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Use of antibodies and immunoconjugates for the therapy of more accessible cancers

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Abstract

There are currently 6 unconjugated antibodies and 3 immunoconjugates approved for use in the Unites States in a variety of cancers, with a considerable number of new agents in clinical testing and preclinical development. Unconjugated antibodies alone can be effective, but more often, antibodies need to be combined with chemotherapy, which enhances the efficacy of the standard treatment. Immunoconjugates tend to be more effective than their unconjugated counterparts, but their increased toxicity often restricts when and how they are used. In order to improve efficacy, a number of immunoconjugates are being examined in settings where the disease is more easily accessible, such as leukemias, or within compartments that allow easier and more direct access to the tumor, such as in the peritoneal cavity or brain, or both locally and systemically, in adjuvant situations, where the disease burden has been reduced by some other means, and with the main goal of these treatments being to kill residual disease.

Keywords

Antibody-drug conjugates; antibody-toxin conjugates; immunotherapy; pretargeting; radioimmunotherapy

1. Introduction

Cancer is characterized by an unregulated cell growth, but the trait that sets it apart from other diseases is the ability of the tumor cells to invade and metastasize [1–4]. If detected early, before it has spread outside the natural tissue boundaries (e.g., stage 1), a tumor usually can be removed surgically without requiring additional intervention, giving the patient an excellent prognosis. However, once the cancer has escaped these natural boundaries, surgery alone will not suffice. A determination of the extent that the cancer has spread is essential for selecting a treatment strategy. A wide range of imaging modalities (e.g., CT, MRI, US, ^{18}F-FDG) are used to probe the body for evidence of disease, but these methods only have the ability to detect tumors of about 1.0 cm in diameter, which already contain nearly 1 billion cells. Also, they often lack the specificity needed to differentiate cancer from other pathology. The most common systemic treatment for disseminated disease is chemotherapy, but over the past 10–15 years, a growing number of biological agents have made a significant impact on cancer

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treatment, not only as therapeutics (e.g., BCG, interleukin-2, interferon-alpha, rituximab, trastuzumab, and other antibodies), but also to help the body counteract some of the harmful side-effects of chemotherapy (e.g., G-CSF, erythropoietin). This article will focus on one group of therapeutic biologicals, antibodies and immunoconjugates (Figure 1 and Table 1), and will evaluate how they may be used to treat more accessible cancers.

2. Antibodies and their immunoconjugates as therapeutics

2.1 Unconjugated antibodies

As an integral part of our immune system, antibodies are capable of killing cells by *complement-dependent cytotoxicity* (CDC) and *antibody-dependent cellular cytotoxicity* (ADCC). In each of these mechanisms, the antibody marks a cell so that other agents (i.e., proteins of the complement cascade or immune cells, respectively) can deliver the cytotoxic effects (Figure 2). In the early 1980's, studies showed that anti-tumor antibodies could elicit cell killing through these traditional mechanisms [5–8], which led to several clinical trials using murine monoclonal antibodies developed against melanoma, gastrointestinal cancers, leukemia, and lymphoma [9–15]. At this same time, other investigations were advancing our understanding of how antibodies could be used therapeutically. Antibodies to growth factors, such as the transferrin receptor and to the epithelial growth factor receptor (EGFR), were found to have an anti-proliferative effect on cells growing in culture in the absence of complement or effector cells [16,17]. These early findings provoked a whole new dimension of antibody therapy, and directly contributed to the development the anti-EGFR antibody, cetuximab. Whereas cetuximab's mechanism of action involved signaling effects on the tyrosine kinase pathway, it also includes ADCC, whereas the anti-VEGF (vascular endothelial growth factor) antibody, bevacizumab, affects tumor growth by binding to VEGF, a growth factor produced by tumors to initiate new blood vessel formation, thereby inhibiting this essential function [18–20]. Since bevacizumab, unlike cetuximab, does not bind directly to tumor cells, ADCC and CMC are not required for its activity. Other antibodies currently approved for clinical use, trastuzumab and rituximab, affect signaling pathways, but also bind directly to the tumor cells, where ADCC and CMC contribute to their anti-tumor activity [21–23].

With such multiple capabilities for disrupting cellular functions and survival, unconjugated antibodies are attractive therapeutics, but overall, they are not very potent, so that most unconjugated antibodies are commonly used in combination with drugs. Rituximab is perhaps the most active of the unconjugated antibodies, with an overall objective response rate of 50%, with only ~10% being complete responses in patients with relapsed non-Hodgkin's lymphoma, when given alone [24,25]. Extending the number of treatments or retreating at the time of progression can be of further benefit in follicular NHL [26,27], but in other forms of NHL, rituximab is best used in combination with chemotherapy [28–30]. For this reason, antibodies are being conjugated to other cytotoxic compounds to enhance their potency.

Antibodies are highly versatile targeting proteins. The past practice of using murine monoclonal antibodies has been replaced with chimerized, and more often humanized, or fully human antibodies prepared in transgenic mice [31,32]. Molecular engineering enables these proteins to be constructed in a number of ways, from modifying the size and pharmacokinetic properties of the construct, to altering the valency of antigen binding, and even altering effector activity. Recombinant fusion proteins with biological response modifiers or toxins have shown promising activity [33,34], and bispecific constructs capable of binding a tumor antigen while also binding another agent (e.g., anti-CD3 for binding T-effector cells, or haptens for binding specialized hapten-peptides) are being explored [35–42].

2.2. Antibody conjugates

In animal models, the unconjugated antibody is most often much less effective therapeutically than the corresponding antibody conjugate. Similar results have been seen clinically. For example, the anti-CD33 antibody, lintuzumab, was ineffective when added to an induction chemotherapy regimen for the treatment of acute myeloid leukemia, but is effective as a stand-alone calicheamicin immunoconjugate (gemtuzumab ozogamicin) [43,44]. The anti-CD20 radioconjugates, tositumomab and ibritumomab tiuxetan, both have been shown to induce a higher rate of complete responses than their corresponding unconjugated anti-CD20 IgG [45, 46]. Thus, conjugates are prepared to enhance the activity of their unconjugated form, but the added potency usually means increased toxicity.

Toxicity is most often the result of an unintended uptake in a non-targeted tissue, such as hepatotoxicity that frequently limits antibody-toxin conjugates because these constructs, like many macromolecules, are naturally cleared from the blood by the liver. Hepatic toxicity has frequently limited the use of toxin immunoconjugates [47]. Most plant toxins are composed of 2 parts, one responsible for cytotoxicity and another component responsible for binding to cells, which was responsible for severe toxicity in the very early work with immunotoxin conjugates [48,49]. Even with the cell-binding component removed, toxin conjugates continued to cause severe hepatic toxicity. More recently, a 3-amino acid motif associated with hepatic binding has been identified for ricin A [50]. A new recombinant ricin-antibody construct had similar activity with less hepatic toxicity in SCID mice [51]. Being able to produce antibody-toxin conjugates by recombinant engineering provides a manufacturing advantage over drug conjugates that are prepared chemically. However, most toxins are foreign proteins that are immunogenic, which limits their use [52–54]. The potential exception may be the RNases [55].

