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Potential of phage-displayed peptide library technology to identify functional targeting peptides

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Abstract

Combinatorial peptide library technology is a valuable resource for drug discovery and development. Several peptide drugs developed through phage-displayed peptide library technology are presently in clinical trials and the authors envision that phage-displayed peptide library technology will assist in the discovery and development of many more. This review attempts to compile and summarize recent literature on targeting peptides developed through peptide library technology, with special emphasis on novel peptides with targeting capacity evaluated *in vivo*.

Keywords

library; peptide; phage display; targeting

1. Introduction

New technologies have reduced many of the earlier limitations of peptides as potential therapeutics. Recent efforts to identify new peptides that bind to their target molecules and to manipulate biologic function of the targets with peptide engineering technologies have brought several potential peptide-based therapeutics to clinical trials. Although several types of combinatorial peptide display technologies have been used to identify ligands and targeting peptides, bacteriophage (phage) display has dominated the field. Using phage-displayed peptide libraries, a number of peptides that interact with protein targets, in some cases with high affinity and specificity, have been found [1-5].

Peptide therapeutics developed through phage-displayed library technology presently in clinical trials include Hematide[®] (Affymex/Takeda), DX-890, AMG-386 and AMG-531. Hematide is a synthetic, dimeric peptide erythropoiesis-stimulating agent covalently linked to polyethylene glycol. The original peptide structure was isolated from a phage-displayed peptide library. After extensive sequencing and architectural modification, the peptide was converted into a stable, potent erythropoietin mimetic. Hematide is being evaluated in patients in a Phase II clinical trial as a once-monthly administration for the correction of anemia associated with chronic kidney disease [6].

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DX-890 is a specific inhibitor of human neutrophil elastase with low picomolar order affinity [7]. The peptide component of DX-890 was initially isolated from a peptide library displayed on a bacteriophage. Neutrophil elastase produced by white blood cells is involved in inflammation and infection, particularly in chronic inflammation of the lungs in patients suffering from cystic fibrosis. DX-890 is in Phase II clinical trials for the potential treatment of cystic fibrosis.

AMG-386, which is in a Phase I clinical trial, is a recombinant Fc-fusion protein containing angiopoietin-2-targeting peptide, which was originally isolated from a phage-displayed peptide library [8]. AMG-386 blocks the interaction between angiopoietin-2 and its receptor, therefore, inhibiting the Tie2-dependent stimulation of endothelial cells.

AMG-531 consists of a carrier Fc domain linked to multiple copies of an Mpl-binding peptide, which stimulates the thrombopoietin receptor and, thus, increases platelet production. The peptide active component of AMG-531 was first isolated from a phage-displayed peptide library [9]. AMG-531 has been investigated for the treatment of idiopathic thrombocytopenic purpura, which is an autoimmune bleeding disorder characterized by an abnormal decrease in platelets. Phase III clinical studies with AMG-531 were initiated in 2005.

The goal of this review is to look back on the use of peptide library technology and to examine the future prospects of the technology in the identification of targeting peptides in drug discovery and development. Phage display technology has been the most widely reported method of peptide library display and, as such, most of this review is focussed on phage-displayed peptide libraries. However, targeting peptides have also been successfully isolated from one-bead-one-compound and bacteria-displayed peptide libraries. This review focuses on the recent literature published from 2005 on and places special emphasis on peptide targeting capacity evaluated *in vivo*.

2. Phage-displayed peptide library technology

Several types of combinatorial peptide display technologies have been used to identify ligands and targeting-peptides. These include synthetic one-bead-one-compound libraries [10-12], cell-free translation systems, such as mRNA display (peptide linked to mRNA by puromycin) [13-15], DNA display (DNA and peptide captured in compartments of water-in-oil emulsions) [16] and ribosome display libraries (peptide linked to mRNA by stalling the ribosome) [17-19], and biologic libraries, such as bacteria display [20-22], adenovirus display [23,24], baculovirus display [24] and bacteriophage display libraries [25-28]. GP Smith reported in 1985 that a bacteriophage could be manipulated to display foreign amino acid sequences on the surface of the phage by fusion to phage coat proteins without losing the ability of phage infectivity [28]. Thus, it has been more than two decades since phage display technology was invented and phage display has, by far, become the most widely used peptide display system in drug discovery. The key features of phage display are: i) the physical linkage between the displayed polypeptide (phenotype/function) and the polypeptide encoding DNA sequence encapsulated in the viral particle (genotype/information); ii) the size of polypeptide library that can be displayed ($10^8 - 10^{11}$ unique amino acid sequences); iii) the use of self-replicating nanoparticles; and iv) the ability to subject these libraries to *in vivo* biopanning. Peptide libraries displayed on phage have been used as a screening resource for identifying peptides bound to any given target and many of these peptides showed pharmacologic effects on the target protein [3,29-31].

Screening a phage-displayed peptide library is accomplished through an affinity-selection process referred to as biopanning. Biopanning consists of: i) incubating the peptide library with the target; ii) washing away unbound phage; iii) eluting the remaining bound phage, and iv) amplifying the eluted phage for subsequent screening rounds. After multiple rounds of

biopanning, the target-binding phage can be enriched and individual phage are isolated and sequenced to reveal any enriched binding motif. This biopanning process can be accomplished both *in vitro* and *in/ex vivo* or by a combination of both techniques [30,32].

