Tumor Targeting Reagents: The Road from Ligand Selections to Clinical Reagents

Kathlynn C. Brown, PhD
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Kathlynn C Brown, PhD
Assistant Professor
Division of Translational Medicine
Departments of Internal Medicine and
The Simmons Comprehensive Cancer Center
University of Texas Southwestern Medical Center
5323 Harry Hines Blvd.
Dallas, TX 75390-9185 USA

Kathlynn.Brown@UTSouthwestern.edu
214-645-6348
Office: ND 2.210F

Dr Brown's research interests include drug targeting and molecular imaging of non-small cell lung cancer, creation of molecularly targeted cancer vaccines. the role of the integrin $\alpha_v\beta_6$ in transformation and metastasis, development of methodology to probe protein-protein and peptide-protein interactions, cancer biomarker identification, and novel library screening methods.

Introduction: The ability to target a particular cell type has been a longstanding objective in medicine. Paul Ehrlich introduced the concept of the magic bullet drug over 100 years ago stating "we have to learn how to aim with chemically"[1]. While significant progress has been made, medicine has not fully realized this goal. Clearly, cancer diagnosis and treatment is an area that can benefit from the generation of cellularly targeted applications. A potential paradigm for the diagnosis and treatment of cancer is to generate a panel of tumor-specific binding reagents. These reagents could be used to "classify" the tumor cell surface phenotype and to customize treatment accordingly by attaching the appropriate cell-targeting ligand to an anticancer drug. This allows a more refined molecular diagnosis of the tumor and creates "smart bullet" drugs that will be more effective and have fewer side effects. This is critical as most available chemotherapeutics are limited by their maximum tolerated dose. Furthermore, many pharmaceutical agents have been developed, only to be abandoned as treatments due to high levels of toxicity *in vivo*. Reduction of the amounts required for treatment could resurrect some of these drugs from the shelves of pharmaceutical companies.

Cancer has become the number two cause of death amongst Americans, killing approximately 1,600 people per day[2]. Development of novel treatments for this disease will be dependent on the ability to modulate cellular pathways that are aberrant in cancer cells as well as the capacity to deliver therapeutics effectively to the tumor. It is apparent that treatment of cancers will require some degree of customization of therapeutic regimens. The success of Herceptin® attests to the viability of personalized therapies for cancer. Herceptin® is a monoclonal antibody that binds to the HER2/neu receptor that is over-expressed on the cell surface of approximately 25% of breast cancers[3]. Only HER2 positive cancers are responsive to treatment. The need for tailored treatments was also made clear in clinical trials of AstraZeneca's drug Iressa®. While highly effective in 10% of lung cancer patients, this drug failed to enhance survival in the other 90%. Patients who respond to Iressa® have a somatic mutation in the tyrosine kinase domain of the epidermal growth factor receptor (EGFR)[4, 5]. Similarly, the EGFR monoclonal antibody Cituxamab® is used for these patients as they frequently have an amplification of EGFR levels as well. These examples stress the need for a detailed molecular diagnosis and a tailored therapy regime[6]. They also indicate the heterogeneity of the disease. Even within a single class of cancer, there is considerable variability and these variations affect treatment plans and clinical outcomes. ***As such, reagents that allow for a molecularly refined diagnosis and treatment are crucial.***

The challenges in developing targeted drug delivery system can be broken down into three main areas. First, one must identify cell specific binding reagents that display high affinity and specificity for the desired cell type. Additionally, if the goal is to deliver a cytotoxic drug, the ligand must mediate both binding and cellular uptake. Second, the targeting ligand must be incorporated into drug systems that allow delivery of molecular cargo specifically to the tumor *in vivo*. This depends on many factors including serum stability, clearance rates, and ability to escape the vasculature and penetrate the tumor core. Finally, the cargo must be released in an active form within the desired cellular compartment.

The bottleneck in targeted delivery and molecular imaging has been the identification of ligands that have the ability to discriminate between closely related cell types and have the required affinity for clinical use. The difficulty of the problem is driven home when one considers that the human body contains 210 distinct cell types and is composed of roughly 10^{14} cells total. The surface of a cell represents an assortment of macromolecules, which provides the cell with a topographical surface that is specific to the type and state of the cell. In cancer, a variety of genetic and epigenetic changes occur, resulting in changes in the proteomic profile of the cell[7]. These genetic modifications can result in a change in the type, number, and arrangement of cell

surface receptors, leading to unique cell surface topographies. This surface profile can be thought of as a molecular address for the delivery of biomolecules. Identification of ligands that discriminate between subtle differences in the cellular landscape with high specificity and affinity is necessary in order to fulfill the goals of targeted therapies and molecular diagnosis, as well as expanding *in vitro* diagnostic capabilities.

Cell-Targeting Exploiting Known Cellular Receptors: In some cases, information is known about the macromolecules expressed on the cell-surface, and ligands can be generated for these cellular receptors. Most efforts in the field have focused on exploiting monoclonal antibodies[8]. There are currently 9 monoclonal antibody (MAb) treatments in the clinics for treatment of cancer (Table I). Most of these antibodies are non-conjugated antibodies for passive therapies; however, there are three therapeutic immunoconjugates available. Bexxar® and Zevalin® are both radiolabeled monoclonal antibodies against CD20 used to treat Non-Hodgkin's lymphoma. Mylotarg®, an anti-CD33-calicheamicin immunoconjugate, is approved for treatment of acute myelogenous leukemia (AML). Of the antibodies in the clinics, only Cituximab® (anti-EGFR) and Avastin® (anti-VEGF) are approved for the treatment of NSCLC. The expanding use of monoclonal antibodies attests to the development of personalized therapies for cancer treatments.