Conjugation of drugs to antibodies can change the toxicity profile. A good example is the BR96 (anti-Lewis Y)-doxorubicin conjugate that was limited not by cardiotoxicity from doxorubicin, but by gastrointestinal toxicity because the antibody also bound to the normal gastrointestinal epithelial cells [56,57]. The hematopoietic specificity of the anti-CD33 gemtuzumab ozogamicin causes severe hematologic toxicity, which is dose-limiting, but severe hepatic toxicities also are associated with gemtuzumab ozogamicin, which is likely caused by calicheamicin delivered to the liver [58]. Hematologic toxicity is dose-limiting for IgG-based radioconjugates because they are cleared too slowly from the blood, thereby continually exposing the radiosensitive bone marrow. While this limitation allows sufficient radiation delivery for successful therapy of NHL, radiation-absorbed doses to tumors rarely exceed 1500–2000 cGy, an insufficient dose for effective therapy of solid tumors [59]. Antibody fragments clear faster from the blood and improve tumor/blood ratios, but because a smaller fraction of the administered dose localizes in the tumor, the total dose delivered to the tumor may not be increased substantially. In addition, many radionuclides of therapeutic interest when conjugated directly to antibody constructs that are <60 kD will become trapped in the kidneys, resulting in unfavorable tumor/kidney ratios.

In contrast to direct conjugation of an antibody with a radionuclide, pretargeting techniques have been developed that separate the antibody and radionuclide targeting steps (Figure 3). In these procedures, a specialized antibody conjugate or construct that has the ability to bind to a tumor antigen as well as another compound is administered. After allowing this construct to bind and clear from the blood, the radionuclide attached to a small compound is administered. This compound is capable of binding to the other portion of the antibody conjugate pre-localized in the tumor. This small molecule rapidly clears from the blood, distributing within the extravascular space, and quickly being eliminated, preferably by urinary excretion and with minimal retention by the kidneys [60]. These procedures are able to deliver a higher radiation

dose to tumor than with directly radiolabeled antibodies, improving efficacy, but with much less hematologic toxicity. Still other pretargeting methods also have been developed [112].

Unconjugated antibodies and antibody conjugates prepared with drugs or toxins must bind physically to each tumor cell to have a cytotoxic effect, whereas radioconjugates have the distinct advantage of being able to kill cells located some distance from where the antibody binds, based on type of decay (i.e., alpha or beta) and its energy. Furthermore, drug and toxin conjugates must deliver a critical concentration of their payload inside the cell, sometimes to very specific compartments, for the effect to occur. In this regard, selecting a target on the cell surface that enables conjugate internalization and subsequent processing would seem to be a prerequisite for these agents. The more efficient the internalization process, the more likely the payload will be delivered at the concentration required to kill the cell. For example, although there are relatively few copies of CD74 (invariant chain, Ii) on the surface of cells, this antigen is readily shuttled from the membrane to the cytoplasm and then quickly recycled back to the membrane, making it accessible again for transporting antibody conjugates inside the cell [61]. CD74 expression on a variety of hematopoietic cells makes it an excellent candidate for the delivery of antibody conjugates [62–66], but studies also have shown this antigen is important for cell proliferation and survival [67], and thus unconjugated anti-CD74 antibody has therapeutic activity [68]. Targets that are internalized less efficiently can be used if they are more highly expressed, and it may be necessary to use a more toxic compound. Many of the antibody-drug conjugates that were first investigated used standard chemotherapy agents, but these were often not potent enough, and therefore antibodies coupled to plant toxins, with the theoretical capability of killing cells with very few copies delivered to the cell, were developed [34,69–71]. So called *ultra-toxic* drugs, such as calicheamicin, maytansinoids, and auristatins coupled to antibodies, have showed improved activity, which has rekindled interest in drug immunoconjugates [44,72,73].

While there is clearly a desire to endow therapeutics with specific binding properties (e.g., antibody, peptide, lectin, etc.), the relatively high endocytic activity of some tumor cells can result in sufficient internalization of even non-targeted conjugates that can kill the cells [74]. For example, the anti-CD33-calicheamicin conjugate, gemtuzumab ozogamicin, is potent in CD33-positive as well as CD33-negative cell lines simply because leukemic cells have a high endocytic activity [75]. There are other situations where a conjugate can be effective without being internalized, as long as its payload is released selectively once localized in tumors. A variety of cleavable linkers have been described [76–81]. Gemtuzumab ozogamicin has a hydrolysable linker that allows the drug to be released, but this property may not always be desirable. For example, the failure in ovarian cancer of a similarly prepared calicheamicin-antibody conjugate was thought to be due to the instability of this bond, but clearly other factors could have contributed [82]. Another technique involves the delivery of an antibody-enzyme conjugate. The tumor-targeted enzyme will cleave a prodrug, allowing the active drug to be locally released within the tumor [83,84]. Additionally, there is an increasing awareness that non-targeted cells in the immediate vicinity of the tumor cells can be killed non-specifically by the *bystander effect* [85]. Because the extent that this effect can significantly add to a response is not known, the objective must remain one of delivering specifically as much targeted therapeutic to as many cancer cells as possible.

3. What is “more accessible” cancer?

The vast majority of tumors exist in the form of a solid mass, except leukemia and other hematopoietic tumors, which predominantly are blood/bone marrow-borne diseases. Leukemia is the most accessible of all tumors, because the vascular compartment and the bone marrow are openly accessible to intravenous therapy. However, this presentation also means that the

leukemic cells could be in essentially every tissue of the body; therefore, successful delivery of therapeutics can be challenging when the target cells are integrated in the various organs.

The most formidable challenge for the delivery of therapeutics is in solid tumors. Since a solid tumor exists outside the vascular compartment, agents must be able to traverse these channels, enter the interstitial space, and then travel to the cancer cells to exert their activity directly on the tumor. When a tumor forms, as a small cluster of cells, it can garnish nutrients and oxygen from the local environment, but it must induce the proliferation of blood vessels if it is to continue to growth. Tumors can produce the necessary signals to stimulate vessel formation, but the vascular channels are poorly organized and there is no lymphatic bed to assist fluidic movement [86]. However imperfect these blood vessels are, they are essential for the continued growth of the tumor. As tumors progress in size, new vessels form at the circumference, leaving the interior portions to become hypoxic and acidic. Despite being more tolerant than normal cells to these harsh conditions, eventually the cells die, forming necrotic regions within the internal regions.

Tumor physiology creates an interesting delivery paradox. For a variety of reasons, these vessels are leakier than blood vessels found in normal tissues, allowing macromolecules to escape easily [87,88]. From a delivery viewpoint, this is ideal, but the hastily formed vascular architecture creates an elevated interstitial fluid pressure within the tumor that impedes convectional flow. Thus, while molecules can easily leave the tumor's blood vessels, they are unable to move efficiently away from the blood vessels. Larger molecules are more dependent on convectional flow than smaller ones, and therefore their movement within a tumor is more restricted.

