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Beyond Peptides and mAbs - Current Status and Future Perspectives for Biotherapeutics with Novel Constructs

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Abstract

Biotherapeutics are attractive anti-cancer agents due to their high specificity and limited toxicity compared to conventional small molecules. Antibodies are widely used in cancer therapy, either directly or conjugated to a cytotoxic payload. Peptide therapies, though not as prevalent, have been utilized in hormonal therapy and imaging. The limitations associated with unmodified forms of both types of biotherapeutics have led to the design and development of novel structures, which incorporate key features and structures that have improved the molecules' abilities to bind to tumor targets, avoid degradation and exhibit favorable pharmacokinetics. In this review we highlight the current status of monoclonal antibodies and peptides, and provide a perspective on the future of biotherapeutics using novel constructs.

Keywords

Antibodies; Peptides; Antibody Engineering; Immunoconjugate

INTRODUCTION

More than a century ago Paul Ehrlich proposed that protein therapeutics could specifically target disease processes¹. This “magic bullet” theory has captured scientific and popular imagination since that time. In the past quarter-century Ehrlich’s dream has begun to be realized as many such reagents have entered into routine clinical practice, with particular utility in major disease areas such as oncology and rheumatology². In this review we concentrate on the principles and applications of targeted protein therapeutics for oncological indications. Biotherapeutics are appealing options for cancer therapy due to their high levels of target specificity and potential for minimal toxicity as compared with small molecules. Monoclonal antibodies have become routinely incorporated into the standard armamentarium of cancer therapies³, as shown in Table 1. Many more novel antibodies and antibody conjugates are in clinical development; hence these drug platforms have proven to be useful and cannot be viewed as transitional treatment strategies that await

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the development of improved small molecules. Remarkably, no clinically approved therapeutic antibody for oncology has ever been significantly displaced from the oncological marketplace by a subsequent competitor small molecule.

Overlooked in the excitement of antibody-targeted therapeutics, another protein platform format – peptide-based therapy – continues to undergo improvements. The potential therapeutic utility of peptides had been discounted in the past due to the low stability of such molecules. These challenges have been overcome mainly by chemical modification, such as incorporation of D-amino acids⁴ or cyclization⁵. Antibodies represent the most widely used and clinically successful class of protein and peptide therapeutics in cancer³. However, the current generation of therapeutic antibodies has properties that may limit utility in some circumstances, such as large size, poor tissue penetration due to steric hindrance and the high cost of antibody manufacturing. During the past two decades antibody engineering efforts have led to the discovery and testing of more than 50 different protein scaffolds^{6–8}. The term scaffold, as used in protein engineering, describes a single chain polypeptide framework typically of reduced size (<200 AA) that contains a highly structured core associated with a variable portion of high conformational tolerance. This allows for engineering of the protein through insertions, deletions, or other substitutions. These scaffolds are either antibody-derived with an IgG backbone, or derived from non-immunoglobulin based proteins⁸. Many of the novel scaffolds under development recognize known and validated disease associated targets such as tumor necrosis factor TNF- α , CD20, vascular endothelial growth factor (VEGF), CD19 and CD3.

The first novel engineered scaffold protein to reach the market was ecallantide (DX-88, Dyax Corporation). It was introduced in December of 2009 as Kalbitor®^{9,10}. This potent inhibitor of human plasma kallikrein was approved for the treatment of patients suffering from hereditary angioedema.

This review will provide an overview of the current status of monoclonal antibody and peptide therapy with a look to the future.

Unconjugated Monoclonal Antibody Therapy

Antibody Structure

Antibodies are secreted by B cells in response to antigen exposure. They are composed of heavy and light chains that make up the functional units of antibodies: the constant region (Fc) and the fragment of antigen binding (Fab) region¹¹ (Figure 1). Antibodies are grouped based on their heavy chain compositions and Fc regions into IgM, IgG, IgA, IgE and IgD. IgG is the most commonly used antibody structure for monoclonal antibody therapy. Antibody functions are specified by their respective domains. The Fab domain determines antibody specificity and antigen recognition; this is accomplished through the variable regions, which consist of three hypervariable complementarity-determining regions (CDRs)¹¹. The Fc domain can interact with various receptors expressed at the surface of several cell types. By binding Fc γ receptors (Fc γ R) on effector cells the Fc domain links the antibody binding regions to immune system activation and effector function, enabling receptor mediated endocytosis to clear antigen/Ab complexes, eliciting Antibody dependent

cellular cytotoxicity (ADCC) or triggering/altering signaling cascades across the plasma membrane¹². The Fc portion can also interact with the neonatal Fc receptor (FcRn) expressed at the surface of several cell types¹³. FcRn engagement plays an important role in IgG homeostasis and has been shown that to extend the serum half-life of IgG from one day to up to several weeks¹⁴.

Monoclonal Antibodies (mAbs)

The development of hybridoma technology in 1975 enabled the production of murine monoclonal antibodies¹⁵. Initially, the clinical application of murine mAbs was limited by their immunogenicity to humans and inability to induce robust human immune effector responses against the targeted tumor antigens and cancer cells. These limitations were attributed to the murine origin of these antibodies^{16,17}. A series of technical advancements, starting in the early 1980s and continuing to this day, have facilitated the design and implementation of modified antibody structures with improved target binding, effector potency and reduced immunogenicity. Examples of these improvements include *chimeric* antibodies, where murine variable regions are grafted onto human Fc regions, *humanized* antibodies, where murine complementarity determining regions (CDRs), are grafted onto a human antibody framework, and *fully human* antibodies derived from xenogeneic mice or by recombinant human antibody display technologies.¹⁸ These modified antibody structures now dominate the human antibody therapy landscape. Today, fourteen conjugated and unconjugated mAbs have been FDA approved for the treatment of cancer (Table 1).