Table I. Monoclonal Antibodies in Clinical Use for the Treatment of Cancer

MAb Name	Trade Name	Indication	Antigen	Conjugated
Rituximab	Rituxin	Non-Hodgkin lymphoma	CD20	No
Trastuzumab	Herceptin	Breast Cancer	HER2/Neu	No
Gemtuzumab Ozogamicin	Mylotarg	Acute myelogenous leukemia (AML)	CD33	Yes Calicheamicin
Alemtuzumab	Campath	Chronic lymphocytic leukemia (CLL)	CD52	No
Irbritumomab tiuxetan	Zevalin	Non-Hodgkin lymphoma	CD20	Yes ^{90}Y, ^{111}In
Tositumomab	Bexxar	Non-Hodgkin lymphoma	CD20	Yes ^{131}I
Cetuximab	Erbitux	Colorectal, Head & Neck cancers	EGFR	No
Bevacizumab	Avastin	Colorectal, NSCLC Advanced breast cancer	VEGF	No
Panitumumab	Vectibix	Colorectal Cancer	EGFR	No

Conjugated antibodies are highlighted in blue. Source: American Cancer Society

While monoclonal antibodies as delivery reagents can display high affinity and specificity, they suffer from certain limitations. Because of their size, immunoconjugates have difficulty penetrating tumor tissue[9, 10]. Furthermore, they suffer from nonspecific uptake by the reticuloendothelial system, which can result in liver and bone toxicities when the antibody is conjugated to a toxin or radioisotope[11]. The in vivo half-life of antibodies is not well matched to the half-lives of common radioisotopes used for positron emission tomography (PET) imaging, resulting in poor tumor contrast. As such, antibodies have not found wide use as molecular

imaging agents. Notably, it is challenging to produce large quantities of monoclonal antibodies, and perhaps more importantly, chemically modifying these macromolecules for downstream applications is difficult.

To overcome these limitations, peptides can employed as targeting ligands. Peptides are smaller than currently used antibody-based targeting reagents and display many favorable characteristics. Specifically, peptides can be synthesized in large quantities and are amenable to regiospecific derivatization[12]. They usually avoid uptake by the reticuloendothelial system. Furthermore, peptides can display high affinities for protein receptors, making them attractive ligands. Recently progress has been made in tuning the in vivo stability of peptides by selective modifications. Known peptidyl ligands that bind to cell surface receptors over-expressed in neoplastic cells can be employed as targeting reagents[13, 14]. For example, bombesin, somatostatin and luteinizing hormone-releasing hormone have been used for the delivery of chemotherapeutics. Octreotide, an octopeptide that mimic of somatostatin, is in the clinics. While it is not used in cancer therapy, it highlights the utility of peptides for clinical applications. However, these known ligands bind to a small fraction of the cell surface proteome.

When no known ligand is available, one must seek non-naturally occurring peptides that bind to the desired target. As rational design in these cases is often unfruitful, peptide chemists have turned to screening libraries of peptides for binding ligands. The advent of phage-displayed peptide libraries facilitated screening large peptide libraries for binding to target proteins[15-17]. This approach allows for the creation of large peptide libraries, on the order of 10^8-10^{12} different peptides. Suddenly, the door was opened to being able to identify peptidic ligands for biomarkers which do not have naturally occurring ligands. This opportunity was not lost on cancer biologist, and peptides were identified for proteins that play a role in cancer[18]. Peptides that bind to tumor antigens HER2/neu[19-21], EGFR[20, 22], Hepsin[23], Tie2[24], Il-6 receptor[25], GRP78[26], CD21[27, 28] , melanin[28], EphA2[29], and MMP-9[30] have been selected.

Non-biased approaches for identifying cancer specific cell-targeting ligands:
Unfortunately, in most cases, the best cellular receptor to target is unknown. In 1996, two seminal papers were published that opened up new avenues for obtaining cancer targeting reagents. Both of these papers employed phage displayed peptide libraries to isolate peptides that could bind to specific cell types. However, instead of panning on purified proteins that were anticipated to be good cellular receptors for targeted therapies, they employed a nonbiased selection on heterogeneous targets. Pasqalini and Rhouslahti reported panning phage libraries in a living animal to obtain peptides that home to the vasculature of specific organs[31]. In the same year, Johnston and co-workers panned on cells in culture to obtain peptidic ligands that bind to and trigger uptake within target cell types[32]. In both cases, the peptidic ligands display specificity for their target cell type over other cells. Importantly these approaches eliminated the need for prior identification and purification of the cellular target. Furthermore, it allows for selection of peptides that bind to their target in a native cellular context. In the case of in vivo panning, this is taken one step further in that the selected peptides must be able to reach their target within an animal, despite many biological constraints, such as serum stability, cellular access, and clearance rates, which are inherent in an animal. Additionally, cell-based biopanning allows for isolation of peptides that mediate cellular internalization, a key feature if the eventual application is drug or gene delivery. While neither of these reports was focused on cancer specific targeting, the approach is ideally suited for this application. Subsequently many papers have been published describing the isolation of tumor targeting peptides from phage displayed libraries. I will focus on advances within the past 5 years in nonbiased phage display selections for the isolation of peptidic cancer targeting ligands and will highlight the novel use of these peptides in different applications.

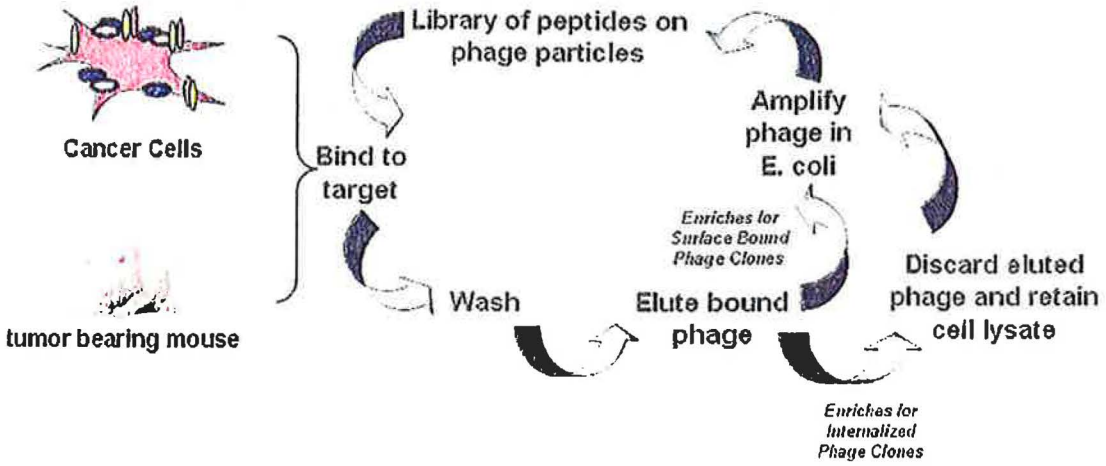


Figure 1. Schematic for the unbiased selection of cancer-targeting peptides from phage displayed-peptide libraries. *Cancer cells in culture or tumors within animals can be used as the bait for the biopanning. Amplification of eluted phage enriches for phage clones that bind the cell surface. Amplification of phage from the cell lysates enriches for peptide that mediate cellular uptake upon binding.*

In Vitro Biopanning using cancer cells in culture : Phage display is a powerful technique for the isolation of peptides that bind to a particular target (reviewed in references [16, 17]) and is typically exploited to identify peptide ligands for purified proteins. However, whole cells in culture can be used as the bait to isolate cell binding peptides. Despite the heterogeneity of the target, this approach has been highly successful in identifying cell binding ligands for a variety of cell types utilizing different phage display formats. Depending on the downstream applications of the ligand, the selection pressures can be varied to isolate ligands that bind to the cell surface or to obtain peptides trigger cellular uptake upon binding. A surprising feature of peptides isolated by the procedure is their cell specificity. In other words, the peptides tend to bind to the cell type for which they were selected against and not other cell type- in some cases very closely related cell types. While a negative selection is employed in some cases to remove ligands that might bind to common cell surface features, in many cases, no negative selection is required.

