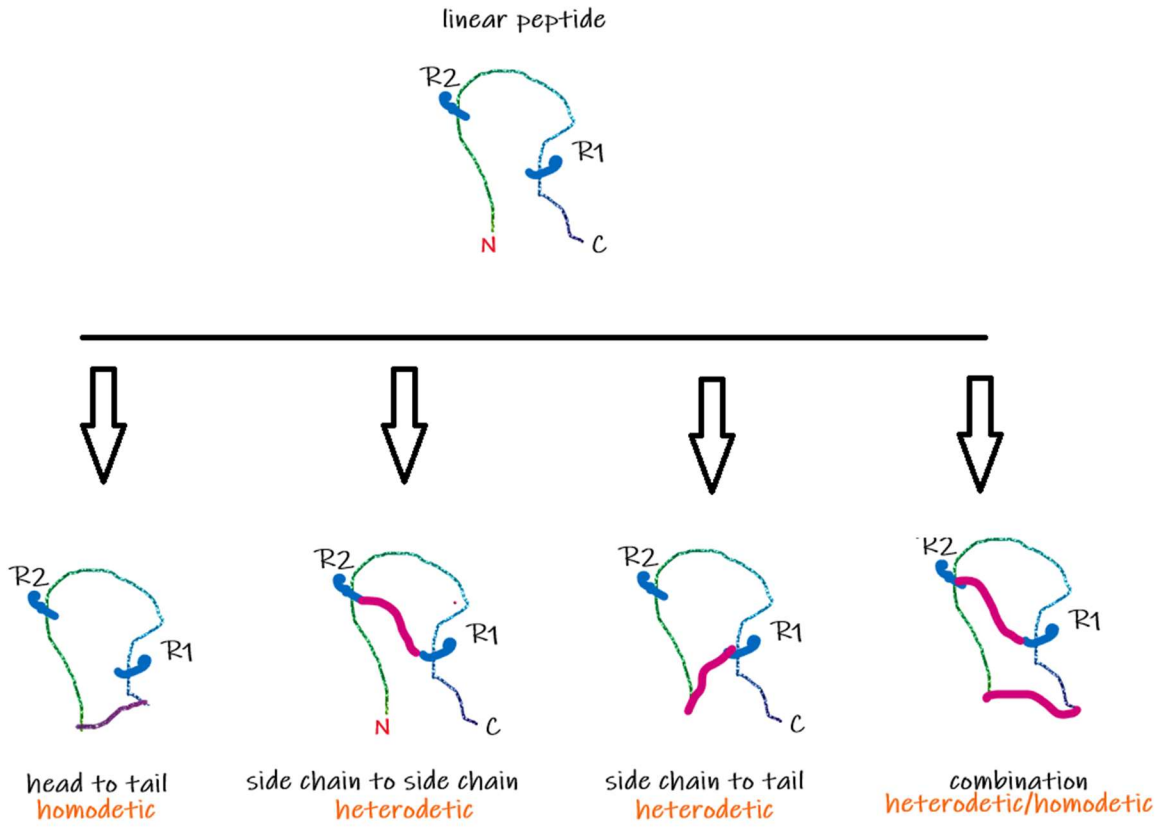

Figure 3. Types of cyclization for a generic peptide chain. Not all aminoacids are shown the backbone is drawn in thin line.

Of all the previously stated strategies, only the ones compatible with Ph.D. technology will be expanded to show how cyclization and display tools can act synergytically to obtain bioactive molecules. A CP per se can act as a scaffold that can be used with Ph.D. But proteinic scaffolds have also been developed with good results and will be reviewed here.

SECTION IV

4. Phage Display Technology

The quest for finding new biologically active molecules with applicability in translational medicine and research has encountered many challenges. For example, the abundant number of candidates and the possible modifications they can undergo to maximize the affinity for the intended target. Ph.D. emerged in 1985 when G. Smith demonstrated that bacteriophages (short phage) could 'display' polypeptides in their capsid proteins (Smith, 1985), and shortly after in the nineties served as an essential start the establishment of Ab engineering (Almagro et al., 2019). Ph.D. offers a significant contribution to overcome some of the challenges to the point of being awarded the Nobel prize in 2018. Bacteriophages are viruses that, in colloquial terms, hijack bacteria.

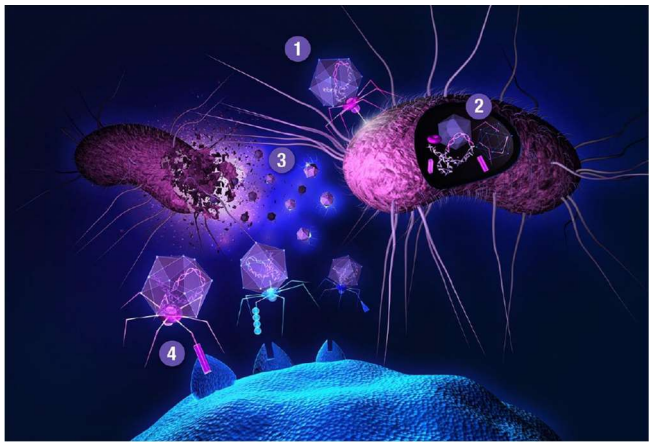

Figure 4. Process of replication of T7 bacteriophage. (1) infection. (2) replication inside the cell. (3) release into the medium. (4). Capturing by the immobilized target. Taken from (Bozovicar & Bratkovi, 2020)

As the name suggests, bacteriophages infect only bacterial cells that readily replicate the viral genetic material, which is susceptible to modification and able to self-assembly (Goracci et al., 2020). The high yield of phages by bacteria (usually *Escherichia coli*) allows a high throughput of clones that express foreign peptides or proteins along with the phage coat proteins (Figure 4). Foreign genetic material is classically inserted into protein

III (pIII) or protein VIII (pVIII) genes that codify the phage capsid. This particularity gives Ph.D. the characteristic of literally linking phenotype to genotype in a specific phage which is of great practical utility during the isolation process. This linkage also ensures that from each clone of *E. coli.* an exact copy of phages will be produced (Bazan et al., 2012).

The library is constructed via cloning of combinatorial DNA sequences. Once the library is created, it must be amplified to facilitate manipulation of a process usually carried out via the polymerase chain reaction (PCR) technique. To complete the selection method, or biopanning process, there are four additional steps to complete. The first step consists of conjugating the prepared Ph.D. library and the target molecule for a determined time. It is expected that only library member clones with the highest affinity are bound to the target molecule. The following procedure is the washing step. It helps get rid of unbound clones. The third step involves three or four rounds of elution that ensures mostly high-affinity bound targets are obtained. In the final step of biopanning, the selected clones or 'hits' are reintroduced in bacteria to amplify them and recover a more 'neat' library that can undergo further maturation processes (Figure 5) (Saw & Song, 2019; C. Wu et al., 2016).

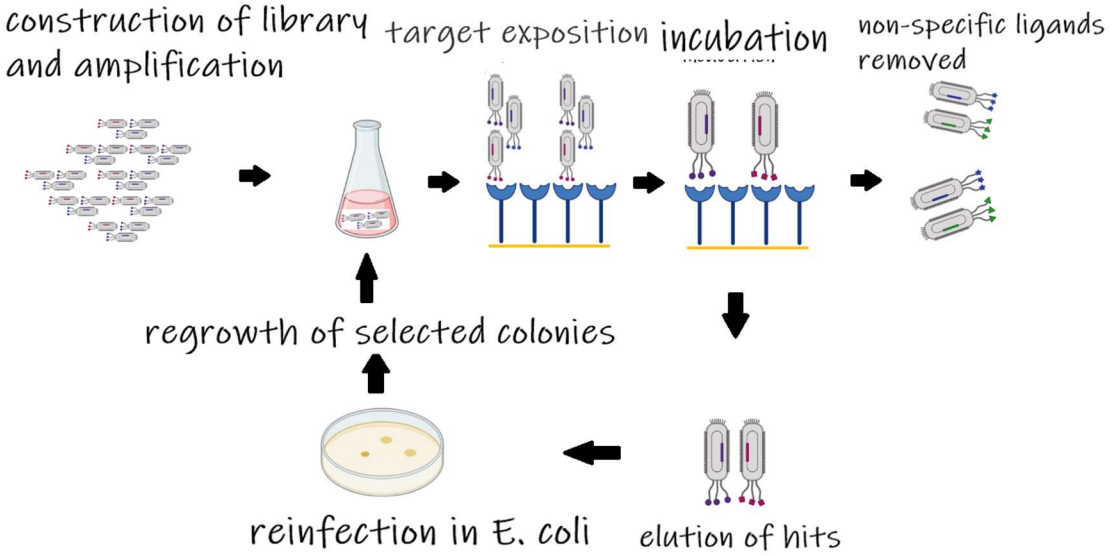

Figure 5. Overview of the library creation and biopanning process.