Filamentous phage (M13, f1, and fd) has been the most common vector used in the construction of peptide phage-displayed libraries [31,33,34], although previous studies demonstrated specific and positional amino acid biases in populations of peptides randomly selected from M13 libraries because of the processes of filamentous phage morphogenesis [35,36]. Alternative phage display systems have been developed using lytic phage such as T7, T4, λ and P4 [37-39]. The prevalence of filamentous phage-displayed peptide library usage may be, in part, due to the initial development of the technology with filamentous bacteriophage, the commercial availability of filamentous phage-displayed peptide libraries or a combination of both or other factors. Recent research in the authors' laboratory indicated that libraries produced with lytic phage could surpass the diversity of filamentous phage-displayed peptide libraries [36]. Peptides displayed on lytic phage do not have to be compatible with host cell synthesis and secretion complexes because lytic phage assembly occurs within the *Escherichia coli* cytoplasm and mature phage virions are released by cell lysis. When performing biopanning, the library should be of sufficient diversity to contain potential binding ligands for the target for successful outcome. Thus, the biologic process of phage morphogenesis should be carefully considered when choosing a phage for the display of peptides.

3. Potential target-specific peptides identified using phage-displayed peptide library technology

A successful outcome of library screening would identify a targeting peptide, which alone could exhibit therapeutic potential (agonists/antagonists). Alternatively, the use of the peptide could be to target a therapeutic to a specific cellular environment. Phage display technology, undoubtedly, has demonstrated its ability to identify such peptides. A search of present literature published between 2005 and 2006 revealed > 30 papers that disclose the identification of peptides using phage display, which were demonstrated to specifically target proteins *in vitro*. The technology has been used in attempts to develop novel targeting therapeutics or diagnostic reagents for human cancers [23,40-58], HIV infection [59,60], human papilloma virus infection [61], obesity [62], bacterial infection [63-66], parasitic infection [67], autoimmune disorders [68], thrombosis and inflammation [69,70], Alzheimer's disease [71] and increasing vaccine efficacy [72]. However, the *in vivo* therapeutic efficacy of these novel peptides remains to be determined.

One promising aspect of phage display technology is the ability to screen peptide libraries *in vivo*, which offers several advantages over *in vitro* screening. In the course of *in vivo* selection, negative selection by untargeted tissue occurs, which decreases background phage in the targeted tissue. In addition, one can screen against diseased tissues in animal models, as such cell lines are often not available for cell culture. Arap *et al.* reported the first *in vivo* screening of a phage-displayed peptide library in a human patient in 2002 [73]. After one round of biopanning with a disulfide-constrained 7-mer filamentous phage-displayed peptide library, phage that targeted different tissues were isolated and it was found that phage binding to these different cell types did not occur by a random process. The group reported peptide motifs that homed to bone marrow, fat, muscle, prostate and skin. Furthermore, the authors evaluated a prostate-homing phage that displayed the peptide CGRRAGGSC, a mimic of IL-11 and showed the phage bound to endothelium and prostate endothelium *in vitro*.

After demonstrating minimal toxicity during *in vivo* screening of phage-displayed peptide libraries in mice, another group received FDA approval for the use of the technique in human clinical trials [74]. Krag *et al.* reported the results of a Phase I clinical trial in 2006 in which

eight stage IV cancer patients received intravenous infusions of filamentous phage-displayed peptide or single-chain antibody libraries [75]. Two rounds of biopanning were performed in two patients and three rounds of biopanning in one patient. The authors disclosed a list of peptides isolated from one breast cancer patient who had received two phage peptide library infusions. In two patients with sufficient tumor nodules amenable to biopsy, the authors were able to validate tumor specificity *in vitro* using a filter-based enzyme-linked immunosorbent assay (ELISA) method. For example, a phage clone displaying the peptide MRIRCAAAWRATGTHCSLRA, which homed to a melanoma in one patient, exhibited binding to patient derived tumor cells in the filter ELISA, but showed little to no binding to normal melanocytes and seven melanoma cell lines. No adverse reactions were reported in this study. However, the authors observed antiphage antibodies after three weeks in some patients and, thus, advised that serial human *in vivo* biopanning should be completed with one week. Thus, phage-displayed peptide library technology has the potential to identify patient-specific targeting peptides by *in vivo* biopanning [76]. This application of the technology is early in development and will be interesting to follow to fruition. It is important to note here that the biodistribution and clearance properties of phage-displayed peptide libraries is dependent on the target organ, strains of mice and types of phage [77] and that researchers may need to try varying *in vivo* screening parameters to achieve success.

4. Targeting peptides evaluated *in vivo*

The ability of a peptide to target a specific protein *in vitro* may not translate to the ability to target a protein *in vivo*. Peptides can have very short half-lives due to proteolytic degradation in serum and may not accumulate to a therapeutic level due to nonspecific uptake and excretion by the kidneys. Furthermore, peptides isolated by *in vitro* biopanning encounter a vast heterogeneity of proteins *in vivo*, the composition of which may differ drastically from *in vitro* screening conditions and, therefore, may not exhibit a satisfactory biodistribution. For these reasons, the authors chose to focus their attention on recent articles in which peptide *in vivo* targeting capacity was evaluated. A list of these novel targeting peptides is given in Table 1.