The general approach, often referred to as biopanning, involves incubating a phage library consisting of random peptides with a cell type of interest (Figure 1). The cells then undergo a series of stringent washes designed to remove unbound or weakly bound phage. Phage can be eluted from the cell surface to obtain the fraction of bound phage and used to infect *E. coli* for phage retrieval and amplification. Alternatively, cell lysates are prepared to isolate all cell associated phage. This process is repeated until the ratio of total phage incubated with the cells compared to the total bound phage no longer increases, approximately 4-6 rounds. As bacteriophage do not exhibit tropism for mammalian cells, the phage that are amplified from the library are either bound very tightly to the cell surface or internalized. Since the only unique component of any phage in the library is the displayed peptide, this peptide should be the factor determining which phage bind to a particular cell type.

Using cells as the bait for the peptide selection has several advantages. First, it keeps the cellular receptors in their native context. The topography of the cell surface is defined by expression levels of the plasma membrane bound protein as well as their arrangement within the membrane; this cannot be mimicked using purified membrane proteins. Second, by changing wash conditions, the selection can be biased towards the isolation of peptides that

mediate cellular uptake. When using purified membrane proteins, the selection process is driven by binding alone. Third, this approach employs an unbiased screen in which there is no selective pressure towards binding a particular macromolecule. This has the advantage that it requires no prior knowledge of the cellular receptor. Thus, targeting peptides can be isolated for cell a type of which little is known about their cellular landscape. Furthermore, it allows for the discovery of cell surface macromolecules that may not have been considered as viable targets or have not yet been identified[33].

The number of cancer specific peptides isolated by this method has expanded as seen in Table II. As observed from the list of peptides, few sequence similarities are observed between the peptides. This most likely stems from the differences in library design, the cell lines used as bait, and panning protocols. Peptide libraries of linear and cyclized peptides have been utilized and the peptide length has varied from 6-20 amino acids. While many of these peptides were isolated by biopanning on cell lines, fresh cells used ex vivo have been employed as well. Recently, human colon cancer cells isolated by laser capture microdissection have been employed for biopanning[34]. This highlights the robustness of the system and the flexibility in the nature of the cellular bait. Although isolated on cells in vitro many of these peptides have been shown to home to tumors in vivo.

While most unbiased biopanning experiments have been performed with cells, unbiased panning can be employed for biological mixtures of proteins found in the tumor stroma. The extracellular matrix in tumors is enriched in fibrin which arises from clotting of fibronectin in plasma that leaks into the interstitial space of the tumor. Taking advantage of this phenomena, Ruoslahti and co-workers have performed biopanning using clotted plasma as the target[35]. Two cyclic peptides were isolated and determined to bind to the fibrin-fibronectin network. These peptides accumulate in the extracellular space of MDA-MB-435 tumors in animal xenografts and bind to frozen sections of clinical breast tumor samples.

The peptides listed in Table II were isolated from experiments in which the selections were pushed to convergence. In other words, the selections were carried out for multiple rounds until the few "best" peptides were identified as defined by binding affinity. However, other selections have been performed on a large set of cell lines or patient samples in which short peptide consensus motifs were identified early in the selection process[36, 37]. While these short peptide motifs are unlikely to be high affinity ligands that are useful for cell-targeting, they can be used to identify cell surface similarities amongst the set of cells used in the experiments. This can provide information about the heterogeneity of the disease as well as an understanding of similarities between cancers arising from different organ sites.

In vivo panning to identify tumor targeting peptides: *In vivo* panning has been employed to isolate organ homing phage from peptide libraries. In this method, first published by Ruoslahti and co-workers, phage libraries are injected into the tail vein of mice[31]. After a short incubation time, the mice are sacrificed and the target organs removed. The organ-associated phage are retrieved and amplified from the homogenized tissue and the panning repeated in another mouse. After 3-5 rounds of panning, several peptide motifs are typically identified for a given organ[38-40]. Using the same technology, tumor targeting peptides can be identified[41]. As the process is dependent on the ability to retrieve infectious bacteriophage, circulation times are generally in the 5 to 15 minute time range. For this reason, i*n vivo* biopanning generally identifies peptides that target vasculature addresses, although several of peptides are found to penetrate within the tumor. This method can be coupled with in vitro panning to enrich for likely peptide candidates before moving to in vivo panning. This approach has been applied to a number of different tumor types (Table III).

