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# Nanomedicine Tumor Targeting

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**Nanomedicines are extensively explored for cancer therapy. By delivering drug molecules more efficiently to pathological sites and by attenuating their accumulation in healthy organs and tissues, nanomedicine formulations aim to improve the balance between drug efficacy and toxicity. More than 20 cancer nanomedicines are approved for clinical use, and hundreds of formulations are in (pre)clinical development. Over the years, several key pitfalls have been identified as bottlenecks in nanomedicine tumor targeting and translation. These go beyond materials- and production-related issues, and particularly also encompass biological barriers and pathophysiological heterogeneity. In this manuscript, the author describes the most important principles, progress, and products in nanomedicine tumor targeting, delineates key current problems and challenges, and discusses the most promising future prospects to create clinical impact.**

## 1. Introduction

Cancer therapy relies on (combinations of) surgery, radiotherapy, chemotherapy, molecularly targeted therapy, hormone therapy, and immunotherapy. The choice of treatment depends on tumor type, location, and stage, as well as on the general well-being of the patient. Complete tumor removal is the ultimate goal of therapeutic intervention and can – in case of early-stage disease – typically be achieved using surgery. In advanced stages, however, cancers invade healthy tissue and metastasize to distant sites, thereby compromising the success of surgery and calling for combination regimens involving systemic drug therapy.

Anticancer drug therapy is often only moderately effective. This is because the majority of drugs do not accumulate well in tumors and metastases. Moreover, anticancer drug treatment typically comes with severe side effects, as the agents have a large volume of distribution and therefore localize in healthy organs and tissues. It is important to note that this situation does not only apply to classical chemotherapeutics, but also to molecularly targeted drugs, like kinase inhibitors, which also present with suboptimal biodistribution and tumor targeting profiles, and which therefore also often show suboptimal therapeutic efficacy and significant off-target toxicity.

To improve the therapeutic index of anticancer drugs, many different drug delivery systems have been designed and evaluated over the years.[1–3] These include liposomes, polymers, proteins, and micelles, as well as nature-derived and inorganic nanomaterials (**Figure 1**). Besides such synthetic nanomedicine formulations, which oftentimes originate from chemical engineering, materials science, and pharmaceutical technology laboratories, also biotechnologically produced delivery systems are evaluated for tumor targeting, represented most prominently by antibody-drug conjugates. The former are mostly used for standard chemotherapeutic drugs, like doxorubicin and paclitaxel, while the latter are traditionally employed for highly potent toxins, such as auristatin and emtansine.[4,5] The former have furthermore been attracting a lot of attention for nucleic acid delivery, as exemplified by the successful clinical development of lipid nanoparticles for siRNA targeting hepatocytes and mRNA-based vaccination strategies.[6,7]

The field of cancer nanomedicine has made significant progress in the last couple of decades. Building upon fundamental liposome and polymer work performed in the 1960s to 1990s, upon the FDA approval of Doxil as the first anticancer nanodrug in 1995, and upon dozens of subsequent success stories and failures, several important steps forward have been made, and a number of important lessons have been learned.[8–12] In the present manuscript, I first re-visits the basic concepts of nanomedicine tumor targeting, subsequently summarizes the current clinical landscape, then address key problems and challenges in cancer nanomedicine development and translation, and finally discuss the – in my opinion – most promising future directions for the use of nanomedicines for cancer therapy.

## 2. Principles

### 2.1. Passive Tumor Targeting

Nanomedicine tumor targeting is traditionally ascribed to the enhanced permeability and retention (EPR) effect. EPR was first described by Yasuhiro Matsumura and Hiroshi Maeda in 1986, who employed six different radiolabeled macromolecules to show that a prolonged circulation half-life time in the blood contributes to increased accumulation in intra/subcutaneous

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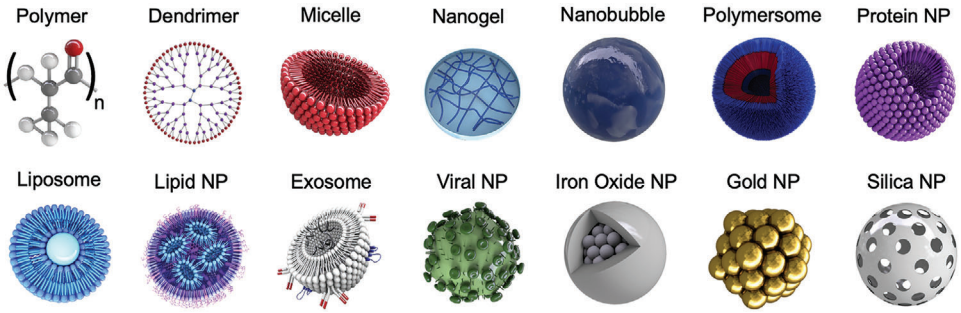



**Figure 1.** Nanomaterials explored for tumor targeting and anticancer therapy. Over the years, many different 1–100(0) nm-sized materials have been designed and evaluated for drug delivery to tumors. These predominantly include nanocarriers based on lipids and polymers, but also protein-based, nature-derived, and inorganic nanoparticles have been evaluated. Image adapted with permission.[13]

sarcoma tumors in mice.[14] In line with work published in the same year by Rakesh Jain and colleagues on tumor microvascular permeability,[15] Matsumura and Maeda also employed Evans blue dye (EBD; which binds to long-circulating endogenous albumin) and postulated that three main features are responsible for efficient macromolecular tumor targeting: 1) Tumor hypervascularization, 2) enhanced vascular leakiness in tumors, and 3) lack of effective vascular and lymphatic drainage from tumors.[14,16] As this type of EPR-based tumor delivery only relies on pathophysiological features, and not on the use of active recognition motifs, it is commonly referred to as passive tumor targeting (**Figure 2a**).

