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Peptide aptamers as guides for small-molecule drug discovery

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Peptide aptamers are combinatorial protein reagents that bind to target proteins with a high specificity and a strong affinity. By so doing, they can modulate the function of their cognate targets. Because peptide aptamers introduce perturbations that are similar to those caused by therapeutic molecules, their use identifies and/or validates therapeutic targets with a higher confidence level than is typically provided by methods that act upon protein expression levels. The unbiased combinatorial nature of peptide aptamers enables them to 'decorate' numerous polymorphic protein surfaces, whose biological relevance can be inferred through characterization of the peptide aptamers. Bioactive aptamers that bind druggable surfaces can be used in displacement screening assays to identify small-molecule hits to the surfaces. The peptide aptamer technology has a positive impact on drug discovery by addressing major causes of failure and by offering a seamless, cost-effective process from target validation to hit identification.

Drug discovery is one of the most challenging scientific endeavors. This subjective statement finds support in the unquestionable productivity crisis of the pharmaceutical industry, which, despite enormous investment in new technologies and partnerships with Biotech companies, currently introduces fewer drugs onto the market than was the case 20 years ago [1]. Many authors have attempted to identify the reasons for this productivity crisis. Because drug discovery follows a highly complex, multistep process, one means of straightforward analysis is to consider the attrition rates at each step of the process, to pinpoint and weigh up the numerous factors that can cause the failure of a drug discovery project [1]. Lack of efficacy and too high toxicity observed in clinical trials remain two major causes of attrition. Although both phenomena are determined by a multitude of factors, the inability to anticipate lack of efficacy and toxicity is most often explained as being a result of one or more of three shortcomings: inadequate animal models, insufficient validation of therapeutic targets and/or insufficient specificity of drug candidates. Although it should be possible to improve the predictive value of animal models for certain pathologies, this task will remain difficult for highly complex, multifactorial diseases such as cancer. Adequate validation of a therapeutic target calls for the introduction of a molecular perturbation in a cellular or animal model. Thus, the major room for improvement concerns the type of perturbation that can be introduced into each model that will most probably remain imperfect. The assessment of specificity (or lack thereof) of drug candidates will increasingly benefit from the continuing progress in proteomics. When combined with impressive new detection technologies to measure binding, the quest for small-molecule drugs that discriminate between closely related proteins (even within single protein families) could be made more fruitful through the targeting of noncanonical molecular surfaces that mediate protein–protein interactions or that are involved in the structural activation of target proteins.

Here, we review recent efforts that have transformed the peptide aptamer technology into a powerful drug discovery approach, enabling a seamless process encompassing high-stringency target identification and/or validation, identification of novel biologically relevant molecular surfaces and discovery of small molecules targeting these surfaces. Because it addresses at least two major causes of attrition, the peptide aptamer technology could have a significant and much needed impact on drug discovery.

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Peptide aptamers

Peptide aptamers are combinatorial protein reagents that were initially developed as research tools to dissect protein function within complex molecular regulatory networks [2], reviewed by Colas [3] and Hoppe-Seyler *et al.* [4] (Box 1). The design of peptide aptamers is inspired directly from the structure of immunoglobulins or T cell receptors, where variable peptidic loops are displayed by constant framework regions (Figure 1). Two fundamental differences distinguish peptide aptamers from these complex naturally occurring recognition molecules. First, peptide aptamers are extremely simple, consisting of a single variable peptide loop constrained within a constant scaffold protein. Second, the double constraint of the variable loop does not depend on disulfide bonds but is simply ensured by an insertion between two closely located residues of a rigid scaffold protein. This double constraint distinguishes peptide aptamers from other man-made combinatorial protein molecules, which often consist of random peptidic sequences fused terminally to a carrier protein or another macromolecule [3]. This design renders peptide aptamers less vulnerable to proteases and enhances the average binding affinity by reducing the conformational freedom of the variable loops. So far, the scaffold protein most often used has been thioredoxin [5] but other scaffolds such as green fluorescent protein [6], staphylococcal nuclease [7] or the protease inhibitor Stefin A [8] have also been explored.

For all of the above-mentioned reasons, peptide aptamers are ideally suited to bind intracellular target proteins, and they can be conveniently selected using intracellular techniques. Yeast two-hybrid methods are most often employed to select peptide aptamers for their ability to recognize target proteins in cells [9]. However, a bacterial display system has also been used to select peptide aptamers against antibodies, proteins and even living cells [10]. Peptide aptamers can also be selected for their ability to confer a cellular phenotype, through a process referred to as 'phenotypic selections' (detailed below).