4.1.Details of the phage structure and display systems

With approximately 10^{31} particles on our planet, bacteriophages constitute the "most abundant and biologically diverse entities" (Dafale et al., 2016). But only five are well-matched to be used in Ph.D. M13 and fd filamentous phage are the preferred option; however, T4, T7, and λ (lambda) phage have been investigated as well. They differ in size and shape. The size of M13 is 900x7 nm; it has an elongated construction that includes filaments in the tail region (Goracci et al., 2020). M13, as previously stated, presents two zones in the capsid open to genetic insertion with tolerable alteration of the structure. These are pIII and pVIII proteins. The display of peptides on each one of these systems has its particularities.

The minor coat protein, pIII, is composed of 406 residues. Ordinarily, there are 3 to 5 duplicates of this protein positioned at the far end of the phage (Bazan et al., 2012). In contrast, pVIII proteins are located along the phage's body and are composed of 50 AAs (Nemudraya et al., 2016). It is estimated that the phage contains around 2700 duplicates of pVIII protein (Aghebati-Maleki et al., 2016). For the display of libraries, fusions with pIII protein are more popular because they allow for more extensive peptides or proteins to be inserted (>100 amino acids) and widely used for Ab display. Whereas pVIII fusions only allow for short peptide insertions (6-7 AAs), cysteine residues must be absent to preserve phage functionality (Bazan et al., 2012; Saw & Song, 2019). This constraint represents a shortcoming for displaying CPs which use cysteine bonding.

The higher number of copies displayed with pVIII proteins offers a higher avidity of binding than pIII displayed libraries. This high avidity impacts target-peptide affinity negatively. Consequently, the dissociation constants of pVIII ligands are between 10–100 μM, and for ligands that use pIII it is 1-10 μM (Noren & Noren, 2001). This translates to ligands having a very low affinity in pVIII display while pIII display offers high-affinity ligands. This aspect has not prevented scientists from building useful libraries based on pVIII display.

The following sections will provide details into how Ph.D. technology has been explicitly combined with peptide libraries. It will show how advancement in research and understanding of CPs contributed to creating different libraries that have encountered practical applications in academia and medicine. Also, a combination of CPs with an underlying protein scaffold can act synergistically to improve drug properties.

$$\Delta G = RT \ln K_d \quad (2)$$

$$K_d = \frac{[R][L]}{[R * L]} \quad (3)$$

An essential specification before describing the subsequent libraries and examples is to define Kd. This value is provided as validation for many investigations that are detailed in the following sections. This element in equation *(2)* does not only serve to offer an insight into how well a cyclization process was performed but also shed light on how strong is the interaction between ligand and target. The lower the value, the stronger the attachment between ligand and receptor and the higher the affinity. This is following the aftermath of the natural logarithm function of Kd: the lower the value, the more harmful it becomes and the more significant the contribution to the spontaneity of the process (Salahudeen & Nishtala, 2017). In equation (3) [R] and [L] are the concentrations of receptor and ligand, respectively; and is another equation to obtain Kd as a metric of ligand quality.

4.2. Isolation methods of hits used with Phage Display screening

Diverse methods are currently used to isolate hits from a pool of peptides using Ph.D. screening. Examples are *in situ, in vivo, ex vivo* and *in vitro*. Development of diverse isolation methods is a field that have been advancing on its own and there is research even around in-human direct selection.

4.2.1. *in situ* screening

This method does not involve any living system such as cell culture or animal model and is the simplest among isolation methods. It only requires the target to be coated on a 96-well (Saw & Song, 2019). As the target does not inherently own the coating, the replicability in a living system can be challenging (Kim et al., 2012).

4.2.2. in vitro screening

Literally meaning 'in glass', these experimetns are designed taking cell's components to the 'pool' of peptides to observe the reaction and determine the feasibility of switching to an *in vivo* setting. *In vitro methods* can also utilize whole cell lines or primary cells to determine wich peptides specifically bind to them. This approach have the advantage that cells and its components maintain its structure and activities. The protocol for this methods is flexible and can be modified to select for internalized peptides in the cell, or also to select for peptides that bind to the surface allowing identification of potentially novel cell surface receptor (C. Wu et al., 2016).

4.2.3. in vivo screening

This selection method was first described in 1996 (Pasqualini & Ruoslahti, 1996). It is performed in a living animal and can be used to determine how the peptide interact in an entire system. Also to check if there is organ specificity which is an important characteristic for a drug and for an imaging agent. The procedure involves the insertion of the peptide pool into the animal via intravenous injection followed by a time window so the peptide can bind to the intended organ (Lo et al., 2008). Tipically, 3-5 roud of biopanning are carried out to reach desired tissue specificity. The major setback of this method is the successful replicability of the experiment in humans due to potentially poor translation among different animal species (C. Wu et al., 2016).

4.2.4. In-human screening

To overcome the replicability of *in vivo* experiments, since 2002 efforts are being put to perfectionate the selection of hits directly in human individuals (Arap et al., 2002). There are FDA approved experiments involving cancer patients that use analogous techniques (Krag et al., 2006).

4.3.Challenges of Phage Display technology

On the other side, Ph.D. is not without drawbacks that significantly affect its use. If the library comprises peptides that contain disulfide bridges as some cyclic and biCPs, it is only feasible to work in a non-reducing environment, meaning that *in-vivo* testing is limited because of its proclivity to undergo reduction (Roxin & Zheng, 2012). The translation of selected clones to the therapeutic application is still a challenge for compounds selected by Ph.D. because some important drug properties such as oral bioavailability, cell permeability, and solubility are not the best. Additionally, Ph.D. is limited to the use of the 20 essential AAs. If further diversity is required, synthetic chemistry techniques should be used.

SECTION V

5. Phage display libraries

5.1.Libraries of linear peptides

When Smith first demonstrated the possibility of displaying molecules on the phage's pIII protein, he made an exhibition of linear peptides. From that moment, the methods used for displaying peptides and proteins using this technology have improved notably. Nevertheless, displaying linear peptides still ‘get the job done’ for many researchers. As will be evidenced in the examples provided, sometimes linear structures are preferred over CPs.

Although there are abundant examples in the literature, some of the results obtained using Ph.D. of linear peptides will be given now. There are multiple linear peptide libraries created by commercial companies like Creative Biolabs, Pepscan, and New England Biolabs structured by as few as 4 residues up to 20. The upper limit on the length of a linear peptide library is placed because for longer polypeptides, the chances it starts to adopt some tertiary structure increases, including some turns and folds. Further details of commercially available libraries will be given in section 6.4

5.1.1. Example

Linear peptide libraries composed of 7 and 12 residues were used along with one CP library of 7 residues. Hartmann and colleagues' aim in the research was to identify peptide mimotopes that could give rise to a similar Ab response as the one elicited with monoclonal antibodies (mAbs) for cancer therapy. Cetuximab and matuzumab are two mAbs that target epidermal growth factor receptors (EGFR) and act by inhibiting the receptor's tyrosine kinase action. Fragments of both mAbs were displayed on the *E. coli.* Cell surface. M13KE, a derivative of M13 bacteriophage, was selected for a library display. Both displaying methods were combined, employing a technique called delayed infectivity panning (DIP). By combining linear and cyclic libraries, they were able to identify two

peptides that were selected using fragments of matuzumab mAbs but which had the particularity of presenting cross-reactivity to cetuximab fragments as well. Common motifs of these peptides were identified as KTL and YPLG, and after performing animal experiments, they concluded that these peptides 'could induce anti-EGFR antibodies with antitumoral activity' (Hartmann et al., 2010). One of these peptides was selected from the linear peptide library, and the second from the CP library highlighting the significance of using a linear peptide library for this research outcome.

