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Type of target molecule influences yield in peptide phage display affinity selections

Peter Molek[1]*, Miha Vodnik[1], Nika Kruljec[1], Jernej Luzar[1], Borut Štrukelj[1,2], Mojca Lunder[1] & Tomaž Bratkovič[1]

[1]Department of Pharmaceutical Biology, Faculty of Pharmacy, University of Ljubljana, 1000 Ljubljana, Slovenia

[2]Department of Biotechnology, Jozef Stefan Institute, 1000 Ljubljana, Slovenia

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In general, relatively few panning attempts using combinatorial peptide phage display libraries yield specific binders. Major factors affecting the outcome of screening are diversity (complexity) of the library, type of randomization and conformation of peptides, quality and purity/homogeneity of the target material, as well as affinity selection strategy and conditions enforced during individual panning steps. In our experience, certain groups of structurally and/or functionally related target molecules have worked out better for the selection of peptide ligands than others. Therefore, we suggest that the type of target molecule itself may also be an important factor limiting isolation of peptide ligands and can even assist in predicting the selection outcome. Here, we summarize data from 87 selections on diverse set of 23 protein targets performed in our laboratory. It appears that especially the antibodies and the enzymes whose natural substrates are peptides or proteins tend to give better yields in affinity selections in contrast to proteins involved in protein-protein interactions or lipid metabolizing enzymes.

Keywords: Peptidomimetics, Phage library, Protein-protein interactions, Selection efficiency

Phage display combined with *in vitro* selection techniques is a widely used method for identifying ligands of soluble proteins[1,2], small synthetic molecules[3] and large biological associates[4-6]. Screening of combinatorial peptide phage display libraries has evolved into a well-established method in drug discovery, since biologically active short peptides can be used in drug target validation or serve as templates for design of peptidomimetics with drug-like properties[7-9]. However, attempts to isolate specific and high affinity binders to a chosen target often fail, suggesting that either not every biomolecule has the ability to bind short peptides or these cannot be identified by conventional panning procedures for some other reason.

Among many factors (e.g., library complexity, conformational restriction of displayed peptides[10] and panning conditions[11]), structure and biological function of the target molecule seem to have a major impact on the outcome of selection. Indeed, in our experience, selections against antibodies have yielded binders in most panning experiments, while attempts to identify peptide ligands of proteins involved in protein-protein interactions (PPI) were mostly unproductive. Here, we compare a diverse set of target proteins in light of outcome of affinity selections, taking the proportion of positive clones identified by phage ELISA as a measure of selection performance. This semi-quantitative "meta-analysis" summarizes data from a series of screening experiments performed in our lab over a period of a decade. Although for this type of analysis unproductive pannings are of relevance, such data are rarely published. Further, to avoid bias towards published successful screening experiments, we have chosen not to include data from other works.

In our study, we have classified the protein targets into groups according to their biological function (Table 1) and have aimed at establishing correlation between the efficiency of selections (screening

*Correspondence:
Phone:+386-1-47-69-619; Fax: +386-1-42-58-031
E-mail: peter.molek@ffa.uni-lj.si

Abbreviations: Ara h 2, major peanut allergen; CCL2, chemokine (C-C motif) ligand 2; Fel d 1, major cat allergen; LR-Fc, leptin receptor extracellular domain-human Fc region fusion protein; mAb, monoclonal antibody; MurD, UDP-*N*-acetylmuramoyl-L-alanine–D-glutamate ligase; MurE, UDP-*N*-acetylmuramoyl-L-alanyl-D-glutamate–L-lysine ligase; pAb, polyclonal antibody; PLA2, pancreatic phospholipase A_2; PPI, protein-protein interactions.