4.1 Cancer-related targets

4.1.1 Breast cancer—In 2001, Zhang *et al.* reported that an *in vitro* M13 phage-displayed peptide library screening against WAC 2 neuroblastoma cell line yielded a linear 12-mer peptide VPWMEPAYQRFL (p160) that also targeted the breast cancer cell line MDA-MB-435, in addition to targeting several neuroblastoma cell lines [78]. Askoxylakis *et al.* determined that the binding affinity of p160 was in the low micromolar range and showed that p160 internalized into MDA-MB-435 cells and clustered at the cellular membrane. Intravenously injected ¹³¹I-labeled p160 was demonstrated to specifically accumulate in Balb/c^{nu/nu} xenografts of the breast tumor as compared with heart, spleen, liver and brain and the perfusion of the mice decreased radioactivity in nonspecific tissue [79].

4.1.2 Gastric cancer—Akita *et al.* reported the tripeptide motif lysine-leucine-proline (KLP) that targeted peritoneal metastases of gastric cancer derived from the AZ-P7a cell line in BALB/c^{nu/nu} mouse xenografts [80]. *In vivo* biopanning with an M13 phage-displayed peptide library was conducted by peritoneal injection of the library and harvesting metastatic nodules for binding phage recovery. *In vitro* and *in vivo* competition experiments showed that synthetic SWKLPPS peptide significantly inhibited SWKLPPS-displaying phage from binding to metastases, indicating that targeting was attributed to the peptide moiety. SWKLPPS-coated liposomes accumulated in the peritoneal metastases more efficiently than non-coated liposomes. Furthermore, treatment with SWKLPPS-coated liposomes containing adriamycin enhanced antiproliferative effects compared with non-targeted control liposomes.

4.1.3 Hepatocellular carcinoma—Li *et al.* screened an M13 phage-displayed peptide library against recombinant human epidermal growth factor receptor (hEGFR) *in vitro* and isolated the phage displaying YHWYGYTPQNVI peptide (GE11) [81]. Synthetic free GE11 peptide and epidermal growth factor (EGF) inhibited phage binding to human EGF receptor (hEGFR) as well as the EGFR-positive human hepatocarcinoma cell line SMMC-7721. These results demonstrated GE11 peptide specificity for EGFR and the overlap of EGF and GE11 binding sites. The dissociation constant for GE11 was determined to be ~ 22 nM. *In vivo* biodistribution studies showed that ^{125}I -labelled GE11 specifically accumulated in SMMC-7721 tumor in mouse xenografts at 4 h post intravenous injection. GE11 peptide internalized into SMMC-7721 cells and lacked mitogenicity when coupled to polyethylenimine (PEI) vector. Li *et al.* further demonstrated that GE11-coated PEI/DNA polyplexes could target SMMC-7721 xenografts *in vivo* and attribute an 18-fold increase in luciferase expression in the tumor as compared with non-coated PEI/DNA polyplexes.

Jiang *et al.* reported the *in vitro* isolation of a 12-mer peptide from an M13 phage-displayed peptide library that bound to human hepatocellular carcinoma cell lines SMMC-7721 and BEL-7402, but not to normal liver cells [82]. To evaluate specificity, the authors showed that HCC79 peptide (KSLSRHDHIIHHH) competed with SMMC-7721-specific HAb25 antibody. Synthetic HCC79 did not inhibit SMMC-7721 cellular proliferation, but did decrease cellular migration in a dose-dependent manner. HCC79 peptide conjugated to toxic shock syndrome toxin 1 (TSST-1) decreased TSST-1 toxicity and inhibited H22 tumor growth in a mouse model compared with TSST-1 alone.

Du *et al.* screened a pool of disulfide-constrained 7-mer and linear 12-mer M13 phage-displayed peptide libraries *in vitro* against the human hepatocellular carcinoma cell line BEL-7402 [83]. WP05 phage, bearing TACHQHVRMVRP peptide, bound specifically to BEL-7402 cells as detected with fluorescent microscopy. Peptide specificity for hepatocellular carcinoma was further demonstrated by showing FAM-labeled WP05 peptide bound to BEL-7402, BEL-7404, SMMC-7721 and HepG2 cells, but not to HL-7702 normal liver cells using fluorescence microscopy and flow cytometry. WP05 phage injected intravenously into mice bearing BEL-7402 tumors accumulated in the tumor as opposed to normal mouse liver tissue.

4.1.4 Leukemia—Peng *et al.* screened a one-bead-one-compound combinatorial 5-mer peptide library for Jurkat T-lymphoid leukemia cell-binding peptides [84,85]. To identify high-affinity targeting antagonists for $\alpha_4\beta_1$ -integrin receptor, BIO-1211, an $\alpha_4\beta_1$ -integrin receptor antagonist, was added during the selection process. LLP2A peptide from the library screening inhibited CS-1 peptide ($\alpha_4\beta_1$ -integrin-binding peptide derived from fibronectin) binding to Jurkat cells with a half-maximal inhibitory concentration (IC_{50}) of ~ 2 picomolar. LLP2A was demonstrated to be specific to $\alpha_4\beta_1$ -integrin-expressing Molt-4 tumors, K562 cells and α_4 -transfected Chinese hamster ovary cells by fluorescent microscopy, but not to five other integrin families. The *in vivo* biodistribution of LLP2A was evaluated by conjugating LLP2A peptide to streptavidin and Alexa680. The compound was then injected intravenously into mice bearing human Molt-4 tumor xenografts. The fluorescent LLP2A conjugate specifically targeted Molt-4 tumors compared with streptavidin-Alexa680 only and a scrambled peptide-streptavidin-Alexa680 conjugate. Accumulation of each conjugate was evident in kidneys, which the authors attributed to streptavidin, but not in other organs such as heart, liver, spleen and lung.