The pharmacology of mAbs is unique among cancer therapies. Pharmacokinetic properties of mAbs are typically nonlinear and, in some instances, the therapeutic half-lives are variable, increasing with subsequent dosing^{19,20}. While some efforts have been made to link therapeutic response with physiologic recovery of the targeted organs following antibody therapy (such as the duration of B-cell repletion in the treatment of hematologic malignancies), it is not clear if these descriptions actually correlate to duration of treatment response^{21–23}. Table 2 summarizes the pharmacokinetic and pharmacodynamic properties of those mAbs with FDA approval, along with the unique adverse effects clinicians need to be aware of when using each agent.

Manipulating mAb Structural Properties

The application and effectiveness of mAb therapy may be influenced by the size of the protein (typically about 150kDa), which hinders tissue penetration and delivery¹⁴, and also by non-specific uptake by the reticuloendothelial system that can limit efficacy and induce dose-limiting toxicity to the liver and bone marrow when the mAbs are conjugated to various toxins²⁴. Modern antibody engineering techniques have made it possible to manipulate the immunogenicity, size, valence, affinity for target antigen and Fc domain-directed interactions to customize antibody properties for particular purposes. Several examples are described below.

Fab Domain Modification—The Fab domain is the antigen-binding domain, and has long been the subject of antibody engineering efforts that have included the creation of antibody-based fragments such as the enzymatically cleaved fragments, Fab and F(ab')₂ and

genetically engineered formats such as scFv, (scFv)₂, diabody and minibody (Figure 1; reviewed by Robinson et al, 2004¹⁸). The usual building block of these structures is a 25-kDa, monovalent single chain Fv (scFv) composed of the variable domains (V_H and V_L) of an antibody derived from either hybridomas, or binding domains isolated from phage- or yeast-display libraries and fused together with short peptide linkers. These flexible building blocks make it possible to create a wide variety of antibody-based structures that have been used to evaluate the impact of antibody size, valence and binding site format on antigen binding, tumor targeting, effector functionalities¹⁸. Moreover, it is possible to employ techniques such as chain-shuffling and site-directed mutagenesis to manipulate antigen-binding properties^{25–27} to define the roles of binding affinity and avidity on *in vitro* and *in vivo* tumor targeting, immune effector functions such as antibody-dependent cell mediated cytotoxicity (ADCC) and therapeutic efficacy. These studies have shown that high affinity for tumor antigen targets leads to impaired tumor penetration due to target-mediated antibody internalization and binding to shed antigen^{28–31}. However, although the actual delivery and penetration of lower affinity antibodies is superior, the higher affinity antibodies have improved therapeutic efficacy in murine models³¹.

Advances in the modification of the Fab domain include the construction of novel multi-specific structures such as bispecific antibodies (bsABs), structures with two Fab domains, or three such domains (triomAbs), creating opportunities for high avidity or multi-specific targeting²⁴. A common target of bsAbs is CD3, the activating receptor for T-cells, as seen with the triomAb, Catumaxomab, which binds 1) the tumor antigen EpCAM, 2) CD3 and 3) innate effector cells through an intact Fc portion³². It's success in a phase II/III clinical trial led to its approval by the European Commission in 2009 for malignant ascites. Catumaxomab's success prompted the generation of other triomAbs directed against the tumor antigens HER2/neu (ertumaxomab), CD20 (Bi20/FBTA05;NCT01128579¹), GD2 and GD3 (Ektomun)²⁴.

Fc Domain Modification—The therapeutic significance of the Fc domain rests in its ability to induce effector functions and to manipulate the serum half-lives of antibodies. Fc recognition by FcγR on immune effector cells such as NK cells, dendritic cells, neutrophils and mononuclear phagocytes can transduce activating signals through immunoreceptor tyrosine-based activation motifs (ITAMs) or deliver inhibitory signals through immunoreceptor tyrosine based inhibitor motifs (ITIMs). Most Fc stimulatory signals are transduced by FcγR1 (CD64) and FcγRIIIA (CD16a). Cross-linking of FcγRs on effector cells promotes antibody-dependent cell-mediated cytotoxicity (ADCC) or activate/ block signaling cascades across the plasma membrane eliciting tumor cell destruction^{12 33}. ADCC has been shown to be an important mechanism for monoclonal antibodies used in cancer therapy^{31,34}.

Two general approaches have been utilized to manipulate Fc domain based effector functions. The first approach entails altering the amino acid structure of the domain to influence binding affinity to Fc receptors^{33–36}. Altered amino acid sequences have led to

¹Information about the referenced clinical trials can be found at the url: <http://clinicaltrials.gov/ct2/show/xxx> where xxx is the specific number/letter code referenced in the text.

the creation of Fc domains with higher affinity for Fc γ RIIIa and enhanced ADCC properties. For example, altering amino acids in the Fc domain or modifying Fc-linked glycosylation of the clinically approved anti-CD20 antibody rituximab yielded its humanized version ocrelizumab, which possesses increased binding affinity to Fc γ RIIIa and promotes enhanced ADCC compared with rituximab^{37,38}. Further modifications were made to create a third generation anti-CD20 antibody, PRO131921. It is an engineered form of ocrelizumab that binds with higher affinity to CD20, and the Fc domain has been engineered to increase binding affinity for both Fc γ RIIIa and C1q as compared with rituximab and ocrelizumab³⁹.

Fc domain oligosaccharide content modification can enhance ADCC as well. For example, antibodies with defucosylated oligosaccharides show enhancement of ADCC *in vitro* and enhanced *in vivo* anti-tumor activity³⁸. Most of the currently used therapeutic antibodies are highly fucosylated. Phase I clinical trials of non-fucosylated antibodies specific for CC-chemokine receptor 4 (CCR4), which is expressed by some lymphoid neoplasms and is used by Treg cells to facilitate their migration into the microenvironment⁴⁰, have shown promise and early data suggest efficacy at significantly lower doses than conventional therapeutic antibodies³⁸.

