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Addressing challenges of heterogeneous tumor treatment through bispecific protein-mediated pretargeted drug delivery

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Abstract

Tumors are frequently characterized by genomically and phenotypically distinct cancer cell subpopulations within the same tumor or between tumor lesions, a phenomenon termed tumor heterogeneity. These diverse cancer cell populations pose a major challenge to targeted delivery of diagnostic and/or therapeutic agents, as the conventional approach of conjugating individual ligands to nanoparticles is often unable to facilitate intracellular delivery to the full spectrum of cancer cells present in a given tumor lesion or patient. As a result, many cancers are only partial suppressed, leading to eventual tumor regrowth and/or the development of drug-resistant tumors. Pretargeting (multistep targeting) approaches, which involves administering 1) a cocktail of bispecific proteins that can collectively bind to the entirety of a mixed tumor population followed by 2) nanoparticles containing therapeutic and/or diagnostic agents that can bind to the bispecific proteins accumulated on the surface of target cells offers the potential to overcome many of the challenges associated with drug delivery to heterogeneous tumors. Despite its considerable success in improving the efficacy of radioimmunotherapy, the pretargeting strategy remains underexplored for a majority of nanoparticle therapeutics applications, especially for targeted delivery to heterogeneous tumors. In this review, we will present concepts in tumor heterogeneity, the shortcomings of conventional targeted systems, lessons learned from pretargeted radioimmunotherapy, and important considerations for harnessing the pretargeting strategy to improve nanoparticle delivery to heterogeneous tumors.

Graphical Abstract

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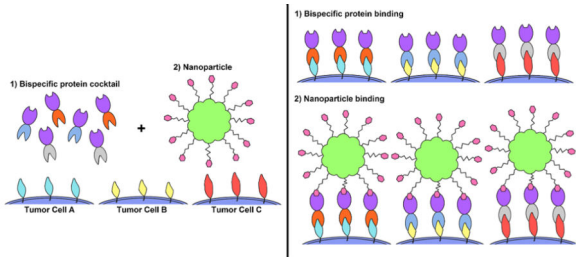

Keywords

pretargeting; tumor heterogeneity; nanoparticles; cancer targeting; bispecific proteins; drug delivery

Introduction

Targeted drug delivery for cancer offers the potential to significantly improve the therapeutic index of anticancer agents by increasing drug concentration at tumor sites while reducing side effects and toxicity in non-targeted tissues. A long-standing approach in the field has been to exploit the leaky tumor vasculature in tumor tissues by encapsulating therapeutic cargo into nanoparticles that remain sufficiently stable when introduced to the systemic circulation in order to reach and extravasate into cancer tissues. To further facilitate selective delivery into cancer cells, many researchers have functionalized nanoparticles with ligands that bind specific receptors on cancer cells, a strategy commonly referred to as “active” targeting [1]. Unfortunately, the accumulation of both ligand-free and ligand-conjugated systems in tumors is generally modest at best, limiting the efficacy of various therapies against cancer [2, 3].

Due to advances in the genetic and phenotypic analysis of tumors, tumor heterogeneity has recently emerged as yet another biological barrier that could limit efficient distribution, retention, and uptake of ligand-conjugated nanoparticles at tumor sites [2, 4, 5]. Tumor heterogeneity also encompasses the highly variable expression of target receptors, both intertumorally between patients or different tumors and intratumorally within a given tumor, and has been reported for a wide range of human tumors [6, 7]. Due to the absence or suboptimal expression of their target receptor on many tumor cell subpopulations, actively targeted drug carriers, which typically consist of single-ligand nanoparticles, are unable to effectively bind and internalize into the full spectrum of tumor cells present in any particular tumor. As noted by Bae *et al*., “aiming at cancer cells with a single surface marker results in aiming at a single population among mixed populations which are constantly changing and moving” [2]. Inadequate drug delivery to all cancer cell subpopulations typically results in only partial suppression of the cancer and eventually leads to tumor regrowth and/or the emergence of therapy-refractory tumor cell populations [8–10]. Thus, targeting strategies that can directly address the challenges associated with tumor heterogeneity and enable effective delivery of nanoparticles are sorely needed.

One promising targeting strategy is to decouple molecular homing and delivery of therapeutics into two separate steps. This approach involves first introducing bispecific

proteins (BsPs) that can specificially bind (i.e., “pretarget”) cancer cells, followed by the administration of a drug-carrying effector such as a nanoparticle that can be captured by the BsPs accumulated on the surface of tumor cells (Figure 1). By introducing multiple distinct BsPs, a single effector nanoparticle could in theory bind with molecular specificity to the full diversity of cancer cells present in any particular tumor. In this review, we will discuss the concept of, important considerations for, and key challenges associated with exploiting the pretargeted strategy to enhance the delivery of therapeutics to heterogeneous tumors.

Conventional cancer targeting strategies: passive targeting

In 1986, Matsumura and Maeda discovered that macromolecules can preferentially accumulate in tumors due to anatomical and pathophysiological differences between solid tumors and healthy tissue [11–13]. Specifically, tumors initiate extensive angiogenesis to maintain their rapid growth, but the newly formed blood vessels display abnormal architecture including fenestrated endothelial lining of vessel walls [12–14]. The more permeable tumor vasculature then allows macromolecules and nanoparticles to extravasate from the bloodstream and accumulate in the tumor [13, 14]. Presumably poor lymphatic drainage further permits enhanced retention of drug delivery systems within tumors [12, 14]. The combination of leaky tumor vasculature and impaired lymphatic drainage constitute the phenomenon termed the enhanced permeability and retention (EPR) effect.

Harnessing the EPR phenomenon simply requires nanoparticles to (i) fall within an appropriate size range and (ii) evade rapid elimination by the mononuclear phagocytic system (MPS). While smaller nanoparticles can naturally extravasate more efficiently than larger nanoparticles, most studies suggest the tumor vasculature in mouse xenografts can permit extravasation of nanoparticles ranging from 10 to 200 nm in diameter [13, 15–17] with some studies reporting EPR of particles up to 500 nm in diameter [14, 18]. In addition to size, prolonged circulation kinetics also directly improve the extent of nanoparticle extravasation through leaky tumor blood vessels by maximizing the number of times a nanoparticle will pass through the tumor vasculature [12, 18]. Polyethylene glycol (PEG) was among the first “stealth” polymers used to extend liposome and other nanoparticle circulation times by minimizing opsonin adsorption and nanoparticle elimination by MPS cells, and PEGylation is the most widely adopted strategy to enhance nanoparticle tumor uptake via EPR [12–14, 18]. Other coating polymers used to improve particle circulation profiles, and thereby exploit the EPR effect, include flexible, hydrophilic polysaccharides such as dextran, hyaluronic acid, and chitosan [19, 20]; synthetic polymers such as polyvinyl alcohol [21] and polyvinylpyrrolidone [22]; zwitterionic polymers [23, 24]; and polyoxazolines [25]. Indeed, dextran-, hyaluronic acid-, chitosan-, and N-(2-hydroxylpropyl) methylacrylamide (HPMA)-coated particles all exhibited improved EPR-mediated tumor accumulation due to prolonged circulation [26–29]. Because nanoparticles of the appropriate size and with MPS-resistant surface chemistry can naturally achieve a low to moderate level of tumor targeting without using specific ligands, these non-molecularly targeted systems are frequently classified as *passively targeted*.

It is important to note that the EPR effect is highly variable and may not be readily exploitable for all tumors [30]. For example, hepatocellular and renal cell carcinomas are

characterized by high vascular density and exhibit increased EPR effects compared to low vascular density pancreatic and prostate cancers that demonstrate diminished EPR effects [30, 31]. Additionally, EPR of drug carriers is not observed homogenously throughout individual tumors, as the central foci of tumors tend to be characterized by necrotic [30, 32], hypoxic [30, 33], and hypovascular areas [12] that do not display the EPR effect [11, 34, 35]. EPR heterogeneity may also vary between primary tumor and metastases [1]. Therefore, harnessing EPR to enhance therapeutic responses in the clinic requires an improved understanding of how tumor heterogeneity impacts the EPR effect both within and between tumors [1, 13, 30, 36, 37].