#### *2.1.1. Expanding Passive Targeting Principles*

Recently, several (patho)physiological features have been added to the historic features of EPR-based tumor targeting. Beyond "passive" vascular leakiness, also "active" energy-dependent transcytosis across endothelial cells has been reported to contribute to nanoparticle entry into solid tumors.[17] In addition, it has been demonstrated that circulating phagocytes can carry nanoformulations to and into tumors. This mainly involves neutrophils and macrophages and occurs predominantly for nanocarriers which are modified with ligands that are recognized by phagocytes, such as the RGD peptide motif.[18,19] Moreover, it has been shown that nanoparticles actually do exit tumors via lymphatics, and it has become appreciated that it is the presence of and nanomedicine uptake by tumor-associated macrophages (TAM; acting as reservoirs) that result in retention.[20–22]

#### *2.1.2. Identifying Passive Targeting Issues*

The EPR effect and passive tumor targeting have been challenged and criticized increasingly extensively over the years.[23,24] First and foremost, it is to be considered that passive tumor targeting differs from tumor type to tumor type, and from lesion to lesion, even within a single patient. This is because – in line with cancer's notorious heterogeneity – not only the vascular permeability (and/or transcytosis) in tumors vary significantly between different lesions, but also features such as vascular density, vascular perfusion, tumor cellularity, and tumor stroma composition are highly variable inter- and intra-individually. Taking this into account, from a less conceptual and more realistic perspective (Figure 2A), one can deduce that tools are needed to address this heterogeneity (see Section 4), and also that additional approaches addressing problematic pathophysiological features may be needed to ensure efficient tumor targeting and antitumor therapy.

#### *2.1.3. Addressing Passive Targeting Issues*

In difficult-to-target tumors, such as pancreatic cancer, complicating pathophysiological features can include inefficient tumor perfusion (due to stroma-induced vessel compression), suboptimal vascular permeability and transcytosis, as well as poor tumor penetration. When injecting fluorophore-labeled 100 nm-sized PEG-liposomes in mice with highly stromal pancreatic tumors, a reasonable degree of EPR-based tumor accumulation is observed at 72 h after i.v. injection, averaging at 1–2% of the injected dose, which is not per se a bad number for passive tumor targeting in mice.[25–27] However, as exemplified in Figure 2b, hardly any of the extravasated liposomes manage to properly penetrate beyond the perivascular space, and they are thus hardly able to interact with cancer cells, which are typically located 2–3 cell layers (plus multiple collagen fiber layers) beyond the vasculature. When using externally applied ultrasound plus i.v. administered microbubbles – together typically referred to as sonoporation or sonopermeation[28,29] – to enhance tumor blood vessel perfusion, permeability, and penetration, it can be seen that via physically massaging the blood vessels and tumor stroma from the vascular side, the extravasation and penetration of liposomes can be substantially improved (Figure 2c). When quantified via 3D fluorescence microscopy analysis, the fraction of liposomes distributing beyond the vascular compartment and penetrating deep into the tumor interstitium was found to be increased from less than 10% for untreated control conditions to more than 50% for sonopermeation priming treatment (Figure 2d).[25]

#### *2.1.4. Strategies to Enhance Passive Targeting*

There are several additional physical and pharmacological tools to improve passive tumor targeting.[30–32] Besides ultrasound and