BOX 1

Peptide aptamer definition

Roger Brent coined the term 'peptide aptamer' by analogy to nucleic acid aptamers, which were developed before peptide aptamers. When used alone, the word 'aptamer' generally designates an RNA or a DNA aptamer. The strict definition of a peptide aptamer is as follows: 'combinatorial protein molecule consisting of a variable peptidic sequence inserted within a constant scaffold protein'. The variable region is thus doubly constrained because both termini are fused to the scaffold (Figure 1). Other types of constrained combinatorial protein reagents have been designed [39] and some of them are being developed as biopharmaceuticals or detection molecules. These alternative recognition molecules are more complex than peptide aptamers because target-binding surfaces consist of noncontiguous peptidic sequences disseminated on several secondary structural elements or across several variable loops. Moreover, many of these molecules require disulfide bonds to fold properly and are thus ill suited to target intracellular proteins. In this review, we focus solely on the use of peptide aptamers in drug discovery.

Target validation

Target validation poses an extremely difficult challenge for two reasons. First, it relates to complex, fundamental biological questions (namely, a thorough understanding of the (dys)function of proteins in normal and pathological situations). Second, target validation is most often achieved through perturbing protein function by various means that are useful for collecting biological information but are inadequate for providing true validation, in that they introduce perturbations that are different from those caused by small-molecule therapeutics. The most popular methods used to validate targets act upon protein expression levels in cellular or animal models. Gene knockouts abolish expression, whereas antisense or RNA interference methods reduce expression

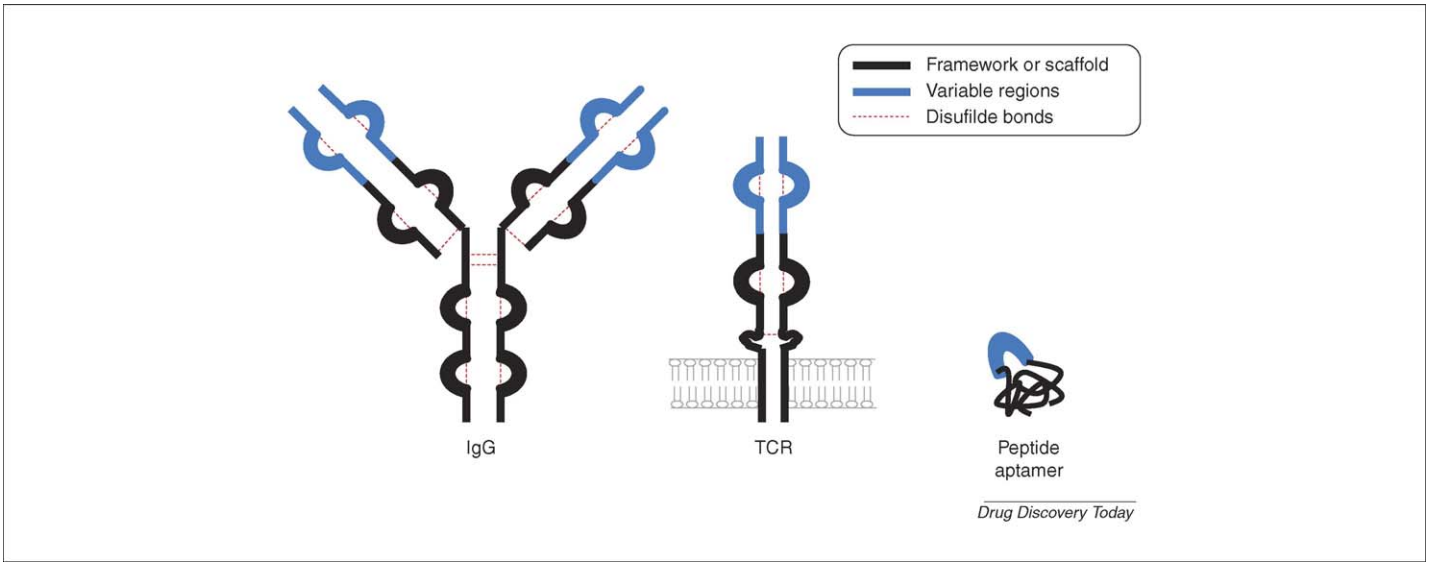

FIGURE 1

Recognition protein molecules. Three major classes of constrained recognition protein molecules are depicted: immunoglobulin G (IgG), T cell receptor (TCR) and peptide aptamer. Black lines represent constant framework or scaffold residues. Blue lines represent variable peptidic regions. Red dotted lines represent disulfide bonds. Major distinguishing features of peptide aptamers include the much smaller size and the absence of requirement of disulfide bonds to ensure structural constraint of the variable region, as opposed to IgG and TCR molecules.