A preclinical study focused on developing active immunotherapy for cancer also used a library of linear peptides using PhD. They target carcinoembryonic antigen (CEA), a protein that shows elevated adenocarcinomas levels, especially in colorectal cancer tissues (Canadian Cancer Society, 2018). The study aimed to develop a vaccine based on a mimotope. This mimotope would be identified using two PhD libraries. These libraries was displayed using pIII display system. As in the previous example, this team used a combination of cyclic and linear libraries. The linear peptide library was composed of nine residues (LL9), and the cyclic library was constrained using disulfide bonds and consisted of 10 residues (CL10). Subsequently, they performed rounds of biopanning with an anti-CEA Ab Col-1. Although the team obtained 7 'hits' that showed specificity to Col-1, only one of them was selected to develop the mimotope. The winner was the one obtained using the linear peptide library, which is named COL2. The result was a functioning inhibitor of tumors with high CEA levels (Brämswig et al., 2007). This is evidence of the feasibility of creating imitative B-cell epitopes that can have candidates develop active immune therapies.

5.2.Libraries of cyclic peptides

The conversion from linear to CP conformation can be bridged using different methodologies, and each one has its advantages and challenges. CPs per se can be used as structural scaffolds in which diverse peptides can be displayed using different technologies

such as Ph.D., mRNA display or split- intein circular ligation of peptides and proteins (SICLOPPS) (Sohrabi et al., 2020). The last two are not discussed in the present work.

The combination of Ph.D. technology and CPs has resulted in many publications and some clinical applications, as evidenced by the examples given in section 3.2.1. Nature evidence that the most habitual form of cyclization comes from disulfide bonds. Libraries based on Ph.D. have used this particularity to improve the performance of this technology. It consists of constraining a peptide, adding two cysteine residues at the borders. If these AAs are placed in a non-reducing environment, they will spontaneously form disulfide bonds (Figure 6). This constituted the earliest cyclic Ph.D. libraries and is still in use.

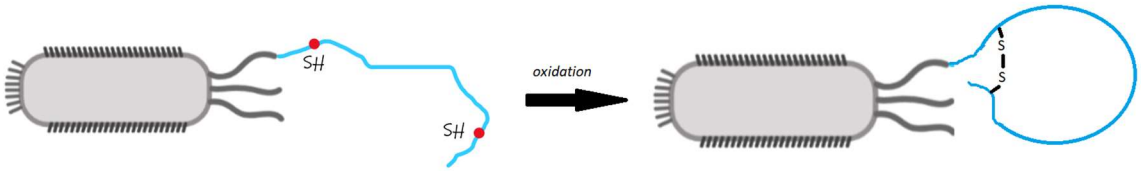

Figure 6. On-phage cyclization through oxidation.

The length of the peptide displayed on the library varies depending on the application. There are documented cases of libraries displaying four to ten residues flanked by a pair of cysteine (Deyle et al., 2017; McLafferty et al., 1993; O'Neil et al., 1992). Frequently the residues were laid out in a $X_lCX_mCX_n$ arrangement in the bacteriophage. X is a genetically encoded AA (proteinogenic); m can take values between 4 and 10. For l and n the value falls between 0 and 4. If l and n are 0 and m equals 7, we get a heptapeptide library (CX_7C) which have been broadly used to identify targets such as enzymes, cytokines, transporter, and receptor proteins (Rubin & Qvit, 2016; Saw & Song, 2019; Sohrabi et al., 2020).

Several biotechnological improvements positively affected the way CPs and Ph.D. work together. From reagents enhancement e.g., DNA polymerases to boost the transformation efficiency of *E.* coli. cells by using electrocompetent cells. Novel cloning methods have also fostered the use of Ph.D. Gibson assembly is a faster and low-cost cloning method

than conventional one as it requires fewer reagents, and a consequence more extensive libraries can be created (Deyle et al., 2017). Cyclization performed on phage is another modification worth mentioning as it allows for the formation of macrocycles in a primary linear peptide (Figure 6). It also opened the way to creating bicyclic peptides, which will be addressed in the coming section. All the previous progress made has resulted in shortening the gap to reach challenging targets and improve the affinity of 'hits.'

5.2.1. Example

Wrighton and colleagues give a very striking example which illustrates the utility of Ph.D.. In this investigation, both display methods that M13 bacteriophage tolerates were employed in different stances (pIII and pVIII display methods). The team wanted to single out a molecule that can elicit a similar response as human erythropoietin (EPO) in the organism, that is, to initiate and regulate erythropoiesis by binding to EPO receptor (EPOR) (Middleton et al., 1999). EPO is a glycoprotein cytokine hormone of around 34 kDa and interacts with EPOR, a transmembrane homodimer protein of 66kDa that is part of a superfamily called cytokines receptors (Suzuki et al., 2015). This process ends with the production of erythrocytes that are in charge of bringing oxygen to tissues and constitutes an essential process for maintaining homeostasis.

Two portions of the intracellular domain of EPOR get closer to each other once the ligand molecule of EPO binds, starting the signaling process in target cells. This interaction is carried out through short epitopes. Therefore, a peptide that interacts with EPOR and produces a similar biological response was desired. During the first phase, where the team had to identify a clone of the motif of EPO, they used a cyclic octapeptide library displayed on pVIII of M13 bacteriophage. A peptide named AF11154 with the sequence CRIGPITWVC was identified and synthetically built. The peptide did compete against EPO for an immobilized soluble EPOR and presented an affinity of around 10 μM. To produce higher affinity ligands, they generated mutagenesis libraries from the AF11154 peptide. As the goal was to increase affinity, the library was displayed using pIII coat

protein. The library ON1963 served to identify 5 peptides that were cyclized and showed 10 - 50 stronger affinity for EPOR than AF11154. Interestingly, when the linear counterparts of these five peptides were tested, they showed 1000x reduced affinity. CPs of around 2 kDa proved to elicit a biological response similar to that of 34 kDa EPO (Wrighton et al., 1996).

To improve the selected peptides' performance and bring them to the clinical onset, they were PEGylated (polyethylene glycol-PEG). The combined compound named peginesatide had improved solubility and longer circulation time in blood. It was administered in patients with renal malfunction once a month. Although initially approved because patients showed normal hemoglobin levels and controlled anemia, the compound was withdrawn from the market due to safety concerns (Deyle et al., 2017). But its example illustrates very well the functionality and versatility of PhD.

5.3.Libraries of bicyclic peptides

Examples of bicyclic peptides exist in nature, e.g., phalloidin, a deadly toxin found in some mushrooms. Other bicyclic peptides make valuable tools for research. They usually have a molecular weight near 1 – 3 kDa (Deyle et al., 2017). Although bicycle peptides can be formed using various chemistries, only those configured using disulfide bonds and chemical linkers will be addressed here.

As mentioned earlier, as the peptide length increases, resemblances of secondary conformations and some folds and turns start to appear. Consequently, linear and monocycle peptides can progressively gain flexibility and become unstable or be enzymatically degraded. By adopting a double ring structure, an oversized unstable monocyclic can reduce its entropy as the original ring size will be narrowed and result in two rings. Each ring's function could be independent of each other as in the case that one serves for cellular access whereas the other does the target critical task (Rhodes & Pei, 2017).

Bicyclic peptides can incorporate additional rigidity to the peptide conformation concerning monocyclic ones, making them even less likely to be broken down by proteases. An increased binding affinity is achieved partly due to a reduction in the entropy involved in the molecule interaction. Besides, the twofold rings broaden the surface of contact with the target and result in a steadier interface (Chen et al., 2013).

The library construction process usually starts from a linear precursor or a monocyclic one. The precursor is then chemically modified by cysteine residues that form disulfide bonds as in monocyclic peptides. Usually, three fixed cysteines are involved in the binding and a chemical linker molecule that ties together the three cysteines. A possible conformation can be ACX_6CX_6CG. The linkers can be compounds such as TBMB, TATA, TBAB, TBMT, TATB. Its structure is schematized in figure 7. Varying the linkers and the quantity of AAs in each ring contributes to increasing the library's diversity. The more diverse the combinatorial library, the better the affinity of the isolated binders (Deyle et al., 2017).

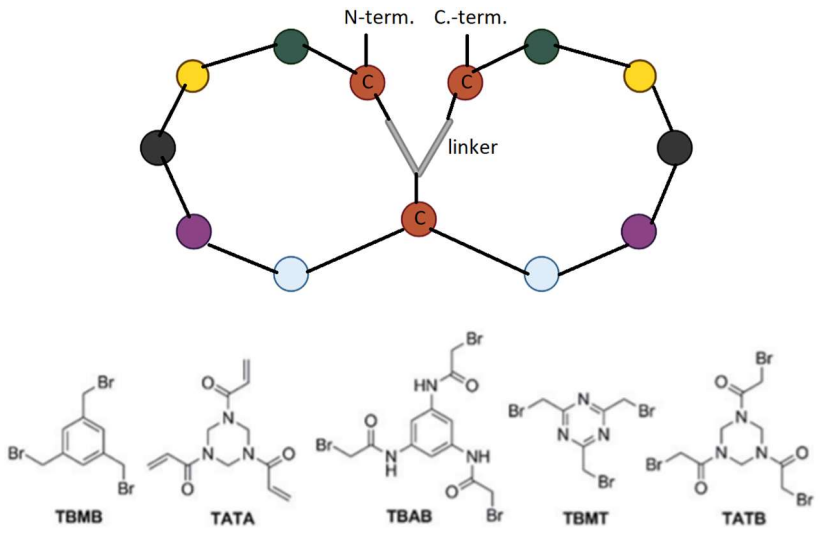

Figure 7. Top: example of a bicyclic library using a linker. Bottom: classical linkers used to obtain bicyclic peptides.