4.1.5 Medullary thyroid carcinoma—Bockmann *et al.* recently reported a deficiency of α_v -integrin expression in human medullary thyroid carcinoma (MTC)-derived cell line (TT) cells *in vivo* compared with expression *in vitro* [86]. In attempts to target adenovirus internalization into MTC cells in a α_v -integrin-independent pathway, a disulfide-constrained

7-mer M13 phage-displayed peptide library screened against TT cells *in vitro* and against a mouse MTC tumor xenograft *in vivo*. The most abundant motif, HTFEPGV, which was isolated from both *in vitro* and *in vivo* biopanning, was specific for TT cells compared with human lung cancer, embryonic kidney cells and fibroblasts and targeted TT cell-derived MTC xenografts *in vivo*. Furthermore, HTFEPGVC-linked adenovirus internalized into TT cells and internalization could be inhibited by synthetic HTFEPGV peptide. Later, the same research group screened another disulfide-constrained 7-mer M13 phage-displayed peptide library in the CT-2A transgenic mouse model of MTC [87]. Two rounds of *in vivo* biopanning afforded a 3000-fold increase in tumor-binding phage and resulted in the identification of the SRESPHP motif, which could specifically target MTC *in vivo*. SRESPHP-bearing phage was demonstrated to internalize into both murine MTC and human MTC xenograft tumors *in vivo*.

4.1.6 Melanoma—Eriksson *et al.* screened a 12-mer M13 phage-displayed peptide library using a combination of *in vitro* and *in vivo* selection in a B16 mouse melanoma model for tumor-targeting phage clones [88]. TRTKLPRLHLS-bearing phage were demonstrated to be specific for melanoma compared MC57 and 293 cell lines and melan-A cells (non-cancerous melanocytes). Infiltration of polymorphonuclear neutrophils and production of T_H1 cytokines was induced in the host by injection of purified tumor-specific phage clones adjacent to tumor. Treatment with tumor-specific peptide phage or antibody phage resulted in similar outcomes of tumor regression and long-term survival in mice.

4.1.7 Pancreatic cancer—Tanaka *et al.* reported that growth factor receptor-bound protein 7 (Grb7) expression was upregulated in 61% of pancreatic cancer specimens and that Grb7 upregulation was correlated with lymph node metastasis [89]. The authors then demonstrated that Grb7 siRNA abrogated Grb7 expression and resulted in reduced pancreatic cancer cell migration into fibronectin. A non-phosphorylated peptide inhibitor of Grb7 SH2 domain G7-18NATE, which was previously identified using filamentous phage display technology [90], was conjugated to a penetratin sequence to facilitate targeting and internalization into human pancreatic cancer cells (Panc-1) for evaluation of metastasis inhibition. Treatment of cells with the peptide conjugate did not alter proliferation, but did reduce migration into fibronectin. To evaluate the peptide inhibitor *in vivo*, BALB/c mice were injected intraperitoneally with Panc-1 cells, followed by peptide inhibitor treatment over 2 weeks. Treatment with the Grb7 inhibitor peptide successfully targeted Panc-1 cells and resulted in drastically limited pancreatic tumor cell migration and metastases.

Huang *et al.* screened a 12-mer M13 phage-displayed peptide library against irradiated Capan-2 human pancreatic adenocarcinoma cells *in vitro*, after which the isolated binding phage were subjected to subtractive screening on non-irradiated Capan-2 cells [91]. Using flow cytometry, synthetic peptide SHGFSRHSMTLI (PA1) was shown to target and internalize into irradiated Capan-2 cells, but not to non-irradiated cells. Furthermore, the authors demonstrated that intraperitoneally injected PA1 could target subcutaneous xenografts of irradiated Capan-2 tumors in mice, but not non-irradiated xenografts. The authors hypothesized that irradiation of the cells induced synthesis of new cell-surface receptor(s), which were responsible for the internalization of the peptide.

4.1.8 Prostate carcinoma—Newton *et al.* screened a 15-mer filamentous phage-displayed peptide library for PC-3 prostate carcinoma targeting phage *in vivo* using a mouse xenograft model [92]. The library was first precleared of non-specific phage clones by injecting the library intravenously and then phage collected from the bloodstream were used for biopanning in mice bearing PC-3 human prostate carcinoma xenograft. After four rounds of biopanning, phage were sequenced and prioritized using a 'two-tier micropanning procedure'. First, clones were evaluated for tumor to muscle specificity and then those with a ratio ≥ 1.5 were evaluated for

binding to PC-3 cells and human embryonic kidney cells *in vitro*. One phage clone was analyzed, which displayed the peptide IAGLATPGWSHW-LAL (G1). Synthetic G1 peptide and G1 phage accumulated at the surface of PC-3 cells and G1 phage also bound to several other prostate carcinoma cell lines *in vitro*, as determined using fluorescence microscopy. *In vivo* biodistribution studies with G1 phage revealed a 10:1 tumor to fat ratio, a 30:1 tumor to muscle ratio and 73% greater binding of G1 phage to tumor compared with wild-type phage in PC-3 xenografts in mice.