Recognition of the neo-natal Fc receptor (FcRn) expressed at the surface of several cell types, including vascular endothelium cells, monocytes and macrophages as well as with barrier sites such as the blood–brain interface, the glomerular filter in the kidneys and the intestinal epithelium has been shown to increase the serum half-life of the antibody molecule¹³.

The Fc domain has been manipulated to increase as well as decrease its affinity for FcRn depending on the desired serum half-life of the antibody¹⁴. Increasing the affinity for FcRn would yield a long serum half-life, which is desirable in therapy, as it would decrease the need for repetitive injections of the molecule to achieve a therapeutically relevant serum concentration⁴¹. Decreasing Fc affinity for FcRn is required in applications such as imaging where short-serum half-lives would allow for the clearance of unbound molecules; this latter approach has yielded improved imaging contrasts for an IgG3 isotype antibody⁴¹.

Modulating the Immune system and Altering Cancer-Related Signaling

Targets of antibody therapy are not limited to the tumor cell; they can also be used to modulate a desired immune response or altering signaling pathways. The attractiveness of the immune system as a therapeutic target stems from the prospect of enhancing anti-tumor response and influencing the outcome of lymphocyte-mediated immune responses by modulating antigen-specific T-cell receptor (TCR) and B cell receptor (BCR) signals¹¹. Modulation is achieved by manipulating entities such as immunoregulatory co-receptors and other cell surface signaling molecules. Ipilimumab is the first FDA approved immune targeting monoclonal antibody against Cytotoxic T-lymphocyte Associated Antigen 4 (CTLA-4), an inhibitory receptor of T-cell activation. Other targets under study that serve as co-stimulatory molecules include PD-1/PDL-1, CD40 and CD137⁴². There are currently 22 antibodies in clinical trials that target the immune system (Table 3) 19 of which are for the treatment of cancer⁴³. Manipulation and stimulation of the immune system can be achieved

not only by mAb but also by structurally modified antibodies such as bispecific antibodies (bsAbs) and trispecific antibodies (triomAb)^{24,32}.

Immunoconjugates

Antibodies' applications in therapy can go beyond directly eliciting anti-tumor effects. They have been utilized effectively against tumors by delivering cytotoxic payloads. The attachment of drugs, toxins, radionucleotides or enzymes to antibodies has generated numerous antibodies or antibody-like molecules applicable in the clinic. Three immunoconjugates are currently FDA approved for clinical use; their pharmacologic properties are also summarized in Table 2.

Antibody-Drug Conjugates (ADC)

Antibody-drug conjugates (ADCs) facilitate the delivery of cytotoxic compounds to tumor cells expressing the antibody-targeted antigen, enhancing their antitumor potency⁴⁴. Gemtuzumab ozogamicin (Mylotarg), which consists of an antibody against CD33 coupled to the DNA cutting drug calicheamicin, was the pioneering, clinically used ADC. In 2000, it was granted accelerated FDA approval for the treatment of relapsed AML⁴⁵. Further post-approval study of gemtuzumab ozogamicin demonstrated higher toxicity rates when compared to standard chemotherapy and no improvement in clinical benefit, leading to the drug's withdrawal in 2010⁴⁶. Ado-trastuzumab emtansine (Kadcyla, T-DM1), an ADC consisting of the human epidermal growth factor receptor 2 (HER2)-targeting antibody, trastuzumab, carrying the maytansinoid, DM1, has since been approved for the treatment of patients with HER2-positive metastatic breast cancer who have received prior HER2 directed antibody therapy with trastuzumab (Herceptin).^{47–49} Ado-trastuzumab emtansine demonstrated a superior progression free survival rate in comparison to the combination of lapatinib and capecitabine; this illustrates the therapeutic potential of ADC in the treatment of patients whose tumors has already been treated with a monoclonal antibody with the same target as the ADC. Brentuximab Vedotin (Adcetris) is an ADC combining an anti-CD30 with an antimicrotubule agent, monomethylauristatin E.⁵⁰ Approved in 2011, this ADC demonstrates impressive response rates, including complete responses, in the treatment of Hodgkin's lymphomas and large cell lymphomas that have relapsed following other standard treatments^{50,51}. A number of other ADCs are in development, and several have reached various clinical phase trials, with encouraging results. Inotuzumab ogazomicin, which links CD22 to calicheamicin, has shown clinical activity for the treatment of B-cell lymphomas and acute lymphoid leukemia⁵². Gembatumumab vedotin has had promising treatment results for the delivery of monomethylauristatin E to solid tumors such as breast cancer and melanoma by targeting glycoprotein NMB^{53,54}. And lorvotuzumab metransine shows clinical promise through the delivery DM1 to a variety of solid tumors expressing CD56⁵⁵.

Immunotoxins

The conjugation of catalytic toxins derived from microorganisms to antibodies has led to the production of immunotoxins that have demonstrated anti-cancer effects. Immunotoxins are different from ADCs in that the therapeutic entity is a toxin that would confer widespread

destruction to the host tissue, if not delivered selectively to the cancer cell via the antibody conjugate. Bacterial pathogens, for example, have been explored for this purpose. The immunotoxin moxetumomab pasudotox consists of an anti-CD22 Fv bound to a modified *pseudomonas* exotoxin. Moxetumomab pasudotox has been well tolerated in early phase trials for the treatment of hairy cell leukemia and is further being explored in the management of other hematologic malignancies^{24,56 57}. Other agents are in preclinical or phase I levels of testing.

Immunocytokine Conjugates(ICs)

Cytokines have established roles as anti-cancer drugs⁵⁸. The therapeutic efficacy of cytokines is limited by their inability to localize to the tumor or tumor microenvironment, resulting in a heavy price of toxicity for any therapeutic benefit^{59,60}. Immunocytokines address this problem by directly targeting the cytokines to the tumor. As with the immunotoxin constructs, immunocytokines largely remain in early clinical trial development, with antitumor activity observed using IL-2, as well as IL-12 and TNF- α . ICs linked to IL2 such as L19-IL2, F16-IL2 and ch14.18- IL2 have demonstrated anti-tumor activity in clinical trials^{59 61}, while other immunocytokines delivering IL12, TNF- α and IL10 have are currently being developed and tested^{62–64}. Immunocytokines have been recently reviewed by Pasche et al, 2012⁵⁹.