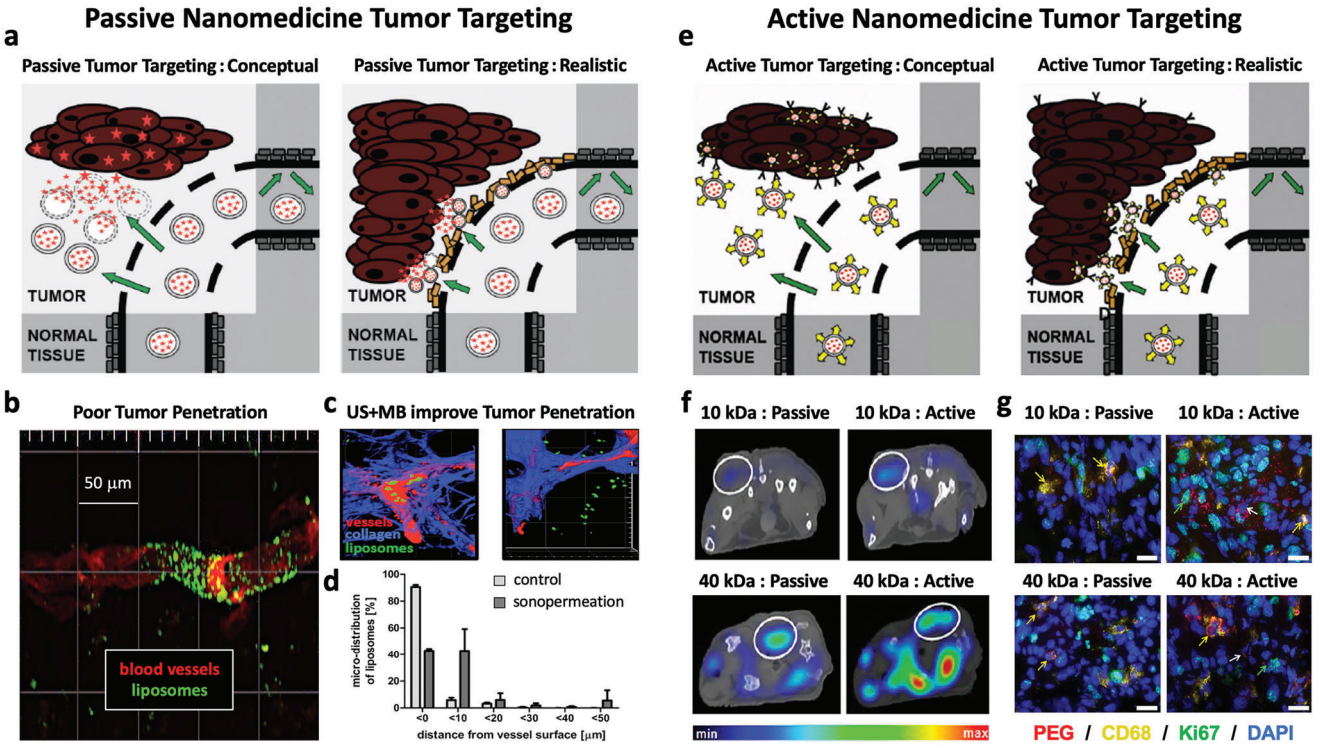



**Figure 2.** Basic principles of nanomedicine tumor targeting. a) Passive tumor targeting is traditionally ascribed to the EPR effect. More recently, also endothelial transcytosis has been shown to play a role. It is important to take into account that in realistic scenarios, not all tumor blood vessels are perfused, not all blood vessels are leaky, that penetration is typically poor, and that the intratumoral distribution of nanoparticles thus tends to be suboptimal. b) Two-photon microscopy image demonstrating how poor liposome penetration beyond the endothelium into the tumor interstitium can be, even in mouse tumors. c,d) Two-photon microscopy images and quantification showing liposome trapping (in green) in the perivascular space in pancreatic cancer xenografts in mice under untreated control conditions versus locally priming tumors with combined ultrasound and microbubbles (i.e., sonopermeation), which substantially enhances liposome extravasation and penetration. e) Basic principles of active nanomedicine tumor targeting. While it is often assumed that active targeting enhances the overall levels of nanomedicine localization in tumors, it in realistic scenarios typically only enhances uptake by target (tumor) cells. Moreover, it is important to understand that active targeting suffers from all the same pathophysiological constraints as passive tumor targeting, such as poor perfusion, poor extravasation, poor penetration, and poor distribution, since active ligand interaction with cancer cells only takes place at the end of the in vivo drug delivery process. f,g) Systematic studies on passive versus active tumor targeting with small (10 kDa; low EPR) versus large (40 kDa; high EPR) show that active targeting only enhances macroscopic whole-tumor accumulation in case of poor passive targeting (i.e., for the 10 kDa polymer). Conversely, at the microscopic level, for both 10 and 40 kDa polymers, active targeting enhances specific target/cancer cell uptake (versus very dominant passive uptake in tumor-associated macrophages). Images reproduced with permission.[9,25,52]

microbubbles, amongst others, radiotherapy, hyperthermia, and photodynamic therapy can help to boost tumor accumulation. Pharmacologically, increasing blood pressure in primary and metastatic liver tumors to promote perfusion and extravasation has been evaluated in animal models and in patients via intra-arterial angiotensin administration.[33] In addition, strategies that pharmacologically promote vascular permeability have been explored, including tumor necrosis factor (TNF), and agents that generate nitric oxide (NO) and carbon monoxide (CO).[34–36] Last but not least, pharmacologically inhibiting the activity of cancer-associated fibroblasts, using agents such as losartan or tranilast, has been shown to potently prime the tumor vasculature and microenvironment for more efficient passive nanomedicine accumulation.[37–39] A key advantage of physical tumor priming is local control over treatment parameters, for example, accurate localization, timing, and temperature/ultrasound exposure. The most important downside is that physical priming can only be applied to locally confined disease, and thus are not very useful in case of metastatic cancer. Conversely, a key advantage of pharmacological priming to enhance passive tumor targeting is that it is more broadly applicable in case of systemic disease. The main disadvantage is suboptimal control over spatial and temporal treatment parameters, due to the inability to control drug concentrations (and concentration windows) in tumors and metastases. In general, it can be concluded that physical and pharmacological priming hold significant promise to improve passive tumor targeting and cancer nanomedicine treatment efficacy.

### 2.2. Active Tumor Targeting

Active tumor targeting is based on the use of recognition motifs that bind to receptors (or other structural features) present on cancer cells, tumor endothelial cells, and/or other cells present within the tumor microenvironment. Also extracellular matrix (ECM) components, like collagen or fibronectin, can serve as structures for active targeting.

#### 2.2.1. Antibodies

Traditionally, antibodies and peptides are employed as carrier materials for active tumor targeting. This has resulted in a number of clinical products. In 2000, gemtuzumab ozogamicin (Mylotarg) became the first antibody-drug conjugate (ADC) to be approved for clinical use. Mylotarg targets the CD33 antigen on myeloid cells in patients with acute myeloid leukemia (AML), and like all ADC, it has a relatively low drug loading capacity (i.e., a drug-to-antibody-ratio (DAR), typically around 2–4). Being a pioneer product, Mylotarg was voluntarily withdrawn from the market for several years, as a result of issues related to both efficacy and toxicity, but it was later re-introduced, at a lower dose, for a different patient population, and as part of a different chemotherapy combination regimen. In the 2010s, a good number of ADCs followed in the footsteps of Mylotarg, and currently, ≈20 different formulations are approved for clinical use[4,5] (which is similar to the number of approved cancer nanomedicines; see Section 3). Initially, ADC almost exclusively targeted hematological cancers, such as leukemias and lymphomas. Over time, paralleled by exponential progress in the development of monoclonal antibody therapeutics, also solid malignancies have gradually become addressed. The most prominent examples in this regard are Kadcyla and Enhertu, which are both based on trastuzumab (Herceptin; which binds to the human epidermal growth factor receptor 2; HER2), and which are conjugated to the highly potent cytotoxic agents emtansine and deruxtecan, respectively. Especially the latter is attracting a lot of attention, as it is able to achieve impressive efficacy results also in tumors with low HER2 expression levels.[40,41]