Zitzmann *et al.* screened a disulfide-constrained 12-mer peptide library displayed on *E. coli* flagella using the FliTrx system for PC-3 human prostate carcinoma cell line ligands [93]. After four rounds of selection on PC-3 cells, bacteria displaying CPGDRGQRRFLFSKIEGPC peptide (MM-2) were enriched. Synthetic MM-2 peptide accumulated in PC-3 cells *in vitro* compared with human umbilical vein endothelial cells (HUVECs) and non-cancerous PNT-2 prostate cells. The authors demonstrated that MM-2 was stable in human serum for > 24 h and showed that MM-2 accumulated specifically in PC-3 xenografts *in vivo* after intravenous injection in mice. MM-2 peptide accumulated in tumor at a rate of 1% injected dose per gram of tissue, which was a greater rate than accumulation in other organs with the exception of kidney and blood.

Zitzmann *et al.* used a 12-mer M13 phage-displayed peptide library to screen for ligands of the DU-145 prostate carcinoma cell line [94]. Biopanning was performed first by negative selection on human embryonic kidney cells and then incubation with DU-145 cells *in vitro*. After six rounds of biopanning, all sequenced phage clones were determined to display the same peptide FRPNRAQDYNTN (DUP-1). Synthetic DUP-1 peptide bound to DU-145 and PC-3 cells, but not to HUVECs or the benign prostate cell line PNT-2 and rapidly internalized into DU-145 cells. The IC₅₀ of DUP-1 for DU-145 cells was determined to be 180 nM. DUP-1 accumulated specifically in DU-145 and PC-3 xenografts *in vivo* and perfusion with sodium chloride solution decreased non-specific accumulation in kidney and blood. The *in vivo* biodistribution of DUP-1 was also evaluated using the AT-1 rat prostate adenocarcinoma model. In rats, DUP-1 accumulated in cancerous prostate tissue threefold compared with normal prostate tissue and muscle. The authors reported that DUP-145 was degraded in human, mouse and rat serum within ~ 10 min and suggested *in vivo* stabilization experiments with this lead structure.

4.1.9 Tumor vasculature—In 1998, Arap *et al.* reported that the integrin-binding RGD and aminopeptidase N-binding NGR peptide motifs mediated the specific drug delivery of doxorubicin to human breast cancer xenografts in mice [95-97]. For a review of recent literature involving RGD peptide-mediated drug delivery the reader is directed to Dunehoo *et al.* [98]. Several recent reports have demonstrated the capacity of NGR peptides for specifically targeting tumor vasculature *in vivo* [99-101]. Curnis *et al.* reported NGR-conjugated IFN- γ stimulated antitumor activity in lymphoma and fibrosarcoma mouse models as compared with IFN- γ alone [99]. The specificity of targeting was demonstrated by showing anti-CD13 monoclonal antibody coadministration diminished NGR-IFN- γ antitumor activity. Sacchi *et al.* evaluated coadministration of NGR-conjugated TNF with several chemotherapeutic drugs in lymphoma and fibrosarcoma mouse models [101]. The authors reported that the NGR-TNF conjugate, when administered 2 h prior, enhanced the efficacy of cisplatin, gemcitabine and paclitaxel.

4.2 Non-cancer related targets

4.2.1 Pancreatic vasculature and islets of Langerhans—Kolonin *et al.* screened a disulfide-constrained 7-mer filamentous phage-displayed peptide library *in vivo* in mice to evaluate a novel screening strategy in which phage clones recovered from multiple organs are

amplified individually (per organ) and then pooled prior to subsequent screening rounds, in combination with statistical prioritization of isolated binding peptides [102]. After each screening round, 96 phage clones were sequenced for each organ studied. The recovered amino acid sequences were analyzed for enrichment of tri-peptide motifs using a Bayesian Beta/Binomial model and the frequencies of which were compared with frequencies of the naive unselected library. The authors then used the Basic Local Alignment Search Tool (BLAST; National Center for Biotechnology Information [NCBI]) to identify motifs that mimicked known receptor ligands and found that several recovered motifs aligned with known proteins. The author reported that ASVL, WSGL and GWSG peptide motifs homed to the murine pancreas and that the motifs could potentially mimic pancreas-signaling peptide hormone. Pooled phage that homed to the pancreas from the second and third rounds of *in vivo* biopanning were screened against a kidney fibroblast-like cell line (COS-1) expressing prolactin receptor (PRLR) and against recombinant PRLR. CRVASV-LPC was discovered as a binding peptide and both CRVASV-LPC and CPLVSAVRC (inverse sequence of the prior peptide) peptides both bound and internalized into PRLR-expressing COS-1 cells. *In vivo* experiments with CRVASV-LPC-phage showed the phage targeted pancreatic vasculature and islet cells, but did not accumulate in skeletal muscle, whereas a skeletal muscle-specific phage accumulated within muscle, but not in pancreatic tissue.