Regardless of size, therapeutics designed to interact specifically with substances found on tumor cells or within their interstitial space face another impediment, known as the *binding site barrier* [89,90]. Once a targeted agent leaves the tumor vasculature and binds to its ligand, its movement within the tumor will be impeded. The higher the binding affinity, the more likely the agent will be localized in the perivascular space, penetrating only a few cells outside the blood vessel [91]. With antibodies, this penetration zone can be expanded by simply increasing the dose [92–96]. As more and more antibody traverses the blood vessel, antigen in the immediate area is bound, allowing the additional unbound protein to move further away from the blood vessel. However, not all agents, particularly antibody conjugates, can benefit from this approach. Dose-limiting toxicity of the immunoconjugate can limit the amount given. Additional unconjugated product can be pre- or co-infused with the immunoconjugate to block some of the antigen in the tumor bed and allow deeper penetration of the immunoconjugate from the vasculature, but this essentially dilutes the moles of active agent in the tumor, which could have a negative impact on efficacy [96,97]. If there is a large antigen sink in normal tissues, administration of an unconjugated antibody may be required to alleviate excessive uptake of the conjugated antibody in the tissue (e.g., several hundred milligrams of unconjugated anti-CD20 antibody are administered prior to the anti-CD20 radioconjugates to reduce binding to a large B-cell sink primarily in the spleen [98,99]). Thus, the targeted delivery of molecules, particularly macromolecules, to solid tumor masses is difficult.

The challenge posed by accessibility is particularly difficult for agents that must be delivered to individual tumor cells to be active, and amplified further for agents that also must be internalized. Radioconjugates were among the first immunoconjugates advanced based on the radioactive decay particle's ability to penetrate from 0.05 mm to as much as 10 mm from where it is deposited (*bystander* or cross-fire effect), reducing the need for the radioconjugate to target individual cells in order to have a cytotoxic effect [59] (Table 2). Indeed, a high concentration of radioactivity targeted to tumor cells in the perivascular space could not only kill the tumor cells, but conceivably could destroy the vasculature in the vicinity, which might cause other

collateral damage within the tumor. However, as with all therapeutics, a critical concentration must be achieved for the desired effect, and therefore the more accessible, the more likely that this critical concentration can be achieved before normal tissue tolerance levels are exceeded. Antigens that are shed or released from within cells when they die often can be detected in the serum, but they also can have a sizeable presence in the interstitial space of the tumor, which can block an antibody from binding to the cell surface. Although binding-site barriers and unfavorable fluid movement within tumors can adversely impact antibody delivery, antibodies targeting intracellular antigens are able to reach necrotic areas of the tumor, where antigen access is no longer impeded by the membrane of intact cells [100]. Thus, these barriers are not impenetrable, but they do place certain limitations on accessibility. For these reasons, there is considerable interest in the selective targeting of the tumor's vasculature. This represents a more accessible target than one that resides on the tumor cell. However, agents that are designed only to destroy or, as in the case of bevacizumab, disrupt tumor vessel formation have not been sufficiently active by themselves to produce significant clinical benefit, but instead need to be combined with chemotherapy [20]. Immunoconjugates made to target tumor blood vessels and then release a cytotoxic payload or deliver a penetrating radionuclide also would likely have a limited impact, unless sufficient concentrations could be locally delivered. There are also formulation options for proteins that could improve their ability to reduce the impact that some of these natural barriers present [101].

When a solid tumor is localized in a fairly confined region of the body, it is considered a more accessible presentation. In this situation, the therapeutic could be delivered locally, either by direct injection into the lesion, into established vascular (or lymphatic) channels of the tissue where the tumor resides, such as hepatic arterial injection to treat hepatic metastases, or within discrete compartments (e.g., intraperitoneal, intrathecal, intracranial) [102–109]. These methods are designed to take advantage of a first-pass effect [110–112], but in each case, the agent must still contend with the barriers existing within each tumor mass for successful delivery. Intralesional injections potentially can mitigate some of these internal barrier issues, but this depends on the size and volume of the mass, and perhaps the rate at which the product is injected. If the injected volume is small in relation to the tumor, the agent could still have a limited capacity to percolate into other regions, and therefore multiple placements would be preferred. These techniques are designed primarily to control the local spread of the disease, and do not address the likelihood that the cancer has already metastasized to other locations in the body, either regional or distant. Nevertheless, the main goal in these approaches is to allow the therapeutic to be in more direct contact with the tumor, raising the chances that it will localize at higher concentrations than if diluted by intravenous injection. In some of these approaches, the therapeutic does escape from these compartments and circulates throughout the body, albeit usually in concentrations too low to optimize efficacy against tumors located elsewhere in the body.

Minimal residual disease, where the tumors are smaller and with less significant barriers than larger tumors, or in an adjuvant setting, where presumably there are only small clusters of cells hidden in the body, could also be considered as more accessible presentations of cancer. Thus, we will focus our discussion on the application of antibodies and immunoconjugates in leukemia, in compartmental settings, such as in peritoneal disease and within the brain cavity, as well as in minimal disease.

4. Antibody-targeted therapy of leukemia/lymphoma

Antibodies have made significant strides in the treatment of hematological malignancies. Rituximab, a chimeric anti-CD20 IgG, is the most successful therapeutic antibody. It was initially approved for use in follicular and transformed (low-grade) non-Hodgkin's lymphoma (NHL) alone and in then combination with chemotherapy in aggressive NHL, but now it is

being examined for activity in other B-cell lymphomas, as well as against non-malignant conditions where B-cells contribute to the disease, such as a number of autoimmune diseases [25,30,113–118]. Rituximab's commercial success can be credited in a large part for the increased interest in antibody therapy for both solid and hematopoietic cancers, unconjugated as well as conjugated. Other antibodies, such as epratuzumab (humanized anti-CD22) and galiximab (chimeric anti-CD80), alone and in combination with rituximab, are also showing promising results in early clinical trials [119–124]. Apolizumab, a human anti-HLA-DR, had been studied in patients with non-Hodgkin's lymphoma, but relatively low doses ($\leq$ 1.5 mg/m^2) resulted in grade 3 and 4 hematological toxicity, and therefore this antibody's toxicity was too severe for continued human use [125].

Ideally, the target should exist on the tumor cells and not on normal cells, but in the case of the hematological malignancies, all of the antibody-targeted therapies also affect antigen-positive normal lymphoid cells to varying degrees. For example, CD20 and CD22 are on tumor and normal B-cells, but are restricted to more differentiated B-cells, sparing the progenitor B-cells. Using these targets should restrict toxicity to only the B-cells, while sparing myeloid lineage cells. CD80 and HLA-DR are found on a number of other differentiated immune cells, and therefore there likely is more collateral damage to other non-malignant immune cells. In acute myelocytic leukemia (AML), antibodies to CD33, CD45, and CD66 have been examined as unconjugated and conjugated products [126–130]. Anti-CD33 and anti-CD66 antibodies target tumor blast cells, as well as normal monocytes/myeloid lineage cells, but are not present on stem cells, while CD45 is also present on stem cells. Because of its reactivity with stem cells, radioconjugates of anti-CD45 antibodies are being used to aid in the elimination of stem cells in patients undergoing transplant procedures [131–134]. In chronic lymphocytic leukemia (CLL), unconjugated anti-CD52 humanized antibody, alemtuzumab (Campath-1H), is active against a wide range of immune cells, causing severe lymphopenia that can result in an increased risk of opportunistic infections. The anti-IL2 receptor (CD25) antibody, daclizumab, expressed primarily on T-cells and currently approved for the treatment of renal allograft rejection, also can be found on B-cells and monocytes/macrophages [135,136]. Anti-CD25 antibodies are being examined in a number of situations requiring immunosuppressive therapy, but also in cancer against T-cell lymphoma and CLL [137]. Zanolimumab, a fully human anti-CD4 IgG, has shown encouraging objective responses in refractory cutaneous T-cell lymphoma (mycosis fungoides and Sézary syndrome) [138].