Table II Peptides Selected by Whole Cell Biopanning

Cancer Type	Cell Line used for Selection	Peptide Sequence[a]	Receptor Identified	In vivo Homing Validated	Used in Imaging Methods	Delivery of Bioactive Molecules
Hepatocarcinoma	BEL-7402	TACHQHVRMVRP[42]		Yes		
	SMMC-7721	KSLSRHDHIHHH[43]		Yes		Yes
	Mahlavu	SFSIIHTPILPL[44]		Yes		Yes
Melanoma	Me6652/4	CTVALPGGYVRVC[45]	GRP78			Yes
	B16-F10	TRTKLPRLHLQS[46]		Yes		
Prostate	Capan-2 (irradiated)	SHGFSRHSMTLI[47]		Yes		
	LnCaP	DPRATPGS[48]				
	DU-145	FRPNRAQDYNTN[49] (DUP-1)		Yes		
Gastric	XGC9811-L4	GRRTRSRRLRRS[50]		Yes		
	GC9811-P	SMSIASPYIALE[51]		Yes		
Colon	HT29	CPIEDRPMC[52]				Yes
	WiDr	HEWSYLAPYPWF[53]		No[54]		
		QIDRWFDAVQWL[53]				
	Human Colonic adenomas	VRPMLQ[55]		Yes	Yes	
	Resected human colon tumors	SPTKSNS[54]				
	SW480	VHLGYAT[56]		Yes		
Head and Neck	MDA167Tu	TSPLNIHNGQKL[57]		Yes		
Nasopharyngeal	NPC-TW 04	RLLDTNRPPLLPY[58]		Yes		Yes
Breast	MDA-MB-321	YQATPARFYTNT[53]				
		CGWMGLELC[53]				
	SKBR3	LTVSPWY[59]				Yes
		WNLPWYYSVSPT[59]				
Neuroblastoma	WAC 2	HLQIQPWYPQIS[70]				
		VPWMEPAYQRFL[60] (p160)		Yes[31]		
Glioma	RG2	VGLPEHTQ[62]				
		ELRGDSLP[62]				
		DSTKSGNM[62]				
		DYDMTKNT[62]				
		DLTKSTAP[62]				
		ESRGDSYA[62]				
	U87-MG	MCPKHPLGC[63]				
Cervical	SiHa	CRLTGGKGVGC[64]				
		CADPNSVRAMC[64]				
		CAAHYRVGPWC[64]				
Medullary Thyroid	TT	CHTFEPVGC[65]				
Rhabdomyosarcoma	RD	CQQSNRGDRKRC[66]	αvβ3			
		CMGNKRSAKRPC[66]		Yes		
Lymphoma	A20	SAKTAVSQRVWLPSHRGGEP[67]				
		KSREHVNNSACPSKRITAAL[67]				
		WLSEAGPVVTVRALRGTGSW[67]				
	Molt-4	CAYHRLRRC[68]				Yes
Leukemia	Kasumi-1	CPLDIDFYC[69]	α4β1			
Lung Cancer	H1299 (large cell)	VSQTMRQTAVPLLWFWTGSL[70] (H1299.1)				Yes
		YAAWPASGAWTGTAPCSAGT[71] (H1299.2)				
		EHMALTYPFRPP[72]				
	H2009 (Adenocarcinoma)	RGDLATLRQLAQEDGVGVR[70] (H2009.1)	αvβ6[33]	Yes	Yes	Yes[73,74]
	A549 (Adenocarcinoma)	MTVCNASQRQAHAQATAVSL[70]				
	CL1-5	TDSILRSYDWTY[75]		Yes		Yes
Bladder	HT-1376 (ex vivo)	CSNRDARRC[76]		Yes		
Pancreatic	Pancreatic ductal carcinomas arising from Kras/p52L/L mice (ex vivo)	KTLLPTP[77]	Plectin-1	Yes	Yes	

[a]Common peptide names that have been used in the literature are indicated in parenthesis next to the peptide sequence.

Table III. Cancer-Targeting Peptides Selected by in vivo Biopanning

Tumor Type	Peptide Sequence	Receptor	Free Peptide Tested	Used in Imaging Applications	Delivery of Bioactive Molecules
Gastric (AZ-P7a)	SWKLPPS[78] (a)	α3β1 (b)	Yes		Yes
Human gastric adenocarcinoma	CGNSNPKSC[79]		Yes	Yes[80]	
Lewis lung carcinoma (irradiated/SU11248)	HVGGSSV[81]		Yes	Yes	
Lung (CL1-5)	SVSVGMKPSPRP[82]		Yes		Yes
Oral (SAS)	SVSVGMKPSPRP[82]		Yes		Yes
	SNPFSKPYGLTV[83]		Yes		Yes
	WDSNTYTPRPLM[83]		Yes		Yes
Nasopharyngeal carcinoma (CNE-1)	EDIKPKTSLAFR[84]		Yes		
Prostate (PC-3)	IAGLATPGWSHWLAL[85]			Yes	
Breast (MDA-MB-435)	CNGRCVSGCAGRC (NGR)[41]	Amino-peptidase N	Yes	Yes	Yes[89]
	CDCRGDCFC (RGD-4C)[41]	αvβ3, α5β1	Yes	Yes[86-88]	Yes[41, 87, 90, 91]
Medullary thyroid carcinoma (RET-C634R transgenic mice)	SRESPHP[92]				
K14-HPV16 mice basal cell squamous carcinoma	CGKRK[93] (c)		Yes		
	CDTRL[93]		Yes		
RIP1-Tag2 mice Pancreatic islets	CRGRRST[94] (c)	PDGFRβ	Yes		
	CRSKG[94]		Yes		
	CKAAKNK[94]		Yes		
	CKGAKAR[94]		Yes		
	FRVGVADV[94]		Yes		
Human myeloid leukemia (HL-60)	KDEPQRRSARLSAKPAPPKPEPKPKKAPAKK (F3)[95] (c)		Yes		
MDA-MB-435 (tumor lymphatic vessels)	CGNKRTRGC (LyP-1)[96] (c)	p32/gC1qR[97]	Yes	Yes[98]	
C8161 Melanoma	CLSDGKRKC (LSD)[99] (c)		Yes		Yes
Prostate Tumors from TRAMP mice	CREAGRKAC (REA)[99] (c)		Yes		Yes
B16-F10	TRTKLPRLHLQS[46] (c)				

[a]Intraperitoneal injections used instead of intravenous injections.
[b]Receptor identified by similarity of the peptide to laminin but not confirmed.
[c]Combination of ex vivo/in vivo biopanning employed.

Different peptides are isolated for different tumor types, even employing the same library, suggesting that the tumor influences the receptor profile of the endothelium. This discrimination can be quit exquisite. For example, peptides have been identified that can distinguish between neovasculature in dysplastic skin and neovasculature in the resultant tumor[93]. Recently, Hallahan and colleagues have identified a peptide that binds to tumors that are sensitive to a combination treatment of radiation and systemic administration of the VEGF receptor tyrosine kinase inhibitor SU11248 while remaining blind to nonresponsive tumors[81]. This demonstrates the specificity that can be achieved with peptides and suggests that peptidic reagents can be developed to access drug responses.

Recently Eriki Rhouslahti's laboratory has employed a combination of *ex vivo* and *in vivo* selections to isolate peptides that bind the tumor lymphatic vessels as opposed to the primary vasculature[96, 98-100]. As the lymphatic vessels in tumors provide a route for cancer cells to escape from the primary tumor, targeting these vessels for the destruction has the potential to inhibit metastasis. Biopanning has isolated a cyclic 9-mer peptide, LyP-1, that binds lymphatic vessels in breast, prostate, and osteosarcoma tumors[96, 98]. The peptide does not home to a melanoma or leukemia xenograft indicating that the peptide is specific for a certain lymphatic

marker present in some tumors. Furthermore it does not bind to tumor vasculature or other tissues in the mouse. Surprisingly, the peptide translocates to the nucleus upon binding. Peptides for tumor lymphatic vessels have been isolated for other tumor types and are indicated in Table III[99]. Again, highly specific peptides can be isolated. For example, peptides have been isolated that can distinguish between lymphatic vessels in premalignant lesions and fully developed tumors in a TRAMP animal model of prostate cancer[99].