to various extents. Overexpression of target proteins is also often performed, either constitutively or in an inducible fashion. However, the overwhelming majority of therapeutic interventions does not affect protein expression levels but rather aims at inhibiting protein function through the use of a specific ligand (small chemical or biotherapeutic molecule). Because of this fundamental discrepancy between the perturbations introduced by most target validation methods and by therapeutic molecules, target proteins can be either falsely validated or falsely rejected. In the first case, the abolition or reduction of a protein expression level might cause a beneficial phenotype that cannot be reproduced by the use of an inhibitory molecule. One obvious reason could be that acting upon expression levels introduces perturbations throughout the molecular network surrounding the target protein, whereas using a specific ligand against the said protein causes more limited perturbations (Figure 2). In the second case, the abolition or the decrease in protein expression levels can trigger compensatory mechanisms or can be masked by functional redundancy within protein families and lead to no conspicuous phenotype, in contrast to the use of a ligand that might either perturb the molecular network without triggering a compensatory mechanism, or that might inhibit different closely related members within a protein family and hence bypass functional redundancy.

For these reasons, a rigorous definition of target validation could be: 'validating a therapeutic target is demonstrating that the use of a ligand against the said target induces the desired phenotype in relevant cellular or animal models of pathological situations'. The closer the properties of the validating ligand are to those of the therapeutic eventually developed, the higher the confidence level can be regarding the protein as an appropriate therapeutic target. No higher stringency test exists until binding to the target is correlated with clinical efficacy during later clinical development. The vast collections of small-molecule inhibitors that could be assembled through the generalization of chemical genomics efforts should offer ideal tools for high-confidence target validation [11]. However, the high cost of screening platforms and the scarcity of key know-how (e.g. medicinal chemistry) and significant collections of chemical compounds within the academic world will probably hinder the implementation of chemical genomics to the extent needed to deliver on its promises. Peptide aptamers already offer a means of high-confidence, ligand-based target validation. Peptide aptamers have been selected against a wide range of target proteins, encompassing cell-cycle regulators (Cdk2, E2F, cyclin J) [2,12–15], viral proteins (human papillomavirus proteins E6 and E7, human hepatitis B virus core protein) [16–18], small G proteins and exchange factors (Ras, Rho–guanine nucleotide exchange factors) [19,20], a receptor tyrosine kinase (epidermal growth factor receptor) [21], a transcription factor (signal transducer and activation of transcription 3) [22] and many more target proteins within the in-house programs of those companies with access to the peptide aptamer technology. In every case, some aptamers among those selected have been shown to interfere with the function of their cognate target when expressed (by transgenesis) or introduced (as recombinant proteins fused to protein transduction domains) in cellular models [12,13,16–22] or in *Drosophila* [14,15]. Three key parameters enable peptide aptamers to exert a wide range of perturbations on protein function. First, their typical binding affinity lies between 1 nM and 100 nM [2] and can be further improved by performing *in vitro* evolution experiments [23]. These binding affinities are significantly better than those of other combinatorial protein reagents [24] and can be explained by the double conformational constraint of the variable peptidic loops. The high binding affinity of peptide aptamers for their cognate targets enables disruption of protein–protein interactions, and such has often been suggested as the basis of their mechanism of action. Other inhibitory mechanisms have also been reported, such as locking the target protein in an inactive state or binding enzyme active sites. Another key parameter that influences the perturbations exerted by peptide aptamers is their specificity of binding. Yeast two-hybrid mating assays have revealed that peptide aptamers can exhibit an acute specificity for their target protein and can discriminate between closely related members within a protein family [2], and even between different allelic variants of a given protein [25]. Interestingly, peptide aptamers can also exhibit different specificity profiles towards a protein family, ranging from an absolute specificity against the target protein to an ability to cross-react with other members of the target family. This particular property might prove to be important because the concept of 'multitargeting' promises to be a fruitful avenue for drug discovery and development, especially when addressing protein families whose members exhibit functional redundancy. Finally, peptide aptamers 'decorate' target proteins by binding to many different surfaces (as detailed below), and thus interrogate the targets with regard to biologically relevant binding sites. The binding site of each bioactive peptide aptamer (an aptamer that generates a desired phenotype when expressed in a cellular or animal model) represents an opportunity to inhibit a particular function or interaction involving the target. In a recent study using a bacterial protein as a test case, we have compared the perturbations exerted by a gene knockout, a dominant negative allele and a set of peptide aptamers selected against