As easily accessible as leukemias are, it is surprising that targeted agents have not been more effective. CD33 expression is very low on most myeloid leukemias, except acute promyelocytic leukemia, where conjugates appear to be more active. Thus, a reduced expression of a target will limit the amount of therapeutic product delivered [126,139]. In the case of CD33, protein doses of only 5 mg/m^2 were able to saturate antigen sites in patients with extensive disease [140]. However, analysis of cells taken from patients undergoing multiple cycles of gemtuzumab ozogamicin have indicated that the conjugate is rapidly internalized and re-expressed, allowing new sites for the conjugate to bind [141]. Radiolabeled anti-CD33 antibodies can reduce the number of circulating blast cells significantly in patients with advanced disease, but without complete responses. However, by minimizing disease burden with cytarabine prior to radioantibody treatment, complete responses were observed [142]. Thus, extensive disease, even if it is easily accessible, is difficult to treat with targeted therapies (or for that matter, non-targeted therapies). Even though CD33 expression is low, the sheer mass of cells in patients with extensive AML can be overwhelming for targeted therapies, particularly when the administered dose is limited by toxicity. For this reason, antibody conjugates will more likely contribute significantly to the clinical outcome when used in a minimal disease setting. Unconjugated antibodies, such as rituximab, can be administered repeatedly without serious complications, but rituximab alone is unable to eradicate all disease in more than 90% of patients. Cells can also become resistant to rituximab, further complicating

its use as a monotherapy [23,143]. Combining rituximab with chemotherapy is more effective than rituximab given alone [144], which has been found for most therapeutic antibodies [40]. Combining antibodies with different specificities has been proposed as a means of targeting a larger fraction of the cells, to target other regions of the tumor (e.g., stroma), or because of additivity/synergistic interactions [121,123,145,146]. Cocktails of antibodies may not improve efficacy if the cocktail requires reduction in the dosage to suboptimal levels of an individual antibody that targets one population of cells. As long as the dose of each agent is at an effective level, then the combination may be more effective. This effect was illustrated recently in a pretargeting procedure that examined individual antibodies and the combination of anti-CD20, CD22, and HLA-DR antibodies [147]. Targeting was best when a single antibody against the more highly expressed antigen in a given cell line was used for pretargeting radionuclides as compared to all 3 antibodies. This study suggests that cancers should be screened in advance to determine the highest target expression, using the antibody to this target for individualized treatment. Other options include sequential administration of multiple agents, especially when there are synergistic or additive benefits, or fractionation, which may aid in delivering an agent at a critical time in the cell cycle to optimize effects, or to reduce toxicity, particularly for drug or isotopic immunoconjugates. These latter approaches can be affected adversely if initial treatments disrupt blood vessels or other means to access the tumor cells for the subsequent doses. Indeed, all of these options do not necessarily solve the problem of targeting tumor cells that are sequestered in less accessible sites.

Since lymphomas typically present as solid masses, the same barriers inhibiting targeted therapeutics would apply. In the case of rituximab therapy, where the amount of protein administered is typically 375 mg/m^2 (~600 to 700 mg) weekly for a minimum of 4 weeks, this amount of protein could conceivably assist the antibody in penetrating more uniformly in the tumor. However, since CD20 is also expressed on normal B-cells found in the blood, spleen, lymph nodes, and marrow, there is a substantial antigen sink that needs to be overcome. Experience with radiolabeled anti-CD20 antibodies, ibrituzumab tiuxetan and tositumomab, has indicated that a pre-infusion of ~300 to 400 mg of the unlabeled antibody is sufficient to reduce uptake in these sites to allow for better targeting of the radiolabeled antibody. Thus, with nearly 2.5 to 3.5 grams of antibody administered over 4 weeks, the antigen sink would be overcome, leaving a considerable amount of antibody available to overwhelm the binding site barrier. However, there are other barriers within the body that cannot be “broken” by merely increasing the protein dose. Rituximab is not as active in extranodal disease, such as in mucosa-associated lymphoid tissue (MALT lymphoma), or when lymphoma is involved in the leptomeningeal cavity, because full antibodies do not penetrate the blood-brain barrier unless this barrier is compromised [148,149]. In the latter case, direct intrathecal injection of just 25 mg of rituximab was shown to be active [149]. Thus, in situations where natural anatomic barriers prevent access, a more local/regional therapy needs to be taken.

5. Intracavitary targeting

Intraperitoneal (IP) spread frequently occurs in ovarian, colonic, pancreatic, and gastric cancers. Under normal conditions, fluids flow freely and quickly through the peritoneum via blood capillaries and lymphatic channels below the diaphragm. Most often, peritoneal tumors present as multiple nodules studding the cavity’s surfaces. As the tumor progresses, the lymphatic channels can become blocked, causing a buildup of fluids (ascites) in the peritoneal cavity. This fluid can become inundated with small clusters of tumor cells that are shed from the tumor bed lining this cavity. Adhesions can form loculated areas of ascites, essentially sealing off various compartments. Thus, intraperitoneal presentation can pose a number of challenging conditions, and for this reason, regional IP therapy is best approached after cytoreductive treatment to reduce tumor burden. This is particularly important for selective therapeutics that can otherwise become overwhelmed by the amount of disease.

The pharmacokinetic advantage for IP therapy has been established for some time [150], and this advantage is particularly enhanced for macromolecules that rely on convectional flow for their fluidic movement, since they will be retained longer in this chamber than smaller molecules. A slow clearance allows a selective targeting agent ample time to localize to the target. However, while antibodies introduced into this compartment may have easy access to the tumor nodules spread throughout the cavity, this does not mean that there is full and open access to all tumor cells. Rather than accessing the tumor from the inside by percolating out of the tumor blood vessels, the antibody in the peritoneal cavity bathes the outside of the tumor and needs to be absorbed within in order to access all the cells. *In vitro* studies have highlighted the difficulty in antibody penetration within tumor spheroids, and *in vivo* studies have emphasized the importance of the tumor's microenvironment in restricting antibody movement into tumors [93,151–154].

The restricted penetration of antibodies raises questions concerning which route of administration might be best for antibody therapy. This issue was evaluated in a clinical study performed in colorectal cancer patients who were co-administered radiolabeled ant-TAG.72 IgG (^{125}I/^{131}I, respectively) either IP or intravenously (iv), and 6–8 days later, they underwent surgery, with uptake measured in the excised tumor and normal tissues. Of the 55 lesions removed, 35 had a 2-fold higher uptake of the IP-administered ^{125}I-IgG, 13 lesions had 2-fold higher uptake of the iv-administered ^{131}I-IgG, and 7 were similar. More detailed pathological examination revealed that true peritoneal implants had a higher uptake with the IP injection, while lesions that were characterized as hematogenous-borne or lymph node metastases, or local recurrences, had higher uptake with the iv-injected antibody [155]. Several patients in this study were co-injected with a radiolabeled non-targeted IgG. In these patients, it took 2 days before peak values (~30% of the injected dose) were found in the plasma [155]. The kinetics of the specific radiolabeled anti-TAG.72 IgG in the plasma was similar to that of the irrelevant IgG, but peak values were only about 10% of the injected dose, indicating that a portion of the dose was retained in the cavity [155]. Because the concentration of the radiolabeled antibody in the blood was substantially lower with the IP route than by intravenous injection, the projected radiation dose to the red marrow was reduced. A similar study was performed using a radiolabeled anti-folate-receptor antibody. It also reported a lower blood concentration of the antibody after the IP injection, but it was found that tumors surgically removed 2 days after the iv injections had 2-fold higher uptake than the IP-administered antibody, and after 6 days, uptake was the same in both groups [156]. Thus, both studies concluded that the main advantage of the IP route of administration was the lowering of activity in the blood, which for radioconjugates is important, since this will lead to less severe hematological toxicity (i.e., allowing higher doses to potentially be given).