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Table 1—Target proteins used in screening phage libraries categorized on the basis of their primary biological function and the nature of native binding partners

Target group	Individual targets
Enzymes involved in peptide synthesis and protein degradation	Papain, UDP-*N*-acetylmuramoyl-L-alanine–D-glutamate ligase (MurD), UDP-*N*-acetylmuramoyl-L-alanyl-D-glutamate–L-lysine ligase (MurE)
Lipid metabolizing enzymes	Pancreatic lipase, lipoprotein lipase, pancreatic phospholipase A_2, ammodytoxin C
Proteins involved in protein-protein interactions	Leptin, ghrelin and des-acyl ghrelin, leptin receptor (LR) extracellular domain-human Fc (IgG_1) fusion protein, human gamma Fc region
Antibodies	Monoclonal antibody (mAb) against leptin, polyclonal antibody (pAb) against leptin, pAbs against ghrelin, pAbs against chemokine CCL2, mAb against chemokine CCL2, pAbs against cat allergen Fel d 1, pAbs against peanut allergen Ara h 2

success rate) and the type of target molecules. Importantly, predicting success rate before embarking on to an affinity selection project could save time and effort considerably, as one might decide on an alternative target. Notably, there are reports of successful selections against cytokine-neutralizing antibodies (serving as molds for cytokine receptors) yielding peptides with cross-reactivity with cognate receptors, whereas direct receptor targeting is notoriously difficult[12,13] (reviewed in ref. 14).

Materials and Methods

The source of target material is listed in Supplementary Table S1. Ph.D. libraries were purchased from New England Biolabs (Ipswich, MA, USA). Microtiter plates used in ELISA assays were F8 MaxiSorp Loose Immuno Module (Nunc, Roskilde, Denmark).

Affinity selections

Altogether 87 selections against 23 targets were performed. Selections differed in screening conditions (e.g., stringency and elution techniques) and strategies employed (i.e., panning against immobilized targets *vs.* solution panning); see Supplementary Table S1 for details. All random peptide libraries employed were based on the same vector (filamentous phage M13KE) and had comparable diversities. Protein targets are listed in Table 1.

Evaluation of individual panning experiments

The proportion of clones from enriched libraries displaying binding affinity to individual targets (as inferred from monoclonal phage ELISA) was calculated and is listed in Supplementary Table S1. All ELISA assays were performed as previously described[15]. Phage clones producing absorption signals of at least 0.3 and twice that of the corresponding negative control (uncoated blocked wells) were automatically considered binders. Some clones did not match up to the set criteria, but were further confirmed to be specific binders by additional assays, such as competitive ELISA or enzyme inhibition assay using the cognate synthetic peptides. The number of subsequently confirmed target-unrelated binders[16,17] enriched in selections against lipoprotein lipase, ammodytoxin, pancreatic phospholipase A_2, and ghrelin was subtracted from the initial number of hits. Whereas the preliminary polyclonal ELISA indicated absence of binders in eluted phage pools, individual clones were not screened for affinity in monoclonal ELISA setting and the selection was regarded unproductive (a value of 0% was assigned to the success rate).

The relative proportion of binders among randomly picked clones following last selection round was considered a semi-quantitative measure of performance rate for the individual target to capture ligands from a diverse pool of peptides. In other words, the higher the proportion of binders, the higher the propensity of the target to enrich binders from random peptide library.

Comparison of affinity selection success rates across target groups

The percentage of positive hits for individual targets was compared to estimate the ability of individual molecules to bind short peptides, regardless of the library type (Fig. 1). At least two structurally different library types (i.e., one containing linear and the other cyclized peptides) were screened against most target molecules, except in few cases where rational reasons for choosing only one library type existed. For example, papain was only targeted with library of constrained peptides as linear peptides would likely be cleaved off the phage by the enzyme. Ghrelin, on the other hand, is a relatively short peptide forming a random coil conformation in solution and was thus probed only with a linear peptide library.