Conventional cancer targeting strategies: active targeting

To further improve nanoparticle-based delivery to cancer cells, numerous investigators have developed nanoparticles decorated with ligands specific to receptors overexpressed on cancer cells, an approach generally termed *active targeting* [1]. Ligands on actively targeted systems are typically grafted to the distal end of polymer chains that are used to coat the particles and provide prolong circulation kinetics [18]. These systems are presumed to effectively extravasate from the tumor vasculature based on the underlying stealth polymer coating, while the presence of ligands can facilitate nanoparticle binding to and subsequent internalization into specific tumor cells expressing the corresponding receptor [18, 38]. Actively targeted systems were thought to directly address the shortcoming of inefficient cellular uptake of passively targeted systems [14, 18]. Numerous targeting ligands have been utilized to actively target nanoparticles to cancer cells, including antibodies and antibody fragments [39, 40], aptamers [41], peptides [42], proteins, sugars [43], and low molecular weight ligands such as folate [44]. For excellent reviews of the features and design of actively targeted systems, please refer to [1, 18, 38, 45]

Unfortunately, active targeting systems face several challenges that may limit their efficacy in practice. The target cell surface receptors must be highly overexpressed or selectively expressed solely on malignant cells, as opposed to healthy cells, to maximize tumor-specific delivery [45–47]. Additionally, the choice and density of ligand are critical to optimizing the effect of the targeting moiety. Greater ligand density was previously assumed to enhance nanoparticle targeting to tumors *in vivo* due to generally observed improvements in cancer cell uptake *in vitro* [46]. Nevertheless, an increasingly number of studies have shown that maximal accumulation of nanoparticles in tumors *in vivo* is typically achieved with an intermediate ligand density [46, 48–51]. For example, increasing the surface aptamer density on polymeric nanoparticles actually resulted in reduced tumor accumulation and increased particle distribution in the liver [48]. The poor *in vivo* performance of particles with high ligand densities was attributed to ligand shielding or adulteration of the underlying stealth polymer coat, leading to rapid MPS clearance and a reduction in the fraction of particles that can reach and extravasate into tumors [46, 47].

Tumor heterogeneity and implications for targeted drug delivery systems

Variations in accumulated genetic mutations, which can be further exacerbated by alterations in the local tumor microenvironment, frequently lead to genomically distinct

subclonal populations within the same tumor or between tumor lesions. This in turn creates a phenomenon termed tumor heterogeneity, which describes the functional and phenotypic profile differences between cancer cells such as cellular morphology, gene expression, metabolism, motility, proliferation, level of drug resistance, and metastatic potential. Additionally, the highly variable presence of stromal cell populations such as fibroblasts, immune cells, and endothelial cells within tumors is critical in shaping the tumor microenvironment [52, 53]. Interactions between the non-tumor cell populations and tumor cells contribute to different tumor phenotypes, impact tumor response to various therapies, and influence disease progression [54, 55].

Tumor heterogeneity (Figure 1) encompasses both (i) intertumoral heterogeneity, which describes differences between tumors in an individual patient as well as clinical response differences between patients with the same tumor subtype, and (ii) intratumoral heterogeneity, which refers to the genetic, epigenetic, and phenotypic features that vary within malignant cell populations of the same tumor mass [56]. Intratumoral heterogeneity is further classified into spatial heterogeneity, which refers to differences between distinct anatomical regions or individual cells within a tumor, and temporal heterogeneity, which refers to changes in a tumor's molecular profile and receptor expression over time. An example of intratumoral spatial heterogeneity is the highly discordant HER2 expression observed in different areas within a single biopsy from HER2-positive metastatic breast cancer patients (Figure 3a) [57]. Temporal heterogeneity can be observed for relapsed lesions that exhibit a disparate molecular profile, compared to their original tumor.

In addition to morphological and spatiotemporal variations within the same tumor or between primary tumors, tumor heterogeneity can also directly result from metastasis. Metastatic heterogeneity (Figure 2) has been observed to include (i) discordant biomarker or receptor expression between metastases arising from distinct subclonal populations in the primary tumor ("intermetastatic" heterogeneity) and (ii) heterogeneity within individual metastases ("intrametastatic" heterogeneity), which may have a substantial impact on therapeutic outcome [7, 52, 56, 59, 60]. For example, Gerlinger *et al*. reported a case of intrametastatic heterogeneity in which significant changes in the mutational profiles of spatially separated biopsy samples from primary renal-cell carcinomas and metastases were identified using next-generation sequencing [61]. Additionally, Albino *et al*. observed intermetastatic heterogeneity in a melanoma patient whose multiple metastases displayed contrasting morphologies and surface antigen expression [60]. Other studies have also investigated variable estrogen, progesterone, and HER2 receptor expression between primary breast tumors and metastases, with discordance rates that varied greatly from 18% to 54% [52, 62, 63]. Additional types of heterogeneity include non-genetic phenotypic and functional heterogeneity [63] and tumor microenvironment heterogeneity [52, 53]. Because tumor cells interact with their environment, tumor microenvironment heterogeneity exerts a crucial influence on disease progression. For example, the heterogeneous distribution of stromal cells, extracellular matrix organization, and especially hypoxic regions within the tumor microenvironment may promote metastasis and development of drug resistance [52].

Tumor heterogeneity has been reported in a wide range of human tumors such as breast [52, 57], non-small cell lung [64, 65], ovarian [52, 58, 66, 67], prostate [52, 68], and lymphoma

[69] and poses a significant challenge for diagnosis, prognosis, and efficacy of molecularly-targeted therapies (Figure 3b) [58, 70]. The presence of heterogeneous cancer cell populations within tumors will likely limit the efficacy of any therapeutics targeted against any single tumor-associated receptor, leading to poor/varied outcomes, including cancer recurrence and therapeutic resistance [6, 56]. For example, the heterogeneous expression of programmed death 1 (PD-1) was reported in two distinct T-cell subpopulations and differentially impacted survival in patients with follicular lymphoma [69]. Similarly, heterogeneous HER2 expression in breast cancer has prompted treatment stratification in the clinic based on receptor expression [52]. Indeed, intratumoral HER2 heterogeneity, both genetic and spatial, affected the trastuzumab treatment responses and survival of patients with HER2-positive metastatic breast cancer [57]. Only a small fraction of trastuzumab-treated patients achieved complete disease eradication, and the majority of patients developed relapsed tumors that were resistant to trastuzumab therapy due to the proliferation of HER2-negative breast cancer cells.

In addition to therapy with monoclonal antibodies such as trastuzumab, variable target receptor expression in heterogeneous tumors also presents a critical bottleneck for actively targeted drug delivery systems. The common active targeting approach, in which drug-loaded particles are surface modified with a single ligand group, cannot target and facilitate intracellular delivery to the full diversity of malignant cells. One potential strategy is the administration of a cocktail of single-ligand particles. Unfortunately, this would pose considerable challenges and substantial cost burden in the context of particle formulation and complexity in clinical evaluation [47], which has generally limited particles to one or two distinct targeting ligand groups. More importantly, a single universal targeted nanoparticle cocktail for all patients is unlikely to succeed due to interpatient heterogeneity; inadequate levels or the complete lack of corresponding target cells for a significant fraction of the ligand-modified particles could lead to increased hepatic and splenic biodistribution and, correspondingly, reduced tumor accumulation. Alternatively, multiple different targeting ligands could be theoretically conjugated onto the surface of a single nanoparticle. However, as discussed above, increased density of ligands beyond a particular threshold will likely trigger rapid MPS clearance of the particles.

Pretargeted radioimmunotherapy (PRIT)

The discovery that human tumor-associated antigens could be used as targets for antibodies to differentiate tumors from normal tissue helped spawn the field of monoclonal antibody (MAb)-based immunotherapy of cancer. The multiple applications of cancer immunotherapy include radioimmunotherapy (RIT) (Figure 1b), which uses radioisotope-conjugated Mabs to treat radiosensitive tumors such as non-Hodgkin's lymphoma (NHL) [71]. Unfortunately, the therapeutic efficacy of RIT is limited by the long circulatory half-life of many MAbs, as well as high non-specific deposition of the MAbs in normal organs, resulting in low tumor-specific delivery of radiation and significant toxicity [72].