Radioimmunoconjugates

Radioimmunoconjugates facilitate the direct delivery of radioactive particles to the tumor site via antibody recognition of antigen on the tumor or the tumor microenvironment. The only current approved radioimmunotherapy agent is (⁹⁰Y)-ibritumomab tiuxetan (Zevalin), which links yttrium-90 to a CD20 monoclonal antibody⁶⁵. This agent has demonstrated activity in patients with non-Hodgkin's lymphoma who are otherwise heavily pretreated. Of note, a second radioimmunoconjugate, iodine tositumomab, was approved for treatment of follicular lymphoma, but was subsequently removed from the market due to infrequent practitioner use, attributed in part to the complicated nature of its administration⁶⁶. At least twelve more radioimmunoconjugates are under development, 10 of which are targeted against solid tumors⁶⁷.

Ig- Like Scaffolds

Deeper understanding of the structure and function of the immunoglobulin molecule has led to the discovery and development of recombinant antibody fragments such as single chain variable domains (scFv) and diabodies. These efforts also have shed light on antibody structural and functional limitations, some of which are described earlier in this review. Such limitations have been addressed by the development of recombinant single chain Ig scaffolds such as nanobodies, domain antibodies (dAbs), bispecific scaffolds (BiTEs, DARTs and other TandAbs) and chemically programmed antibodies (cpAb). Interestingly, related structures have been found to be naturally occurring in living organisms such as camelids⁶⁸ and sharks⁶⁹ with similar solubility and antigen binding properties to the conventional IgG structures^{68,70}.

Nanobodies

Nanobodies are unique heavy chain antibodies (HCAB) consisting of only one heavy chain with a single variable domain (e.g., VHH) (Figure 2a). They are produced in camelids along with normal antibodies containing both heavy and light chains. The VHH in a HCAB is the structural and functional equivalent of the Fab fragment of conventionally structured IgG antibodies⁶⁸. Compared with typical VH segments the camelid VHH is exceptionally long, containing as many as 24 amino acid residues⁶⁸. The long VHH is an appealing structural feature, enabling nanobodies to potentially reach structurally hidden targets that are inaccessible to conventional antibodies. This feature has stimulated its commercial development and production in bacteria and yeast to accommodate large-scale fermentation. Phage display libraries have also been utilized to select recombinant single variable camelid VHH domains to yield antigen-specific antibodies or nanobodies. The advantages of these nanobodies include ease of manipulation by genetic fusion; the combination of identical or different antibodies can generate avidity effects or bispecificity/bifunctionality. Properties such as specificity, stability, thermo-tolerance and low immunogenicity and ability to cross the blood brain barrier increase the attractiveness of nanobodies as potential drugs. There are currently six nanobodies undergoing clinical trials, none of which are for anti-cancer purposes⁷¹.

Domain antibodies (dAbs)

dAbs or engineered antibody domains (eAds) represent the smallest engineered human antibody-based binding domains. They are based on either the variable domain of an antibody heavy chain (V_H domain)⁷² or the variable domain of an antibody light chain (V_L domain)⁷³. They are small monomers (11 to 15 kDa) that can be modified and fused to yield a stable compound with unique biophysical properties that include reduced aggregation, thermal stability and reversible unfolding properties most of which are attributed to their small size⁷⁴. Currently one dAb is undergoing clinical trials; CEP-37247 is an anti-TNF α bivalent dAb fused to a human IgG1 Fc region⁷⁵. This dAb that has completed a phase II clinical trial for the treatment of sciatica (CEP-37347/NCT01240876).² The therapeutic efficacy and application of dAbs in cancer therapy is yet to be established. The small size and ease of modification of dAbs make them attractive candidates for pharmaceutical development.

Bispecific scaffolds: BiTEs, DARTs and other TandAbs

Genetic engineering has been used to produce multifunctional protein scaffolds derived from the scFv antibody fragment (Figure 2b). Clinical success has been demonstrated in several bispecific formats^{76–80}, the most successful of which is the BiTE (Bispecific T cell Engager). BiTEs are able to stimulate T-cell immunity directly by targeting CD3 and tumor antigens such as CD19, EpCAM or EGFR. The most clinically advanced BiTE is blinatumomab (MT-103), which targets CD19 and CD3 for the treatment of acute lymphoblastic leukemia and Non-Hodgkin's lymphoma⁷⁹. Additional bispecific scaffolds under development include the DART (Dual Affinity ReTargeting)^{81 82} and the TandAb

²Information about the referenced clinical trials can be found at the url: <http://clinicaltrials.gov/ct2/show/xxx> where xxx is the specific number/letter code referenced in the text.

(tetravalent tandem antibodies)⁸³ platforms. They are closely related to BiTes, but differ in how the heavy and light chain variable domains are linked. AFM13 is a TandAb that targets CD30 and CD16A (FcγRIIIa) to activate NK cells, dendritic cells and macrophages^{83,84}. A phase I clinical trial has been completed, showing safety and tolerability of AFM13⁸⁵. No clinical trials of DARTs have yet been reported.