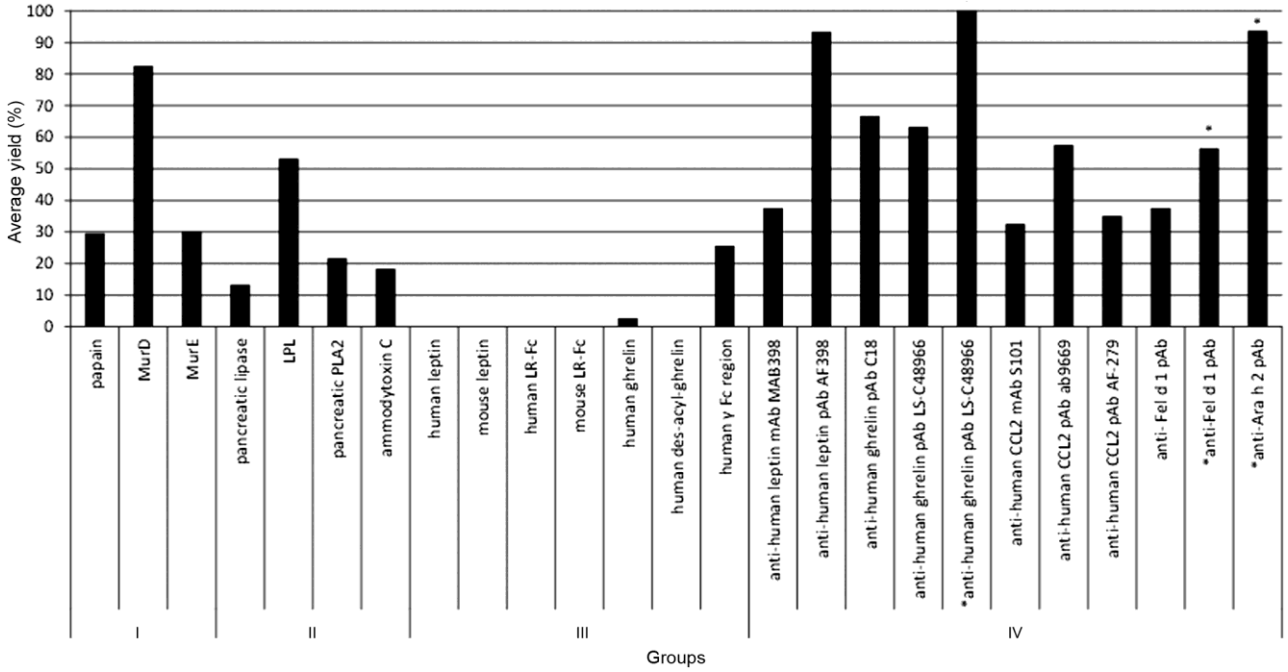

Fig. 1—Depiction of the percentage of positive hits among all clones screened for individual target molecules regardless of the library screened [Group-I: Enzymes involved in peptide synthesis and protein degradation; Group-II: Lipid metabolizing enzymes; Group-III: Proteins involved in protein-protein interactions; and Group-IV: Antibodies] Note that various screening strategies were employed for most targets. Asterisk (*) above the bar indicates that anti-human ghrelin pAb LS-C48966, anti-Fel d 1 pAb, and anti-Ara h 2 pAb were additionally immunogen-affinity purified before panning

The average values for individual target groups may indicate the influence the protein's nature has on the capability to enrich peptide ligands in general (Fig. 2). Additionally, we analyzed whether specific conformation of library peptides (i.e., cyclized *vs.* linear peptide) was preferrred for individual target molecules as well as for groups of similar targets (Supplementary Fig. S1). For most phage binders, the oligonucleotide sequences encoding displayed peptides were sequenced and consensus peptide motifs for individual targets (if present) were determined.

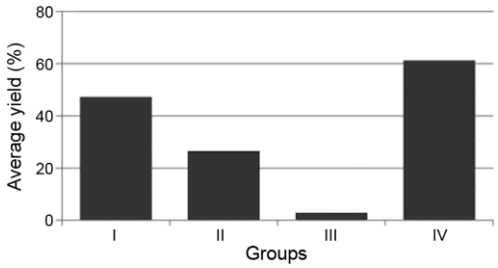

Fig. 2—Average screening yields for each target group. [Group-I: Enzymes involved in peptide synthesis and protein degradation; Group-II: Lipid metabolizing enzymes; Group-III: Proteins involved in protein-protein interactions; and Group-IV: Antibodies]

Results and Discussion

Grouping of target molecules

Twenty-three target molecules were sorted into four groups based on their biological function and the chemistry of their ligands or substrates. According to this principle, each of the four groups comprises molecules of similar nature. Assorting protein macromolecules on the basis of ligand similarity rather than structural homology has been applied before; Lin *et al.*[18] have recently classified structurally distant G protein-coupled receptors (GPCRs), as well as diverse non-GPCR targets according to ligand similarity and have validated their approach by experimental confirmation of predicted common ligands within a class.

Comparison of affinity selection success rates across target groups

The relative proportion of clones possessing detectable affinity towards specific target in the set of all clones tested (a semi-quantitative rank of selection performance for a given target) is shown in Supplementary Table S1 (Fig. 1 depicts summary of data). The average values within the same group of target molecules represent a mean performance of screenings against target molecules of similar nature (Fig. 2). Such information

can be helpful in predicting the outcome before beginning a panning process using a new target molecule.