#### 2.2.2. Peptides

Besides antibodies, multiple other biotechnological carriers have been explored for active targeting. These include peptides, single-chain antibodies, affibodies, nanobodies, diabodies, and aptamers. Peptides have clinically had the biggest impact in oncology, albeit almost exclusively for radionuclide delivery, and not for classical drug delivery. The best-known compound in this class of peptide receptor radionuclide therapeutics (PRRT) is LutaThera, which is based on the 8-amino-acid targeting ligand oxodotreotide coupled to the chelator DOTA, which can complex both gallium-68 (for radionuclide imaging) and lutetium-177 (for radionuclide therapy).[42] In a typical clinical scenario, patients with neuroendocrine tumors are first administered the peptide-based imaging agent, employing positron emission tomography (PET) to visualize and quantify the extent of tumor (and metastasis) targeting. This guides decision-making with regard to whether or not the peptide-based therapeutic agent Lutathera is given. This is a classic example of theranostics, and it exemplifies how imaging biomarkers can be used for patient stratification, thereby contributing to more individualized interventions and improved treatment outcomes.[43,44]

#### 2.2.3. Active Targeting in Nature

Active targeting has, for obvious reasons, become very popular in nanomedicine and tumor targeting. One needs to consider, however, that active targeting of antibodies and peptides is fundamentally different from active targeting of nanocarrier materials, particularly if the latter are typically way beyond 35 nm in diameter. A suitable starting point for appreciating and understanding these differences can be obtained by looking at and learning from nature. In our bodies, evolution has generated IgG antibodies – which are ≈15 nm in size – for antigen targeting in the bloodstream, in the lymphatic system, and in extracellular fluid in tissues. Conversely, IgM antibodies – which are ≈35 nm in size – are almost exclusively localized and active in the bloodstream and in the lymphatic system, which aligns with their naturally evolved function as the first antibodies produced by the body in response to new infections. Consequently, it appears as if nature, via many years of evolution, has designed antibody vectors in the range of 10–15 nm for whole-body multi-compartment targeting, while vectors with larger sizes (upwards of 35 nm) appear to be primarily generated for targeting antigens and cells in the systemic circulation.

#### 2.2.4. ADC versus Nanomedicines

Theoretically, based on the European Science Foundation definition,[45] ADC can be categorized as nanomedicines. This is because their size dimensions are in the right 1–100(0) nm range and because they contain both a carrier moiety and an active pharmaceutical ingredient. Historically, however, the ADC field and the nanomedicine field have been conceived, perceived, progressed, and translated very differently. More details on this are provided in Section 4. Of relevance in the current context is that the size of ADC (≈15 nm) is typically smaller than that of classical nanomedicines, that is, liposomes, protein condensates, and micelles, which tend to be ≈50 to 150 nm in diameter. In addition, ADCs are always "single molecules", that is, formulations in which the carrier and the drug are chemically covalently conjugated. Certain types of nanomedicines are also single molecules, for example, pegylated proteins, like pegaspargase (Oncaspar), which is used for the treatment of acute lymphoblastic leukemia,[46] or some of the traditional PHPMA- or PGA-based polymer-drug conjugates that have been evaluated in phase I-III clinical trials in the 1990s-2000s.[47–50] The sizes of these formulations typically are in the same range as those of ADC, that is, 5–15 nm.

#### 2.2.5. Active Nanomedicine Targeting

Drawing parallels from nature to nanomedicine, it seems obvious that for active targeting to extravascular structures, such as (receptors expressed on) cancer cells, particularly small-sized carrier materials are optimal, in the size range between 5 and 35 nm. In this context, one has to take into account that materials that are smaller than 5–10 nm are typically rapidly cleared from the bloodstream via renal filtration, and materials that are larger than 35 nm may not extravasate and penetrate tumors well enough to efficiently engage with extravascular receptors.[51,52] Consequently, when actively targeting cancer cells with materials such as liposomes, which typically have sizes of 80–150 nm, not much improvement in total tumor levels is to be expected, as the

initial phases of the delivery process, that is, prolonged circulation, extravasation, and penetration are completely independent of the presence of the targeting ligand (Figure 2e). Only after such relatively large nanocarriers have completed these initial steps, active ligand-receptor interactions can start setting in, resulting at best in 1) an increase in target cell uptake, versus otherwise predominant uptake by tumor-associated macrophages (TAM) in case of solely passive targeting and 2) an increase in tumor retention, by binding to and/or internalization by target cells, versus otherwise mainly retention mediated by uptake in TAM.

#### 2.2.6. Active Nanomedicine Tumor Targeting – Conceptual Studies

The above notions are in line with the observations obtained in key pioneering studies on active nanomedicine targeting in the literature, showing, for example, that 1) transferrin-modified gold nanoparticles and non-modified gold nanoparticles present with similar levels of overall tumor accumulation, but demonstrate a different level of cancer cell uptake and intratumoral distribution,[53] and that 2) HER2 antibody-modified PEG-liposomes have similar tumor accumulation kinetics and peak levels as compared to normal passively targeted PEG-liposomes, with the only added value of active HER2-targeting being slightly slower clearance of the liposomes from tumors, as evidenced via a 5–10% increase in tumor area-under-the-curve (AUC).[54] Of importance in this regard is that these semi-positive results for active targeting only apply to situations where the presence (and/or the density) of the targeting ligand on the surface of the nanoparticle does not negatively affect its long circulation times. If that is the case, then the initial passive phases (i.e., circulation, perfusion, extravasation) of the combined passive plus active targeting process are affected to such an extent that overall tumor accumulation is decreased, rather than increased. One of the first (and unfortunately few; assumingly because many negative-results stories on failed active targeting remain unpublished) studies systemically showing this reported that trastuzumab modification of gold nanoparticles substantially reduced the blood circulation half-life times of the particles. As a consequence, less than half of the actively targeted gold nanoparticles ended up in HER2-positive tumors as compared to non-modified passively targeted particles.[55] In the decade that followed these initial active targeting studies, many papers have been published with more positive claims. However, in the majority of cases, the underlying rationale(s) for how active targeting actually improves (nano-)drug delivery and therapeutic efficacy are not properly touched upon and conceptually oversimplified, and they typically do not make much sense, at least not when considering the anatomical, physiological and pharmacokinetic issues discussed above. Several examples of such considerations are discussed and referenced below. Altogether, these notions to a large extent explain why thus far, hardly any actively targeted nanomedicines have made it into advanced stages of clinical trials.

