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Original article

# Cell-based peptide screening to access the undruggable target space

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ABSTRACT

Only 20–30% of drug target proteins can be accessed by common drug classes, like small molecules or therapeutic antibodies. The vast majority of the remaining proteins are considered “undruggable” and include drug target proteins, like transcription factors, scaffold or adapter proteins, which play important roles in disease. However over the last years innovative compound classes including nucleotide derived drugs (e.g. siRNA, antisense), macrocyclic compounds and cell-permeable peptides matured significantly and hold now the potential to modulate these hard to access target proteins for therapeutic use.

This article will focus on the discovery of cell-permeable peptides and discuss intracellular screening systems for peptides, which yield highly relevant peptides, because peptide selection takes place in eukaryotic cells, under conditions, which are very similar to the later therapeutic use.



## 1. The undruggable target space

Over the last decades traditional drug discovery approaches have focused on small molecule drugs. A major pharmacologic advantage of this drug class is oral availability, systemic exposure and cellular uptake. It became evident that obeying certain physicochemical properties, known as Lipinski rules and their improvements [1,2], would increase the probability of a chemical compound to achieve drug-like properties. However these chemical properties (e.g. molecular weight <500) limit the use of small molecule drugs to mostly enzymatic proteins and certain membrane proteins (e.g. G-protein coupled receptors). In order to bind strongly and provide specificity small molecule drugs need to make extensive contacts to the target protein. This is best achieved in deep, mostly hydrophobic pockets of the target proteins. Another important drug class, therapeutic antibodies, act exclusively on target proteins on the outside of cells. Bioinformatic analysis suggests that <10% of the human proteome is residing on the cell surface or is being secreted [3]. This leaves a considerable part of those proteins difficult to access, which reside inside of cells and which are hardly accessible by small molecule drugs. This set of drug target proteins includes protein classes like transcription factors, adapter and scaffold proteins. These non-enzymatic target proteins have mostly flat surfaces which make it difficult for small molecule drugs to bind with high affinity. There are many analyses about the size of the “undruggable” target space [3–5] ranging from 70 to 80% of the human genes, thereby constituting the majority of the human target proteins.

Since most “undruggable” targets are non-enzymatic, modulation of their activity has to be achieved by other ways than the classical blocking of an active enzymatic center. A given protein's activity can be interfered with by lowering the abundance of the protein's mRNA (e.g. by siRNA, antisense) or protein amount (e.g. by targeting it to degradation, [6]). Also it may be inhibited by a classical protein binding compound, which blocks important protein–protein interactions and/or triggers an allosteric response of the target protein [7,8].

## 2. Cell-permeable peptides as drugs

Peptides are inherently unable to enter cells and would therefore not be able to access, bind and modulate an intracellular “undruggable” target protein. However there are different ways to aid the membrane passage of peptides, like the addition of a cell penetration peptide tag (e.g. Tat, octa-arginine and others; [9]). A

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common way to link such cell penetrating peptide tags to a given peptide is to synthesize a linear bipartite peptide by adding the penetrating peptide sequence C- or N-terminally to the peptide of interest [10].

Also peptide cyclization by means of an intramolecular hydrocarbon bridge (olefinic "stapling", [11,12]) may confer cell permeation to such constrained peptides [13]. Other possibilities are chemical and structural modifications (e.g. N-methylation of the peptide backbone), which increase the hydrophobic properties of a given peptide and mask its solvent H-bonding [14,15]. This strategy is used by some natural cell permeable peptides, like cyclosporin A [16].

These modifications led to the development of a variety of cell-permeable peptide modulators, which showed efficacy in tissue culture and in various animal models, like a BH4 anti-apoptotic peptide preventing cardiac ischemia-reperfusion injuries [10] or an MDM2/MDMX modulating stapled peptide for p53-dependent tumors [17].

In addition several clinical trials of CPP-peptides have been successful (see below). Cell-penetrating peptide drugs (cyclosporin A and Depsipetide/Romidepsin) which were derived from natural products even progressed through pharmaceutical registration and are sold as drugs on the market [18].