To overcome the shortcomings of radioimmunotherapy (RIT), many researchers have adopted a multistep approach (Figure 1d) to more specifically deliver radionuclides to tumor cells by first injecting BsPs that contain a tumor cell binding domain and an effector binding

domain. Subsequently, radiolabeled effector molecules are introduced and interact with BsPs bound on the surface of tumor cells. Such an approach has been termed pretargeted radioimmunotherapy (PRIT) [73, 74]. Because the BsPs are non-radioactive and the radiolabeled effector molecules typically consist of modified small molecule metal chelators that can be rapidly cleared, PRIT can significantly improve the therapeutic index of radioisotope treatment compared to RIT [72, 75, 76], as well as increase the maximum tolerated dose for radionuclides [74]. Pagel *et al*. demonstrated that anti-CD45 PRIT improved the specificity of radiation delivery to leukemia in a rodent model, delivering twice as much radiation to bone marrow and five times more activity to the spleen than conventional RIT [77, 78]. *In vivo* PRIT was able to mediate broad tumor growth suppression and prolonged survival with the use of BsPs against receptors expressed at different levels on lymphoma cells, with CD20 and HLA-DR proving to be superior targets compared to CD22 [79, 80]. CD38-specific PRIT achieved tumor-to-blood ratios as high as 638:1 after 24 hours for a multiple myeloma model, compared to a ratio of ∼1:1 with conventional RIT [81]. Subbiah *et al*. reported that treating athymic mice bearing Ramos human Burkitt's lymphoma xenografts with a pretargeted system consisting of anti-CD20 scFv-conjugated streptavidin (SA) and ^{90}Y-DOTA-biotin cured 100% of mice with allowable toxicity, whereas conventional RIT with ^{90}Y-1F5 at the same dose produced no cures, generated profound pancytopenia, and was lethal to all mice [82]. Zhang *et al*. demonstrated that both ^{90}Y-DOTA-biotin and ^{213}Bi-DOTA-biotin could both be used in combination with anti-CD25 scFv-conjugated SA for PRIT of a murine T-cell lymphoma xenograft model, with the beta-emitter ^{90}Y curing 10 of 10 mice and alpha-emitter ^{213}Bi curing 7 of 10 mice [83].

These encouraging results with PRIT studies in animal models led to clinical studies of PRIT, which have yielded promising results with reasonable tumor response rates and limited toxicity [84]. Forero *et al*. evaluated the pharmacokinetics and immunogenicity of an anti-CD20 scFv-SA conjugate in 15 patients with NHL [85]. Although the complete remission rate was low (2 of 15), the majority (12/15) patients exhibited no signs of hematologic toxicity, suggesting that the dose of radionuclide could be further increased. Another phase I/II PRIT clinical trial was performed using a chimeric anti-CD20 IgG-SA in combination with ^{90}Y-DOTA-biotin. Six of seven NHL patients demonstrated significant tumor regression, with an estimated tumor-to-whole body dose ratio of 38:1. While six of the ten patients developed humoral responses to streptavidin, the transient nature of the responses appeared to result in no significant long-term effects [86, 87]. Kraeber-Bodere *et al*. evaluated the therapeutic efficacy of PRIT using a bispecific monoclonal antibody that binds to carcinoembryonic antigen (CEA) and to a ^{131}I-labeled effector molecule for PRIT of medullary thyroid cancer. Of the 17 patients treated, 4 reported pain relief, 5 demonstrated minor tumor responses, and 4 achieved biological responses (decrease in thyrocalcitonin); however, 9 patients also generated human anti-mouse antibodies [88–90].

While PRIT has led the way in preclinical and clinical studies of pretargeting, it is important to note that the applications for pretargeted strategies extend far beyond radiotherapy. For example, solid cancers, which will account for more than 90% of all newly diagnosed cancer cases and deaths in the United States in 2015 [91], are significantly more resistant to

radioimmunotherapy compared to hematological malignancies such as NHL. To date, little is known about whether the pretargeting approach can enhance the delivery of other therapeutic agents such as nanoparticle drug carriers that can encapsulate and slowly release chemodrugs to solid tumors.

Pretargeted drug delivery to heterogeneous tumors

The growing interest in precision/personalized medicine, coupled with the incomplete treatment of heterogeneous cancers using common passively or single-ligand targeted therapies that can give rise to recurrent, more aggressive, and/or drug-resistant tumors [7, 56] highlights the need for alternative nanoparticle targeting strategies to improve treatment responses. The modular nature of pretargeted systems is particularly useful in addressing the challenge of and many barriers to effective drug delivery to heterogeneous tumors [52] because it enables pretargeted systems to be targeted to new or different tumor antigens by simply modifying the tumor binding domain of BsPs, as opposed to direct, ligand-based targeting systems that would require the formulation of a new nanoparticle system. This flexibility is expected to markedly reduce the production costs and complexity, as well as the potential regulatory burden, for pretargeted nanoparticles. Another equally appealing feature of pretargeting is the ability to pretarget multiple receptors simultaneously. The administration of a cocktail of pretargeting BsPs that can all bind to the same drug carrier could in theory enable the delivery of a drug carrier to the full spectrum of a patient's cancer cells (Figure 4). Drug cocktails containing mixtures of different MAbs have already been applied to cancer therapy, with one combination of pertuzumab, trastuzumab, and docetaxel significantly improving the overall survival of patients with HER2-positive breast cancer [92]. Antibody mixtures have also been used for *in vitro* and *in vivo* imaging and diagnosis of tumors [93, 94]. Additionally, pretargeting with individual or mixed BsPs was able to differentially label a range of human tumor cell lines *in vitro* (Figure 5a) [95, 96]. To our knowledge, no studies have been published on the simultaneous use of multiple pretargeting BsPs to enhance nanoparticle delivery to date, although Khaw *et al*. did report the receptor-dependent efficacy of doxorubicin nanoparticles pretargeted with anti-HER2 affibody-based BsPs in a dual tumor model [97]. In that study, tumor growth inhibition was achieved for HER2-positive BT-474 breast cancer tumors, while the HER2-negative BT-20 breast cancer tumors were simultaneously unresponsive to the treatment, further emphasizing the opportunity for improved cancer treatment through appropriate targeting of all tumor cell populations.

Biological and pharmaceutical aspects and considerations of pretargeted drug delivery

As multicomponent systems, the potential arsenal of pretargeted therapies is sizeable and highly diverse. Thus, many features (e.g., choice of target receptor/antigen, binding pair technology, and drug carrier) must be taken into account when developing a pretargeted drug delivery system to maximize transport of drug cargo to target cells and overall therapeutic efficacy. Because only a few publications have evaluated the use of pretargeting for nanoparticle delivery, the majority of the current knowledge about optimal pretargeted conditions have been gleaned from *in vivo* PRIT studies, but, due to the overlap of

components for multistep targeting approaches (Figure 1d & e), many of the lessons learned from PRIT likely apply to pretargeted nanocarriers.

Binding pairs

A key consideration of any pretargeted delivery approach is the binding interaction between the pretargeting BsP and nanoparticle effector, as the affinity of the binding pair directly influences the capture and retention of the drug carrier at the tumor site. In addition, the immunogenicity of the BsP and its interactions with endogenous ligands can also alter the efficacy of pretargeted therapies [71].

The first binding pairs used in pretargeted systems were based on antibody-hapten interactions. In 1985, Reardan *et al*. reported the development of antibodies against indium chelates of EDTA, and suggested the possibility of bispecific antibodies that can simultaneously recognize target antigens and metal chelates [72, 98]. Soon afterwards, Goodwin *et al*. developed an early pretargeted imaging approach using a murine tumor model through the injection of anti-chelate antibodies, followed by the administration of a radiolabel [99]. Since then, a number of antibodies against various haptens have been utilized for binding to effector molecules, including anti-DTPA complex [100, 101], anti-peptide [102–104], anti-methotrexate [105], and anti-cotinine antibodies [106]. In addition to bispecific antibodies [101, 102, 107], a range of antibody fragments and derivatives have been developed as pretargeting BsPs [71, 106, 108]. Most antibodies, including those used to capture radioisotope-carrying effector molecules in PRIT, exhibit nanomolar to high picomolar affinity ($K_D \sim 10^{-7}$–10^{-10} M) for the antigen target on the surface of cancer cells [98, 105, 109]. Because PRIT typically uses single radionuclide-loaded agents, the improvement of antibody-hapten binding through multivalency can significantly enhance the specificity of radioisotope localization and retention in tumor sites [102, 108, 109]. For example, the application of pretargeted bivalent haptens, termed the affinity enhancement system (AES), was able to improve the tumor biodistribution of bivalent ^{111}In-diDTPA by more than 7-fold compared to monovalent ^{111}In-DTPA (tumor biodistribution: 52.9% vs. 7.6% ID/g at 1 h and 92.5% vs. 0.9% ID/g at 72 h, respectively) [110].