#### 2.2.7. Active Nanomedicine Tumor Targeting – Systematic Follow-Up Studies

In the past decade, besides many oversimplified active targeting papers, also a number of systematic studies have been published on the principles and potential of active nanomedicine tumor targeting. Some of these papers focused on specific ligand-mediated targeting to receptors specifically expressed by cancer cells, while other reports focused more broadly on targeting structural features present in multiple cell types and/or more widely available within the microenvironment of tumors. We mainly focused on the latter, to try to exploit the potential added value of active targeting to the fullest. To this end, in the first exemplary study, we generated polymeric nanocarrier materials that are within the ideal size range for active targeting (i.e., above 5–10 nm and below 35 nm), and we modified them either with RGD and NGR peptides (for integrin and aminopeptidase targeting on endothelial, tumor cells and in the microenvironment) or with vitamin B2 (i.e., riboflavin; for targeting tumor cells, cancer stem cells and endothelial cells).[56–59] For RGD and NGR peptide-mediated active targeting of 67 kDa (10–20 nm) pHPMA-based polymeric nanocarriers, we observed rapid and efficient binding to angiogenic blood vessels in two different tumor models. However, due to presence of peptides, the polymeric nanocarriers were excreted more rapidly from the systemic circulation, and their long-term levels of tumor targeting (i.e., AUC) were lower than those of non-peptide-modified control polymers.[56] This shows that RGD- and NGR-targeting of angiogenic endothelium does work, but does not result in long-term enhancement of tumor accumulation. In a similar study, we employed 10 kDa (≈7 nm) and 40 kDa (≈13 nm) star-PEG-polymers, which are within the optimal size range for nature-inspired active targeting to extravascular structures, to explore riboflavin targeting.[58] This study design allowed us to demonstrate that for small and fairly rapidly excreted nanocarriers, that is, for the 10 kDa star-PEG polymer ($t_{1/2}$ = 1 h), active targeting does make a difference in terms of total tumor retention, whereas for the larger and longer circulating 40 kDa star-PEG polymer ($t_{1/2}$ = 13 h), there was no difference at the whole tumor level between passive and active targeting (Figure 2f). Importantly, however, in line with the above-mentioned conceptual studies on active versus passive tumor targeting, a clear difference was found at the level of nanocarrier uptake by cancer cells versus tumor-associated macrophages, with much higher fractions of the actively targeted polymer taken up by target/cancer cells (Figure 2g).

#### 2.2.8. Active Nanomedicine Tumor Targeting – Clinical Translation

In the last decade, scientists have become increasingly aware of both the conceptual shortcomings and the potential added value of active tumor targeting. The former can be indirectly exemplified by multiple clinical trials on actively targeted nanomedicines failing at the phase II level. Important examples of this are BIND-014 (ACUPA-targeted PLA nanoparticles loaded with docetaxel; targeted towards the PSMA receptor but clinically mostly explored for non-prostate cancer applications[60–62]) and MM-302 (single-chain anti-HER2 antibody-targeted PEG liposomes loaded with doxorubicin; targeted to the HER2-overexpressing tumor cells, but clinically only explored in trastuzumab-refractory breast cancer patients[63,64]). As alluded to below, the clinical trial performance of the former could have likely been improved if imaging or biopsy biomarkers had been employed to perform

patient stratification, in order to only include patients with high levels of PSMA expression in tumor lesions, thereby enriching the trial for potential responders. In case of the latter, one could argue that if the HER2-binding single-chain antibody fragment on the Doxil-like liposomes has pharmacological activity, it would then make sense to perform a clinical trial in patients that are (still) responsive to HER2 inhibition, rather than in patients which are refractory to trastuzumab. In such a setup, much like some of our own older work on intrinsically active anti-EGFR nanobody-modified polymeric micelles loaded with doxorubicin,[65] anti-HER2 antibody-fragment modified doxorubicin-liposomes could act as a double-drug therapeutic, with a receptor-inhibiting anti- or nanobody moiety on the surface, and with a small molecule chemotherapy drug entrapped in the core of the nanoformulation. Via such combination therapy strategies, significant added value in terms of increased therapeutic efficacy can be realized via what was initially conceived as an active targeting strategy.

#### *2.2.9. Active Nanomedicine Tumor Targeting – Future Directions*

Beyond active targeting-based combination therapy, active nanomedicine targeting is increasingly being realized for certain specific applications. A nice first example of this is the development of materials for the delivery of nucleic acid therapeutics into target cells. For hepatocytes and antigen-presenting cells (APC), this already works quite well without the presence of a targeting ligand on the surface of the nanoparticles. In the case of delivery to and into hepatocytes, which express the LDL receptor, one could argue that a targeting ligand, that is, apolipoprotein E, is physiologically added in vivo in the bloodstream, as part of the LNP's protein corona formation.[6] When in the future aiming to address cancer cells with nucleic acid-loaded nanoparticles, there seems to be hardly any way around including targeting ligands serving as cancer cell recognition and internalization motifs in the nanoparticle shell. Accordingly, the earliest pioneering efforts in the area of siRNA delivery to solid tumors in humans were based on nanoparticles incorporating transferrin as a targeting ligand.[66,67] Without such recognition motifs, nucleic acid-containing nanoparticles would – even if they manage to reach tumors in good amounts – not end up in cancer cells, but predominantly in tumor-associated macrophages, where siRNA-mediated gene knockdown or mRNA-mediated protein expression does not make much therapeutic sense (unless, of course, specifically aiming for specific modulation of gene expression in TAM). A second key example of directions in which active nanomedicine targeting to malignant cells is increasingly realized is in the area of hematological cancers. These advances conceptually overlap with the early-day developments of antibody-drug conjugates, which were initially also mainly explored for leukemias and lymphomas. As a key example, using modular antibody-functionalized LNP for nucleic acid delivery and bispecific antibody-modified liposomes for small molecule delivery, promising in vivo proof of concept for efficient active drug targeting and effective drug treatment has recently been achieved in diseases such as mantle cell lymphoma and high-risk childhood leukemia.[68,69]