An alternative method developed to obtain bicyclic peptides libraries is that starting from a sequence like XmCXnCXoCXp, bicyclic peptides can be pulled out because a fourth

cysteine can appear from any of the X positions. Statistic states that for a library with CX_6CX_6C the probability of the peptide having four cysteines is 26% (Chen et al., 2013). The four cysteines then can bind and form disulfide bonds which simplify the procedure of creating a library of bicyclic peptides and makes them accessible to less specialized laboratories.

5.3.1. Example

Urokinase-type plasminogen activator (uPA) is a serine protease that is involved in cancer metastasis. More specifically, it contributes to the deterioration of the extracellular matrix, which facilitates the migration of cancerous cells away from the first point of defect to reach normal tissue (Mahmood et al., 2018). When uPA interacts with its receptor uPAR, they form a system that is thought to be involved not just as a proteolytic entity but rather accompany the tumor process from the beginning to the point of metastasis (Duffy, 2005). Identification of high levels of uPA in cancerous tissues has led researchers to target this protein.

It was desired to have bicyclic peptides inhibitors for uPA. A library was designed with the configuration ACX_6CX_6CG and displayed using the pIII coat protein. For the chemical cyclization, the linker TBMB was used (Figure 8). There were 19 peptides identified that had two consensus sequences, one at each ring, respectively. This was a positive characteristic because it indicates a reasonable specificity of the targets isolated (Deyle et al., 2017). Ligands bound to the second consensus group presented a stronger binding. The selected peptide named UK18 had an inhibitor constant (K_i) of 53nM (Angelini et al., 2012).

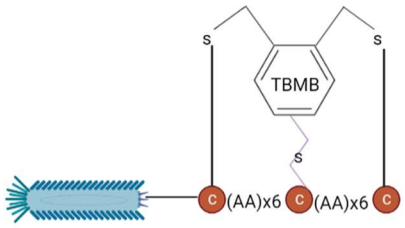

Figure 8. The schematization of the bicyclic library is displayed on pIII. Taken from (Sohrabi et al., 2020)

As the findings of this research experiment showed promise, in-vivo experiments were carried out. For bicyclic peptides, this was the first time being tested in-vivo. Linear, monocyclic and bicyclic versions of UK18 were incubated in blood plasma. An essential portion of the bicyclic UK18 kept its original conformation. This was not the case for the other two analogs. The relevant point was that UK18 was less stable when the rings were cyclized independently. This leads to presume that each ring synergistically contributes to the overall stability of the bicyclic molecule (Deyle et al., 2017). When UK18 was tested with tumor tissues, the peptide's functionality and conformation were excellent, but unfortunately, tumor growth was not stopped (Pollaro et al., 2015). This experiment shed important information on the nature and particularities of bicyclic peptides and how to combine them with Ph.D.

A clever technique was developed in another very recent experiment to introduce ‘unprecedented’ variability to macrocycle libraries' scaffolding (Kale et al., 2018). As a result, high-affinity ligands for plasma kallikrein were isolated using Ph.D. The novel technique consisted of using 4 cysteines, which will form disulfide bonds through two chemical linkers on a library of CXmCXnCXoC where m, n, and o are the random numbers AAs. Scaffold diversity is expanded if AAs are displayed, exploiting all the possible combinations and because the two chemical linkers can connect two pairs of cysteine in three alternative ways. By applying this methodology to a nonapeptide, 63 possible macrocyclic scaffolds in the library use two chemical linkers (Figure 9) (Kale et al., 2018).

Plasma kallikrein levels can rise in the body and cause an excess in the release of bradykinin to the point of causing hereditary angioedema responsible for swelling episodes (Banerji et al., 2017). Using the novel technique previously described, the team could isolate a very active inhibitor denominated PK2 (Ki=0.5±0.1 nM) (Kale et al., 2018). This peptide's particularity is its low molecular weight (~1kDa) and high stability compared to other inhibitors that are either bigger or present a decreased binding affinity. Thus, it is a potential candidate from which oral drug delivery could be plotted.

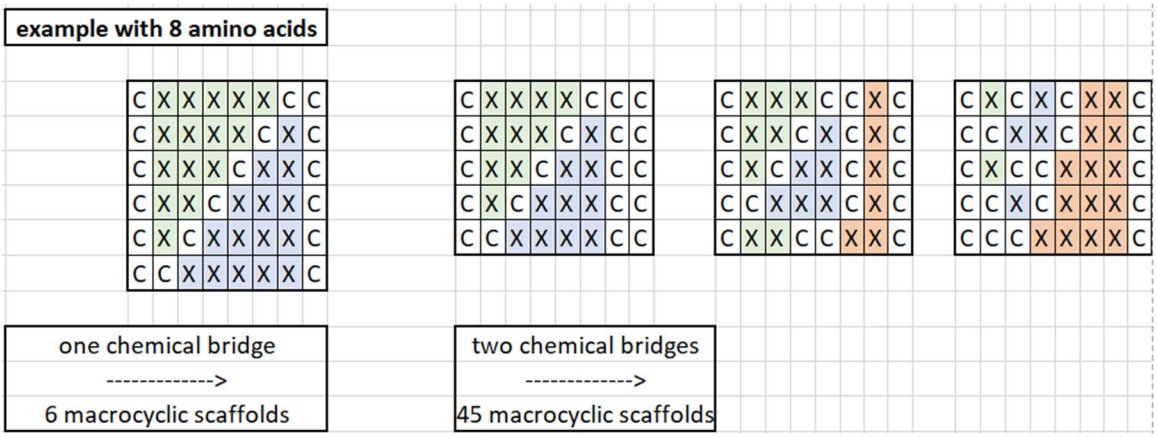

Figure 9. Comparison of using one vs two linkers in a double-bridged nona-peptide. Light colors serve to more easily identify where the cysteine residue is alternating and offer the advantage of adding this aminoacid to form two chemical bridges.

SECTION VI

6. Protein scaffolds

The term protein scaffold can comprise multiple proteomic structures that have been used in combination with PhD technology, including the well-known Igs that have proven very useful for biotechnological applications. Protein scaffolds must retain a fixed folded structure to serve as a framework in which peptides (or other molecules) can be displayed; this implies that a specific motif in the scaffolds has to allow an affinity function, preferably on an exposed surface. The present section will address non-Ig proteins which have been used as scaffolds for Ph.D. applications.

Although immunoglobulin (Ig) molecules have the great advantage of covering a significant part of the spectrum of recognition to the most sought-after targets, its development is accompanied by high costs. This is due to the problematic modifications such as glycosylation, bridging through disulfide bonds implied in the manufacturing. The relatively bigger size of Ig proteins is prone to elicit undesired immunogenic responses and a decline in cell permeability (A. M. Wu & Senter, 2005). Protein scaffolds have been raised as a promising alternative approach that can potentially improve *in vivo* stability of displayed elements. But the challenge of reaching the combinatorial diversity of Ig molecules is patent.

6.1. Properties of protein scaffolds

A stable 3D structure and a clear function are inherent properties of proteins. This stability, often reinforced by steady disulfide bonds, makes the protein resistant to protease degradation. There have to be specific protein segments susceptible of modification, and the introduction of variation is expected not to alter the overall structure and folding of the scaffold, which will, as a consequence, affect its stability. If the scaffold is derived from a natural source such as animal, plant, bacteria, etc., the original function of the protein scaffold is often lost or does not bind to the original ligands (Hosse, 2006). But it is a valid

approach as long as the scaffold's configuration is maintained despite being recombined with phage capsid proteins and at the same time can proficiently display peptides.

The insertion of mutations in the scaffold, which make possible the elaboration of a library, can be achieved through distinct methodologies. One or more loops can be randomized to increase the surface of binding, a tethered motif variation instead of thorough variation, point-specific mutations that ensure an adequate binding (Hosse, 2006). The number of mutations inserted in the scaffold and the insertion site is restricted by its structural stability, prone to misfolding.