As a binding pair with one of the strongest noncovalent binding affinities ($K_D \sim 10^{-14}$ 10^{-15} M), the streptavidin (SA)-biotin system was quickly adopted by the pretargeting field [111, 112]. Additionally, SA is a tetravalent protein and could enable the capture of multiple biotinylated drug molecules. SA and biotin can be attached to tumor-specific pretargeting proteins and/or effector molecules through a variety of methods, including direct conjugation [82, 113, 114], genetic engineering of fusion proteins [115–117], and enzymatic conjugation [118]. While SA-based PRIT systems have demonstrated increased tumor specificity and higher therapeutic indices relative to directly targeted systems [82, 117] and have even been evaluated in clinical trials [85, 114, 119], the immunogenic nature of SA, a bacterial protein, represents a major challenge to widespread clinical use of SA-biotin binding pairs [85, 87]. The immunogenicity of SA can be reduced through site-specific mutations [120, 121], although it remains unclear whether these SA mutants will be sufficiently hypoimmunogenic to allow for repeated dosing in humans. The problem of interference from endogenous biotin [122], which necessitates the use of biotin-free feed for

in vivo studies, could also be potentially addressed by SA mutants that selectively bind bis-biotin instead of biotin [123, 124]. Other proteins that naturally bind to specific substrates (e.g., enzymes) can also be modified to bind to exogenous molecules for use in pretargeting, although only a few such systems have been reported in the literature [125, 126].

The majority of published pretargeted and multistep targeting systems utilize antibody-hapten or protein-ligand interactions, but research in areas such as complementary synthetic nucleic acids and peptides and bioorthogonal chemistry continues to generate novel classes of binding pairs. Morpholinos (MORFs) are the most popular class of synthetic nucleic acid analogs for pretargeting using complementary nucleic acids and have been evaluated preclinically in combination with tumor-specific pretargeting antibodies and a variety of radionuclides [127–131]. In addition to relatively low immunogenicity, optimized complementary morpholinos exhibit high specificity and binding affinity [132], and the use of bivalent MORFs may further enhance affinity [133]. Bioorthogonal chemistry comprises reactions that can rapidly occur in a living system with high selectivity and without any off-target reactions or toxicity. These properties enable pretargeting using small molecule binding pairs with low immunogenicity, although the relative merits of different bioorthogonal chemistries vary based on reaction kinetics, complexity of synthesis, and stability of the resulting conjugate (see refs [71, 134, 135]). Rossin *et al*. demonstrated the feasibility of using “click” chemistry for pretargeting of radioisotopes *in vivo* by treating tumor-bearing mice with an anti-TAG72 antibody (CC49) modified with *trans*-cyclooctene (TCO), which then reacted with ^{111}In-tetrazine (^{111}In-Tz) administered 24 h later [136]. The CC49-TCO predosed mice exhibited a tumor uptake of 4.2% ID/g and tumor-to-muscle (T/M) ratio of 13.1, compared to tumor uptake and T/M ratios of 0.3% ID/g and 0.5 and 1% ID/g and 2.1 for unmodified CC49 and control Ab-TCO groups, respectively. Further preclinical studies have confirmed the utility of TCO-tetrazine and other bioorthogonal chemistries for tumor imaging and treatment [71, 137–139].

While all of the aforementioned classes of binding pairs have also been used in the pretargeting of nanoparticles and other potential drug carriers [95, 140–142], the ability of pretargeted systems to actually deliver therapeutics to tumor cells has only been evaluated in a few studies [97, 143–145]. Pretargeted poly-lysine polymers [146], liposomes [147], and carbon nanotubes [148] have been used to deliver higher doses of encapsulated or conjugated radionuclides to both solid tumor and hematologic cancer cells, suggesting that the application of nanocarriers could further improve the efficacy of PRIT. In the context of cancer chemotherapy, pretargeted biotinylated polymeric nanoparticles loaded with pactilaxel (PTX) increased the *in vitro* cell killing of glioma and breast cancer cells, relative to free drug or Taxol and nontargeted nanoparticles [143, 144]. The injection of an anti-HER2 affibody-anti-DTPA Fab complex (BAAC) 8 h prior to the administration of ^{99m}Tc-DTPA-succinylated polylysine enabled the specific labeling of tumors (5.3% ID/g vs. 0.5% ID/g for anti-DTPA Fab-pretargeted particles) [97]. BAAC pretargeting of doxorubicin- and DTPA-conjugated polyglutamic acid produced tumor growth inhibition results that were similar to those of free doxorubicin, but pretargeting through the combination of BAAC and polymer-drug conjugate minimized weight loss in mice relative to the free drug treatment

[97], underscoring the ability of pretargeting to improve the therapeutic index of chemotherapeutics *in vivo*.

Although BsP considerations such as immunogenicity and competition with endogenous ligands apply to both PRIT and pretargeted nanoparticles, other features and characteristics of binding pairs required for pretargeted drug delivery systems may differ from those for pretargeting based on small molecule effectors. Nanoparticles are inherently highly multivalent due to their large surface area, which allows the grafting of tens to possibly thousands of a given binding partner moiety. Thus, BsPs with lower affinity to a hapten may still be able to capture hapten-coated nanoparticles with high avidity compared to individual radiolabeled haptens. However, as is the case with actively targeted systems, the incorporation of peptides, nucleic acids, proteins and other macromolecular components onto drug carrier particles could negatively impact their circulation kinetics and efficiency of extravasation into tumors. For highly asymmetric binding pairs that consist of a large protein and a smaller moiety (e.g., SA-biotin, antibody-hapten), the smaller, the more immunologically inert moiety should be assigned to the effector nanoparticle, rather than the BsP, to minimize MPS clearance. Steric considerations may further support the modification of drug carriers with smaller BsP-binding components. For instance, Haun *et al*. reported that, in addition to relatively to providing a 10- to 15-fold increase in cell binding relative to directly targeted iron oxide nanoparticles, a pretargeted antibody-TCO/Tz-NP system demonstrated significantly higher fluorescent labeling of various tumor cell lines, compared to an antibody-biotin/avidin-NP system [95]. The authors attributed this difference to the large footprint of avidin (∼67 kDa) on the particles, which likely resulted in the reduced accessibility and valency of biotin-binding sites. The use of a PEG spacer for TCO-antibody modificiation also improved the pretargeting of quantum dots by reducing masking of reactive groups [149].

Target antigen(s)

A diverse array of receptors and other antigens overexpressed on tumor cells have been exploited for active targeting of nanoparticles and for RIT [1, 150]. In contrast, the number of target cancer antigens/receptors suitable for pretargeted approaches is certainly more limited. The multistep nature of pretargeting requires that the tumor cell-binding BsP must remain on the tumor cell surface to capture subsequently injected effector drug carriers. Indeed, the majority of PRIT studies to date utilize BsPs that target epitopes generally considered to be non-internalizing, including CD20, CD45, TAG72, and CEA [75, 85, 115, 133, 142, 145, 148]. However, Liu *et al*. observed the fairly rapid internalization of radiolabeled anti-TAG72 and anti-CEA antibodies, with about 60% of the antibodies internalized by LS174T colon carcinoma cells after 5 h [128]. Similarly, although HER2 is thought to be an internalizing epitope, pretargeting using bispecific antibodies against HER2 mediated enhanced tumor accumulation *in vivo* [97, 106, 151]. Whether these apparently counterintuitive results are due to differences in antibody internalization kinetics between *in vitro* and *in vivo* conditions (e.g., differences in receptor density, receptor turnover rates, and/or endocytosis and cell signalling pathways), dosing of the pretargeting molecules at sufficiently high levels that compensate for loss due to antigen/BsP internalization, or other factors remains unknown.