Yao *et al.* performed mouse *in vivo* biopanning with a disulfide-constrained 7-mer filamentous phage-displayed peptide library in combination with laser pressure catapult micro-dissection to screen for peptides which bound to vascular receptors in the pancreatic islets of Langerhans [103]. Two of the binding peptides (CVSNPRWKC and CHVLWSTRC) showed sequences with homology to ephrin A-type ligands. Confocal microscopy verified that binding of these peptide phage *in vivo* was localized at blood vessels in the pancreatic islets. Binding of both phage and antibodies to a receptor for ephrin-A ligands (EphA4) was significantly enhanced in blood vessels of pancreatic islet tumors in RIP-Tag2 transgenic mice, which spontaneously develop pancreatic islet tumors, indicating that endothelial cells of blood vessels in pancreatic islets preferentially express EphA4 receptors and this expression is increased in tumors.

Samli *et al.* reported screening a filamentous phage-displayed 20-mer peptide library on freshly excised non-diabetic rat pancreatic islets [104]. Two phage clones displaying peptides RIP1 and RIP2, LSGTPERSGQAVKVKLKAIP and GAWEAVRDRIAEGSWGIPS, respectively, were recovered. RIP1 and RIP2 phage clones localized at rat islets *in vivo* and immunostaining revealed binding overlapped with insulin expression, which suggested phage clone specificity for β -cells. However, the authors noted that RIP1 phage demonstrated enhanced islet-specific accumulation compared with RIP2 phage. Furthermore, the authors tested whether RIP1 phage retained the ability to target pancreatic islets in a rat model of Type 2 diabetes (ZDF rat). RIP1 phage failed to specifically accumulate in diseased islets, which suggested that RIP1 phage could discriminate between normal and diseased islets *in vivo*.

4.2.2 Endothelium—Work *et al.* reported the *in vivo* biopanning of a disulfide-constrained 7-mer M13 phage-displayed peptide library in Wistar Kyoto rats (WKY) rats [105]. After three rounds of biopanning, phage clones specifically homing to lung and brain endothelium were recovered. CVNTANSTC- and CQPEHSSTC-bearing phage targeted the lung and brain, respectively, compared with a no-peptide control phage. When displayed on the surface of adeno-associated virus (AAV), these peptides specifically target AAV delivery to the lung and brain. Furthermore, at 28 days postintravenous infusion of targeted AAV, AAV DNA and AAV-directed transgene expression of β -galactosidase was more prevalent in the tissues to which the peptide targeted the virus, compared with non-targeted AAV.

Zhang *et al.* screened a T7 phage-displayed disulfide-constrained 7-mer peptide library for phage binding to mouse cardiac endothelium [106]. Three rounds of biopanning on murine

cardiac cells *ex vivo*, in combination with recovery of phage clones bound to cardiac endothelial cells by selection with anti-CD31 magnetic beads, afforded an enriched population of phage clones that were subsequently used in *in vivo* biopanning. Peptide-encoding DNA from phage clones that homed to the heart were used as bait in a bacterial two-hybrid system in conjunction with a mouse heart cDNA library and several cardiac-specific protein target and receptors were identified. The biodistribution of T7 phage clones displaying the homing peptides was evaluated *in vivo* and most were discovered to colocalize with cardiac endothelium. The authors also validated several receptor–ligand interactions by demonstrating phage clones bound specifically to 293T cells transfected with their cognate receptor cDNA and the interactions could be inhibited by synthetic peptides. Furthermore, the authors showed that a synthetic fluorescein-conjugated CRPPR peptide, which homed to cardiac epithelium and endocardium *in vivo*, co-localized with CRIP2, the cognate receptor of which was identified using bacterial two-hybrid analysis.

Kelly *et al.* screened a M13 phage-displayed disulfide-constrained 7-mer peptide library on murine cardiac endothelial cells (MCECs) under physiological flow conditions and isolated a family of endothelial vascular adhesion molecule-1 (VCAM-1)-mediated cell-internalizing peptides after four rounds of biopanning [107]. One specific sequence (CVHSPNKKK), having homology to the α -chain of very late antigen (VLA), bound VCAM-1 and inhibited leukocyte–endothelial interactions. CVHSPNKKK peptide showed 12-fold enhanced fluorescent labeling of MCECs as compared with a VCAM-1 monoclonal antibody. A CVHSPNKKK-modified magnetofluorescent nanoparticle (VNP) accumulated in VCAM-1-expressing endothelial cells specifically compared with macrophages. The VNP-targeted VCAM-1-expressing endothelial cells in an inflammatory mouse model and co-localized with VCAM-1-expressing cells in atherosclerotic lesions present in cholesterol-fed apolipoprotein E ApoE^{-/-} mice. These authors indicated that the VNP could be useful for targeted *in vivo* imaging of endothelial markers by MRI and fluorescence imaging and possibly for targeted therapeutic delivery.