In vitro and in vivo biopanning can lead to the isolation of different peptides, even when the same library and cell line are employed. The 12-mer library available from New England Biolabs was biopanned against the CL1-5 NSCLC cell line in vitro as well in vivo using CL1-5 tumor xenographs[75, 82]. The in vitro panning isolated the peptide TDSILRSYDWTY (SP5-2) while the in vivo experiment resulted in the selection of the peptide SVSVGMKPSPRP (SP5-52). Both experiments were preformed in the same laboratory. As anticipated, SP5-52 targets the vasculature while SP5-2 penetrates into the tumor results in a more diffuse binding pattern throughout the tumor mass. This emphasizes that these two approaches to biopanning are targeting different molecular features.

It is important to remember that the tumor vasculature arises from mouse endothelium. As such, the peptides isolated by this method may not translate to human vasculature if the peptide binding does not cross species. This limitation can be overcome by identifying the cellular target of the peptide and determining if its homolog is expressed in human tumor vasculature. The isolated peptide sequence can be used as a starting point for mutagenic optimization for binding to its human counterpart. Alternatively, the biopanning can be performed in humans. Arap and Pasqualini first demonstrated that phage could be injected into terminal human patients and retrieved from human tissues[101]. High density sequencing was performed to identify tripeptide motifs that segregate to different organ vasculature. This was followed by a report by the Krag laboratory in which serial panning/biopsies were performed in patients[102]. While promising, biopanning in humans has not yet been performed to identify high affinity tumor targeting reagents that can be used clinically.

Use of cancer-targeting peptides for drug delivery: Clearly one of the primary uses for these tumor targeting peptides is cell-specific delivery of chemotherapeutics. Tumor specific delivery of therapeutics can increase efficacy of the treatment while decreasing untoward side effects, thus widening the therapeutic window of the therapeutic. There are two primary formats for targeted drug delivery. In the first, the targeting peptide is directly conjugated to the chemotherapeutic target. In the second configuration, the targeting moiety is attached to a drug carrier[103]. A variety of drug carriers have been developed including polymeric drug scaffolds[104], micelles[105], dendrimers[106, 107], and liposomes[108]. The advantages and disadvantages for each drug delivery approach are outlined in Table IV.

Chemotherapeutics can be covalently coupled to a targeting reagent for drug delivery. This approach requires that chemically compatible sites exist on the drug molecule and the ligand. In most cases the drug must be released to be functional, yet the linkage must be stable until its reaches its tumor target. Most efforts have focused on utilizing acid labile linkers that are stable at pH 7 but upon internalization free drug will be released from the conjugate at the acidic pH found in the lysosome. Ester and carbamate linkages can be employed as well [89, 109, 110] as they are generally stable in serum but cleaved when internalized within the cells as a result of the dramatic increase in esterases. Doxorubicin has been attached via a hydrazone linker to two non-small cell lung cancer targeting peptides resulting is cell specific death in vitro[73]. While the therapeutic window was dramatically widened, the targeted drug was less effective that free doxorubicin. This may stem from low drug uptake, inefficient drug release from the peptide

Table IV. Comparison of Different Drug Carrier Platforms as Therapeutics

	Advantages	Disadvantages
Direct-Conjugate	• Better escape from the vasculature • Better diffusion through interstitial space of tumor • Able to control peptide-drug ratio • Less non-specific uptake in non target cells	• Drug Release is Necessary • Lower Drug Load • Rapid Clearance Rate
Liposomal Formulation	• Higher drug load • Drug molecule is unmodified • Single coupling chemistry for different drugs • Can incorporate multiple therapeutic reagents in a single nanoparticle • Takes advantage of the enhanced permeability and retention (EPR) effect of the tumor • Longer circulation time • Can incorporate multiple peptides on liposome surface	• Less efficient escape from the vasculature • Large size reduces diffusion through the tumor • Higher background uptake in non-targeted cells
Polymeric Micelle Formulation	• Moderate drug load • Drug molecule is unmodified • Single coupling chemistry for different drugs • Can incorporate multiple therapeutic reagents in a single nanoparticle • Reduced renal clearance • Moderate circulation time • Can incorporate multiple peptides on liposome surface	• Less stable than liposomes. • Concentrations must remain above critical micelle concentration. • Currently limited to the encapsulation of hydrophobic drugs • Increased background uptake due to polymer
Polymeric Drug Carriers	• Increased drug load • Takes advantage of EPR effect • Reduced renal clearance • Longer circulation time	• Difficult to control polymer chain length • Difficult to control drug load • Requires direct conjugation of the drug • Polymer must be biodegradable

carrier, or incorrect cellular trafficking. These problems can be overcome by increasing the drug load of the conjugate, changing the linkage of the drug to the targeting agent, or employing a more potent drug. In contrast, taxol conjugated to a melanoma targeting peptide is more efficacious in inducing apoptosis than free taxol[45].

The proapoptotic peptide KLAKLAKKLAKLAK has been fused to several cell-targeting peptides to create a chimeric peptide[52, 68, 99]. This peptide is nontoxic to cells until internalized, at which point it disrupts the mitochondrial membrane resulting in cell death. The peptide can be synthesized using D-amino acids rendering it protease resistant while maintaining its ability to induce apoptosis. The chimeric peptides have been found to affect highly specific cell death of the target cell type. While it is unlikely that the proapoptotic drug will replace small molecule chemotherapeutics, it serves as a useful tool to assess the utility of the peptide.

Direct peptide-drug conjugates have the advantage of high specificity and are predicted to penetrate the tumor better than a larger nanoparticle[111]. On the other hand, drug carriers such as liposomes, micelles, and polymers can carry more drug molecules per targeting event and

have longer circulation times. Peptides isolated from phage-displayed selections have been incorporated into doxorubicin loaded liposomes[44, 58, 75, 78, 82, 83, 112, 113]. In all cases, the peptide-targeted liposome was more effective in reducing tumor growth and enhancing survival when compared to a non-targeted liposome. For example, the peptide TDSILRSYDWTY was incorporated into liposomes containing doxorubicin or vinorelbine[75]. Both formulations were more effective in reducing tumor growth and increasing survival times than non-targeted liposomes or the free drug. Correspondingly, nuclear doxorubicin was greater than 2-fold higher in tumors treated with the targeted liposome compared to the non-targeted. Recently, the tumor binding F3 peptide was incorporated onto the surface of a multifunctional micelle containing fluorescent quantum dots, iron oxide nanoparticles, and doxorubicin[114]. This peptide facilitated delivery of the micelles into the targeted MDA-MB-435 cells. Polymers can also serve as drug carriers and addition of a targeting peptide can increase intracellular delivery to a targeted cancer cell[27, 86, 104]. For example, we have recently incorporated a NSCLC targeting peptide into a polyglutamic acid polymer carrying doxorubicin[74]. The targeted polymer results in 2-fold greater uptake and a corresponding reduction in cell viability.