While the pretargeting molecule should initially remain non-internalized, many therapeutics require intracellular delivery to be effective and/or exhibit maximal potency; thus, the ideal pretargeted nanoparticle must be internalized only after binding of the drug carrier (Figure 1). Although internalization mediated by a non-internalizing pretargeting molecule may appear paradoxical, cellular entry could be achieved by relying on the eventual endocytosis of bound receptors or, more preferably, through multivalent nanoparticle binding effects such as crosslinking of receptors. Mulvey *et al*. observed that anti-A33-MORF conjugates remained stably on the surface of LS174T cells for up to 24 h, and that the addition of complementary MORF-modified carbon nanotubes resulted intracellular punctate staining indicative of internalization (Figure 6) [148]. In contrast, free complementary MORFs failed to induce internalization (Figure 6). Gunn *et al*. similarly reported that iron oxide nanoparticles pretargeted to CD20-expressing cells were found in endosomes, as visualized by transmission electron microscopy [140]. These results suggest that BsPs that bind non-internalizing epitopes can still facilitate pretargeted intracellular delivery of nanocarriers.

Pharmacokinetics and biodistribution

The theoretical improvements in the therapeutic index of drug delivery systems that can be achieved using pretargeting are based on the decoupling of the tumor targeting vs. drug-carrying functions. This in turn implies that the efficacy of a given pretargeted system is dependent on the pharmacokinetics and biodistribution of each component. One of the most important requirements is that the pretargeting BsPs are maximally cleared from systemic circulation prior to the administration of the drug carrier, particularly for pretargeted systems based on high affinity binding pairs such as SA-biotin. Indeed, SA-coated liposomes were detectable in circulation for at least 24 h after i.v. administration in mice, whereas SA-coated liposomes premixed with biotinylated anti-Thy1.2 antibodies prior to dosing were rapidly cleared within 4 h [145], illustrating the potential problem of circulating BsP binding to effector nanoparticles before the particles can extravasate into the tumor. Correspondingly, Karacay *et al*. found that, while an anti-CEA IgG x anti-DTPA Fab' conjugate demonstrated superior tumor labeling relative to F(ab')$_2$ x Fab' and Fab' x Fab' constructs, the F(ab')$_2$ x Fab' conjugate provided better pretargeting of a divalent DTPA peptide due to the high residual blood concentration of IgG x Fab' even 6 days after administration [100]. In order to simultaneously optimize tumor distribution and retention along with systemic clearance, a variety of techniques have been used to modify the size, valency, and composition of pretargeting BsPs, including the "dock-and-lock" method [152, 153] and fusion protein engineering [85, 115, 125].

An alternative approach to ensure elimination of residual pretargeting molecules from the systemic circulation is the use of clearing agents (CAs) prior to the dosing of nanoparticles or therapeutic effector molecules. These multivalent agents are generally designed to bind tightly to the pretargeting molecules and are sufficiently large enough to be rapidly cleared from the systemic circulation without extravasating into tumors. Previously reported CAs include secondary antibodies [154] and avidin [155], as well as biotinylated and galactosylated human serum albumin [156] and dendrimers [78, 115]. The use of a CA can effectively purge circulating BsP molecules (reducing blood concentrations by up to 10-fold) without affecting the tumor accumulation of pretargeting molecules [115, 155, 157].

The potential drawback of CA use is the addition of yet another dose and wait step to the course of therapy. For example, the use of CAs with the sequential combination of biotinylated antibodies, SA, and finally biotinylated radionuclide resulted in a *5-step* PRIT strategy (biotinylated MAb/avidin CA/streptavidin/biotinylated CA/biotinylated radiolabeled chelate) [158, 159] Although the radioimmunotherapy was well-tolerated and effective in glioma patients, with a median survival of 33.5 months (compared to 8 months for untreated control patients) in a nonrandomized phase I/II study, the need for several parenteral injections to deliver a single dose of radiation or drug not only introduces a high degree of complexity but also increases the cost of therapy. A simpler 2-step approach is likely far more preferable, particularly when using antibody-hapten binding pairs. These pretargeted systems appear to better tolerate the presence of minute amounts of uncleared pretargeting BsP, possibly due to the lower affinity and the dissociation of BsP-effector complexes formed in the blood [160].

The pharmacokinetics of the pretargeted drug carrier must also be taken into consideration. Because the commonly utilized pretargeting molecules are generally much smaller than nanoparticle drug carriers, the overall tumor distribution and accumulation of pretargeted systems is therefore limited by the circulation and extravasation kinetics of the drug carrier. To minimize premature elimination from the circulation and maximize tumor accumulation, drug carriers should be effectively coated with stealth polymers, whereas the use of of bulky, charged, and/or hydrophobic moieties to facilitate particle binding to the pretargeting molecule should be avoided if possible. As noted in the previous section, this latter requirement may affect the choice of binding pair technology for pretargeted nanoparticle systems, as well as the assignment of binding pair components to the BsP and effector particle. For example, if using a SA-biotin binding system, the nanoparticle should be biotinylated, with the SA component in the pretargeting molecule, rather than vice versa.

Challenges and unknowns

The combination of a bispecific pretargeting cocktail with nanoparticle drug carriers is a promising but vastly underexplored approach to targeting nanoparticles to heterogeneous tumors. Thus, many aspects of this proposed strategy must be rigorously evaluated to confirm its suitability for clinical applications.

One of the major challenges is that a greater dose of pretargeting BsP could potentially reduce nanoparticle binding and accumulation to tumor cells. Because tumor receptor expression varies both spatially and temporally, and receptor testing is typically performed on primary tumor biopsies obtained close to the time of diagnosis, “personalized” pretargeting cocktails based on those patient biopsy results is unlikely to capture the full heterogeneity of cancer cells in a patient over time, particularly for relapsed and/or highly metastatic tumors [6]. Thus, truly personalized pretargeted therapy would greatly benefit from improvements in noninvasive molecular profiling of cancers [56, 161]. As an alternative to the fine-tuning of individual pretargeting cocktails, the properties of BsPs could be optimized to allow rapid elmination of non-binding BsPs from the circulation. The mechanism, rate, and extent of pretargeting BsP clearance with and without the use of clearing agents must be carefully investigated, particularly since Pagel and colleagues

observed that the administration of high doses of MAb-SA conjugates specific to receptors poorly expressed on certain lymphoma tumors overloaded the capacity of mice to hepatically clear MAb-SA/CA complexes, resulting in low tumor-to-normal organ biodistribution ratios and toxicity [79, 162]. The increased doses of total protein required for a cocktail pretargeting approach may also affect the immunogenicity of the pretargeting BsPs used.

Additionally, the limited number of appropriate target receptor/antibody combinations that have been evaluated for pretargeting to date may hinder the development of useful pretargeting cocktails. The main driving forces behind the discovery of novel tumor-specific receptors and their corresponding ligands/antibodies are diagnostic biomarkers, imaging applications and targeted drug and MAb therapy. Unfortunately, few of these studies focus on *non-internalizing* antibodies, a critical requirement of pretargeting. However, the use of (pre)clincially validated ligands and therapeutic MAbs could lead to fortuitous combinations for pretargeting. For example, anti-CD20 MAbs can induce apoptosis clinically [163], and anti-CD20 Fab' fragments linked to MORFs have been found to also induce apoptosis of B-cell lymphomas *in vitro* and inhibit development of diffuse tumors *in vivo* upon crosslinking by cMORF-modified polymers [141]. The use of antibodies with inherent therapeutic efficacy for pretargeting of drug carriers could allow for synergistic treatment effects. Improvements in the generation of diverse bispecific proteins and antibodies will also certainly expand the diversity of available pretargeting molecules [152, 153, 164].

Other concerns regarding the application of pretargeted drug delivery systems include the clinical feasiblity of multistep parenteral injections and the poor tumor accumulation of many drug carriers in patient tumors. Similar to passively and actively targeted nanoparticles, the tumor accumulation of pretargeted drug carriers would still rely on the EPR effect [145], which has been found to be highly variable [36].