Although preclinical testing of antibody-drug and toxin conjugates has been conducted in IP models, no clinical studies with these agents injected into the peritoneal cavity have been reported to date. Perhaps because of penetration issues, clinical studies have instead focused on the use of radiolabeled antibodies. With non-antibody radionuclide IP therapy, clinical studies have found no survival advantage as compared to standard of care [157,158]. Responses have been reported in a number of Phase I/II trials using several antibodies radiolabeled with different radionuclides administered by IP or intravenous routes [159–165]. However, only one agent, a ^{90}Y-labeled anti-MUC1 IgG, was tested intraperitoneally in a randomized Phase-III clinical trial [166]. Patients were enrolled after responding to primary platinum-based therapy, and upon second-look surgery, had no evidence of disease. The patients were randomized to receive a single injection of 18 mCi/m^2 of the ^{90}Y-murine anti-MUC1 IgG plus standard care or standard care only (no active therapy). After a median follow-up of 3.5 years, no difference was found in the overall survival or time to relapse. In a follow-up assessment of this study, Oei et al. [162] found that significantly more of the patients given the specific targeted therapy IP recurred outside the peritoneal cavity, and that the time to IP recurrence

was also significantly longer than in the control arm. This analysis provides some indication that IP-administered radiolabeled antibodies are effective in controlling local lesions, but illustrates the problem in a cavitary treatment when in fact the disease likely spread elsewhere. A recent Phase III clinical trial found that administering combination chemotherapy by both IP and iv routes was more efficacious than just the IP route in ovarian cancer, emphasizing the importance of treating systemically as well as locally [167]. Additional preclinical studies have indicated that antibody conjugates prepared with lower energy beta emitters and alpha emitters would be more suitable to the treatment of microscopic disease that might be present after cytoreductive surgery/effective primary chemotherapy than the ^{90}Y-conjugate employed in the Phase III trial [168–173]. It still seems likely that locoregional IP therapy with antibody conjugates could be more effective with careful planning of the appropriate construct/conjugate, and if efforts were undertaken to combine localized targeted therapies with some form of systemic therapy, especially when occult metastataic disease is likely to be present. It is noteworthy that a different method for targeting radionuclides, known as pretargeting, has been reported to be more effective than directly radiolabeled antibodies, with significantly less hematologic toxicity [60]. Thus, combinations of systemic pretargeted radionuclides with locally administered, directly radiolabeled, IgG might be possible for enhanced responses to extraperitoneal and peritoneal disease.

Depending on their location, brain cancers also can be accessible to localized treatment. Primary brain cancers seldom metastasize outside the central nervous system (CNS), reducing the need for a systemic treatment approach, but heightening the need for effective directed therapy to the primary mass and the eradication microscopic disease wherever it might be present. The first option for primary brain cancers is to remove the mass surgically, which leaves a cavity in the space occupied previously by the tumor. Localized radiation is typically administered afterward to kill any residual cancer, but the cavity also serves as a reservoir for the introduction of therapeutics. Metastatic cancers to the brain occur more frequently than primary brain cancers, and are most often treated with external beam radiation. However, stereotactic radiosurgery has become an option for those with evidence of solitary metastases [102,174–179]. Intravenous antibody therapy with trastuzumab suggests that the blood-brain barrier impedes movement of these antibodies, since there is a relatively high incidence of solitary breast cancer metastasis to the brain in these patients [180]. Thus, for cancers residing in the brain or within the CNS, intracranial or intrathecal routes of administration of targeted macromolecules will be required.

As with regionalized IP treatment, most of the clinical studies for intracranial treatment of primary brain cancers have used radiolabeled antibodies [181]. Radiolabeled anti-tenascin antibodies are the most studied and more advanced in clinical testing [175,176,182–190]. Two Phase-II studies with a single injection of the ^{131}I-murine 81C6 in the surgically-created resection cavity of patients with either newly diagnosed [183] or recurrent malignant gliomas [184] have been promising. Of the 32 newly diagnosed patients that received a full dose (120 mCi) of ^{131}I-m81C6, most also received additional external-beam radiation therapy (29 patients) and systemic chemotherapy (30 patients). In the recurrent population, patients first had a gross resection of the primary tumor to create the cavity where the antibody was administered; within 4 weeks of this treatment, they were also allowed to receive adjuvant chemotherapy. The Phase-II efficacy results are highly encouraging, with evidence that the overall survival increased over currently approved therapy regimens. These 2 experiences are good examples of how regionally targeted therapy should likely be applied, even in cancers that do not spread outside the central nervous system. Early clinical studies have also shown responses when the radiolabeled anti-tenascin antibody was administered intrathecally [191], providing evidence that disease that might spread within this cavity could be treated successfully with this approach.

Eligibility for the intracranial trials mentioned above required an assessment of the cavity to show no leakage, essentially ensuring optimal retention within the cavity. This raises a question whether the radionuclide's attachment to the antibody is necessary or whether any contained radioactive source could prove beneficial. Traditional brachytherapy has not been beneficial in this situation [192], but initial reports of success have appeared with a brachytherapy method that traps the radionuclide inside a balloon that expands to conform with regional boundaries of the cavity [193,194]. Nontargeted ^{90}Y or ^{32}P colloidal or microsphere therapy has been given intratumorally or locally for a variety of solid tumors and in brain cancer with some success [195–200]. This form of non-targeted therapy is effective in reducing the size of cystic craniopharyngiomas [199–203], which appears to be related to destruction of the epithelial cells that line the cyst wall, replaced with a collagen layer that inhibits fluidic movement [204,205]. However, these studies also concluded that these methods would not be effective in treating solid tumors. Preclinical studies with the radiolabeled anti-tenascin antibody showed significantly-enhanced therapeutic benefit over an irrelevant antibody in rats bearing intracranial tumors, but the therapeutic was administered intravenously [206]. Initial studies in patients demonstrated the specificity of the murine anti-tenascin antibody as compared to an irrelevant antibody after intracarotid artery injection, with the specific targeting measuring 1.5 to 4-fold higher than the irrelevant antibody (e.g., first-pass) [207]. While the value of using a specific radioantibody for targeting cancer has been demonstrated repeatedly preclinically and clinically, none of these studies specifically addresses the uniqueness of the intracranial cavity. Impressively, dosimetry studies showed that the half-life for the anti-tenascin antibody in the cranial cavity is the same as the physical half-life of ^{131}I (~8 days) [208], but it is still unclear whether this extended retention time was related to the antibody's ability to bind and hold the radioactivity to the tumor cells, or simply the patency of the cavity. Radioactivity bound to a specific antibody would undoubtedly be deposited on tumor lining the resection margin, providing an important local boost in the radiation dose that could enhance overall survival, whereas radioactivity attached to a non-binding compound might tend to be distributed uniformly in the cavity. Radiological/pathological studies have indicated that viable tumor primarily resides along the lining of the cranial cavity [209], and therefore focal concentrations of radiation in these regions should provide some advantage.