Antibody-Peptide fusion

The fusion of antibody to peptide or small molecule can result in creating a new entity with favorable features. There are two types of antibody-peptide fusion molecules peptibodies and chemically programmed antibodies(cpAb), both of which have the Fc fragment as the minimum antibody requirement and the non-binding Fab portion is modified with or replaced by a peptide or synthetic component (Figure 2c)⁸⁶. Peptibodies are composed of only Fc domain fused with a bioactive peptide (reviewed by Wu, 2013)⁸⁷. While cpAb are composed of an antibody component and a synthetic component (peptide or small molecule) (reviewed by Rader C, 2014)⁸⁸. The antibody component (Fc) contributes the half life, bivalence and effector function of conventional antibodies, while the peptide/synthetic component is responsible for antigen recognition and binding^{86,87}. Romiplostim is the first peptibody approved by the FDA for the treatment of chronic immune thrombocytopenia⁸⁹, trebananib on the other hand is a peptibody currently undergoing clinical trials for treatment of solid tumors NCT01204749, NCT01281254, NCT01493505³. The only cpAbs undergoing clinical trials are based on CovX-Body platform (e.g., mAB h38C8) pioneered by the late Carlos Barbas III. Four CovX-bodies were investigated in clinical trials, but development of these reagents was halted. Despite this, cpAbs as well as Peptibodies remain promising due to the numerous possibilities of small molecule attachments that can provide specificity, affinity and stability.

Peptides

Peptide therapeutics are based on the use of short sequence of amino acids as drug or delivery vehicles that can be produced biosynthetically via natural or recombinant microbial fermentation or chemically through mechanisms such as solution based or solid-phase peptide synthesis. Historically, the concept of peptides as therapeutics has been challenged by peptide instability, susceptibility to degradation, size and consequent limitations in delivery method (low oral bioavailability). These factors overshadowed the advantages of peptides' small size, ease of synthesis and modification, tumor-penetrating ability and good biocompatibility. Chemistry advances have resulted in stabilization methods such as cyclization and the introduction of D-amino acids to protect peptides from peptidase attack⁹⁰. Currently, about 60 approved peptide drugs are on the market for the treatment of cancer, diabetes, and cardiovascular diseases. Peptides can be used for cancer treatment directly as drugs or as carriers of cytotoxic compounds, ribonucleotides, hormones or vaccines. Approved peptides used in cancer treatment include peptide hormone therapy, peptide-conjugated radionucleotides and the peptide-related drugs bortezomib and mifamurtide⁵.

³Information about the referenced clinical trials can be found at the url: <http://clinicaltrials.gov/ct2/show/xxx> where xxx is the specific number/letter code referenced in the text.

Peptide hormones: LHRH agonists and antagonists

The LHRH (luteinizing hormone-releasing hormone) agonist family of peptides acts by targeting follicle stimulating hormone (FSH) and LH as well as testosterone production. Schally et al. first introduced this idea of down-regulation of LHRH in the pituitary for androgen deprivation as a therapy for prostate cancer patients^{91–93}. Since then a number of LHRH agonists have been developed, followed by the discovery of LHRH antagonists⁵.

Somatostatin Analogues

Somatostatin receptors are overexpressed on the surface of neuroendocrine tumors, making them attractive therapeutic targets. Two somatostatin analogues are approved for cancer therapy – octreotide (Sandostatin®) and lanreotide (Somatuline®). Octreotide is used for the treatment of acromegaly, gigantism, diarrhea and flushing episodes associated with carcinoid syndrome, and for diarrhea associated with vasoactive intestinal peptide-secreting tumors (VIPomas)^{94,95}. Lanreotide is used in the management of acromegaly and symptoms caused by neuroendocrine tumors, most notably carcinoid syndrome and VIPomas⁹⁶. Peptide receptor radionuclide therapy (PRRT) entails the chelate-mediated conjugation of a somatostatin analogue to a radionuclide yielding a radiopeptide. The most commonly used radionuclides used in radiopeptides are ¹¹¹In, ⁹⁰Y and ¹¹⁷Lu⁵. Somatostatin receptor 2 (sst2) is the most highly expressed receptor in neuroendocrine tumors; binding of this receptor by a receptor agonist results in rapid and efficient internalization of ss2 into the cell. A radiolabeled somatostatin analogue thus acts as a ss2 antagonist, resulting in the delivery of radioactive target particles to the target cell⁹⁷. This approach to targeted radiotherapy of neuroendocrine tumors is undergoing clinical evaluations^{98–101}.

Peptide Vaccines

Peptides derived from tumor-specific or tumor-associated antigens have been studied extensively as components of vaccines designed to stimulate adaptive anti-cancer immune responses (reviewed in Aranda et al, 2013¹⁰²). A detailed discussion of this area is beyond the scope of this review, but notable examples include identification of melanoma antigens MART-1, Melan-A and GP100^{103,104}, MAGE-3 and related oncofetal antigens^{105–108}, HER-2/neu peptides used in breast, lung or ovarian cancer¹⁰⁹, Mucin-1 (MUC-1, peptide in breast or colon cancers^{110,111} and carcino-embryonic antigen (CEA) in colorectal, gastric, breast, pancreatic and non-small cell lung cancers¹¹². The major impediments to efficacy have been the relatively low immunogenicity of peptide vaccines; suggesting the need for improved adjuvant strategies and the concomitant disabling of various tumor-derived immunosuppressive factors¹¹³.

Homing peptides

The in vivo administration of phage displayed peptide libraries has identified specific peptides that are able to specifically home to normal organs or diseased tissues^{114–116}. The first homing peptides to be discovered were the RGD (Arg-Gly-Asp) and NGR (Asn-Gly-Arg) recognizing receptors on tumor surfaces that seem to be upregulated during angiogenesis. In vivo and ex-vivo phage display peptide libraries^{117–119} were later utilized to discover several other homing peptides (Reviewed by Svensen et al, 2012)¹²⁰. Homing

peptides have been used as delivery vehicles for imaging agents, drug molecules, oligonucleotides, liposomes and inorganic particles to tumors and other tissues^{116,121}. For example, the conjugation of TNF- α to RGD and NRG homing peptides has been shown to decrease the off-target toxicity of the compound, by delivering higher concentrations to the target without free compound¹²².