Conclusion

Despite marked advances in biotechnology, nanotechnology and drug delivery, effective therapy for cancer remains exceedingly challenging, with few treatment options that can provide durable suppression or elimination of the tumor without resulting in eventual recurrence and/or the development of drug-resistant tumors. Emerging insights into tumor physiology have underscored tumor heterogeneity as one of the key bottlenecks to targeted therapy. The concept of pretargeting using a cocktail of bispecific pretargeting proteins combines the strengths of precision medicine and personalized medicine by offering the potential to deliver nanoparticle therapeutics to diverse cell populations while avoiding the pharmacokinetic pitfalls typically associated with actively targeted nanoparticles. Although the radioimmunotherapy field has offered substantial evidence supporting the pretargeting strategy, its application for enhancing targeted delivery of nanoparticle therapeutics remains underexplored to date. We believe further rigorous evaluation of pretargeted NP systems is both warranted and needed to confirm whether pretargeting can indeed prove superior to current passive and active targeting approaches.

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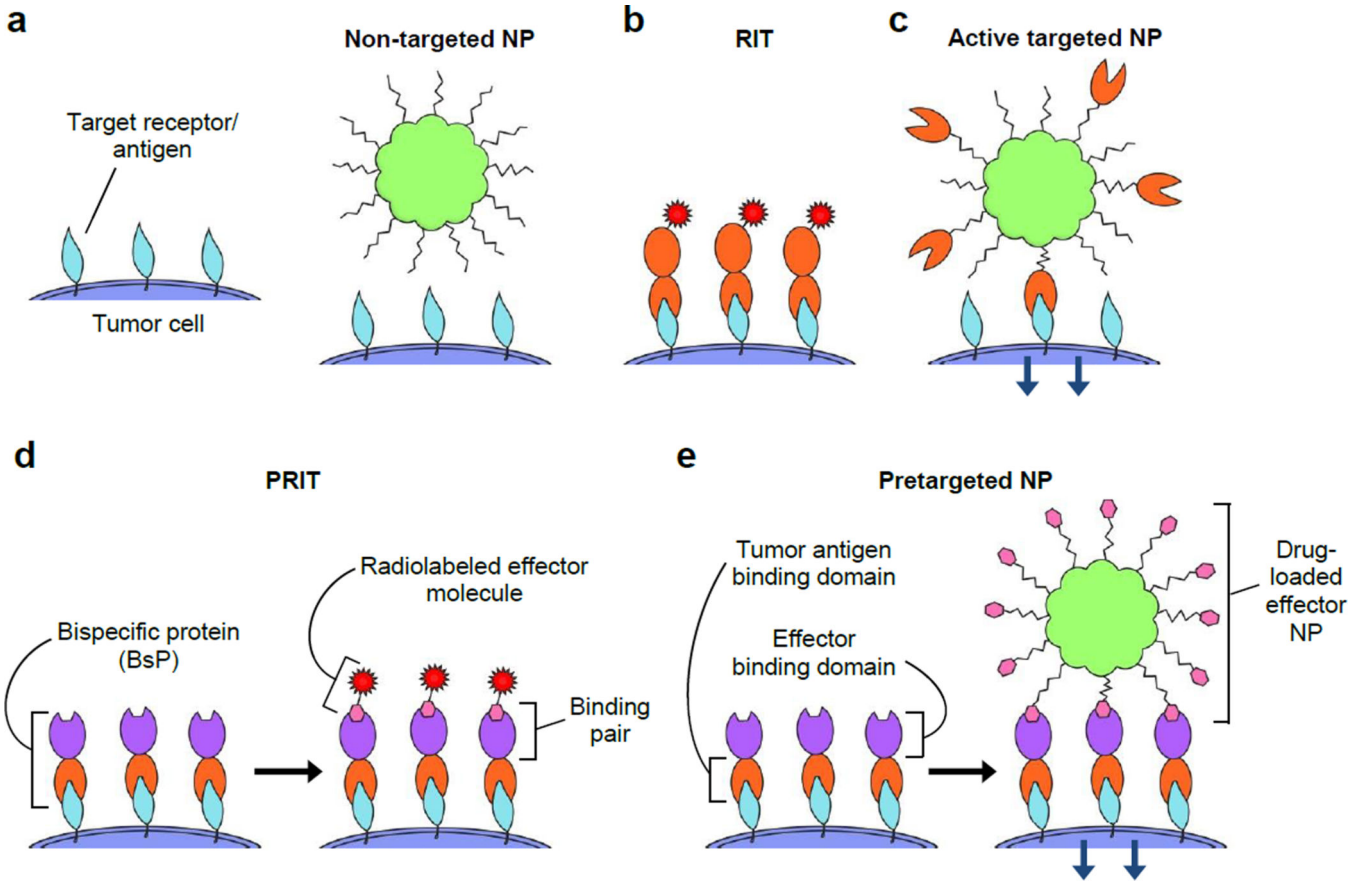

Figure 1.

Strategies for the delivery of nanoparticle drug carriers and/or radioisotopes to tumor cells include **a**) non-targeted, **b** & **c**) directly targeted (1-step), and **d** & **e**) pretargeted (multistep) approaches. **a**) Passively targeted nanoparticles coated solely with stealth polymers typically do not exhibit specific interactions with tumor cells. **b**) Radioimmunotherapy (RIT) uses radiolabeled tumor receptor-specific antibodies to deliver therapeutic doses of radiation to target cells. **c**) Modification with receptor-specific ligands allows the active targeting of nanoparticles (NPs) to tumor cells, which commonly induces receptor-dependent internalization. **d**) Pretargeted radioimmunotherapy (PRIT) splits tumor targeting and radioisotope delivery into sequential steps: 1) binding of bispecific proteins (BsPs) to target receptors and 2) binding of radiolabeled effector molecules to the BsPs. **e**) For pretargeted drug delivery systems, 1) bispecific proteins (BsPs) bind target receptors, and 2) a drug-loaded effector nanoparticle binds to the BsPs, which should ideally result in internalization.

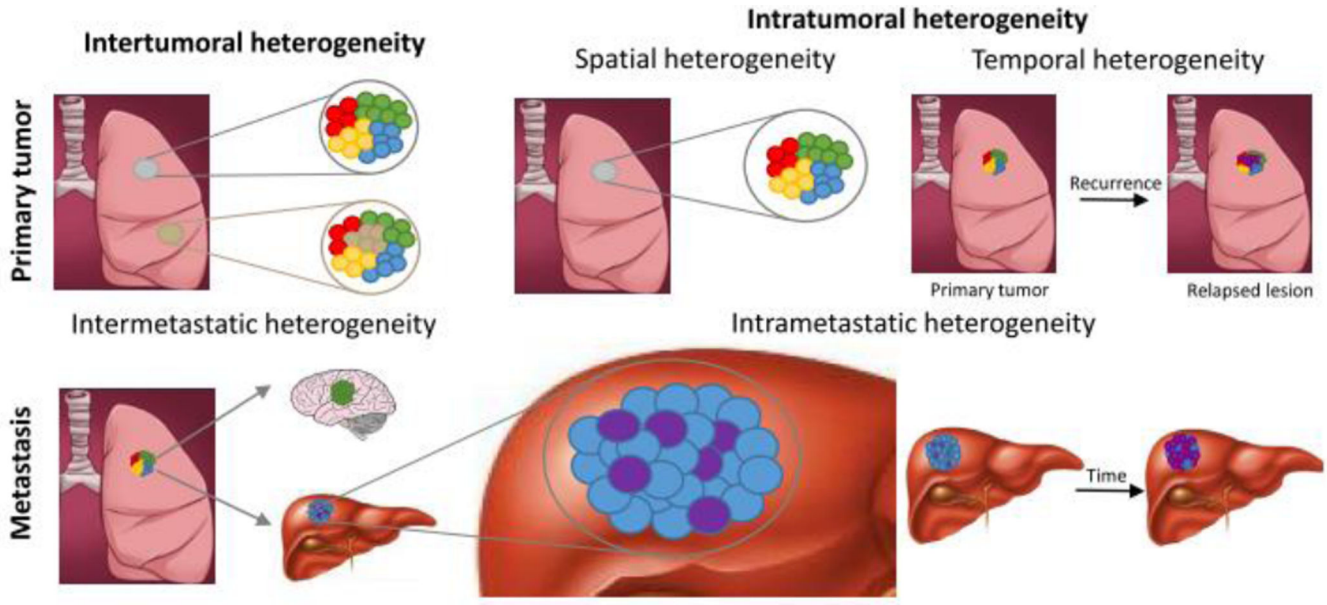

Figure 2.