6.2. Advantages of using protein scaffolds

Among the enhanced aspects of the pharmacokinetic profile, using a protein as a scaffold for Ph.D. offers, the most notable is the *in-vivo* stability. This stability translates into an efficient selection of 'hits' with good affinity and selectivity inside an environment that resembles natural conditions that facilitate safety, toxicity, and efficacy assessment. The improved kinetic profile and an adequate size of the protein scaffold exhibit targeted bioaccumulation (Schmidt & Wittrup, 2009).

Using protein scaffolds for Ph.D. has also proved useful when combined with molecular imaging technologies. Recent development in molecular imaging methods focalized in tumor metabolism such as single-photon emission tomography (SPECT) and positron emission tomography (PET) has been used in combination with traditional anatomical imaging methods systems such as magnetic resonance imaging (MRI) or computed tomography (CT). It positively impacts the decision-making process, resulting in an improved preclinical and clinical evaluation of the novel compound, enabling better monitoring, detection times, and improved therapies. (Willmann et al., 2008).

6.3. Classification of protein scaffolds

Although more than one taxonomy has been proposed for classifying protein scaffolds (Hosse, 2006; Simeon & Chen, 2018; Zoller et al., 2011), the present work will abide by characterization made more recently by Simeon and Chen. They proposed that depending on the location of the region where the variability will be inserted and the ligand will attach; there are two categories: (i) scaffolds where variated residues are placed in flexible loops and (ii) scaffolds where residues are located in secondary structures (α-helices and β-sheets).

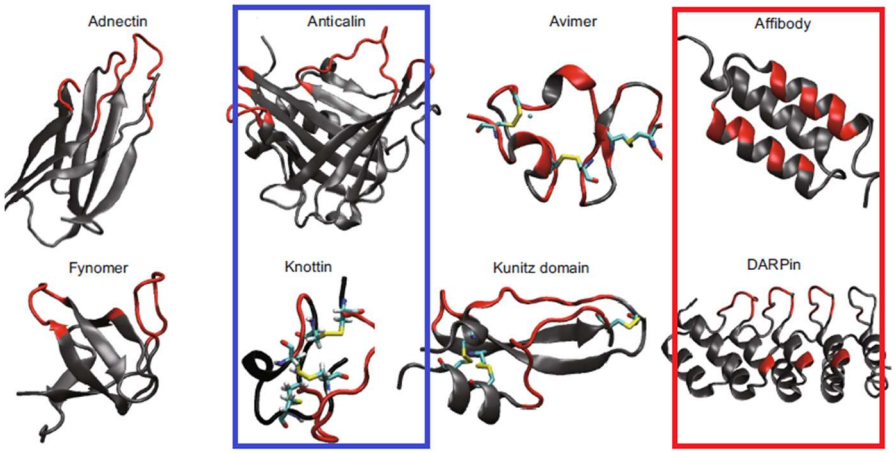

Figure 10. A representative set of non-Ig protein scaffolds. Highlighted in blue are the scaffolds described here belonging to category (i) and in red the ones belonging to category (ii). Variable regions that serve for antigen recognition are depicted in red and framework AAs in gray. Modified from (Simeon & Chen, 2018)

6.3.1. Category one: Ligand-binding residues in exposed loops

6.3.1.1. Anticalins

Product of an engineering and design process, this group of proteins is derived from a natural family of lipocalins proteins. Lipocalins present in eukaryotic and prokaryotic organisms (Goetz et al., 2000) have a role in transporting hydrophobic molecules and storing biological compounds. In humans, it takes part during normal physiological processes such as retinoid-binding (Lepperdinger et al., 1997) and some maladies that

include cancer. There are at least 37 identified lipocalins in humans (Du et al., 2015). The engineered version, anticalins, is intended to contribute to the making of alternatives to Ab proteins for medical and biotechnological applications.

Anticalins are smaller (~20 kDa) than monoclonal antibodies and are devoid of disulfide bridges or glycosylation (Simeon & Chen, 2018). Although the structure is less sophisticated than antibodies, the functionality can be comparable when binding antigens, deficient molecular weight or hapten-like ones (Hosse, 2006). The structure facilitates the synthesis and favors the combination of other proteins to increase the field of anticalins applications further. In combination, size and structure allow anticalins to have better tissue penetration and suitability for bacterial production e.g. *E. coli.* (Skerra, 2008). Therefore, it has also been used in combination with Ph.D. Before entering into the detail of how this is carried out, a specific description of anticalins' structure will be provided.

6.3.1.1.1. Structure

Anticalins deviate structurally from their parent protein lipocalins only in four specific loops close to the ligand-binding site (Skerra, 2008). Eight β-strands organized in antiparallel arrangement combine and a βbarrel-like conformation emerges. The four loops afore mentioned are hypervariable in anticalins and constitute the access to the ligand-binding site positioned close to the protein's surface (Hosse, 2006). Each extruding loop can tolerate a variability of 16 to 24 residues, and in total, the biomolecule consists of 160 to 180 residues (Gebauer & Skerra, 2012). In the context of Ph.D. library creation, the number and location of residues selected for randomization vary depending on the target molecule's nature. If the target is a protein, all the variability tolerated will be utilized in each loop; but if the target is a small molecule, only randomization, in particular, AAs near the ligand-binding site cavity, will be enough (Hosse, 2006).

6.3.1.1.2. Example

A natural protein that takes up an essential quota of the total proteins present in human tears belongs to the lipocalin family. The wild type version of human tear lipocalin (Tlc) is brutal to play a role in tear viscosity and acts as an antiviral and anti-inflammatory agent (Dartt, 2011). This polyfunctionality is probably attributable to the fact that Tlc poses broader access to the ligand-binding sites than other characterized lipocalins (Breustedt et al., 2005). A group of researchers reengineered Tlc to obtain an anticalin that selectively targeted vascular endothelial growth factor A (VEGF-A) (Gille et al., 2016).

The VEGF family of proteins regulates angiogenesis, a central process that touches virtually every stage during cancer. The VEGF-A member of the family is considered one of the most binding ligands in solid tumor angiogenesis (Innocenti et al., 2018). Although monoclonal Ab such as bevacizumab has already been developed and FDA approved to neutralize VEGF-A, there is evidence of severe side effects such as intestine perforation and platelet aggregation leading to thrombotic complications (Meyer et al., 2009; Shojaei & Ferrara, 2007). For this reason, anticalins' structure and immunogenic profile stand as a good alternative.

The anticalin library was created by selectively mutating 18 residues located in the four loops constituting the ligand-binding site entrance. Additionally, four N-terminal, two C-terminal residues were eliminated, and both cysteines involved in a disulfide bridge were exchanged (Breustedt et al., 2005). One PCR fragment was used to encode the first pair of loops along with the variable codons. For the second pair of loops, a second PCR fragment was used in an analog manner. Four rounds of selection against a synthetic fragment of VEGF-A as the target produced an initial set of 'hits' that were recovered using streptavidin paramagnetic beads and a series of washings steps. Using high-throughput (HT) ELISA and other techniques, one ligand, named PRS-050, was selected that showed affinity to all the splice forms of VEGF-A used in the experiment. Maturation step procedures raised the affinity from KD = 400 nM to 25 pM (Gille et al., 2016).

In subsequent experimentation *in-vivo* with local and systemic administration confirmed the capacity of PRS-050 to neutralize VEGF-A. Moreover, to improve the anticalin's biological capabilities, it was PEGylated (Roberts et al., 2012) by inserting one cysteine residue in the surface of the lipocalin. This procedure resulted in an extension of the plasma half-life from < 1 hour to about 28 hours (Gille et al., 2016). The first application of PRS-050 is in cancer. Using mice for a 20-day treatment, it was shown how tumor growth curves corroborate the action of the anticalin. A second application tested was for inhibiting the breakdown of the retinal-blood barrier in rabbit eyes. The low molecular weight and high specificity favor delivering a higher stoichiometric prescription and is, therefore, a good candidate for treating age-related macular degeneration (AMD).

6.3.1.2. Knottins

It is also called inhibitor cystine knot; it belongs to a group of proteins denominated disulfide-rich proteins (DRP). According to the structure exhibited, they constitute a family of natural proteins that have been adapted to be used in combination with various screening technologies such as yeast, bacterial, and Ph.D. (Barkan et al., 2016; Getz et al., 2011). It is naturally found in animals, plants, and fungus playing defense mechanisms roles as protease inhibitors, toxins, and antimicrobial agents. (Zhu et al., 2003).