Several other antibodies have been examined clinically for the treatment of primary brain cancers, but these studies involved systemic delivery. An unconjugated anti-EGFR antibody, mAb 425, was ineffective when given systemically, but as an ^{125}I-labeled conjugate given systemically with surgery, external radiation therapy, and chemotherapy, it appeared to have some benefit [210]. Intravenously administered bevacizumab is being used successfully to treat radiation necrosis in the brain [211]. Several clinical studies have reported promising preliminary data with systemically-administered cetuximab as a monotherapy or in combination with radiation and chemotherapy [212,213]. Intravenously administered anti-tenacin IgG used for pretargeting ^{90}Y-biotin also has been examined in several Phase I/II trials [174,175,188]. Imaging studies confirmed the localization of the radiolabeled biotin following the pretargeted localization of the anti-tenascin antibody, and several patients had objective responses with reasonably good durations.

6. Systemic therapy of minimal/micrometastatic cancer

Once an antibody or antibody conjugate is proven to be effective in treating established disease relapsing after initial therapy, inevitably efforts will be undertaken to determine if its use can be expanded to a more naive treatment setting (i.e., front-line vs. second- or third-line), or as an adjuvant/neoadjuvant therapy. For unconjugated antibodies that have minimal long-term risk, use in an adjuvant setting is attractive. For example, randomized studies have shown that trastuzumab (administered every 3 weeks for 1–2 years) added to an adjuvant treatment (given concomitantly or after completion of chemotherapy) improved disease-free survival [214,

215]. Other antibodies, such as cetuximab, panitumumab, and bevacizumab, are being explored in adjuvant settings [216–218]. The anti-CD20 radioconjugates, tositumomab and ibritumomab tiuxetan, originally approved as second-/third-line monotherapies for follicular NHL, are being examined in a front-line setting or in combination with other agents [219–224].

With the lack of Phase-II trials indicating appropriate efficacy for radioconjugates administered systemically in solid tumors, most efforts have been focusing on intracavity applications. However, our early studies in animal models with radioconjugates emphasized that smaller tumors were more likely to be eradicated than larger tumor [225]. In addition, the presence of a large tumor mass could adversely influence the successful treatment of co-existing micrometastatic disease, emphasizing the importance of debulking [226]. Furthermore, in an animal model of microdisseminated human colon cancer in the lungs, specific targeting with radiolabeled antibody IgG could cure a substantial portion of the animals as long as treatment was given before the tumor was too advanced [227,228]. These studies clearly emphasize that radioimmunotherapy is more likely to have its greatest impact in situations where there is minimal disease. Thus, while the results of many Phase-I/II radioimmunotherapy trials in patients with advance solid tumors have been disappointing [59], efforts to examine systemically-administered antibody conjugates in more appropriate settings should be undertaken.

Indeed, administration of an ^{131}I-humanized anti-CEA humanized antibody (labetuzumab) as an adjuvant treatment for colorectal cancer patients after salvage resection of liver metastases has been studied [229,230]. After a minimum 5-year follow-up, post-surgical treatment with the radioconjugate nearly doubled the median overall survival time for these patients as compared to a contemporaneous control group not given radioimmunotherapy [229]. Radioconjugates are also showing promise in medullary thyroid cancer. Investigators found a survival advantage for a subset of patients given a bispecific antibody, pretargeted radionuclide therapy, who had a more rapidly progressing serum calcitonin level [231]. It was suggested that this advantage could be attributed to the eradication of micrometastatic bone marrow involvement.

There also have been a number of studies in animal models indicating that chemotherapeutic agents can be given with radioimmunoconjugates to enhance the radioconjugate's efficacy [232–237]. In these circumstances, the therapeutic agent may be given at doses that are not designed to optimize its activity, but just enough to be a radiosensitizer. Given the poor response rate with radioconjugates in established solid tumors, the gain seen in preclinical models in this setting may not translate well to improved clinical results, unless used in minimal disease or intracavity settings, where a reasonable baseline response with the radioconjugate alone is observed. Several clinical studies have combined directly radiolabeled antibodies with chemotherapy in advanced cancer, but these trials still failed to produce substantive responses [238–241].

A different approach would be to add antibody conjugates to treatments that already have proven efficacy, but with a need for further improvement. Gold et al. showed that a relatively small, fractionated dose of a radioimmunoconjugate could be added to a full human equivalent dose of gemcitabine (~1000 mg/m^2) that was given in 3 cycles of 3-weekly injections and improved therapeutic outcome in animals bearing a human pancreatic cancer cell line [242]. A significant obstacle for this approach is that radioconjugates frequently share a similar dose-limiting hematological toxicity with the chemotherapy. In the previous preclinical study, animals did not experience the same severe hematological toxicity associated with a full course of gemcitabine as is found in patients given this drug. To the extent that a similar procedure can be duplicated in patients will require clinical testing. However, pretargeting procedures

can deliver similar radiation doses to tumors as a directly radiolabeled antibody, but with substantially less radiation dose to the red marrow, thereby reducing hematological toxicity [40,60]. Thus, these methods would be more amenable to combinations with chemotherapeutic agents less limited by myelosuppression, which is the major side effect of radioimmunotherapy.

7. Concluding remarks

Antibodies and immunoconjugates are just beginning to have an impact on the treatment of not only cancer, but many other diseases [40]. However, as presented in this overview, even in situations where the cancer is considered to be more accessible, complete, long-lasting responses are difficult to achieve, and in most instances, the tumor burden must be substantially reduced for meaningful responses to occur. In this regard, antibodies are finding a niche in cancer treatment, but more often they are being used in combination with other agents. There are examples where immunoconjugates have been shown to be more effective than their respective unconjugated antibody, but immunoconjugates have additional side effects that can influence their acceptance and adoption. Biologics are also inherently more expensive than traditional chemotherapeutic agents, and with the need to be repeatedly administered, substantial costs are added to treatment regimens. Thus, while there continues to be considerable interest and activity in developing antibodies and immunoconjugates for therapy, it will be increasing important to select appropriate clinical settings where these agents can have the greatest impact on disease progression and survival.