## 3. Strategies and screening systems to identify peptidic modulators

In principle there are at least three ways to identify peptide binders or modulators:

a) Designed peptides

After analysis of structural data e.g. from interacting proteins found in protein complexes, structural elements found at the binding interface, e.g. an alpha helix, are used as starting point for peptide development. This approach takes advantage of existing structural information, which gives valuable information for the optimization of the molecule (improvement of binding is supported by structural modeling of optimized versions). However there are also drawbacks because this approach is based on a reliable hypothesis, namely the function of the interacting proteins and the causal role of the protein complex in disease. The starting point is normally a single or a limited number of independent hit molecules, which bears the risk of limiting backup molecules during peptide development. There are many examples of this approach, (e.g. Ref. [19]). Usually these peptides need to be structurally constrained (e.g. by cyclization; [13]) or are being grafted onto a scaffold protein [20].

b) *In vitro* screening for peptide binders

*In vitro* screening systems, like phage display, reviewed in Ref. [21], ribosome display, reviewed in Ref. [22] and mRNA-display, reviewed in Ref. [23] use recombinant peptide libraries and often purified recombinant target proteins for peptide binding. These systems enrich for improved peptide binders in successive cycles of peptide library binding, washing away low affinity or unbound peptides, peptide elution and propagation of the enriched peptide binders [24]. General principle is the fact that the expressed recombinant peptides are linked to their encoding DNA/RNA (in a phage particle or by a stalled ribosome bound to the peptide encoding RNA). An advantage is the vast complexity of the libraries and the number of peptides, which can be screened *in vitro* (phage display:10E9 to 10E11 phages). Always challenging is the selection of the screening buffer composition (e.g. salt concentration, pH, redox potential, co-solvents), which has a significant impact on the type of selected binders. Therefore the specific binding conditions used for selection may limit the resulting spectrum of the isolated binders and the targetable epitopes.

c) Cell-based screening

There are various peptide or protein fragment screening systems which are based on a two hybrid principle in different organisms (bacterial and yeast based; [25]). Quite common are two hybrid systems with transcriptional read-out. These systems are prone to the generation of false positive candidates resulting from transactivating peptides contained in the peptide library. A novel peptide screening system, based on Recruitment Screening Systems [26,27] has been developed in the author's lab. This system functions with high stringency in the cytoplasm via the mitogenic RAS signal transduction pathway.

## 4. Recruitment Screening Systems

The increasing interest in transcription factors and their interacting proteins led to the development of a novel screening system, whose selection mechanism was not disturbed by the use of proteins involved in transcriptional control, like the conventional two hybrid system. This novel recombinant screening system for protein interactions, termed Sos- or Ras-Recruitment-System, SRS/RRS [28,29,26] is functioning in the yeast *S. cerevisiae*. Upon interaction of two proteins the mitogenic Ras-signaling pathway is specifically activated leading to proliferation of yeast colonies containing the interacting partners. This screening system has been used successfully for the identification of novel protein interaction partners in the lab of the author as well as others [30–32,28,33–35]. SRS/RRS systems take advantage of the intracellular milieu for protein expression, folding and interaction and allow selection under natural intracellular conditions. Interestingly cell-based screening systems can exploit the presence of additional cellular proteins, which support the formation of certain protein interactions/complexes. In an RRS protein–protein interaction screening with cyclin dependent kinase 4 (CDK4) several CDK-inhibitor proteins (the tumor suppressor proteins p15, p16, p21) were identified (Bachmann, M, doctoral thesis, University of Essen-Duisburg, Germany). It is known that the identified CDK-inhibitor proteins make only rudimentary contacts with CDKs but more extensive contacts with cyclins. This suggests that the eukaryotic yeast cell supplies the cyclin component of the complex, which leads to bridging of CDK and CDK-inhibitor proteins resulting in activation of the screening system. Another beneficial aspect of using cell based screening systems is taking advantage of covalent modifications, which take place spontaneously or are driven by co-expressed modifying enzymes, like protein kinases. The recombinant tethering of the upstream MAP-kinase MKK4 to the stress kinase JNK1 led to JNK phosphorylation, which in its phosphorylated form recognized the transcription factor TCF4 in an RRS-screening. This led to the identification of the linkage between Wnt and MAP kinase pathways via phospho-JNK1 and TCF4 [36].

The use of the SRS- and RRS-screening systems showed the following features for the *de novo* identification of interaction partners. Both systems use a stringent and titratable selection mechanism which is not dependent on transcriptional read-out. This avoids false positives from spurious transactivating sequences contained in peptide libraries. Yeast cells provide the environment for the expression and folding of both protein interaction partners in eukaryotic cells. The selection of interaction partners takes place in the cytoplasm of eukaryotic yeast cells, providing the proper binding milieu. Yeast cells support the

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