6.3.1.2.1. Structure

As the name suggests, these proteins possess a motif that resembles a ‘knot’ in the core that results from the bridging of six cysteine residues that form the distinctive three disulfide bridges, which is the minimum number of required bridges to be called knottins. The interweaving is formed by a coil formed in the two disulfide bridges and the protein backbone (green in figure 11); the remaining disulfide bridge (blue in figure 11) engages in this coil, and the knot is formed (Figure 11). Therefore, the beta-strands are provided with excellent stability that confers the entire molecule of resistance to adverse basic/acidic, thermal, or mechanical conditions (Daly & Craik, 2011; Postic et al., 2018).

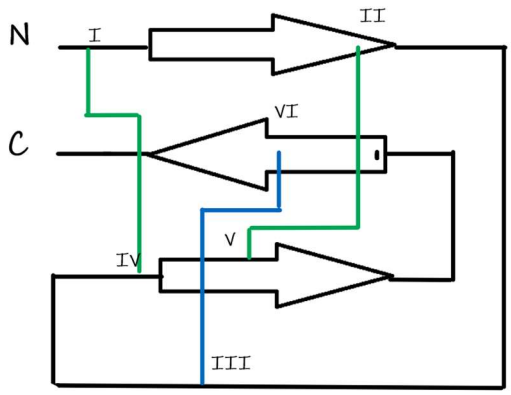

Figure 11. The detailed structure of knottins showing specifically wich aminoacid positions are used to form the interweaving

Knottins, which are approximately 30 residues long (Simeon & Chen, 2018), has been exploited for biomedical purposes, e.g., as drug design and imaging in medicine. The plethora of functions this protein has been given, such as neurotransmitters, anti-erectile dysfunction, analgesics etc. (Postic et al., 2018) has motivated the creation of a database created in 2004 and upgraded in 2017 (https://www.dsimb.inserm.fr/KNOTTIN/index.php). One outstanding example of these molecules is the FDA-approved drug linaclotide which disulfide bridges are ligated as follows: C1–C6, C2–C10, and C5–C13. This 14-residues drug is orally available and is used to treat chronic constipation and irritable bowel syndrome (Zorzi, Deyle, et al., 2017).

6.3.1.2.2. Example

Fibronectin 1 is an extracellular matrix protein present in healthy as well as in tumorous tissue. One isoform of fibronectin, called extra domain B fibronectin (EDB-FN), is an oncogenic protein unusually present in non-cancerous tissue (Han & Lu, 2017), a characteristic that makes it attractive for targeted applications in cancer treatment and imaging. This isoform contains the domain EDB linked to many indicators of tumor aggressiveness such as metastasis, proliferation, epithelial-mesenchymal transition (Han et al., 2018; Petrini et al., 2017). EDB is powerfully expressed in a type of breast cancer but

is also present in lung, brain, colorectal, ovarian, and thyroid cancers (Vaidya et al., 2019). Being a very well conserved sequence among species, the 91 AA EDB qualifies as a tumor marker (Lui et al., 2020). Therefore, Ph.D. technology can be used to obtain high-affinity ligands to EDB.

The knottin libraries created in this very recent investigation (Lui et al., 2020) were synthesized from an open-chain variant of *Momordica cochinchinensis* trypsin inhibitor II (MCoTI-II), which in turn was isolated from the fruit Gac. Two Ph.D. libraries that used the same M13 bacteriophage were made. The first, called MCopt 1.0 employed the major coat protein (pVIII) for display, and the varying portions were located in three regions. Two of these regions were allocated in two loops out of five the knottin presented. A third varying region was located next to the N- termini of the knottin. The second Ph.D. library called MCopt 2.0 used the minor coat protein (pIII) for display and had a single portion of the variable region located in the first loop.

The ligands were obtained after three screening processes, and 46 of these were selected for sequencing. Three clones from the library MCopt 1.0 (MC-FN-010, MC-FN-020 and MC-FN-030) and three of the MCopt 2.0 (MC-FN-040, MC-FN-050, and MC-FN-060) were further analyzed. One particularity was that except MC-FN-020 all the rest exhibited a motif R-I/V-R-(L) that alanine substitution lowered the affinity and proved the importance of this motif for binding. SPR technology showed that MC-FN-010 alone displayed binding activity while lacking any tags, which confirmed its potential as an imaging tool. Alanine scanning also revealed that a derivative of the SPR selected ligand, MC-FN-016, had a specific residue substitution that had performed better for selective linkage to primary amines, a desirable property for imaging agents.

When both MC-FN-010 and MC-FN-016 were trimerized through oligomerization for *in-vivo* testing, the affinity improved from the nano to the pico-molar range without affecting other binding properties. This was confirmed through immunofluorescence staining of tumor and standard brain tissue sections. The improvement was 1500x to 3600x times

stronger and was comparable to the binding of an Ab fragment previously developed (Pini et al., 1998). The fine biodistribution in the liver and kidneys using the xenograft mouse model was also promising. Taken together, these results show promise for further development of knottins-based ligands for imaging or drug delivery.

6.3.2. Category two: Ligand-binding residues in secondary structures

6.3.2.1. Affibodies

This engineered molecular scaffold is derived from one of the five domains of protein A. Each domain is a three α-helical structure which contains 57-60 AA. Protein A resides in the Staphylococcus aureus membrane and anchors the Fc portion of Ig G (Alonso & Daggett, 2000). Domain B was distinguished from the rest for being thermodynamically stable and due to its remarkable folding reaction time (Myers & Oas, 2001) and was selected to undergo chemical modifications that resulted in a protein named Z-domain. This protein maintained an affinity for the Fc fragment of IgG and has been used in therapeutic endeavors to block PPI and strategies for payloads and increased selectivity (Löfblom et al., 2010). Table 1 shows several affibody molecules that have reached pico-molar affinities to different targets; some entered clinical trials.

6.3.2.1.1. Structure

Construction of affibodies-based libraries that use Z-domain as scaffold make use of 13 residues open to variation out of 58 that constitute the protein fragment. It is spread in two of the three α-helices (Hosse, 2006; Löfblom et al., 2010). The absence of disulfide bonds and relatively small size (6.5 kDa) are favorable properties of these libraries. They have been successfully used to isolate ligands with remarkable affinities (μM to ρM) in areas such as imaging, therapy, and biotechnological development. Some examples of targets are tumor necrosis factor-α, interleukin-8, envelope glycoprotein gp120, Cluster of Differentiation 28 (CD28), human epidermal growth factor receptor 2 (HER2), and epidermal growth factor receptor (EGFR) (Nygren, 2008).

Table 1. Examples in the literature of affibody pico-molar affinity ligands.(ECD): extra cellular domain Selected from (Ståhl et al., 2017)

Target	Target size (kDa)	Highest affinity (pM)	Reference
Epidermal growth factor receptor (EGFR)	69 (ECD)	160	(Andersson et al., 2016) www.clinicaltrials.gov/NCT02901925
Human Epidermal growth factor receptor 2 (HER2)	70 (ECD)	22	(Sörensen et al., 2016) www.clinicaltrials.gov/NCT01858116
HER3	69 (ECD)	21	(Malm et al., 2013)
Interleukin-17 (IL-17)	30 (dimer)	0.3	www.clinicaltrials.gov/NCT02690142
Staphylococcal protein A Domain	7	16	(Lindborg et al., 2013)
Tumor necrosis factor-a (TNF-a)	54 (trimer)	95	(Löfdahl & Nygren, 2010)

6.3.2.1.2. Example

The application of affibodies for imaging is exemplified by developing a radioiodinated Ph.D. selected affibody to achieve high contrast imaging in overexpressed HER2 tissues (Orlova et al., 2006). HER2 is a transmembrane protein, and its dimerization with related protein receptor family leads to cell multiplication, migration, and invasion of other tissues (Hayes, 2019). It is present in around 15% of breast cancers (Sapino et al., 2013). Thus, it's a potential marker for cancer imaging applications that can make possible more individualized treatment options for the most commonly diagnosed cancer in women worldwide (Sung et al., 2021). For imaging application, the agent's high affinity and specificity make possible only diseased structures to detain the agent (in this case, an affibody), whereas normal tissue does not. This resulted in high contrast images and reduced injection-examination time (Orlova et al., 2006).