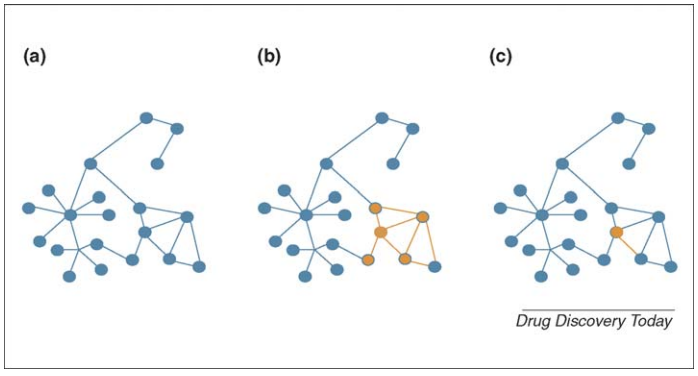

FIGURE 2

Perturbations on molecular regulatory networks. (a) An imaginary molecular regulatory network is depicted, where the balls represent proteins and the lines linking the balls represent protein interactions. **(b)** Perturbations exerted by popular methods that decrease or abolish protein expression. The target protein is colored in orange. As it interacts with, and acts upon, more than one protein (a common scenario), different molecular pathways, leading to different cellular responses, are affected. **(c)** Perturbation exerted by a peptide aptamer or a therapeutic molecule. The peptide aptamer or the molecule binds to a specific surface on its target protein, preventing the target protein from interacting with one of its partners or inhibiting a specific function, without affecting the 'hardware' of the regulatory network. Consequently, a more targeted perturbation is achieved.

the protein. We have shown that the peptide aptamers introduced a wider range of perturbations with regard to protein function than more classical reverse genetics approaches.

Target identification

The identification of therapeutic targets relies more on observational rather than experimental methods. Human genetics, gene profiling and proteomics, when combined with clinical studies, establish correlations between mutations or expression levels and disease. Proteome-scale protein interaction mapping also points to putative therapeutic targets by unraveling the molecular networks of which the proteins found mutated or differentially expressed in pathological situations are a part. As a correlation is not a causal link, the putative targets identified by such means must be subsequently validated.

Recent developments of libraries of transdominant genetic agents have made it possible to use experimental methods to identify targets with good therapeutic potential. The principle consists of applying the library to an appropriate cell or animal system and identifying the agents responsible for conferring a given phenotype. The identity of the cognate target proteins is then determined. A significant advantage of these perturbational methods is that the target proteins are already (pre)validated, even before they are identified, because the selection endpoint is the desired phenotype, such as neuronal survival or tumor cell growth arrest. Libraries of complementary DNA fragments [26] or short hairpin RNAs [27] have been constructed and used for phenotypic selections. Determining the selected coding sequences reveals the identity of the proteins whose inhibition or downregulation produces the selected phenotype. Retroviral libraries of combinatorial peptides have also been used to perform phenotypic screens and to identify target proteins [28]. In an in-house phenotypic selection program, we have constructed different retroviral libraries of peptide aptamers, which we have used successfully to select aptamers that inhibit tumor cell growth. In contrast to a nucleic acid-based approach, the identity of the target proteins is not immediately revealed by the sequence of the selected peptides or peptide aptamers, which are subsequently used as baits to identify the targets, either by yeast two-hybrid screening experiments [7,29,30] and/or by protein pull-down assays followed by mass spectrometry [31]. However, the use of libraries of combinatorial protein reagents in phenotypic screens presents two considerable advantages. First, these reagents might inhibit, but might also activate, the function of their cognate target proteins, offering enhanced opportunities to identify targets of interest and reveal new mechanisms of action. We have shown that an aptamer selected for its ability to inhibit tumor cell proliferation binds and, indeed, activates rather than inhibits the enzymatic activity of its target. Second, as discussed above, the degree of target validation provided by these protein ligands is higher than that provided by other methods because they act by binding the target, as opposed to lowering its expression level.