A word of caution about targeting larger drug carrier molecules to tumors is required. Tumors possess a disordered and leaky vasculature which allows for large particles to extravasate from the vasculature. This coupled with a dysfunctional vasculature results in retention of nanoparticles of 50 to 400 nm in the tumor[111]. This effect, known as enhanced permeability and retention (EPR) can complicate our understanding of the active targeting by the peptide. Mathematical modeling of tumor targeting suggests that passive targeting is the driving force for tumor accumulation of nanoparticles[115, 116]. While increased efficacy of targeted liposomal drugs is observed over non-targeted liposomal formulation, the reason for this is debatable. In some cases, especially in vasculature targeting, it appears that more liposomes accumulate in the tumor when targeted[75, 113]. In others, it appears that the targeting ligand does not increase the liposomal delivery to the tumor but it facilitates cellular uptake of the drug as well as increasing distribution of the liposome throughout the tumor[117, 118]. This stresses the need for appropriate controls in these experiments. Additionally, total drug delivery as well as tumor localization should be determined for targeted therapies.

Use of peptide ligands for diagnostic applications: The ability of these peptides to distinguish between tumor and normal tissues make them ideal as diagnostic reagents. The peptides can be used for in vitro characterization of cancer cells or tumor samples. The peptides can be used as antibody replacements for immunohistochemistry on fixed tumor samples. This has the advantage that peptide binding can be directly tested on a patient's tumor in order to determine which targeting peptide should be employed for in vivo applications. However, as the molecular target for most of these peptides is unknown, they are unlikely to replace antibodies for pathological classifications. Peptides can also serve as capture reagents. Peptides selected for binding to a B-cell lymphoma cell line can enrich for the cancerous cells out of a background of normal B-cells[67]. Although, peptides as affinity reagents have not been widely used to date, these tumor specific peptides may be able to enrich tumor cell from biological fluids. Additionally, the targeting peptides can be used for in vivo diagnostic applications. Peptides have been attached to a variety of dyes or fluorescent nanoparticles, such as quantum dots, for in vivo optical fluorescence imaging[77, 114, 119]. While progress is being made in the development of near infrared red reagents[120], whole body fluorescence imaging in humans is currently not feasible due to high background fluorescence, poor light penetration, and inherent light scattering[121]. However, it is valuable research tool in animal models. In a novel diagnostic application, a 6-mer peptide selected for binding to adenomas of the colon, was fluorescently labeled and administered topically to patients undergoing colonoscopy[55]. Using a fluorescent microendoscope, dysplastic regions of the colon could be distinguished from normal tissue with

sensitivity and specificity of greater than 80%. This success highlights the potential use of peptides in clinics for early diagnosis of cancer.

Positron Emission Tomography (PET) imaging has high sensitivity; as such it is an ideal platform for molecular imaging. The low spatial resolution of PET has been compensated for by the generation of PET/CT scanners that combine the sensitivity of PET with the anatomical resolution of CT. Guided PET reagents will increase the sensitivity of detection while providing molecular information about the tumor without biopsy. Information obtained from PET imaging would aid in determining which ligand(s) should be employed for tumor targeting for an individual patient. Of importance, PET imaging can provide whole body biodistribution which is crucial in optimizing targeted therapies[122].

Compared to antibodies, peptides are more amenable to harsh conditions for chemical modifications and labeling [123, 124]. Unlike antibodies, the in vivo half-life of the peptides is well matched to the half-life of most commonly used PET radionucleotides. Despite these advantages, few peptides isolated from phage-displayed libraries have been used for PET imaging. Instead, most peptide-based PET imaging has been performed using naturally occurring peptidyl ligands such as bombesin and somatostatin[125, 126]. Several $\alpha_v\beta_3$-binding peptides, including the RGD-4C peptide[86, 87], have been used in several applications to image angiogenic tumor vasculature[127, 128]. Clearly, this is an area of research that is under-represented. This may stem from the lack of available resources (small animal PET or CT/PET) imagers. Additionally, development of molecular reagents for PET imaging requires a fine balance between clearance rates, tumor retention, and nonspecific uptake in other tissues. Small changes in the peptides, linkers, chelator, and isotope can dramatically affect the biodistribution. The empirical process involved in optimizing PET reagents is time consuming and costly. As such, the potential of many of these peptides as molecular PET reagents has yet to be explored.

Magnetic resonance imaging yields high resolution images but current contrast reagents suffer from a lack of sensitivity. It is challenging to target high enough T1 contrast reagent, such as gadoteridol, to a tumor to achieve a reasonable signal. For this reason, many have turned to iron oxide particles which are a highly sensitive T2 reagent that darkens the signal in regions in which the nanoparticles accumulate[129]. A plectin-1 binding peptide that homes to pancreatic ductal adenocarcinomas has been coupled to fluorescent cross-linked iron oxide nanoparticles[77]. This peptide-nanoparticle homes to pancreatic ductal adenocarcinoma as determined by intravital fluorescence microscopy and ex vivo MRI. Using the versatile Huisgen cycoladdition, better known as click chemistry, the LyP-1 peptide has been attached to fluorescently labeled, dextran encapsulated iron oxide particles. Stressing the issue of "targeting" of nanoparticles, the authors found the total amount of non-targeted and targeted nanoparticle in the tumor to be the same yet the targeted-particle penetrated within the tumor while the naked nanoparticle remained localized around the tumor blood vessels. In both of these examples, the peptides home to the appropriate tumors in animals as assessed by ex vivo fluorescent imaging but in vivo MR imaging was not performed[130].