Different types of tumor heterogeneity. Spatial heterogeneity refers to differences between distinct anatomical regions or individual cells within a tumor, while temporal heterogeneity illustrates changes in a tumor's molecular profile over time. Intermetastatic heterogeneity arises from distinct subclonal populations in the primary tumor, and intrametastatic heterogeneity reflects the discordant molecular profiles of cells within individual metastases.

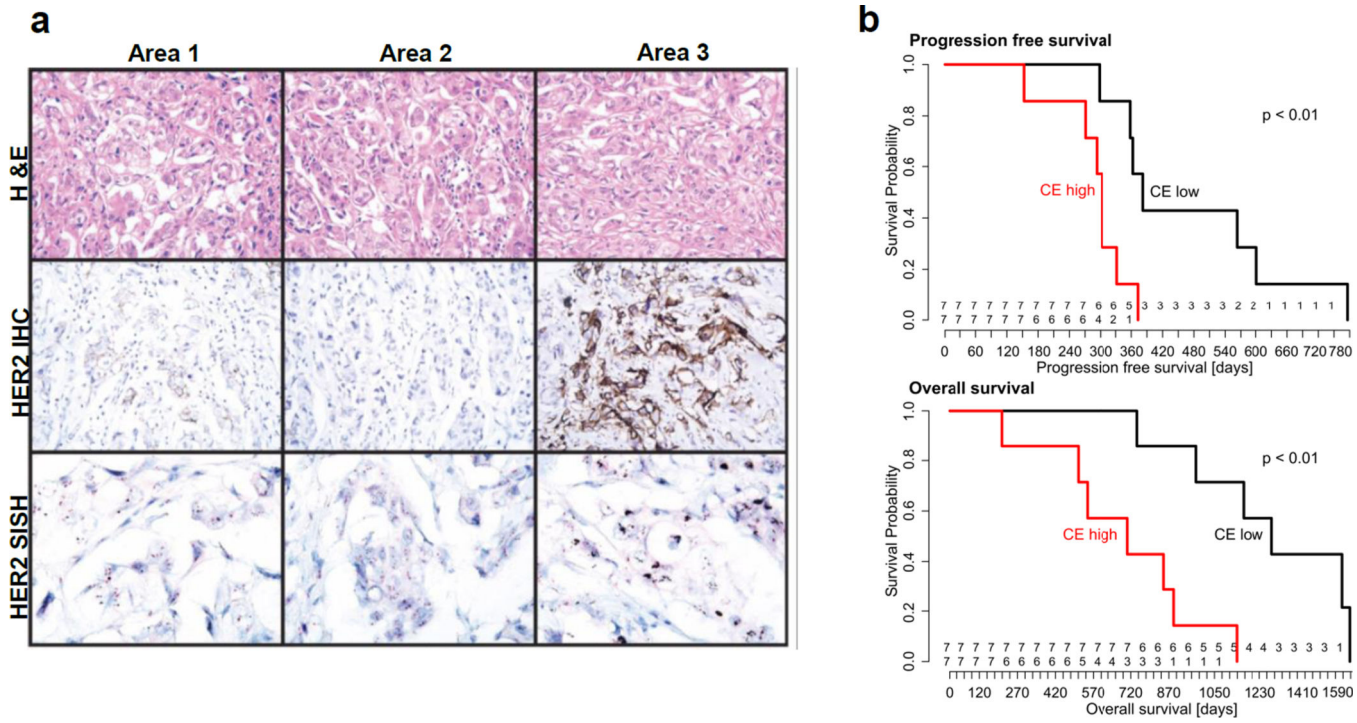

Figure 3.

a) Spatial heterogeneity in HER2 expression between three different areas of an invasive ductal carcinoma biopsy sample. HER2 amplification was confirmed using immunohistochemistry (IHC) and silver in situ hybridization (SISH). H&E, ×200; IHC, ×200; SISH, ×400. Reprinted with permission from Lee *et al*. [57]. **b**) Progression free survival (top) and overall survival (bottom) of high-grade serous ovarian cancer patients treated with platinum-based chemotherapy and surgery, stratified by degree of clonal expansion (CE). CE reflects the accumulation of mutations that promote cell expansion into varying subclonal populations from the original cell. Higher CE is correlated with divergent subclonal populations and thus greater tumor heterogeneity. Reprinted with permission from Schwarz *et al*. [58].

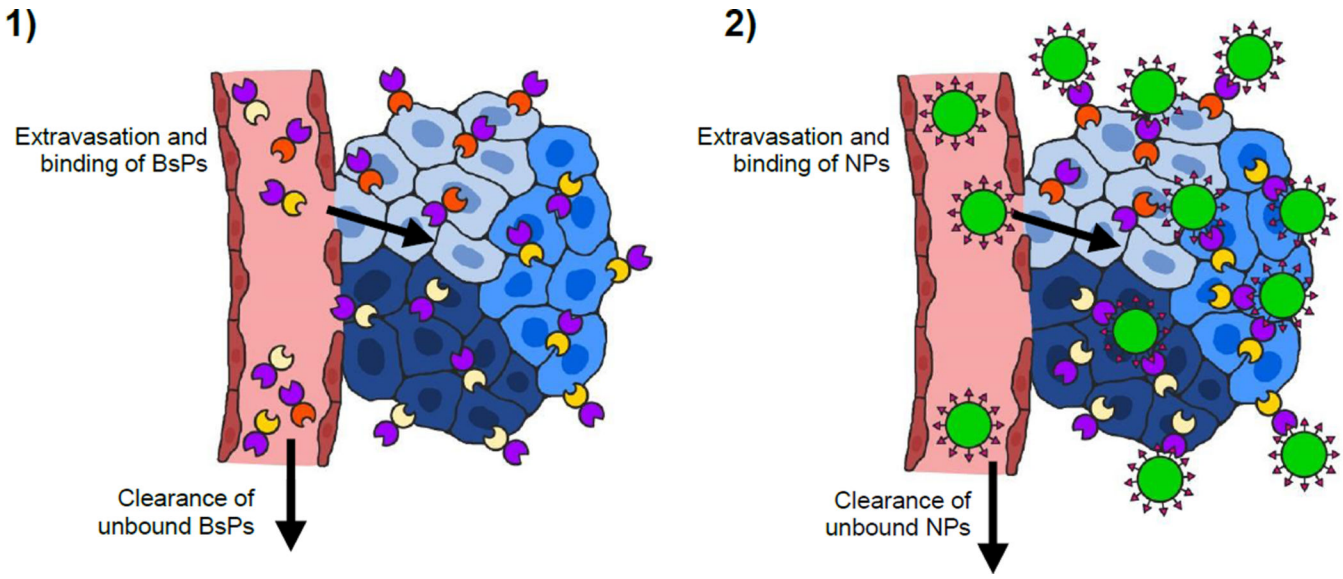

Figure 4.

Pretargeted delivery of nanoparticles (NPs) to heterogeneous tumors. 1) A cocktail of bispecific proteins (BsPs) is administered and allowed to fully clear from systemic circulation prior to 2) dosing with nanoparticles that can be captured by BsPs on the tumor cell surface. To enable effective targeting of multiple tumor cell subpopulations using a single nanoparticle, the tumor antigen-binding domain (Figure 1e) of the BsPs can be modified to reflect the full diversity of tumor cells, while the effector (NP)-binding domain remains the same for all BsPs.