Among individual target molecules, best efficiencies were achieved in pannings against antibodies (relative proportion of positive hits 33-100%) and the enzyme Mur ligase D (83%). Focusing on performances of entire groups of target molecules, the antibody group reached the highest value (61%), followed by the enzymes involved in peptide synthesis and protein degradation (47%) and lipid metabolizing enzymes (27%). On the other hand, the group of proteins participating in PPI was associated with the lowest average yield (3%), comprising targets for which we got no peptide binders (namely, leptin, leptin receptor and des-acyl ghrelin).

The results collectively indicated that some molecules represented excellent targets in selection of phage displayed peptides, while others were apparently incapable of trapping short peptides. Considering only target binding, the most successful selections were achieved against the enzymes papain, Mur ligases D and E and lipoprotein lipase and in general against all antibody targets. However, whereas peptides selected against antibodies either clearly mapped to the primary structure of cognate antigens or displayed a common amino acid motif (the same holds true for papain and Fc binders), the lack of consensus motif in peptides identified as ligands of Mur ligases[19-24] and lipid metabolizing enzymes (with the exception of some screening against lipoprotein lipase) indicated enrichment of target unrelated peptides.

Indeed, in a number of screenings, peptides adhering to polystyrene or phage clones with propagation advantages prevailed over selective target binders[16]. These were not considered positive hits and were therefore excluded from data analysis. Furthermore, in at least one case (selection against MurD), the identified peptide HSSWYIQHFPPL turned out to be promiscuous, although it inhibited MurD, displaying IC_{50} in a mid-micromolar range[19]. The same peptide has also been selected as a binder of a mAb against hepatitis C virus envelope proteins[25] and carbon nanotubes[26]. Promiscuity might have arisen from the highly hydrophobic character and structural flexibility of the linear peptide.

As it was difficult to pin the promiscuity label to an affinity-selected ligand, we decided to treat the peptide as a positive hit. Other peptides, especially those enriched in screenings against lipid metabolizing enzymes may represent promiscuous ligands as well. Notably, only one peptide (i.e., CQPHPGQTC) inhibited its target enzyme[27], whereas others displayed no biological activity. With that in mind, the average selection efficiencies (Fig. 1) were likely somewhat overestimated for Mur ligases and the entire lipid metabolizing enzymes group.

Antibodies as targets

Antibodies usually have well-exposed and compact hypervariable regions with a cleft-like structure, evolutionary optimized for binding antigens, which are mostly peptides and proteins[14,28]. Both linear and discontinuous epitopes typically comprise only a small set of protein antigen's amino acid residues, which can be excellently mimicked by short peptide fragments[14,29]. However, the antibody downstream processing is of significant importance for the successful selection of peptides that can compete with the cognate antigen for binding. For example, the antihuman ghrelin pAb LS-C48966 was first used as a target in crude form (i.e., isolated from immunized rabbit serum by protein A chromatography) and was later immunogen-purified using magnetic beads with immobilized ghrelin. As a result, the proportion of isolated binders was raised from 63% to 100% and *in vitro* biological activity from 0% to 100%. Significantly increased proportion of binders (from 38% to 56%) was also observed after similar immunogen-affinity purification of anti-Fel d 1 pAbs, indicating the importance of target material quality (Fig. 1).

Enzymes as targets

The active sites of enzymes are usually located deep within the molecule, most often at the bottom of a large pocket, and existence of such cavities seems to be the major requirement for efficient peptide trapping. High yield achieved with a protease and amino acid ligases can be further explained by the relatively low conformational specificity of such enzymes possessing cavities and grooves perfectly shaped and sized for a polypeptide chain to fit in[28,30]. However, peptides targeting lipid-metabolizing enzymes had no detectable inhibitory activity *in vitro*, suggesting that either catalytic sites remained accessible to substrates after peptide binding or that peptides targeted areas distinct from the active sites.