Identification and validation of druggable sites

We have developed an approach (called AptaPrint^{TM}) that enables us rapidly to map the target sites recognized by the peptide aptamers (Figure 3). The prerequisite is that the structure of the target protein is available or can be modeled by homology. The first step consists of analyzing the structure of the target and localizing salient features such as enzyme active sites, sites mediating protein interactions, or pockets that can be predicted as being druggable. In a second step, amino acid substitutions are introduced at these different sites by directed mutagenesis to assemble a panel of target mutants. Yeast two-hybrid mating assays are then performed between this panel and the collection of peptide aptamers selected against the wild type target. Those aptamers that no longer bind a mutant are most likely to bind the surface subjected to the mutation [12]. A possible caveat of this approach is that some amino acid substitutions can introduce

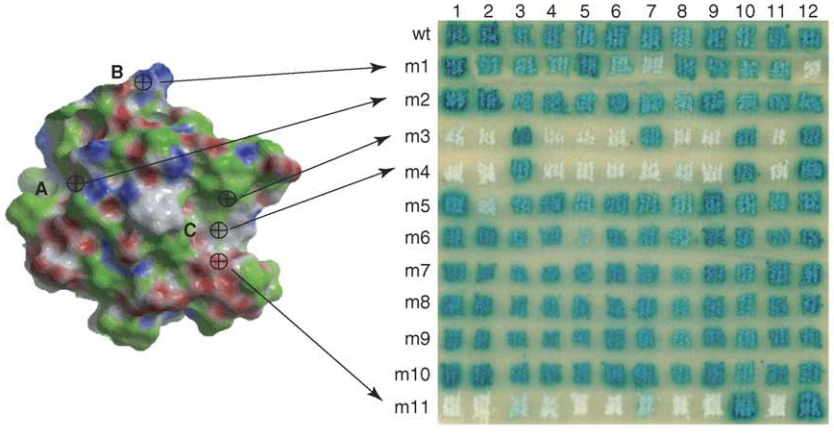

FIGURE 3

AptaPrint^{TM} – mapping peptide aptamer-binding sites on target proteins. In this analysis, 12 different peptide aptamers were analyzed against a panel of 11 mutated forms of their cognate target protein, in a yeast two-hybrid mating assay. The reporter gene used in this experiment is *lacZ*. Pockets A, B and C were interrogated by mutations m2, m1 and m3/4/11, respectively, whereas other molecular surfaces were interrogated by mutations m5 to m10. Here, no peptide aptamer binds to surface A, whereas aptamer 12 binds to surface B and many aptamers bind to surface C. Interestingly, their precise binding mode shows subtle differences (compare profile of aptamers 6 and 7). Further mutagenesis experiments would be needed to map the binding site of aptamer 10. Abbreviation: wt, wild-type target protein.

delocalized structural modifications, by inducing allosteric mechanisms or by affecting the proper folding of the protein. This problem can be overcome by performing a high-resolution mutagenesis of the site of interest and of its vicinity. Another possible caveat relates to the imperfect accuracy of the software used to build structural models by homology, when working on protein targets whose structure has not yet been determined. We have applied this approach to many diverse target proteins. In every case, we have observed that our collections of selected peptide aptamers bound to many different surfaces, located on different functional domains of the proteins. Although peptide aptamers are protein molecules, we have observed that aptamer-binding sites are not necessarily flat surfaces (known to mediate most protein–protein interactions) but rather are structurally diverse and include molecular pockets.