Kelly *et al.* later screened a linear 7-mer filamentous phage-displayed peptide library *in vivo* in ApoE^{-/-} mice for aorta plaque-binding peptides [108]. Thirty families of binding peptides were recovered and the isolated binding peptides exhibited sequence identity to VCAM-1 ligand VLA-4, leukemia inhibitory factor, transferrin receptor and several other peptides exhibited no sequence identity to known proteins. The authors synthesized a magnetofluorescent nanoparticle coated with 12 copies of the VLA-4 mimetic VHPKQHR peptide (VINP₂₈-NP) for further evaluation. The VINP₂₈-NP exhibited a 20-fold increase in endothelial binding to MCECs as compared to previously evaluated VNP [107]. The authors attributed the enhancement in affinity to avidity effects as higher numbers of linear peptides as compared to cyclic peptides can be incorporated onto the surface of the nanoparticles (12 linear peptides versus 4 cyclic peptides).

4.2.3 Fibrinogen and fibronectin—Pilch *et al.* screened a disulfide-constrained 8-mer T7 phage-displayed library for peptides that bound to human clotted plasma [109]. After three rounds of selection on clotted plasma, CGLIIQKNEC (CLT1) and CNAGESSKNC (CLT2) peptides were identified, synthesized as fluorescein conjugates and injected intravenously into mice with MDA-MB-435 xenografts and also into mice with Lewis lung carcinoma. The peptides stained tumor tissue in a pattern the authors described as a fibrillar network. The authors showed that the CLT peptides colocalized with fibrinogen and fibronectin. In fibrinogen and fibronectin knockout mice bearing B16F1 tumors, CLT peptides did not form a fibrillar network at the tumor site, which indicated CLT peptides target fibrinogen and fibronectin. Furthermore, the authors showed that CLT peptides accumulated at sites of tissue injury. The authors postulated that the peptides interact with the fibrinogen/fibronectin network caused by clotted plasma at sites of injury and vascular extravasation at tumor interstitium and could be utilized to develop reagents for targeting tumors and wounds.

5. Expert opinion and conclusion

The search for agents that can specifically target a population of cells for discriminatory drug delivery has mainly focussed on peptide and antibody-based therapeutics [3,29]. Both classes of molecules have benefits and limitations as therapeutic agents. Peptide-based therapeutics have recently received attention for having promise in the clinic due to their small size, ease of derivatization and low cost of manufacturing compared with monoclonal antibodies. However, peptide therapeutic efficacy can be limited by relatively low affinity, proteolytic degradation, potential immunogenicity and rapid renal filtration. Peptide display technology has afforded researchers plausible targeting lead structures from which effective therapeutics can be developed.

Several methods have been used to improve the pharmacodynamic properties of peptide therapeutics. Peptide stability has been enhanced by peptide cyclization, blocking of N- and C termini, use of scaffold proteins and substitution of L-amino acids with D-amino acids, unnatural amino acids and chemically modified amino acids [2,29,84,110-113]. Conjugation of the peptide to polyethylene glycol and polysialic acids has led to increased peptide half-life, stability and reduced immunogenicity [114]. Furthermore, the affinity of peptides has been improved by conducting secondary biased library screenings and peptide multimerization [48,108,115,116]. Thus, the ability of phage-displayed peptide library technology has been proved to identify peptide leads that can be engineered into efficient therapeutics and targeting moieties. A potentially useful method of developing peptides of enhanced therapeutic efficacy could involve initially screening a phage-displayed peptide library to identify targeting moieties and subsequently screening a secondary synthetic combinatorial peptide library [117], which incorporates the targeting moiety [1]. The use of secondary synthetic peptide libraries could provide additional screening flexibility through the ability to incorporate modified amino acids. We envision that this combination of library screening technology could increase the success rate of targeting peptide identification and development.

Phage-displayed peptide library technology has been used to identify peptides with targeting specificity in drug development and the technology has been extensively applied in the search of novel treatments for many human diseases and illnesses. The identified peptides have either served as leads for development into independent therapeutics or diagnostic reagent, or were manipulated to target a unique molecular entity for specific and discriminatory drug delivery. Research with the RGD and NGR tumor vasculature-targeting peptides, which were identified through phage-displayed peptide library technology, offered proof-of-principle that peptides can mediate specific *in vivo* delivery of therapeutics. Review of the literature published within the last 2 years yielded reports that at the least, 22 novel peptide moieties identified through peptide library technology in which *in vivo* targeting capacity was validated. These leads could be used directly in additional preclinical studies and with the assistance of affinity maturation and stabilization techniques, the authors envision that many of these targeting peptides will be of use in the treatment or diagnosis of disease. Furthermore, > 30 reports disclosed the identification of novel peptides, which were demonstrated *in vitro* to specifically target their receptors. As targeting capacity *in vitro* does not always equate to targeting capacity *in vivo*, further studies will need to be completed before these peptides can be validated as possessing targeting potential.

In the authors' opinion, phage-displayed peptide library technology will increasingly become a more integral part of drug development in the near future as novel disease markers are identified and applications of phage display technologies are expanded. Protein interactions mediate many biologic processes and, therefore, it is rational to believe that new interactions in disease progression could be identified and/or manipulated using phage display technology. Presently, most peptide-based therapeutics target cell surface proteins and receptors. However,

recently cell-penetrating peptides and protein transduction domains have been discovered and could be utilized to initiate screening for intracellularly-focussed peptide therapeutics. Phage display technology could be developed to contribute significantly in developing organelle-specific therapeutics. In addition, phage display technology has only recently been used in human *in vivo* biopanning, with the first clinical trial results reported in 2006. Having demonstrated clinical safety, we envision that peptides identified through phage display technology could be used *in vivo* to diagnose, confirm and possibly to customize patient-specific therapeutics.