Proteins involved in protein-protein interactions as targets

Intuitively, one might expect that targets interacting with peptides or proteins in their natural

environment would hold a better chance to yield peptide ligands in affinity selections. However, the mere fact that a target molecule used in selection naturally binds a protein or a peptide does not necessarily mean that it will also bind a short peptide and consequently represent a reliable target for panning a peptide phage display library. Indeed, molecules involved in PPI usually have large, flat and featureless interfaces, lacking special characteristics for binding small or intermediate sized molecules, which makes selecting short peptides from phage display libraries difficult[30-33].

To be able to speculate about the possibility of target molecule-short peptide interaction, generally one would have to look deeper at the three-dimensional structure of a target and search for potential cavities capable of capturing smaller molecules. Here, the flexibility of a target molecule at molecular dynamics level is also important, since it may allow for the formation of transient grooves or pockets at apparently flat surfaces[30,31]. Furthermore, there can be several distinct hotspots scattered over a relatively large protein surface. If so, the total binding energy of the natural ligand is at least equal to the sum of the contribution of each individual hotspot interaction due to the avidity effect[34]. Short peptides are generally too small to cover more than one hotspot, making their potential interactions too weak to remain attached to the target during washing steps. Structural flexibility of short peptides, especially non-constrained is another plausible cause for their low-affinity interactions due to entropic effects[31,35]. All these phenomena may explain the low yield in selections against peptide hormones and their receptors.

Notably, in parallel screenings utilizing either a neutralizing antibody or a receptor, both binding the same natural ligand, we could not isolate a single peptide binding to the recombinant human leptin receptor (hLR-Fc), while we easily identified specific and high-affinity ligands for both monoclonal and polyclonal neutralizing antibodies against human leptin. Yet, none of the peptides showed the ability to cross-interact with the leptin receptor, demonstrating that a peptide selected for binding an alternative target (e.g., neutralizing antibody) cannot necessarily be considered a fully functional mimetic of its macromolecular counterpart, binding a primary natural target (e.g., membrane receptor).

Proteins with non-peptidic binding partners as targets

There were also some highly successful selections utilizing target molecules, whose binding partners were not of peptidic origin. Probably, the most outstanding was a routine isolation of the peptide motif HPQ from peptide phage display libraries involving streptavidin as a target molecule. HPQ mimics biotin moiety in binding a well-defined pocket on streptavidin[1,36]. However, it should be emphasized that in this case, elution strategy was crucial for obtaining high affinity binders; biotin effectively displaced HPQ-bearing phages (in our hands selection performances approached 100%; data not shown), while unspecific acidic elution worked poorly, if not combined with additional interventions[37]. Furthermore, we successfully recovered peptide ligands of pancreatic and lipoprotein lipases, demonstrating that other factors besides the origin of target's substrate determined the ability of binding short peptides. As discussed above, ligands of phospholipases A_2 might display promiscuity. The efficiencies of selections against these targets were, however, in most cases relatively low.

Binding affinity *vs.* biologic activity

Binding affinity should not be confused with biological activity of isolated peptides, which is undoubtedly even more difficult to predict. Judging from our experience, the majority of isolated peptides (with the exception of those selected against antibodies and the enzymes with peptide substrates) possessed no biological activity (specifically, they did not displace the natural binding partner nor inhibit enzymatic activity of a target). This was true especially if none of the measures was carried out that would otherwise increase the chances for isolating ligands oriented to the biologically relevant (active) site on a target molecule. Probably, the most beneficial intervention for isolation of bioactive peptides was specific elution of bound phages, which could be carried out by competitive displacement using a known natural ligand, such as inhibitor or substrate. However, even specific elution did not improve selections against leptin and leptin receptor. Earlier, We have discussed the influence of factors other than target nature on phage display selections[14] and will therefore not address them in this report.

We confirmed biological activity of peptides binding to papain[15], Mur ligases[19], porcine pancreatic lipase[27] and poly- and monoclonal antibodies (against

ghrelin, leptin, chemokine CCL2 and allergens Fel d 1 and Ara h 2). Although the binding affinities and inhibitory activities of peptides were generally relatively low, they could be improved by rational modification of primarily isolated bioactive peptides[15] or screening of secondary libraries designed on the basis of initial hits[14].