To further facilitate attachment of peptides to iron oxide nanoparticles, a one-step procedure for the surface functionalization of super paramagnetic iron oxide (SPIO) with a targeting peptide has recently been developed[131]. The hydrophobic surfactants on the SPIO nanoparticles can be displaced through ligand exchange with a peptide containing a C-terminal poly(ethylene glycol)-tethered cysteine residue. The resulting SPIO particles are biocompatible and demonstrate high T2 relaxivity. Attachment of the αvβ6–binding peptide, H2009.1, results in specific targeting of αvβ6–expressing lung cancer cells as demonstrated by in vitro MR imaging and Prussian blue

staining. This surface chemistry may expand the use of SPIO for MR imaging.

Use of peptidic ligands for delivery of oligonucleotides: Peptides can deliver oligonucleotides for gene therapy. Direct conjugation of an oligonucleotide is possible[59] but recent efforts have focused on using targeted carriers that are loaded with the oligonucleotide or gene of interest[132, 133]. For example, the RGD-4C peptide has been incorporated into lipid-protamine-DNA lipopolyplexes for transformation of αvβ3–positive cells[133]. Using a different scaffold, a quantum dot nanoparticle has been functionalized with a tumor targeting peptide (F3) and a siRNA duplex that serves to reduce expression of a reporter protein, EGFP[134]. The siRNA is attached via a disulfide so that it is released intracellularly allowing it to reach its mRNA target. Indeed, the peptide mediates cellular uptake of the functionalize nanoparticle resulting in a decrease in green fluorescence from the EGFP and an increase in red fluorescence from the Qdot.

Lessons from viral gene transfer can be used as guides in the development of effective peptide-targeted gene delivery. Phage clones that internalize into mammalian cells can be utilized for DNA transfer; however, the transfection efficiency is low[135-141]. Selected tumor targeted peptides have been grafted onto eukaryotic viral vectors which have high transfection efficiencies. However, this requires the removal of the native viral tropism in order to redirect the gene transfer to the targeted cell type[142, 143 144]. Recently, Wadip and Pasqualini took a novel approach to the problem; Instead of grafting the peptides onto the viral vector, features of the viral vectors were incorporated into a fd-tet bacteriophage[88, 145, 146]. In this method, a mammalian transgene cassette from adeno-associated virus (AAV) is inserted into a non-coding region of the bacteriophage genome. This cassette is flanked on either end by inverted terminal repeats allowing for improved expression of the transgene. When incorporated into the phage clone displaying the RGD-4C peptide, this chimeric virus mediates efficient transfection in cells expressing αv integrins. Importantly, the chimera enables functional gene transfer specifically to tumor vasculature and adjacent tumor cells. Remarkably, no significant gene expression is observed in the liver. Systemic delivery of the chimeric phage encoding the *tHSVtk* gene results in expression of herpes simplex virus type-1 thymidine kinase selectively in the targeted tumor. Gene expression can be imaged with PET by administration of 2'-[^{18}F]-fluoro-2'-deoxy-1-β-D-arabino-furanosyl-5-thyl-uracil ([18F]FEAU)[147]. This novel approach opens up new avenues of gene therapy and molecular imaging.

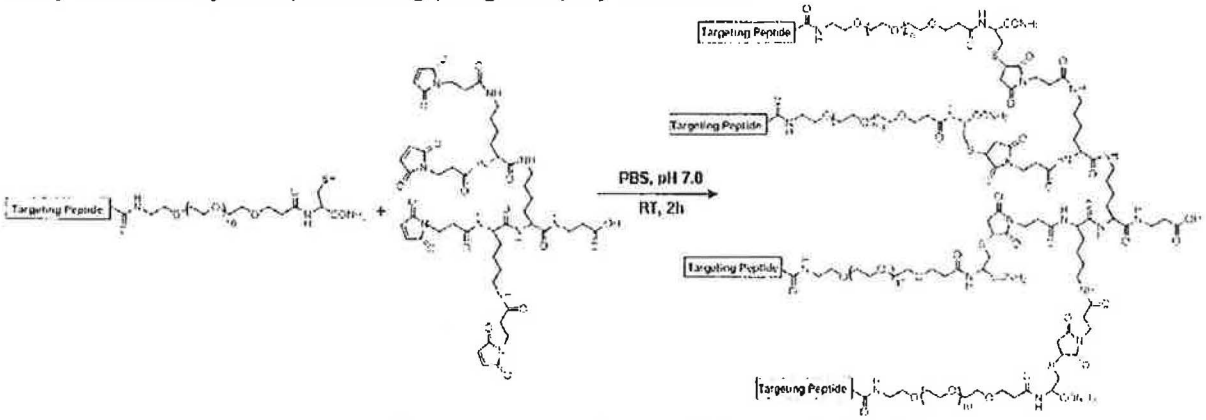
Peptides with cellular effects: In the field of targeted delivery, the assumption has been that the targeting reagents, be they antibodies or peptides are benign delivery vehicles having no effect on the targeted cell. Once their cargo is delivered, little thought is paid to the homing agent. Yet, cell surface receptors are known to modulate a variety of cellular behaviors including proliferation, adhesion, migration, and invasion. As such, it is possible that binding of the peptide may modulate these cellular activities. Such biological activities have been observed for several of the peptides isolated[43, 48, 50, 51]. For example, the peptide SMSIASPYIALE which was selected for binding to the GC9811-P gastric cancer cell line is able to block cell adhesion on a variety of extracellular matrix proteins and reduced cell invasiveness into matrigel[51]. Importantly, the peptide reduces the number of disseminated peritoneal nodules in an animal model. Similar affects on adhesion, migration, and metastasis were observed for the peptide GRRTRSRRLRRS that binds gastric cancer cells that form metastatic liver lesions in vivo[50]. The tumor lymphatic targeting peptide LyP-1 induces apoptosis specifically in cells which have affinity for this peptide[98]. Systemic administration in animals bearing MDA-MB-435 tumors results in a decrease in tumor lymphatic and a reduction in tumor growth rates. In sum, many of these peptides have cellular effects that may act in concert with targeted delivery of therapeutics. To date, no peptides identified by biopanning have stimulated tumor cell growth or

promoted aggressive phenotypes.

Increasing the affinity of the isolated peptides: Retaining the affinity and activity of peptides selected from phage displayed libraries outside of the context of the phage scaffold has been an obstacle. While many reports utilized the monomeric peptide, the affinities of these peptides are typically in the micromolar range which is unsuitable for most clinical applications[45, 51, 52, 69]. In most cases, the peptides are displayed in multiple copies on the phage, thus the peptides may bind to their cellular target via a multivalent interaction. The increased affinity due to the multivalent binding is lost when the peptides are used in their monomeric forms. Additionally, many endocytotic processes are triggered by receptor multimerization of the cell surface. If internalization of the peptide is desired, the free peptide must facilitate this interaction on the cell surface.