## 3. Products

A decent number of nanomedicine formulations have received regulatory approval and are routinely used in the clinic for disease treatment. Depending on how strict the definition of a nanomedicine drug product is applied, this number currently varies from 32 to 100.[70–72]

### 3.1. Nanomedicine Drugs – Definition

Properly defining what a nanomedic(in)al drug is, and what not, is more difficult than one may think. Consequently, current definitions are neither very strict, nor very clear, and they can therefore exclude (or include) formulations that obviously are (or are not) nanomedicines. Some scientists have postulated a maximal size dimension of 100 nm, or at least one dimension of the product being less than 100 nm. That would exclude products such as Abraxane, which is about 125 nm in all three dimensions (at least prior to intravenous administration). According to the 2005 European Science Foundation Forward Look on Nanomedicine, a nanomedicinal drug should have a carrier component and a pharmacologically active component (typically referred to as an API, active pharmacological ingredient).[45] That would exclude intrinsically active nanoformulations, such as iron oxides, which are widely used for anti-anemia interventions, and to a lesser extent also for anticancer hyperthermia therapy. Moreover, as already alluded to above, the ESF definition would include antibody-drug conjugates, as these are in the right size range, and as they do contain a carrier moiety and an API.

In a concerted answer to the question "What do we mean when we say nanomedicine?", the editors of ACS Nano recently wrote, "Nanomedicine can be broadly defined as the branch of medicine that makes use of nanotechnology for disease prevention, monitoring, and intervention, through new modalities for imaging, diagnosis, treatment, repair, and regeneration of biological systems. Whether this relates to new (or improved) therapies or diagnostic methods, or the development of more efficient biomaterials for tissue regeneration, the goal of nanomedicine research is to reach the clinic and improve the patient's health or quality of life".[73]

In its most simple and straightforward definition, nanomedicine refers to the "application of nanotechnology in medicine". Narrowing this down – given the focus of the present paper – to anticancer therapy, I propose that all formulations that are >1 nm and < 1 μm in all three size dimensions, and that for improved diagnostic or therapeutic performance depend on size-specific formulation features, should be considered cancer nanomedicines.

### 3.2. Cancer Nanomedicines – First Clinical Development

In 1905, Paul Ehrlich coined the term "Zauberkugel", which in German means "magic bullet". He thereby pioneered the concept of developing drugs that are able to specifically target and eliminate pathological cells (in his case bacteria), while avoiding drug localization in and/or activity against healthy cells.[74] Ninety years after Ehrlich introduced the magic bullet concept,


**Figure 3.** Cancer nanomedicine products. Overview of nanomedicines approved for clinical use. Note that 1) not all formulations are approved globally, that is, some are only approved in certain countries; 2) multiple generic versions of liposomal doxorubicin are approved; 3) some formulations have been taken off the market during the course of time (e.g., DepoCyt; due to production robustness issues).

Doxil, which is a pegylated long-circulating version of liposomal doxorubicin, was approved in the United States as the first tumor-targeted anticancer nano-drug.[8] In 1996, the same drug product was approved in Europe under the trade name Caelyx. As depicted in **Figure 3**, two additional anthracycline-containing liposomes were marketed in the years that followed, which were both non-pegylated. While it is nearly impossible to trace down the reasons for not pegylating these liposomes, it appears that issues associated with patents and intellectual property rights (IPR) are to be held responsible. In the years that followed, that is, between 2000 and 2010, several additional liposome products were approved for clinical use, as were several pegylated proteins, in which the PEG polymer served to improve protein stability, circulation time, and/or target site accumulation, as well as to suppress immunogenicity.

### 3.3. Cancer Nanomedicines – Follow-up Developments

In the 2000s, the first nanomedicines based on albumin-condensates and polymeric micelles gained approval. Both formulations primarily aim to overcome adverse effects associated with the use of the non-ionic surfactant Cremophor (i.e., ethoxylated castor oil), which is extensively used as an administration aid to assist in the i.v. application of the potent but also highly hydrophobic taxane drug paclitaxel. The polymeric PEG-b-PLA micelle formulation Genexol-PM is only approved in Korea, while albumin-based Abraxane is used globally, and extensively. Abraxane creates significant added value for patients as compared to standard Cremophor-based Taxol because it can be administered in a shorter period of time (30–60 min vs 3–4 h), at a higher dose (225 vs 175 mg $m^{-2}$), and without corticosteroid and/or antihistamine co-medication.[75]

In the 2010s, several novel types of cancer nanomedicines reached the market. These include inorganic nanoparticles, such as Nanotherm and Hensify, which are not loaded with API but are themselves serving as therapeutics. Both formulations are not designed to systemically target tumors upon i.v. injection, but they need to be injected into tumors, and they rely on activation via external physical means (i.e., alternating magnetic fields and radiotherapy, respectively) for their therapeutic efficacy.

### 3.4. Cancer Nanomedicines – Current Clinical Landscape

When looking at the performance of the cancer nanomedicines that have been approved between the mid-1990s and today, it needs to be noted that they have thus far mainly contributed

to reducing side effects, rather than to improving therapeutic efficacy. As a key example, Doxil/Caelyx significantly reduces cardiomyopathy and several other side effects associated with the use of free doxorubicin, for example, alopecia. Hair loss is oftentimes not considered a very important side effect, and it will indeed never be dose-limiting. However, when considering that doxorubicin (which in free form massively induces alopecia) and Doxil/Caelyx are both approved for the treatment of advanced breast cancer, including for certain genetically induced subtypes that already hit patients in their end-20s, 30s, or 40s, one can easily appreciate why there is a significant added value for patients in terms of improving their quality-of-life when using nanomedicines, since liposomal formulations barely induce hair loss. This implies that females do not have to paint facial hair and/or wear wigs to not publicly disclose they are being treated for a severe disease. These notions are in line with the abovementioned added value achieved by Abraxane, which also mostly benefits patient treatments by controlling the side effect profile and practicality of drug administration, and not that much by promoting improved therapy responses.