Libraries of constrained *vs.* linear peptides

Obviously, selection strategy, the number of selection rounds and conditions enforced during individual steps of panning were of crucial importance for the outcome of panning and affected the percentage and heterogeneity of isolated binders. We, however, found that modifying the selection protocol did not drastically improve the odds of isolating binders, indicating that the panning approach might be of secondary importance, affecting the rate of success only slightly rather than making it possible at all. This seemed especially true for selections against leptin and leptin receptor, where altering selection strategy and library type (i.e., linear *vs.* cyclic peptides) did not help in identifying ligands, and highly successful pannings against antibodies which appeared to be very robust.

Yet, even with antibodies, there were two deviations from this observation. In the case of neutralizing mAb against CCL2, we could only identify linear peptide mimetics of the native antigen, but no cyclic peptides. This was attributed to the fact that the antibody S101 recognized a stretched conformational epitope on CCL2 (as inferred from computational prediction[38] of the epitope based on the peptide extracted from a phage display library) that short cyclized peptides were unable to mimic. An opposite situation was encountered with a neutralizing mAb against human leptin, where antigen mimetics could only be derived from a library of cyclic, but not linear peptides. Here, mAb MAB398 recognized a cluster of residues that was closely matched by the conformation of a cyclized peptide. This demonstrated the importance of library design (i.e., conformational flexibility *vs.* restriction) for the outcome of panning against monoclonal antibodies. Conversely, it seemed that polyclonal antibodies could accommodate conformationally diverse peptide ligands.

Based on the success rates of affinity selections against non-antibody targets, any generalization on the library type (cyclized *vs.* linear peptides) that holds the highest potential for successful panning cannot be made. As shown in Supplementary Fig. S1, library of constrained peptides (Ph.D.-C7C) worked out better in case of both groups of enzymes, which was contrary to our expectations; i.e., one could hypothesize that more flexible linear peptides should have better chances to accommodate into the enzyme's active sites, which are often characterized as pockets or grooves of various architectures. It was possible that most cyclic peptide binders actually interacted with an enzyme surface distinct from its active site. This was supported by the fact that with the exception of few papain, MurD and pancreatic lipase-binders, most selected enzyme ligands did not show any detectable biological activity. By focusing on the group of enzymes with peptide substrates, the average success rate (shown in Supplementary Fig. S1 B) appeared to be equal for both library types (cyclic and linear peptides) and based on our findings we cannot recommend which library type to use with specific target enzyme.

For the group of lipid metabolizing enzymes, library of cyclic peptides seemed to work out better, but such generalization was questionable, since the majority of isolated ligands lacked biological activity. In few successful selection attempts using proteins involved in PPI, libraries of cyclic peptides worked out better, as judged from the proportion of isolated binders and their apparent affinities (reflected by ELISA signals, not shown). This was in line with expectations due to the much more favorable entropic cost upon target binding for structurally constrained cyclic peptides, compared to flexible linear peptides. However, since chances of isolating binders of target molecules of this type are low, we recommend screening both types of libraries.

As shown for individual mAbs (Supplementary Fig. S1 A), it depends on the type (linear *vs.* discontinuous) and structural characteristics of their corresponding epitopes (curved *vs.* flat cluster of residues) to speculate which library would have a greater chance of productive panning experiment. When such structural information is not available, it is recommended to perform screenings with both type of libraries; i.e., constrained and un-constrained. In case of polyclonal antibodies, selections of peptides appeared to be very robust and based on our experience, the success rates of screening libraries of both linear and cyclized peptides were similar for most pAbs.

In conclusion, any estimation of selection outcome would be desirable before embarking on to a phage display project. As demonstrated, one of the major

factors impacting the likelihood of successful course of panning seemed to be the target itself. Here, we show that some types of molecules, such as enzymes involved in peptide synthesis and protein degradation, and antibodies represent excellent targets, while proteins involved in protein-protein interactions do not. This phenomenon seems to be independent of the selection strategy/conditions and elution principle. Especially selections against polyclonal antibodies seem to work well, regardless of the conformation of library peptides. On the other hand, we[27] and others[1] have shown that peptide ligands of targets naturally binding non-peptide molecules can in principle be selected from phage-displayed peptide libraries. Although one cannot reliably foretell the outcome of panning, generally the enzymes with peptide substrates and antibodies seem best suited as targets. Speaking more generally, based on our observations neither peptide nature of target's natural binding partners nor favorable topological features of the target molecule (e.g., presence of clefts or pockets) guarantee successful selection of short peptides, while promising outcome of panning may be expected with greater probability, when both of the aforementioned conditions are met.

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