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Table 1
Summary of targeting peptides either isolated by *in vivo* biopanning or evaluated *in vivo*

Target	Selection method(s)	Peptide characteristics	Reference
Cancer-related			
Breast cancer	M13 Phage display; mouse xenograft	p160 (VPWMEPAYQRFL) targeted and internalized into MDA-MB-435 cells, $IC_{50} = 0.6 \mu M$, $K_d = 0.86 \mu M$	[78,79]
Gastric cancer metastases	M13 Phage display; mouse xenograft	SWKLPPS-coated liposomes targeted peritoneal metastases of AZ-P7a cells and enhanced adriamycin anti-cancer activity	[80]
Hepatocellular carcinoma	M13 Phage display; mouse xenograft	EGFR-selected GE11 (YHWYGYTPQNV1, $K_d = 22 \text{ nM}$) and GE11-polyethylenimine vectors targeted SMMC-7721 cells	[81]
	M13 Phage display; mouse model	SMMC-7721-selected KSLSRHDHIIHHH suppressed tumor migration and when conjugated to TSST-1 superantigen, inhibited mouse H22 tumor growth	[82]
	M13 Phage display; mouse xenograft	TACHQHVRMVRP targeted BEL-7402 tumors	[83]
Leukemia	One-bead-one compound; mouse xenograft	Jurkat cell-selected LLP2A peptide targeted Molt-4 tumors; $\alpha_4\beta_1$ -integrin receptor ligand, $IC_{50} = 2 \text{ pM}$	[84,85]
Medullary thyroid carcinoma	M13 Phage-display; mouse xenograft	HTFEPGV phage targeted human MTC-derived TT cells and directed adenoviral binding and internalization	[86]
	M13 Phage-display; mouse <i>in vivo</i> biopanning; transgenic mouse model	SRESPHP phage specifically targeted and internalized into MTC cells	[87]
Melanoma	M13 Phage display; mouse <i>in vivo</i> and <i>in vitro</i> biopanning; mouse model	TRTKLPRHLQS phage targeted B16 tumors and enhanced host immune response resulting in tumor necrosis and increased survival	[88]
Pancreatic cancer	fUSE 5 Phage display; mouse xenograft	Penetratin sequence-conjugated G7-18NATE peptide decreased cell migration and metastasis; non-phosphorylated inhibitor targeted Grb7 SH2 domain	[89,90]
	M13 Phage display; mouse xenograft	SHGFSRHSMTLI targeted irradiated Capan-2 tumors	[91]
Prostate carcinoma	fUSE 5 Phage display; mouse xenograft	IAGLATPGWSHWLAL phage targeted PC-3 cells	[92]
	Bacterial display, FliTrx; mouse xenograft	MM-2 (CPGDRGQRRFLFSKIEGPC) targeted PC-3 cells	[93]
	M13 Phage display; mouse xenograft and rat model	DUP-1 (FRPNRAQDYNTN) targeted DU-145 cells, $IC_{50} = 180 \text{ nM}$	[94]
Tumor vasculature	fUSE 5 Phage-display, mouse models	NGR-conjugated IFN- γ and NGR-conjugated TNF- α enhanced antitumor activity in T lymphoma (RMA), WEHI-164 fibrosarcoma, TS/A adenocarcinoma	[97,99-101]
Non-cancer-related			
Pancreas	fUSE 5 Phage display; mouse <i>in vivo</i> biopanning	CRVASVLPC phage targeted prolactin receptor in pancreas vasculature and islets	[102]

Target	Selection method(s)	Peptide characteristics	Reference
Endothelium	fUSE 5 Phage display; mouse <i>in vivo</i> biopanning	CVSNPRWKC and CHVLWSTRC, ephrin A-type ligand homologs, homed to pancreatic islets	[103]
	fd-tet Phage display	RIP1 phage clone (LSGTPERSGQAVKVKLKAIP) targeted rat β -cells in islets	[104]
	M13 Phage display; rat <i>in vivo</i> biopanning	VNTANST and QPEHSST targeted adeno-associated virus to lung and brain endothelium, respectively	[105]
	T7 Phage display; <i>ex vivo</i> and <i>in vivo</i> mouse biopanning	CRPPR peptide, and CKRAVR, CRSTRANPC, CPKTRRVPC phage targeted cardiac endothelium	[106]
	M13 Phage display; mouse inflammatory and atherosclerotic model	CVHSPNKKC-coated superparamagnetic fluorescent nanoparticle targeted VCAM-1-expressing endothelium and atherosclerotic lesions	[107]
Fibrinogen/fibronectin	M13 Phage display; mouse atherosclerotic model	VHPKQHR-coated superparamagnetic fluorescent nanoparticle targeted VCAM-1-expressing endothelium and atherosclerotic lesions	[108]
	T7 Phage-display; mouse xenograft and model	CGLIIQKNEC,CNAGESSKNC targeted clotted plasma proteins and tumor tissue	[109]

MTC: Medullary thyroid carcinoma; TT: Medullary thyroid carcinoma cells.