Along the same line of thinking, multiple recent developments in the cancer nanomedicine field relate to the ability to broaden the clinical use of highly efficacious but at the same time very poorly tolerable chemotherapy agents, such as irinotecan. This drug works well against several difficult-to-treat cancers, such as colon, lung, and pancreatic cancer, but patients barely tolerate it. Consequently, formulating irinotecan in liposomes helps to promote the use of this very potent drug, with fewer side effects, and in multi-drug regimens.[76] Another important recent development is the approval of liposomes for multi-drug delivery. By co-loading cytarabine and daunorubicin in a fixed 5:1 ratio, synergistic drug effects can be achieved, resulting in improved therapeutic management of certain forms of acute myeloid leukemia.[77] Recently, also a second albumin-based nano-drug has gained approval, which is termed Fyarro. This formulation contains the mTOR inhibitor sirolimus as an API, is used in the clinic for the treatment of relatively rare advanced perivascular epithelioid tumors (PEComas), and may in the future become much more widely applied in a cancer type-agnostic and biomarker-controlled manner in tumors with mTOR-activating TSC1/2 mutations.[78]

### 3.5. Cancer Nanomedicines – Promising Platform Technologies in Clinical Trials

In the past two decades, prominent progress has been made with a next-generation liposome formulation termed ThermoDox. This lysolipid-containing doxorubicin-liposome is temperature-responsive in the clinically relevant range of 37–41 °C, enabling site-specific drug release upon locally heating tumors. In initial trials in patients with hepatocellular carcinoma (HCC), ThermoDox was combined with radiofrequency ablation (RFA). RFA is based on the use of needles which are injected into tumors, and which locally ablate tumors by inducing significant hyperthermia, up to 50 °C. The initial rationale for combining RFA with ThermoDox was that the liposomes could be used to target the tumor margins surrounding the site of RFA application, in which temperatures are typically not high enough to directly kill cancer cells. While initial clinical trials evaluating this concept were unsuccessful – for a number of reasons – it is expected that future optimization will result in clinical implementation.[79,80] In this context, it is likely that cancer types beyond HCC will also be evaluated, that the heating procedure will be optimized (e.g., replacing RFA by focused ultrasound) and that the liposome formulation will be further fine-tuned. An example of the latter is the Thermosome, which has a lipid composition optimized for temperature-triggered intravascular drug delivery.[81,82] More in-depth reflections on intravascular triggered drug release are provided below, in the section on future prospects.

A second class of cancer nanomedicines that have prominently moved into clinical trials in the past decade is polymeric micelles. As for liposomes, several generations of micelles have been developed, starting off with relatively straightforward non-stabilized self-assemblies, upon until core-crosslinked formulations, in which metal complexation or conjugation chemistry is employed to stabilize the hydrophobic core.[83–86] Prototypic examples of polymeric micelles evaluated in the clinic are based on block copolymers of PEG and poly(amino acids), and of PEG and p(HPMA)-Lac. An advantage of core-crosslinked micelles is the ability to control multiple important parameters, for example, size, drug release kinetics, and micelle degradation kinetics.[87] To date, several advanced polymeric micelle formulations have progressed up until phase II and III clinical trials, but none of them has managed to gain regulatory approval to date, in spite of extensive proof-of-concept in mouse models, and documented prolonged circulation times and tumor targeting in patients. As discussed in more detail in the next chapter, key reasons for this are – as for cancer nanomedicines in general – high heterogeneity in tumor physiology and tumor targeting, and the lack of protocols for patient stratification.

## 4. Problems and Challenges

There are multiple problems and challenges associated with the clinical translation of cancer nanomedicines. Not only are several elements related to their production, upscaling, and regulation not yet properly established, but also optimal protocols to ensure their successful use in patients are lacking. Below, several key conceptual, translational, and industrial challenges are discussed, and potential solutions are proposed.

### 4.1. Conceptional Challenges

Cancer nanomedicine is an interesting and interdisciplinary field. In the 20+ years the field exists, it has probably always encompassed (way) more people from material science and chemistry, than people from pharmacy and medicine. This may at first glance seem remarkable, but it is actually to a certain extent understandable, because drug delivery and cancer therapy are both very attractive topics to work on and because nanoparticles are widely claimed to be useful for this purpose. The problem of this field-specific development is that there has gradually become a disconnect between what the real aim of the research is, and what people are producing. One could argue that there is (way) too much focus on generating novel nanoparticle designs, while

there is insufficient attention on how and when nanoformulations are actually accumulating in tumors, and for identifying and overcoming the reasons why nanomedicine drugs are failing in clinical trials.[88,89]