Cell penetrating peptides

(CPPs) are small peptides (< 30aa) which are able to gain access into cells upon contact¹²³. CPPs are able to recognize specific molecular tags, serving as a homing signal, to which they are recruited to, facilitating internalization which is cell type specific¹¹⁶. The first CCPs to be discovered were sequences from HIV-1 encoded TAT protein, TAT(48–60), which are able to efficiently facilitate delivery through cell membranes of cultured mammalian cells¹²⁴. Since then many natural and synthetic CPPs have been identified or developed, and are now utilized as facilitators of cellular uptake, alone or conjugated to cargo (recently reviewed by Farkhani et al, 2014¹²⁵)^{126,127}. In cancer therapy, conjugation of CPPs with anti-cancer agents such as paclitaxel¹²⁸, methotrexate¹²⁹, and doxorubicin¹³⁰ have shown improved activity compared to their unconjugated precursor drugs. The ability of CPPs to increase cellular membrane permeability makes them appealing for anti-cancer drug conjugation, as they can potentially decrease toxicity associated with drug therapy and overcome drug resistance mechanisms such as multi-drug resistance¹³¹.

Peptide Based Drugs

Bortezomib, is an N-protected dipeptide composed of pyrazinoic acid, phenylalanine and leucine with the substitution of the carboxylic acid with a boronic acid. It is the first therapeutic proteasome inhibitor to be tested in humans^{93,132,133} and was approved for the treatment of relapsed multiple myeloma and mantle cell lymphoma in 2003. New synthetic technologies have improved productivity and reduced metabolism of peptides. In addition to their high specificity, peptides are now easily synthesized and very amenable to site-specific modification¹³⁴.

Non-Ig based protein scaffolds

Advances in genetic engineering has lead to the development of several novel non-Ig based protein scaffolds. The novel structures are designed as derivatives of naturally occurring substrates or proteins. Anticalins¹³⁵, adnectins¹³⁶, Designed Ankyrin Repeat Proteins (DARPs)¹³⁷ and Avimers¹³⁸ are amongst the most developed non-Ig based protein scaffolds summarized in table 4. The progress in the development of these structures has yet to yield a scaffold with anti-tumor applications.

Anticalins

Anticalins are non-natural lipocalin derivatives engineered with novel-binding functions^{139,140}. They resemble immunoglobulin binding sites and have a simple structure allowing for ease of manipulation to achieve novel binding application^{141,142}. In cancer therapy anticalins have been developed in both monovalent and bivalent forms. Monovalent anticalins have been designed as antagonists of growth factors, receptors or

immunoregulatory molecules. Anticalins have been produced against CTLA-4 (PRS-9), VEGF-A (PRS-050) and c-met oncogene (PRS-110)^{143,144}. Anticalins can also be genetically fused to other anticalins (e.g., Duocalin)¹⁴⁵, to create Ig fragments or immunotoxins. Currently, no Anticalins are approved for therapy. These structures have been more fully described in a recent review (Richert and Skerra, 2014)¹³⁵.

Adnectins

Adnectins represent a new family of therapeutic proteins based on the tenth human fibronectin type III domain ¹⁰F_n¹⁴⁶. Their attractiveness stems from the adnectins' structural similarities to antibody variable domains, their ability to multimerize into multifunctional molecules and their potential for high affinity binding and specificity. They are predicted to have low immunogenicity due to the abundance of fibronectin in humans, and possess desired biophysical properties such as high thermostability, small size compared to mAbs, solubility and stability¹⁴⁷. Adnectins have been developed using yeast surface display of a F_n3 library^{148,149}. The first adnectin to be tested in humans was Angiocept® (CT-322=BMS-844203)¹⁵⁰, a single domain inhibitor of the VEGFR2 pathway¹⁵¹. Ongoing phase II clinical trials are evaluating the efficacy of CT-322, in combination with chemotherapy or with chemotherapy and radiation, in glioblastoma multiforme, non-small cell lung cancer and metastatic colorectal cancer¹⁵². Advanced library designs and screening methods¹⁵³ continue to generate high-potency adnectins with excellent biophysical properties. Multi-domain adnectins are being developed, with each domain having a different target and binding specificity¹³⁶. As with the anticalins, adnectins possess a combination of modularity, small size and high stability that should be amenable to future pharmaceutical applications.

Designed Ankyrin Repeat Proteins (DARPsins)

DARPsins are synthetic scaffolds replicates of ankyrin repeat proteins (AR) engineered to phenocopy AR's natural regulation of protein-protein interactions¹⁵⁴. In therapeutic applications monovalent DARPsins can be used to deliver cytotoxic payloads, or can be structurally modified to yield biologically active multivalent DARPsins. Examples of payload-fused DARPsins are DARPin C9 and DARPin Ec4, both of which target epithelial cell adhesion molecule (EpCAM) with distinct effector payloads. C9 is a carrier of siRNA against *bcl-2* mRNA, a pro-apoptotic factor¹⁵⁵, while DARPin Ec4 is fused to a truncated form of *Pseudomonas* exotoxin A (ETA'')¹⁵⁶. Exposure to DARPin C9 decreases *bcl-2* in EpCAM-positive cells, and DARPin Ec4 has demonstrated anti-tumor effects in xenograft studies¹⁵⁶. In an effort to engineer DARPsins with their own effector functions Boersma *et al.* constructed a tetravalent bispecific DARPin E69_LZ3_E01 combining two DARPsins selected against EGFR that recognize different epitopes, linked by a leucine zipper. DARPin E69_LZ3_E01 phenocopies and in some respects exceeds the effects of cetuximab¹³⁷. Linking of effector domains may allow for the discovery of easily manufactured novel structures with high yields in bacteria. The first DARPin to enter clinical trials was a VEGF-A inhibitor MP0112 for the treatment of wet macular degeneration and diabetic macular edema, but those studies were terminated¹⁵⁷.