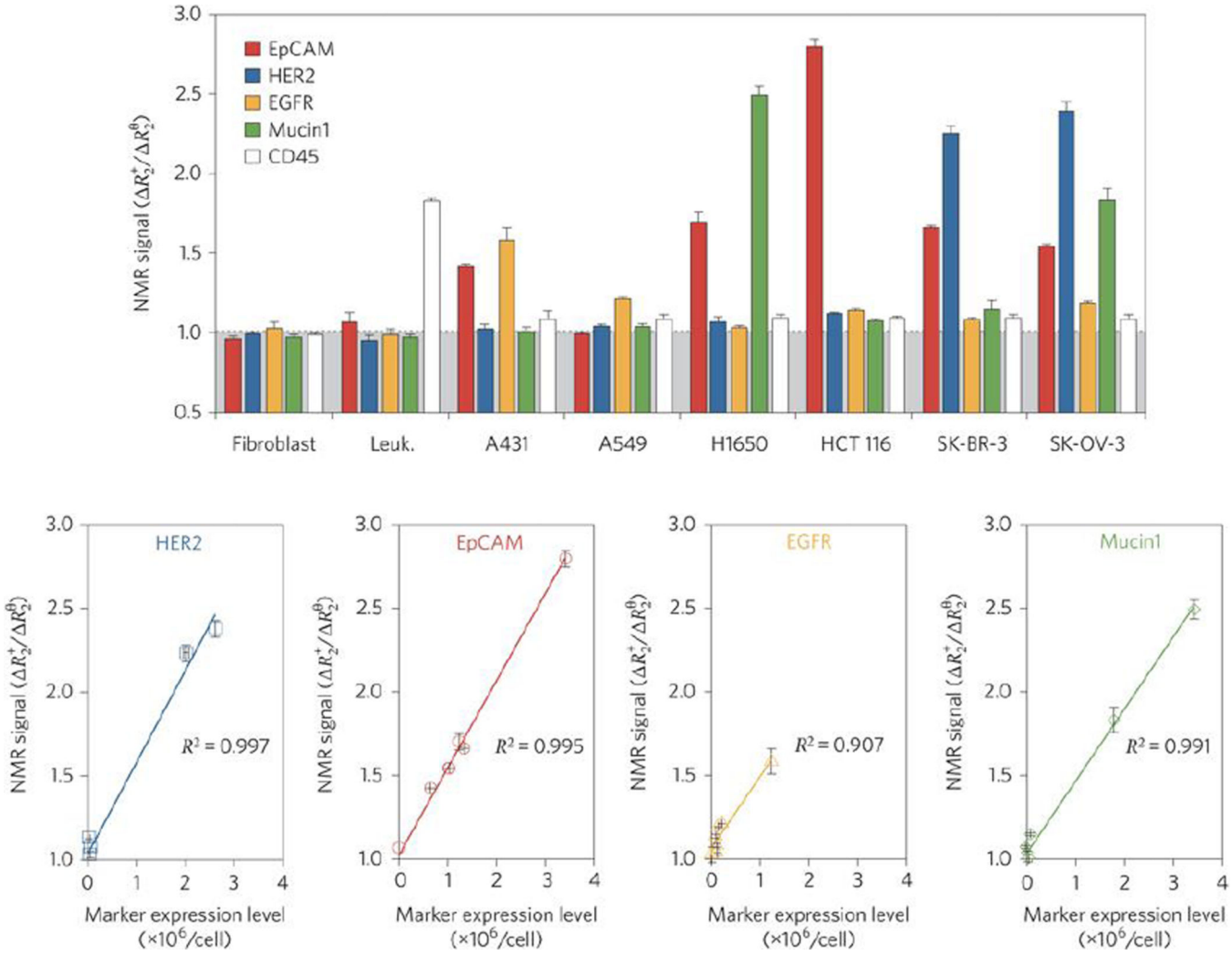

Figure 5.

Diagnostic magnetic resonance profiling of human tumor cell lines, fibroblasts, and leukocytes using a pretargeted approach *in vitro*. The cells were labeled using various *trans*-cyclooctene (TCO)-conjugated antibodies followed by tetrazine-modified magneto-fluorescent nanoparticles (Tz-MFNPs) prior to the measurement of the transverse relaxation rate (R_2). Figure reprinted with permission from Haun *et al*. [95].

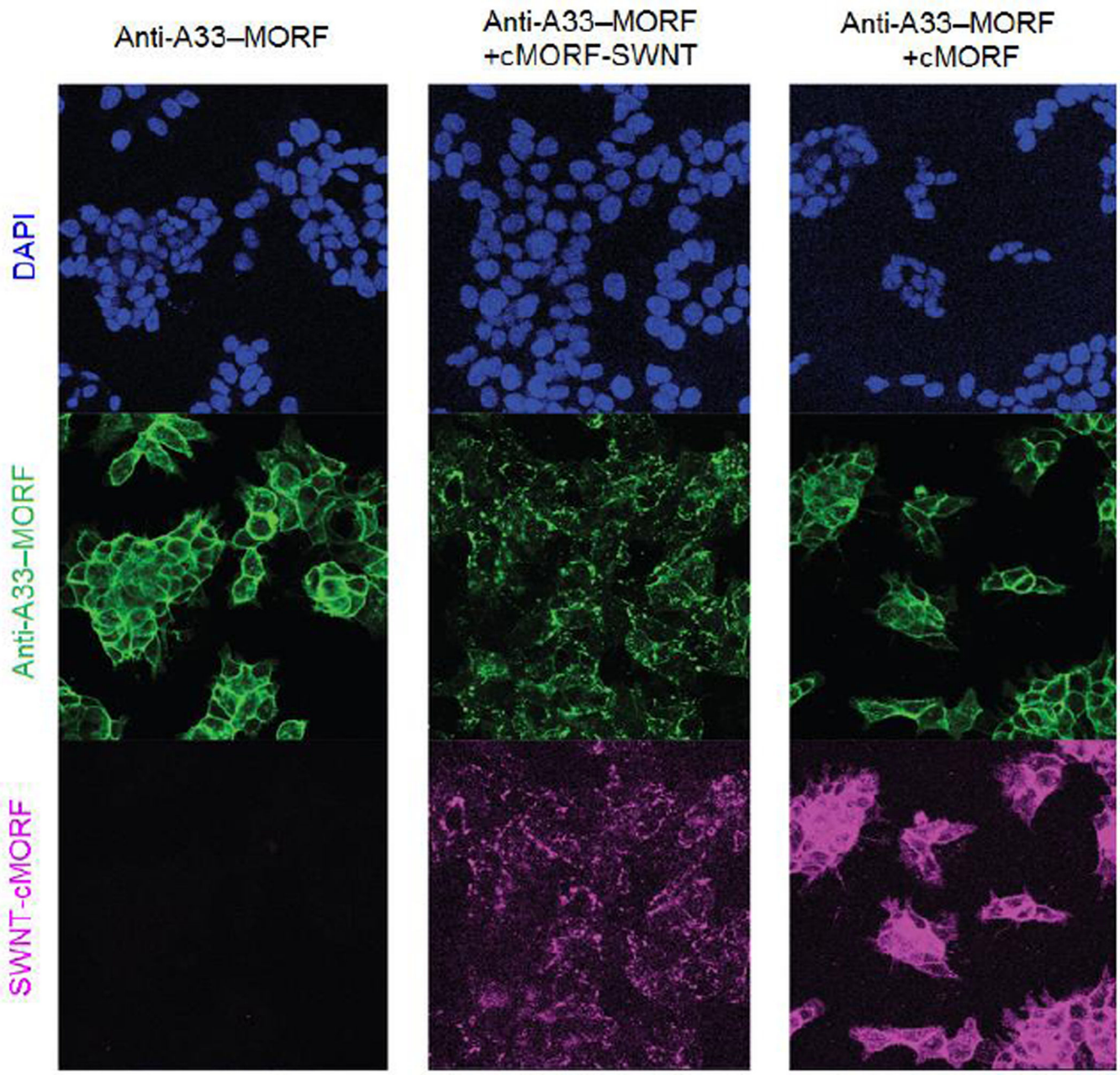

Figure 6.

Internalization of pretargeted single-walled carbon nanotubes (SWNTs). LS174T (A33-positive) colon carcinoma cells were preincubated anti-A33 antibodies conjugated to morpholino oligonucleotide (anti-A33-MORFs) for 4 h prior to washing and further incubation with complementary MORF (cMORF)-SWNT-AlexaFluor 647 or free cMORF-AlexaFluor 647. Figure reprinted with permission from Mulvey *et al*. [148].