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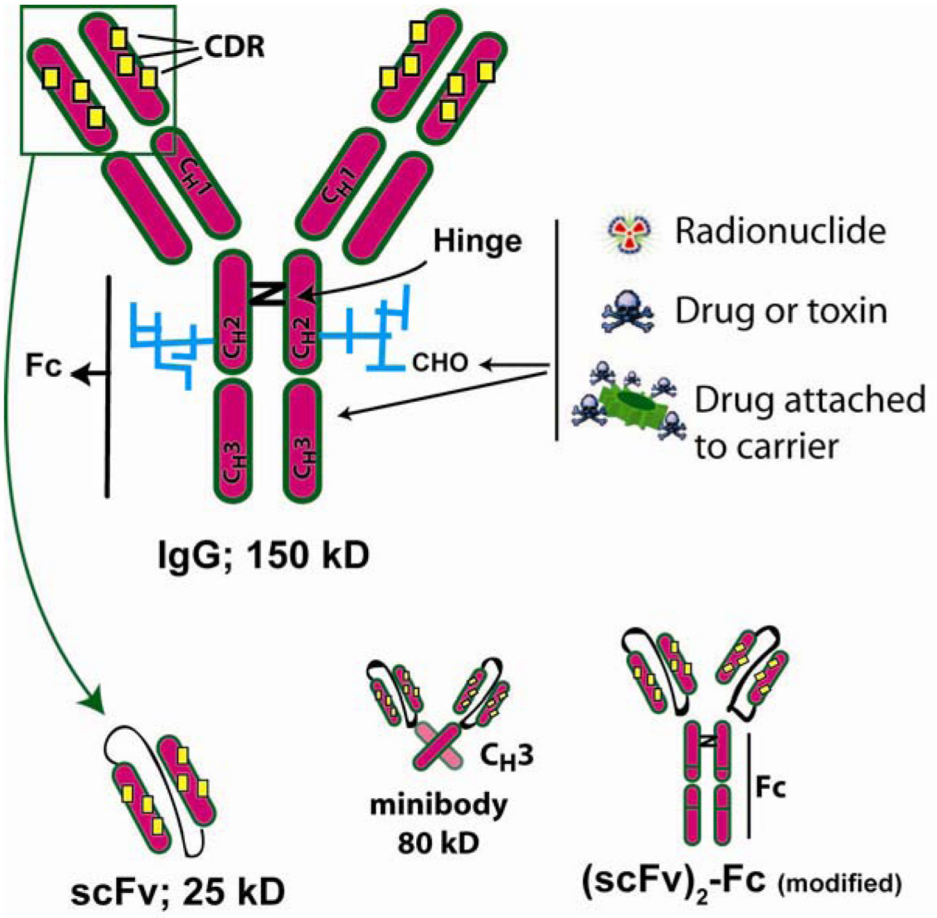

Figure 1. Schematic representation of IgG and several molecularly engineered fragments

Antigen binding is based on the unique 3-dimensional orientation of the CDRs (complementarity determining regions) interacting with the framework regions within the V_H and V_L regions of the molecule, while effector activity (CDC and ADCC) are defined in the Fc-portion of the molecule. The molecule is glycosylated (CHO), which can affect the antibody's blood clearance and function. Immunoconjugates can be prepared by chemically attaching a radionuclide, drug or toxin to the IgG's amino acids or the carbohydrate side chains. Drugs have often been preloaded on a carrier before this attachment to increase the substitution level. The smallest antigen-binding fragment is a single chain (scFv), which essentially represents the V_L and V_H chains (collectively known as the Fv) that are tethered together with a 15–18 amino acid linker. The scFv is monovalent, but will form more complex multivalent molecules (diabodies, tribodies, etc.) as the length of the linker is shortened. Single chains are also used in recombinant antibody-toxin constructs, and have been joined to C_H3 to make minibodies or to a genetically modified Fc fragment to provide a divalent binding fragment with altered pharmacokinetic properties ($scFv_2$-Fc-modified) [73].

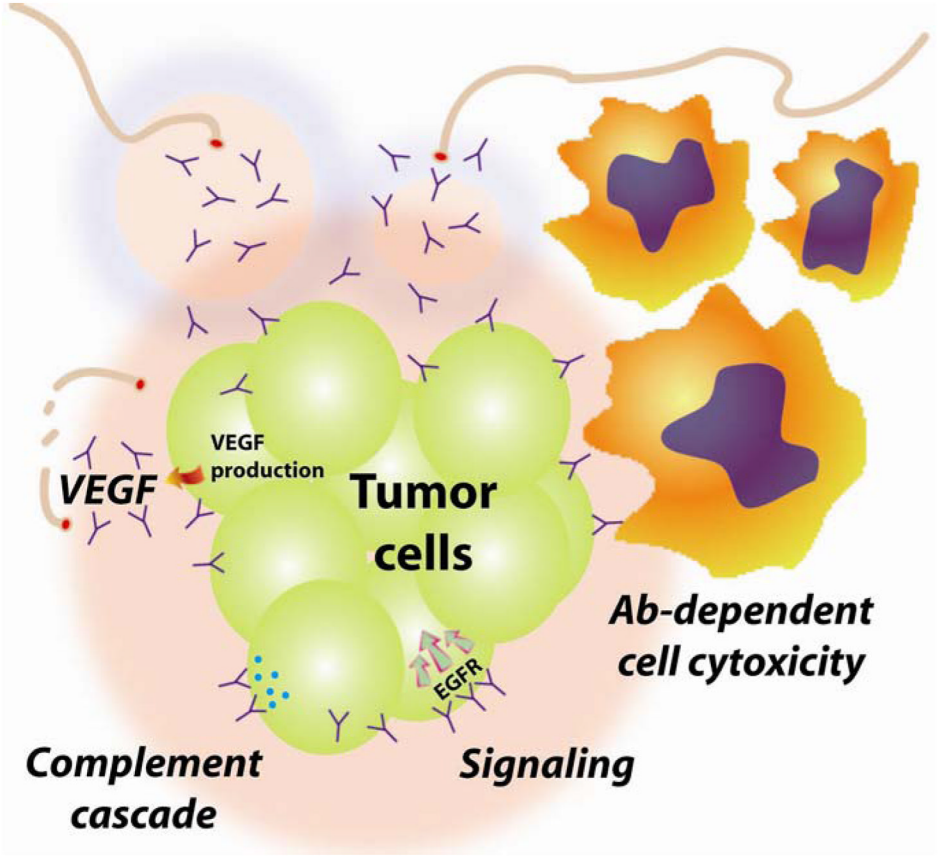

Figure 2. Mechanisms of action for unconjugated antibodies

Traditional mechanisms of action include antibody-dependent cell cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). Both require the Fc-portion of the IgG to activate effector cell binding or complement proteins that can lead to the death of the cells. However, antibodies that bind to growth factor receptors on the surface of the tumor cell (such as EGFR) can block critical functions and send signals that will lead to the cell's death. Alternatively, antibodies can bind to substances produced by tumors, such as VEGF, that are essential for stimulating new blood vessel formation, thereby inhibiting new tumor growth.