Avimers

Avimers are single domain multimers designed to increase binding avidity: hence the term, “the **avidity multimer**”. They are created by genetic fusion of small protein domains called A-domains that can be found in various cell surface receptors such as the low-density lipoprotein (LDL) receptor¹³⁸. Screening of A-domain libraries created by exon shuffling yielded multiple A-domains recognizing different epitopes that can be fused to form a high avidity avimer. The only reported avimer is C326, which targets the cytokine IL-6, and is being evaluated in Crohn’s disease patients¹³⁸. No anti-tumor applications of avimers have been reported.

Future Opportunities for Protein and Peptide Therapeutics

Inner space – namely the cellular interior – represents the next frontier for protein and peptide therapeutics. Internalizing antibodies and peptides can be designed to traffic to the cell cytoplasm and to specific subcellular compartments where they might either manipulate intracellular processes or deliver toxic payloads that cause targeted cell death. Recent results with antibody-drug conjugates demonstrate the potential utility of these approaches, but as cell-penetrating peptides and intracellular targeting motifs gain in sophistication, the potential therapeutic applications of proteins, their engineered fragments and peptides will expand. In the meantime, the current and immediate next generations of antibodies and peptides will find new therapeutic uses in cancers and other diseases.

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Figure 1. Structures of enzymatically derived and genetically engineered fragments of Ig molecule

The native IgG molecule was initially enzymatically digested to create Fab and $(\text{Fab}')_2$ fragments. These and the other molecular variants described on the figure now are readily manufactured through recombinant antibody engineering techniques.

Figure 2. Schematic representation of Ig like scaffolds

(a) Heavy Chain Antibody (HCAB) and VHH derivative (nanobody) (b) Bispecific scaffolds BiTes, DaRTs and TandABs (c) Chemically programmed Antibodies (CpAbs), composed of an antibody component and a synthetic component (peptide or small molecule).

Table 1

FDA Approved Antibodies for the Treatment of Cancer

Generic name (trade name)	Origin	Isotype (conjugate)	Cancer(s) treated	Initial Approval	Reference
Unconjugated MABs					
Rituximab (Rituxan)	Chimeric	IgG1	B-Cell Leukemias and Lymphomas	1997	157-161
Ofatumumab (Arzerra)	Human (XenoMouse)	IgG1	Chronic Lymphocytic Leukemia	2009	162
Alemtuzumab (Campath-1H)	Humanized	IgG1	Chronic Lymphocytic Leukemia (Not currently available for clinical use)	2001	163
Trastuzumab (Herceptin)	Humanized	IgG1	Breast, Gastric	1998	164,165
Pertuzumab (Perjeta)	Humanized	IgG1	Breast	2012	166
Cetuximab (Erbix)	Chimeric	IgG1	Colorectal, Squamous Head/Neck, Lung	2004	167-170
Panitumumab (Vectibix)	Humanized	IgG2	Colorectal	2006	171
Bevacizumab (Avastin)	Humanized	IgG1	Colorectal, Non- Small Cell Lung, Glioblastoma	2004	172,173
Ziv-aflibercept (Zaltrap)	Humanized	IgG1	Colorectal	2012	174
Denosumab (Xgeva)	Human	IgG2	Bone-metastasis skeletal-related events	2010	175
Ramucirumab (Cyramza)	Humanized	IgG1	Gastric	2014	176,177
Immunocojugates					
IbritumomabTixetan (Zevalin)	Murine	IgG1 (⁹⁰ Y)	Non-Hodgkins Lymphoma	2002	65
Brentuximabvedotin (Adcetris)	Chimeric	IgG1 (MMAE)	Hodgkins Lymphoma, Systemic Anaplastic Large Cell Lymphoma	2011	51

Generic name (trade name)	Origin	Isotype (conjugate)	Cancer(s) treated	Initial Approval	Reference
Ado-trastuzumab emtansine (Kadcyla)	Humanized	IgG1 (DM1)	Breast	2013	178

MAb, monoclonal antibody; IgG, immunoglobulin-G; 90Y, yttrium-90 ; 131I-Iodine-131; MMAE, monomethylauristatin E ; DM1, Mertansine; SRE, skeletal-related event; ADT/AT , androgen deprivation therapy aromatase inhibitor ; GTCB, giant cell tumor of bone.

Table 2

Pharmacologic Properties of Antibodies

Generic name (trade name)	Pharmacokinetics	Mean Half Life	Antibody target and cellular effect	Side effect profile	References
Unconjugated MAbs					
Rituximab (Rituxan)	Linear	76.3 hours (first infusion) 205.8 hours (fourth infusion)	Targets CD20, depleting B-cells, 16–24 weeks from treatment completion until B-cell repletion.	Risk of increased infection Risk of Hepatitis B reactivation Infusion- reactions	19–23,179
Ofatumumab (Arzerra)	Nonlinear	30.4 hours (first infusion) 326.5 hours (fourth infusion) *2000mg dose	Targets CD20, depleting B-cells, 24 weeks from treatment completion until B-cell repletion.	Infusion-reactions Infection Neurotoxicity Richter's Transformation	180–183
Alemtuzumab (Campath-1H)	Nonlinear	11 hours (first week) 144 hours (twelfth week)	Targets CD52, resulting in T- cell depletion of variable duration	Autoimmune disease reactivation	19,184,185
Trastuzumab (Herceptin)	Nonlinear	28.5 days	Inhibition of proliferation of HER2+ overexpressing tumor cells	Cardiotoxicity	19,186–188
Pertuzumab (Perjeta)	Linear	11.6 hours	Dimerizes HER2 antibodies thus inhibiting proliferation of HER2+ overexpressing tumor cells	Cardiotoxicity	189,190
Cetuximab (Erbfix)	Nonlinear	71 hours *250mg/m ² dose	Binds to EGFR, preventing ligand-binding stimulation of cellular proliferation.	Acneiform rash Hypersensitivity	19,191
Panitumumab (Vectibix)	Nonlinear	7.5 days	Binds to EGFR, preventing ligand-binding stimulation of cellular proliferation.	Acneiform rash Hypersensitivity	192,193
Bevacizumab (Avastin)	Linear	20 days	Binds VEGF-A, preventing its receptor binding and thus tumor angiogenesis	Bleeding Hypertension Proteinuria Bowel Perforation	19,194
Ziv-aflibercept (Zaltrap)	Linear	6 days	Binds VEGF-A and VEGF-B, preventing their receptor binding and thus tumor angiogenesis	Bleeding Hypertension Proteinuria Bowel Perforation	195
Denosumab (Xgeva)	Nonlinear	2.7 days	RANKL binding that reduces fracture rate associated with cancer	Infection	196,197