Although other technologies have been developed that bridge ligand-based validation of therapeutic targets to small-molecule discovery [32], a distinguishing feature of the peptide aptamer

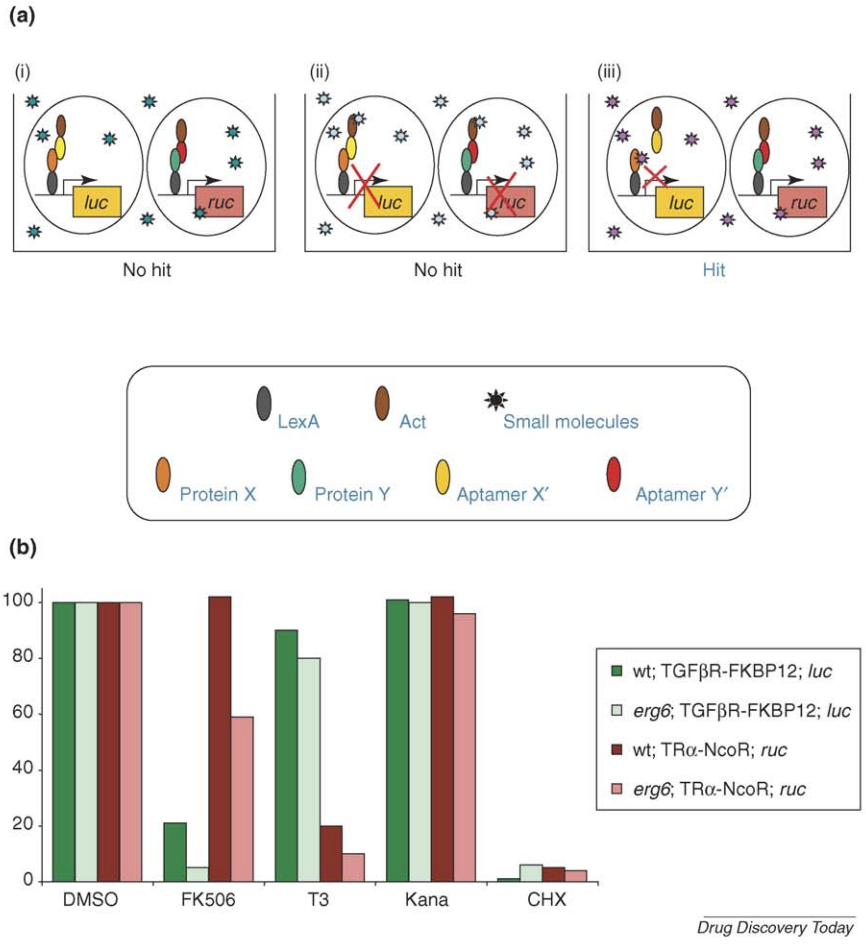

FIGURE 4

AptaScreen™ – a yeast two-hybrid peptide aptamer displacement screening assay. (a) Principle of the assay. Two yeast strains are used, each hosting a protein–peptide aptamer interacting pair. Target proteins are expressed as 'baits' – that is, are fused to the DNA-binding domain of a transcription factor (in this case, LexA). Peptide aptamers are expressed as 'preys' – that is, are fused to a transcriptional activation domain, which recruits the RNA polymerase machinery. The protein–peptide aptamer interaction brings the activation domain into the vicinity of the DNA-binding domain, which reconstitutes an active transcription factor that directs the transcription of either the firefly (*luc*) or *Renilla* (*ruc*) luciferase, the two reporter genes used in this assay. The panels represent three wells from a 384-well plate, in which different small molecules are screened. In (i), the added molecule produces no effect on either yeast two-hybrid phenotype. In (ii), the added molecule inhibits both phenotypes, either because it interferes with the yeast two-hybrid assay (as depicted) or because it is toxic to yeast. In (iii), the added molecule inhibits one of the two yeast two-hybrid phenotypes, and is therefore scored as a hit candidate, whereas the two other molecules are not considered further. **(b)** Experimental validation of the screening assay. Two protein interactions were used, for which known small-molecule inhibitors were available. Transforming growth factor β–FK506-binding protein (TGFβR–FKBP12) and thyroid hormone receptor–nuclear receptor co-repressor (TRα–NcoR) interactions are inhibited by FK506 [36] and tri-iodothyronine (T3) [37], respectively. Kanamycin (Kana) and cycloheximide (CHX) were used as test molecules, showing no toxicity or a strong toxicity for yeast, respectively. The experiments were performed using a wild-type and an *erg6* yeast strain, a mutation known to enhance yeast permeability to small molecules [38]. This result shows that the assay can detect the inhibition of both protein interactions by their cognate inhibitors, and that toxic molecules such as CHX can be immediately identified and discarded. It also shows that the detected inhibitions are more pronounced in the *erg6* than in the wt strain, as can be expected from an enhanced permeability to small molecules. The experiment was performed in a 384-well plate, whereby wild-type strains or *erg6* strains were mixed in five different wells. Small molecules were tested at a concentration of 10 μM. Luminescence values were normalized at 100% in the presence of dimethyl sulfoxide (DMSO).