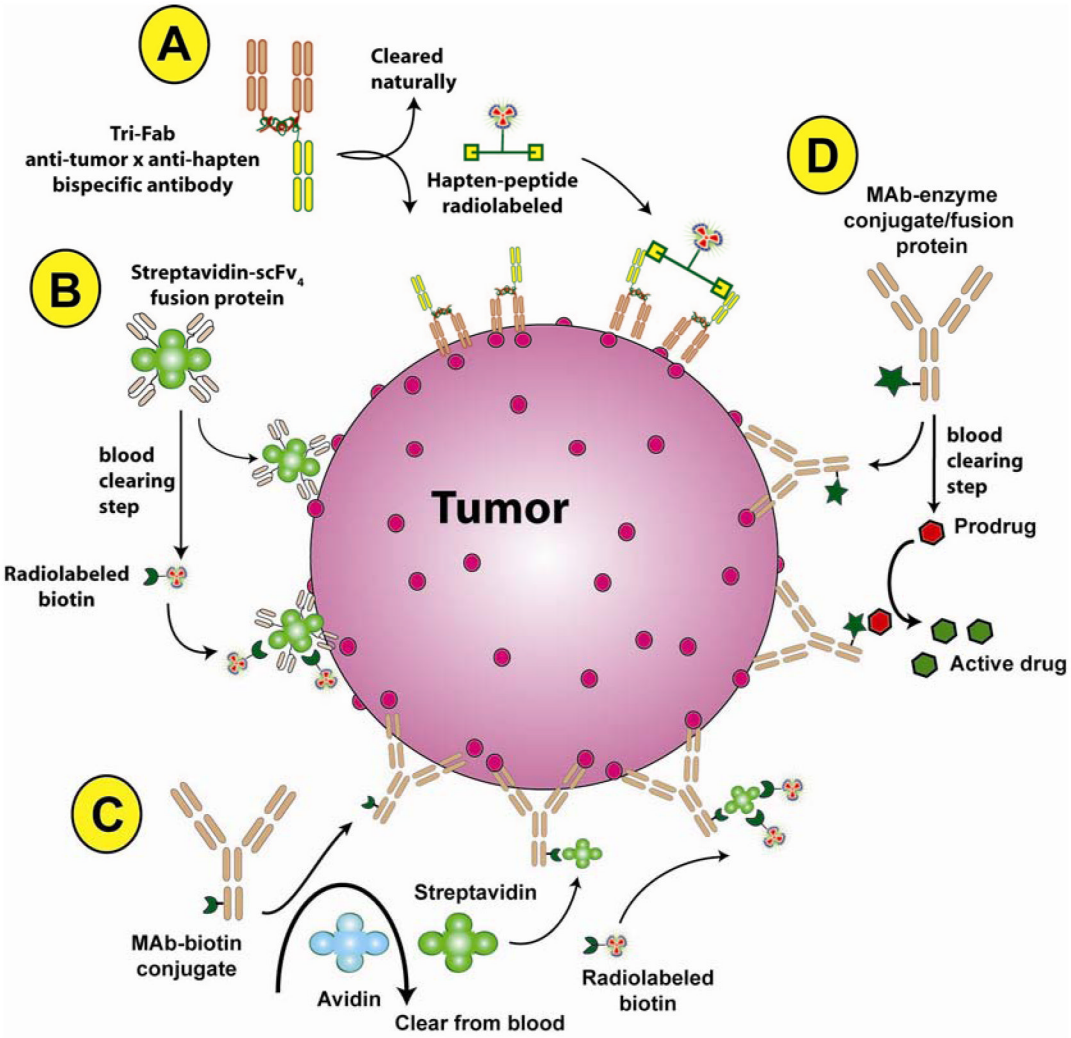

Figure 3. Examples of pretargeting methods used in cancer treatment

These multi-step processes most often have been used to localize a radionuclide (A–C), but locally activated drug delivery has also been investigated [60,81]. (A) Bispecific antibodies, such as a trivalent Fab' construct [39], that can bind to a tumor antigen and a hapten is administered first. Once it localized in the tumor and has cleared from the blood sufficiently, a radiolabeled divalent hapten-peptide is given, which quickly binds to the anti-hapten binding arm of the pretargeted bispecific antibody and clears from the body by urinary excretion. Other pretargeting approaches, such as those shown in B and C, make use of the bacterial protein, streptavidin that has an extremely high binding affinity for biotin. (B) A scFv-streptavidin fusion protein is administered, but due to a slow blood clearance, a separate clearing step is included to remove the construct from the blood where it is metabolized in the liver. Once the excess construct is removed from the blood, radiolabeled biotin is given that will co-localize on the scFv-streptavidin pretargeted to the tumor, with the majority being cleared rapidly from the body by urinary excretion. (C) Another method first injects a biotin-IgG conjugate, which is subsequently cleared from the blood after it localizes in the tumor using avidin (avidin is glycosylated and is readily removed by the liver). A second chase, this time using streptavidin is given, and streptavidin will bind to the pretargeted biotin-IgG. The streptavidin clears from the blood within a day, and then the radiolabeled biotin is given. This technique is possible

due to the multivalency of streptavidin that has 4 biotin-binding sites. (D) Antibody-enzyme conjugates can be pretargeted to tumors and then removed from the blood with a clearing agent. The targeted enzymes are specifically able to interact with a prodrug that is subsequently given, with the release of an active drug within the tumor.

Table 1

More commonly studied antibody-conjugated therapeutic radionuclides in patients.

	Energy (MeV) β_{max}	Penetration (mm in soft tissue)	Physical half-life
Beta-emitters			
^{131}I	0.61	2.3	8.0 days
^{90}Y	2.27	11.3	64.1 hr
^{177}Lu	0.50	1.8	6.7 days
Alpha-emitters			
^{213}Bi	8.3	0.08	46 min

Table 2

Antibodies and immunoconjugates approved in the US for human use in treating cancer.

Generic name	Tradename (specificity)	Approved Use
Unconjugated antibodies		
Alemtuzumab	**Campath** (humanized anti-CD52)	second-line treatment of chronic B-lymphocytic leukemia who failed fludarabine.
Bevacizumab	**Avastin** (humanized anti-VEGF)	combined with intravenous 5-FU-based chemotherapy for first or second line therapy of colorectal cancers. combined with carboplatin and paclitaxel for first line unresectable, locally advanced, recurrent or metastatic non-squamous, non-small cell lung cancer
Cetuximab	**Erbitux** (chimeric anti-EGFR IgG)	combined with irinotecan for metastatic colon cancer or alone in patients unable to tolerate irinotecan. combined with radiation therapy in head and neck cancer (locally or regionally advanced) or as a single agent in patients with recurrent, or metastatic squamous carcinoma of head and neck who failed platinum-based therapy.
Panitumumab	**Vectibix** (human anti-EGFR IgG)	second-line in patients who failed FOLFOX or FOLFIRI chemotherapy regimens
Rituximab	**Rituxan** (chimeric anti-CD20 IgG)	relapsed low-grade or follicular non-Hodgkin's lymphoma (NHL) first-line treatment follicular NHL in combination with CVP chemotherapy first-line treatment of diffuse large B-cell NHL in combination with CHOP or other anthracyclinesbased chemotherapy; low-grade NHL with stable disease or who achieve a partial or complete response following CVP chemotherapy
Trastuzumab	**Herceptin** (humanized anti-HER2 IgG)	single agent in patients with metastatic breast cancer that overexpress HER2 and who have received one or more chemotherapy regimens; combined with doxorubicin, cyclophosphamide, and paclitaxel in an adjuvant setting for nodepositive breast cancer; combined with paclitaxel in patients with metastatic breast who have not received treatment for their metastatic disease.
Radiolabeled antibodies		
Ibritumomab tiuxetan	**Zevalin** (murine anti-CD20 IgG-DTPA conjugate; radiolabeled with ^{90}Y or ^{111}In; rituximab pre-dose)	treatment of relapsed or refractory low-grade, follicular or transformed B-cell NHL.
Tositumomab	**Bexxar** (murine anti-CD20 IgG; radiolabeled with ^{131}I; tositumomab pre-dose)	treatment of patients with refractory, low-grade, follicular, or transformed NHL, including patients with rituximab-refractory NHL
Antibody-drug conjugate		
gemtuzumab ozogamicin	**Mylotarg** (humanized anti-CD33 IgGcolecheamicin	acute myeloid leukemia in first relapse and patients ≥60 yr old who are not candidates for other cytotoxic therapy.