Generic name (trade name)	Pharmacokinetics	Mean Half Life	Antibody target and cellular effect	Side effect profile	References
Ramucirumab (Cyramza)	Nonlinear	200–300 hours	Binds VEGF-2 preventing its ligand binding and thus tumor angiogenesis	Hypertension Proteinuria	198
Immunocojugates					
Ibritumomab Tiuxetan (Zevalin)	Not Reported Presumeably nonlinear	27 hours	Antibody to CD20 radiolabeled with Y90, delivering this isotope selectively to these hematologic cells	Low grade cytopenias are typical	19,199,200
Brentuximabvedotin (Adcetris)	Not Reported Presumeably nonlinear	4–6 days	Antibody to CD30 linked with MMAE, delivering this pharmacologic selectively to these hematologic cells	Neuropathy Cytopenias	201,202
Ado-trastuzumab Emtansine (Kadcyla)	Nonlinear	6 days	Antibody to Her2 linked with DMI delivering this pharmacologic selectively to these tumor cells cells	Fatigue Cytopenias	203,204

Table 3

Antibodies targeting the immune system in clinical trials

Name	Antibody Type	Target	Activity	Disease Treated	Trial phase
Tremelimumab	Human IgG2	CTLA4	T cell priming and activation	Solid tumors	II
Galkinab	Chimeric IgG1	CD80	B cell proliferation	Lymphoma	II
BMS-936558	Human IgG4	PD1–B7H1, PD1–B7DC	T cell activation and tolerance	Multiple cancers; HCV	III
CT-011	Humanized IgG1	PD1–B7H1, PD1–B7DC	T cell activation and Tolerance	Solid tumors; HCV	II
MK-3475	IgG4	PD1–B7H1, PD1–B7DC	T cell activation and tolerance	Solid tumors	I
AMP224	Fusion protein (B7DC+ IgG1)	PD1–B7H1, PD1–B7DC	T cell activation and tolerance	Multiple cancers	I
BMS-936559	Human IgG4	PD1–B7H1	T cell activation and tolerance	Solid tumors	I
MPDL3280A	Engineered human IgG1	PD1–B7H1	T cell activation and tolerance	Solid tumors	I
MEDI4736	Engineered human IgG1	PD1–B7H1	T cell activation and tolerance	Solid tumors	I
MGA271	Humanized IgG1	B7H3	T cell activation and tolerance	Solid tumors	I
IMP321	Fusion protein (LAG3+ IgG1)	LAG3–MHCII	DC maturation and T cell activation	Multiple cancers	I/II
BMS-663513	Human IgG4	CD137	T cell activation	Solid tumors	I/II
PF-05082566	Human IgG	CD137	T cell activation	Lymphoma	I
CDX-1127	Human IgG1	CD27	T cell activation	Multiple cancers	I
Anti-OX40	OX40-specific mouse IgG	OX40	CD4 T cell activation	Prostate cancer	II
TRX518	GITR-specific humanized IgG1	GITR–GITRL	T cell activation	Solid tumors	I
CP-870,893	Human IgG1	CD40	APC activation and B cell maturation	Multiple cancers	I
Lucatumumab	Human IgG1	CD40	APC activation and B cell maturation	Lymphoma and leukaemia	I/II

Name	Antibody Type	Target	Activity	Disease Treated	Trial phase
Dacetuzumab	Humanized IgG1	CD40	APC activation and B cell maturation	Lymphoma and multiple myeloma	II
Blinatumomab	scFv fusion protein (α-CD3/α-CD19)	CD19/CD3	T cell activation and B cell recognition	non-Hodgkin's lymphoma Acute Lymphoblastic Leukemia	I II/III

B7H1, B7 homolog 1 (programmed death-1 ligand-1), B7-DC, (B7 dendritic cell=programmed death-1 ligand-2); CTLA4, cytotoxic T lymphocyte antigen 4; GITR, glucocorticoid-induced TNFR-related protein; GITRL, GITR ligand; HCV, hepatitis C virus; IgG1, immunoglobulin G1; LAG3, lymphocyte activation gene 3; MHCII, major histocompatibility complex class II; OX40L, OX40 ligand; PD1, programmed cell death protein 1.

Table 4

Most advanced Ig-Based and Non-Ig based protein scaffolds

Scaffold name	Derivative of	Size	Refs
Ig-Based scaffolds			
Nanobody	VHH (camelidIg)	~100 AA	71
dAb	VH or VL domain	100–130 AA	74
BiTE	Bispecificdiabody	~ 55 kDa	79
DART	Bispecificdiabody	~50 kDa	82
TandAb	Dimerizedbispecificdiabody	~ 100 kDa	205
Non-Ig Scaffold			
Anticalin	Lipocalins	160–180 AA	142
AdNectins	10th FN3 (Fibronectin)	94 AA	146,206
DARPin	AR proteins	[67+33n]	207
Avimers	Domain A/LDL receptor	~[35 AA]n	137

dAb, domainantibodies; BiTE, Bispecific T cell Engager; DART, Dual Affinity ReTargeting; TandAb, tetravalent tandem antibodies; cpAb, chemically programmed antibodies.