technology lies, on the one hand, in the ease of mapping aptamer-binding sites through AptaPrint™ and, on the other hand, in the great structural diversity of the said aptamer-binding sites. At least two reasons can be evoked to account for this diversity. First, peptide aptamers are protein molecules and they can thus interrogate molecular surfaces that mediate the interaction of their target proteins with their natural partners. Conceivably, peptide aptamers can either accurately mimic naturally occurring protein interactions by contacting the entire protein-binding surfaces, or they can bind to portions of the said surfaces, such as 'hot spots' that contribute the most to binding energies. Second, in many cases, target-binding surfaces are confined to the variable regions of the peptide aptamers and do not involve scaffold residues. Over the past few years, we have gradually reduced the size of the variable regions, from the initial 20 amino acids [2] to 13 and even eight amino acids in our most recent peptide aptamer libraries. We believe that peptide aptamers endowed with shorter variable regions comprising the entire target-binding site are better suited to interrogate molecular pockets or clefts and identify those most likely to be druggable.

The druggability of a protein determines the chances to discover small molecules that bind to it and modulate its function. It has long been considered that druggable sites are generally pockets as opposed to flat surfaces, and a spectacular demonstration has recently been obtained by the use of a computational algorithm [33]. The fact that peptide aptamers can bind to druggable sites is a key fundamental strength of the technology. Beyond providing high-confidence target validation, bioactive peptide aptamers identify and validate molecular surfaces on therapeutic targets. Those surfaces that are deemed to be 'sufficiently druggable' can be subjected to an aptamer displacement screening assay to identify small molecules targeting the said surfaces.

Discovery of small-molecule agonists of peptide aptamers

We have developed a high-throughput screening assay (called AptaScreen™) to identify small molecules that target aptamer binding sites, on the premise that peptide aptamers and small molecules can trigger the same biological effects when binding to the same target sites. AptaScreen™ is based on an automated dual-luminescence yeast two-hybrid assay, performed in 384-well plates [34]. The firefly and the *Renilla* luciferases (*luc* and *ruc*, respectively) are used as reporter genes, enabling a rapid and quantitative detection of the two-hybrid phenotypes caused by the interaction between two target–aptamer pairs, hosted by two yeast populations mixed in each well. This scheme presents two major advantages. First, the screening capacity throughput is doubled because two interactions are screened simultaneously. Second, because the assay aims at identifying small molecules that decrease the two-hybrid signal (as a result of a decreased target–aptamer interaction), it is important to be able immediately to discard toxic molecules that decrease the signal by killing the yeast. Those molecules that decrease both two-hybrid signals are considered to be toxic or to interfere with the assay, whereas those molecules that inhibit only one of the two signals are hit candidates (Figure 4). AptaScreen™ is derived from the interaction trap, a yeast two-hybrid assay in which prey expression is controlled by a galactose-inducible promoter [35]. This important feature