The team aim was to perform affinity maturation of a previously identified affibody called $Z_{HER2:4,}$ which showed 50nM affinity (Wikman et al., 2004). A secondary phage library was constructed using two of the three α-helices of the Z domain. A 129 nucleotide template was employed to encode the motif open to variation and the invariant residues of the two α-helices. In total, six residues at selected locations were open to randomization. The remaining invariable α-helix was encoded by a phagemid vector (pAffi1) and ligated to the oligonucleotide. For the selection procedure, the M13K07 from NEB was used. The target was determined to be a 624 AA portion of the extracellular domain of HER2.

After five rounds of selection with biotinylated HER2, 160 clones were chosen arbitrarily. The criteria for scaling down this number were based on two techniques. First, they subjected the clones to an ELISA test, and the majority showed higher absorbance values than the reference $Z_{HER2:4.}$ And second, a clustering method that is based on an average-link hierarchical procedure. As a result, 10 clones were selected. Parameters such as solubility, melting temperature, along *in-vivo* assays facilitated the further reduction of candidates up to 2 ligands ($Z_{HER2:342}$ and $Z_{HER2:477}$). Finally, it was found through a kinetic investigation that although both affibodies present affinities in the range of the picomolar, for $Z_{HER2:342}$ the dissociation equilibrium constant K_D value was proximate to 22 pM and better than the 32 pM of $Z_{HER2:477}$.

The selected affibody showed similar fluorescent properties for imaging than the commercially available monoclonal Ab trastuzumab. Then, to radiolabel the affibody, ^{125}I was used. The team tested through Biacore technology that the binding properties were intact. Therefore $Z_{HER2:342}$ is an excellent candidate for radionuclide in-vivo imaging (Orlova et al., 2006).

Affibodies have not only found application in imaging but also as therapeutics with the purpose of discover alternatives to existing monoclonal antibodies to treat inflammatory condition but require high delivery doses and even intravenous delivery wich impacts

negatively patient compliance. An affibody based therapy, taking advantage of its small size, can increase by ten fold the dose in the same volume compared to a monoclonal based therapy (Frejd & Kim, 2017). An example is the development of a complement 5 (C5) protein inhibitor. The complement system belongs to the innate immune system. Ph.D. was used to identify hits in the nanomolar range. This affibody molecules were futher modified to match the charactersitics of specific diseases. One pegylated affibody molecule fused to albumin binding domain (ABD) was used to target the eye and reached clinical trials but was discontinued (Berglund & Stromberg, 2016). This evidenced the ongoing research efforts that are in course to find more ways affibodies can be used as targeted therapy.

6.3.2.2. Darpins

Again, nature provides a model to create new approaches for library construction. Some families of proteins naturally assemble by joining repeats of certain sections of AAs. Ankyrin repeat domains are a family of proteins belonging to this class and is among the most abundant (Simeon & Chen, 2018). They are present in various organisms other than humans and are involved in different intracellular processes, including tumor suppression (Mosavi et al., 2004). Inside the cell, it has been found in the cytosol and also in the nucleus suggesting a functional versatility to switch surroundings. This is partly because it lacks disulfide bonds that allow it to preserve the conformation in the cytoplasm's reducing environment. At the same time, is compatible with high throughput amplification in *E. coli.* for the creation of libraries using Ph.D (Hosse, 2006).

6.3.2.2.1. Structure

Ankyrin repeat domains possess a tandem-like structure in which each repeat unit has 33 residues consisting of a pair of antiparallel α-helices linked by a β-turn and, lastly, a coil that serves as linkage to the neighboring repeats or the N/C-termini of the protein. To translate these existing properties into the creation of a library, an engineering process through consensus design led to creating a library that allows the randomization of 6 of the

33 total residues in each consensus repeat (Forrer et al., 2004; Killias, 2010). Up to four repeats have been reported, excluding the N and C termini repeats (Binz et al., 2003). This modular conformation (Figure 12) of the engineered scaffold is termed Designed Ankyrin Repeat Domains (DARPins) and offers exciting characteristics to Ph.D. based libraries. Some improvements of DARPins scaffolds consisted of redesigning one of the capping repeats to confer the entire molecule more stability (Kramer et al., 2010).

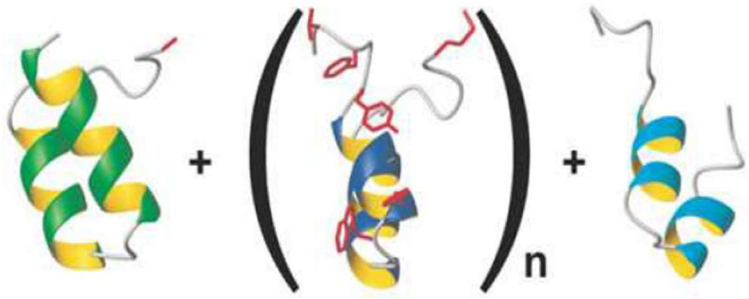

Figure 12. Representation of DARPin library structure. The N-terminal capping is shown in green. In light blue the C-terminal. In blue internal repeats along with randomized AA in red. Taken from (Killias, 2010)

DARPins application has been manifested in cancer research and therapy. Its relatively small size, great binding affinity and specificity, flexibility to introduce peptide configuration distributed along with the repeat units are characteristics that make DARPins suitable for targeted therapies, especially for cancer. Some examples of Ph.D. selected ligands are the Fc domain of human IgG, ErbB2 (HER2), ErbB1 (EGFR), ErbB4 (HER4), and TNFα (Killias, 2010; Steiner et al., 2008).

6.3.2.2.2. Example

Although in the following example, ribosome display was also involved during the research, the selected ligand came from Ph.D. technology. It is worth mentioning how these two technologies complement each other at specific points during the research process. The investigation had two purposes: first, engineer a DARPin molecule with the capacity to bind to the epithelial cell adhesion molecule (EpCAM); and second, to use the selected

ligand to transport and deliver the toxin pseudomonas exotoxin A (ETA) to tumor specific sites (Stefan et al., 2011).

EpCAM is a type-1 glycoprotein found in approximately 39-42 kDa (Stefan et al., 2011). If higher than average expression levels are found, it is associated with adenocarcinomas and squamous cell carcinomas (Went et al., 2004). Therefore, it is considered a tumor-associated antigen with low occurrence in healthy tissue and a cancer stem cell marker (Aiman Mohtar et al., 2020; Killias, 2010). ETA is one common polypeptide used for directed cell intoxication and is classically conjugated to engineered antibodies in order to create immunotoxins. The second generation of immunotoxins makes use of smaller scaffolds such as DARPins.

To obtain well suited DARPins candidates that target EpCAM, two technologies were used. Ribosome display had already been successfully used to isolate 'hits' for cancer ligands (Amstutz et al., 2006; Zahnd et al., 2006), but being a technology that can only be carried out *in-vitro,* it must be provided of conditions such as buffered non-denaturing environment, low temperature and assure the nonexistence of any RNases (Killias, 2010). On the contrary, the conventional procedure of filamentous Ph.D. relies on transforming *E. coli.* for the library construction. This *in-vivo* step limits the library size and restricts protein display only to those with favorable translocation to the periplasm and folding speed. Moreover, the functional size of the library is even smaller, that is, the fraction of the whole library that is viable for display due to its correct folding, size, and stability while conjugated to the bacteriophage and in the bacteria medium (Steiner et al., 2008).

A clever, efficient, and straightforward solution to the problem of premature cytoplasmic folding implemented in Ph.D. was to switch from the post-translational Sec protein-translocation pathway to the signal recognition particle (SRP) translocation pathway. This change counteracts insufficient translocation into the bacteria periplasm, and the protein display levels increase evidently (Steiner et al., 2006). Ph.D. technology proved one more time its versatility to adapt, in this case, to DARPins scaffolds.

The DARPin Ph.D. library had the conformation N3C; that is, besides the N and C capping repeats, 3 more repeats were inserted in a tandem conformation. These DARPin composed of 5 repeats in total was amplified by PCR and subsequently cloned in pQE30-derived vectors provided by Qiagen. The biotinylated extracellular domain of human EpCAM was immobilized to serve as a target. Three rounds of selection showed apparent augmentation of ligands showing specificity toward EpCAM. After sequencing 20 clones, EPh1 clone showed dominance and presented the particularity of having two residues deleting the intersection between the N-terminal repeat and the first repeat. The restoration of these two residues reduced the affinity for EpCAM, which showed the importance of these two AAs' absence (Stefan et al., 2011).