Through empirical testing, it has found that multimerization of the cell-targeting peptides on a trilysine core is a useful scaffold for retaining the peptide activity outside of the context of the phage (Scheme I)[70, 148]. The trilysine scaffold mimics the presentation of the peptides on the pIII protein of the phage in both valency and the orientation of the displayed peptides. We have found the tetrameric trilysine framework to be a general platform for cell-targeting peptides selected from bacteriophage displayed peptide libraries[67, 71, 149, 150]. Tetramerization of the peptides increases the affinity of the peptide for its target cell 25-100 fold when compared to the monomeric peptide, indicating the importance of multivalent binding.

To take full advantage of these multivalent peptides, a synthetic route that takes advantage of the chemoselective reaction of a cysteine with a maleimide to synthesize the tetrameric peptide has been developed (Scheme I). This chemistry is facile and is not restricted to labs with expertise in synthetic peptide chemistry. As such, this approach is useful to all performing phage display selections. The chemistry allows for a variety of chemical moieties to be placed in the peptide in a regiospecific fashion, expanding the utility of the peptide. This route will be of utility to the many labs performing phage display selections.



Scheme I. Convergent Synthesis of Tetrameric Peptides

Bead-based peptide libraries as sources of tumor targeting ligands: While I have focused this discussion on the use of phage-displayed peptide libraries as a source of cancer specific binding ligands, it should be noted that a variety of different formats of peptide libraries exist. These methods have been reviewed previously[151] and are not the focus of this review as few of them have been utilized for unbiased biopanning. However, I would like to highlight the use of bead-based peptide libraries for the selection of cell-binding peptides. Like phage display, bead-based libraries are well-suited for selections using intact cells[152]. While bead-based

libraries do not have the diversity represented in phage displayed libraries, they do have the advantage that non-natural amino acids can be incorporated into the peptides. Lam and co-workers have pioneered this approach[153, 154]. Using this method a peptide that promotes adherence and growth of the lung adenocarcinoma cell line, A549[153] was selected from a one-bead-one compound library. This cyclic 6-mer peptide binds to two other NSCLC cell lines, Calu-1 and H178 but not to a normal human bronchoepithelial cell line. The receptor for this peptide has been identified as the integrin $\alpha_3\beta_1$. Similar methodology was used to isolate peptides that bind preferentially to malignant lymphocytes[155, 156], breast cancer cells[157], glioblastoma cells[158], and ovarian adenocarcinomas[159]. Several of these peptides have been utilized for near-infrared imaging of tumors in vivo.

Moving from peptides, the Kodadek laboratory has generated peptoid libraries that can be screened for cell binding[160]. Peptoids are N-substituted oligogylcine polymers that have many features of natural occurring peptides but are protease resistant. Using a two color cell-based screening method, peptoids were isolated that bind specifically to cell expressing vascular endothelial growth factor receptor 2 (VEGFR2) but not the isogenic cell line lacking the receptor. While this screen was biased towards selecting a ligand for a particular receptor, it is clear that a similar screen can be performed using a cancerous cell type and a corresponding nonmalignant cell line.

Future Challenges: Despite the success of phage display for identifying peptidic tumor-targeting ligands, there are still challenges in the field. A major challenge in the field is identification of the cellular target of the selected peptides. This is driven home when one considers that of the peptides listed in Tables I and II, the receptors for these ligands have been identified for only 15%. Although these ligands can be used for drug delivery without knowledge of the cellular receptor, there are several reasons that receptor identification should remain a priority. First, receptor identification can provide information about changes in the cell surface profile during carcinogenesis, tumor maintenance, and metastasis. This opens avenues of new basic research on the role of the receptor in the disease. Second, once identified, new ligands can be generated for the receptor. While peptides might be appropriate in some applications, antibodies, peptoids, or small molecules may be better choice for others. Third, moving the ligand towards clinical use will be facilitated by an understanding of its binding partner.

Most efforts towards receptor identification have focused on biochemical affinity purification or protein cross-linking followed by mass spectrometric identification of the isolated protein species[45, 77]. The reasons for the low success rate are partly due inherent nature of membrane proteins. They are present in low abundance and solubility is an issue. Additionally, my laboratory has found that many of the targeted receptors are found in lipid raft domains which are notoriously difficult to solubilize. This makes affinity purification and mass spectrometric identification difficult. Due to the difficulties with affinity purification of membrane protein, genetic methods are emerging as means to identify cellular targets. Subtractive hybridization cloning can be used as well to generate a set of potential receptors[69].

It is important to remember that the cell surface has topography in which proteins can multimerized with binding partners or cluster within microdomains. This surface landscape can contribute to the specificity of the peptidic ligands. In other words, the specificity may not arise from absolute protein expression levels but an arrangement of the receptors on the cell. This level of information can be lost upon the preparation of membrane protein for affinity purification and is not born out in mRNA levels. It is also important to note that while the assumption in the field has been that the peptides bind protein receptors, they may be binding sugar moieties of glycoproteins or glycolipids, or phospholipids.

Protein databases can be searched for sequence similarity to the peptide. This has yielded candidate receptors for a few isolated peptides[33, 36, 94]. For example, homology of a lung adenocarcinoma binding peptide to the GH viral coat protein of foot and mouth disease virus led to the identification of as the cellular receptor for this peptide[33]. However, most phage-displayed peptide libraries are chemically synthesized and do not originate from biological sources. Furthermore, the complete sequence coverage of the longer peptides is limited. As such, the probability of the peptide sequence matching a biologically derived sequence is statically low. Furthermore, many matches do not provide biological insight into the potential receptors. In sum, new techniques are needed to identify the receptor partners for the selected ligands. A combination of cell biology, proteomic and genomic approaches will be needed to tackle this difficult problem.

Conclusion: Over the past 10 years, phage displayed peptide-libraries have proven to be a rich source of cancer targeting ligands. The peptides can have antibody-like affinities and cell specificity. The chemistry is in place for regiospecific modification of peptides; as such they can easily be manipulated for different purposes. The goal is to now to optimize these peptides and utilize them for clinical applications. This is of high priority for cancer patients, clinical practitioners, and scientists alike. More and more, the barriers towards using peptides as drugs or drug delivery reagents are dissolving[161]. This is driven home by the increasing number of peptide pharmaceutical on the market and the number of companies focusing on peptide formulations[162, 163]. It is likely that pre-clinical and early phase clinical trials using some of these peptides as drug delivery reagents or molecular imaging probes will begin within the next few years.

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