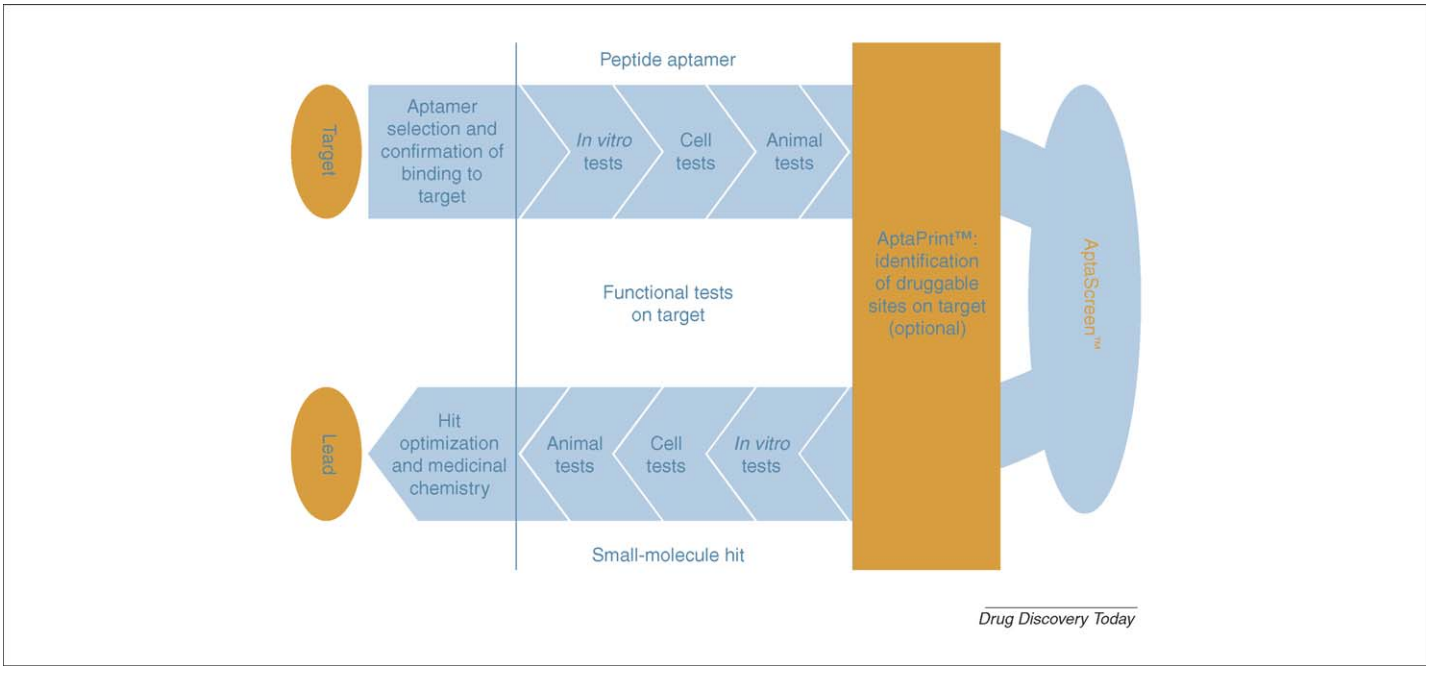

FIGURE 5

The peptide aptamer technology is a seamless drug discovery engine. This flow chart presents the seamless sequence of experimental steps performed at Aptanomics, from peptide aptamer selection to drug discovery. Peptide aptamers are selected against a known target protein by yeast two-hybrid screening, and their binding is confirmed *in vitro*. The peptide aptamers are then tested for their ability to interfere with the function of their target *in vitro*, in cultured cells and sometimes in animal models. Their binding sites on the target are mapped by AptaPrint™. Those peptide aptamers that are bioactive and that bind a druggable site are then used in an AptaScreen™ to discover small-molecule agonists. The hit candidates are confirmed using *in vitro* interaction assays and they are then tested in cellular and animal models. The bioactive hits are then optimized to obtain lead molecules.

improves hit rates because the expression of the aptamer is induced after the addition of the small molecules to the plates. Therefore, small molecules able to prevent target–aptamer interactions but that would be too weak to disrupt already formed complexes can be identified in the assay. AptaScreen™ has been automated to reach a throughput of 15,000 molecules per day, corresponding to 30,000 screening data points. By way of example, in one of our in-house discovery projects, we have screened our collection of 70,000 diverse molecules and we have identified a highly potent protein kinase inhibitor (IC_{50} <10 nM) that shows a strong anti-proliferative effect on human tumor cells (unpublished).

Conclusion

As discussed here, peptide aptamer technology addresses major shortcomings that are considered to have a significant impact on the success rate of drug discovery. It enables a ligand-based, specific perturbation of protein function, thereby providing a higher confidence level in target validation than is the case with more popular methods that affect protein expression. The unbiased combinatorial nature of peptide aptamers makes it possible to interrogate the biological significance of a large number of molecular surfaces on the targets. This approach can guide small-molecule discovery towards given target sites that are deemed to be worth pursuing on the basis of their druggability index and their biological relevance. Of equal importance, peptide aptamers can also guide small-molecule discovery away from target sites that are considered to be undesirable – for example, on the basis of their ubiquitous existence among a given protein class, making it difficult to discover specific small-molecule hits (e.g. ATP-binding sites on protein kinases). Finally, a major strength of peptide aptamer technology lies in the continuity between target identification, target validation, identification of biologically relevant druggable sites and discovery of small-molecule hits against these sites (Figure 5). This seamless process is expected to improve cost-effectiveness by expediting significantly these early phases of drug discovery.

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