Ribosome display ligands were obtained using N2C and N3C libraries conformation. The seven clones selected differed in the randomized positions from those of EPh1 clone. The affinity maturation process involved using error-prone PCR of clones selected by both technologies and ribosome display select new ones. Further adjustments such as reengineering of the C-termini capping repeat of the DARPin binders (Interlandi et al., 2008), competition ELISAs, analytical size-exclusion chromatography, and flow cytometry analysis were made to obtain five binders derived from EPh1 and two from the ribosome display selected ligands. Among the five was Ec4, which showed picomolar range affinity to EpCAM (Kd=917.2 ±77.8 pM) and, even better, was internalized once bound to EpCAM (Stefan et al., 2011). After fusing ETA to Ec4 it showed a similar affinity to free Ec4 and proved to be cytotoxic to various tumorous cell types (Martin-Killias et al., 2011).

As a summary of the 2 categories of scaffolds, Table 2 show details.

Table 2. Summary of scaffolds

	Scaffold name	Residues	MW (kDa)	Structure	Parental protein
Cat. 1	Anticalins	160-180	~20	B-sheet and α- helical terminals	Lipocalin
	Knottins	~30	<4	B-sheet and disulfide bridges	Peptides
Cat. 2	Affibodies	58	~6	α-helical	Protein A
	DARPins	67+(n*33)	14-20	α-helical and B-turns	Ankyrin repeats

6.4. Provider companies

Commercially available libraries of the linear, cyclic, bicyclic peptide and protein scaffolds are offered by some companies such as Creative Biolabs, New England Biolabs (NEB) Pepscan, among others. For linear peptide libraries, NEB offers the M13KE cloning vector to build customized libraries and provides premade linear libraries. The NEB instruction manual specified that they offer one library made up of 7 residues (heptapeptide) and another library made of 12 (dodecapeptide). The projected diversity for both libraries is in the order of 10^9 independent peptides. This means that not all possible peptide arrangements will be achieved (GmbH, 2009; NEB, n.d.). In the case of Creative Biolabs, on the institution website, they advertise three premade linear peptide libraries composed of nine, sixteen, and twenty residues, respectively. These libraries are designed for an *ex-vivo* selection and do not encode Cys codons that can contribute to the formation of unwanted constraints in the peptide displayed, causing unpredictability while screening the library. Detailed information and characteristics of the libraries can be found on the institution's website (Creative_Biolabs, n.d.).

Regarding protein scaffolds, Creative Biolabs contains a complete set of scaffolds that are compatible not only to Ph.D. but to the ribosome, mRNA, and cell surface display and can be revised on its website (*Engineered Protein Scaffold Library Construction Service* -

Creative Biolabs, n.d.). For anticalin libraries, the German pharmaceutical company Pieris offers libraries with a diversity of up to 1 x 10^{11} (*Libraries :: Pieris Pharmaceuticals, Inc. (PIRS)*, n.d.).

SECTION VII

7. Applicability in the Ecuadorian context

CPs have an exciting potential to develop antivirals. To target dengue virus (DENV) proteases, some approaches that do not involve Ph.D. have been tried, such as using CPs through molecular docking techniques to find inhibitors (Tambunan & Alamudi, 2010). In another approach, a team used conotoxins as a library and was able to identify inhibitors against NS2B-NS3 protease (Xu et al., 2012). These efforts have focused mainly on targeting nonstructural proteases NS2B and NS3.

In the Ecuadorian context, in 2019 and 2020, the country experienced a large dengue outbreak of 8416 cases (Navarro et al., 2020) that accompanied the COVID-19 pandemic, outclassing the capabilities ever further of the health care system. Another latent danger comes from the Zika virus (ZIKV). In 2016, after the earthquake in the coastal region, an outbreak of ZIKV proved to be a national threat (Fors et al., 2018; Pacheco Barzallo et al., 2018). Interestingly, ZIKV shares several commonalities with DENV.

DENV and ZIKV belong to the family *flaviviridae*, genus Flaviviruses. Both have single-stranded positive-sense RNA (ssRNA). It's generally accepted that DENV has 4 different serotypes, known as DEN-1, DEN-2, DEN-3, and DEN-4, although there is evidence pointing to a fifth serotype DEN-5.(Mustafa et al., 2015) ZIKV also has one serotype that has been classified into African, Asian, and American lineages due to genetic variation.(Gutiérrez-Bugallo et al., 2019). The four serotypes of DENV share an average of 73.4% of genetic material, and ZIKV lineages are 99.2% identical.(Elong Ngono & Shresta, 2018). DENV and ZIKV are arboviruses meaning the transmission is performed by blood-feeding arthropods. These vectors belong to the genus Aedes: Ae. aegypti and Ae. Albopictus. DENV uses both vectors. ZIKV is transmitted to humans mainly by Ae. aegypti (Shragai et al., 2017).

7.1.Virology of DENV and ZIKV

Considering the mechanism at the molecular level of interaction between virus-vector and virus-humans, we can find some similarities. It is known that a virus is composed of many types of proteins that mediate its attachments to host cells and replication. There are structural, non-structural, regulatory, and accessory proteins. The virology and cellular tropism of DENV and ZIKV, which belong to the same family (*flaviviridae*), are very similar and share some infection mechanisms.

Flaviviruses possess a relatively tiny genome of around 10.7 Kilobases. This translates into an initial single polyprotein that contains the structural and non-structural proteins. This polyprotein is cleaved by host and viral proteases (Elong Ngono & Shresta, 2018). The precursor molecule is broken apart by the protease NS2B–NS3 (NS2B–NS3pro) into three structural proteins and seven nonstructural ones that include the proteases NS1(A. Sharma & Gupta, 2017). In the case of DENV, the NS2B–NS3pro, which are very important for viral replication, has focused on developing antiviral drugs (H. Wu et al., 2015).

7.2.Phage Display libraries contribution

Recently a group of researchers decided to switch the focus from NS2B–NS3pro to the nonstructural protein NS1 which was essential for virus survival (Songprakhon et al., 2020). They used a twelve amino acid length (12mer) Ph.D. library obtained from NEB. Using the pIII display system of M13 phage, they selected ligands that attach to NS1 of DENV-2. An initial set of 11 ‘hits’ was scaled down to 4, and its interaction was analyzed through SPR. They found that these 4 ligands showed inhibition not only with DENV-2 but also with DENV-1 and DENV-4 but not DENV-3.

This result highlights the importance and need of further development of Ph.D. technologies. There is no literature available on the use of libraries of Ph.D. that use scaffolds proteins or CP so far. By combining these technologies, it has been shown here

that exciting and surprising results can be obtained that offer a contribution to present challenges that DENV and ZIKV presents.

SECTION VIII

8. Conclusions and Recommendations

This thesis work has reviewed different examples that show the vast contribution of Ph.D. through constrained peptides to academia, the pharmaceutical industry, and medicine. Therefore it can be concluded that:

- The superiority of a constrained setting for a peptide over linear counterparts has positive implications in binding to its intended target due to a thermodynamically favorable profile that results from a reduction in entropy.

- Disulfide bridged peptidic structures exist in nature, and technologies like SPS have facilitated its creation. Monocyclic peptides offer improved stability over linear ones. Bicyclic peptides increase even further stability and provide a steadier contact interface with the target. The production of bicyclic peptides using chemical linkers and disulfide bridges has facilitated the creation of Ph.D. libraries to treat hereditary angioedema and cancer, among others.

- Protein scaffolds combined with Ph.D. make peptide interaction even more practical because of size, structure, affinity, and natural origin. This also has favorable implications regarding immunogenicity. Although the family of protein scaffolds comprises more examples than the ones reviewed here, they have offered insight into the working mechanisms and hopefully reassure the necessity of maintaining the endeavors to research new ways to target a determined biological interaction. Protein scaffolds show good potential to give birth to first-in-class drugs.

- Ab engineering has shown precise results, as in the case of monoclonal antibodies, some have been FDA approved, but Ab technology is not without challenges mentioned in this work. Protein scaffolds still have improvements to

match the properties of antibodies, but the goal may not replace Abs but find ingenious solutions where Ig technology does not reach the desired outcomes. Or to find a low-cost alternative solution to make it accessible to a broader population. Protein scaffolds have improvements in fronts like serum half-life and tissue penetration.

- In the Ecuadorian context, Ph.D. libraries can be used to find antivirals that target proteases or non-structural proteins of DENV and ZIKV. Both of these have tested the local healthcare system's capacities due to natural disasters or the COVID-19 pandemic. There is no literature available on the use of libraries of Ph.D. that use scaffolds proteins. By combining these technologies, it has been shown here that exciting and surprising results can be obtained that offer a contribution to present challenges that DENV and ZIKV presents.

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