### 4.2. Nanomedicines versus ADC

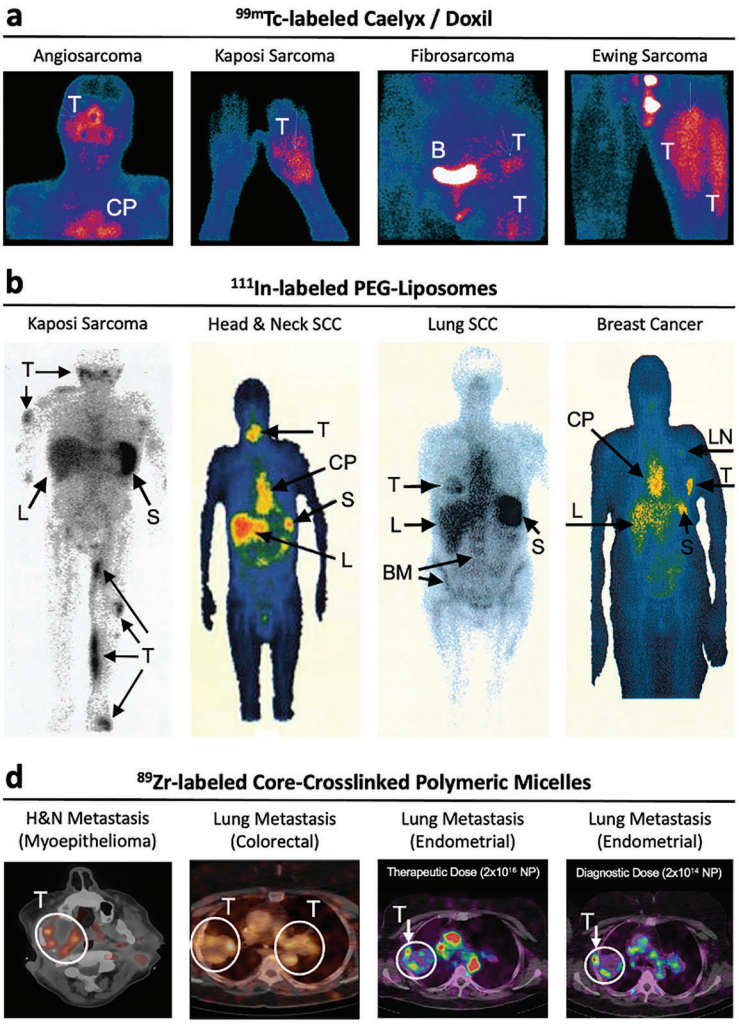
When comparing nanomedicines to antibody-drug conjugates (ADC), it is remarkable to note how different the fields are perceived, and how differently they are progressing. This goes beyond the size- and chemical-conjugation-dependent differences discussed above, in Section 2. The former field is producing millions of papers per year, and mainly shooting for the stars and for very difficult-to-treat cancers (e.g., pancreatic and brain), the latter produces way fewer papers, typically in lower-impact journals, and aims mainly for low-hanging-fruit cancers (e.g., lymphomas). As a result of this divergence, the cancer nanomedicine field has been receiving a lot of visibility, media attention, and also criticism, while the ADC field has been developing a bit more in the background. At this point in time, the number of approved products is almost identical for cancer nanomedicines and anticancer ADC (both ≈20). When visualized and quantified via nuclear medicine (PET/SPECT) techniques, also the tumor-targeting potential is comparable for nanomedicines and ADC. The percentages of the injected dose per kilogram tumor tissue (%ID $kg^{-1}$) and the specific uptake values (SUV) – which are not identical based on definition, but overall well-comparable – are typically in the range of 2–10 for both types of formulations.[90–94] Apart from these two similarities, the nanomedicine and ADC fields could not be more different. They could have learned a lot from each other if they would have interacted more. A very important advantage of anticancer ADC over cancer nanomedicines is that the former are intrinsically coupled to a biomarker, which guides patient stratification and helps to ensure successful clinical translation. Regardless of whether CD30, CD19, HER2, Trop-2, or other overexpressed surface markers serve as the receptor target for ADC, patients are only included if the receptor is confirmed to be present on the malignant cells. In the case of cancer nanomedicine, no such biomarkers are available for patient stratification. Cancer nanomedicines are essentially given to all patients, without any upfront pathophysiological information on whether or not the treatment may work.[88,89]

### 4.3. Translational Challenges – Cancer Nanomedicines Need Biomarkers

Since nanomedicines' mechanism of action is primarily based on their ability to improve the target site localization of entrapped or conjugated drugs, it would be good if biomarkers were available to assess (predict) their ability to promote tumor targeting. Given that cancer is a very heterogeneous disease, it is not surprising that tumor-targeted drug delivery is also very heterogeneous. While this has been known for many years, it is hardly ever taken into account, in spite of likely being the #1 reason for the thus far fairly disappointing and inefficient clinical translation of cancer nanomedicines. In this context, it needs to be taken into account that for almost all new anticancer agents that have made it to the clinic and to the market in the past couple of years (i.e., for antibody therapeutics, kinase inhibitors, checkpoint blockers, and beyond), biomarkers are available for patient stratification. These are typically based on histopathological or genetic analysis of tumor tissue, and sometimes also on liquid biopsies, for example, assessment of genetic mutations in circulating tumor cells. In the cancer nanomedicine field, as discussed in more detail below, it is considered to be truly crucial to identify similar ways forward toward patient stratification.

### 4.4. Translational Challenges – Tumor Targeting is Heterogeneous

From the few studies that have been published over the years on nanomedicine (as well as on free drug[95,96] and ADC[90–92]) tumor targeting in patients, it has become obvious that there is a high variability in how efficient drugs and drug delivery systems accumulate at sites of malignancy. Among the most insightful results reported with regard to nanomedicine tumor targeting are the papers published by Koukourakis and colleagues,[97] and Harrington and colleagues.[93] The former group of clinical scientists evaluated $^{99m}$Tc-labeled pegylated liposomal doxorubicin tumor targeting in seven patients with different types of sarcoma, while the latter studied the accumulation of $^{111}$In-labeled pegylated liposomes in 17 patients with head and neck, lung, breast, cervical, and brain cancers. Representative images obtained in both clinical studies are provided in **Figure** 4a,b. They disprove provocative statements made in the literature claiming that "the EPR effect works in rodents but not in humans" and that "tumor targeting cannot be proved in the clinic".[23] While one can indeed argue that the EPR effect may mechanistically not be completely correct – besides vascular leakiness, active transcytosis potentially playing a role in nanocarrier accumulation, and besides compromised lymphatic drainage, uptake by tumor-associated macrophages (TAM) contributing to nanocarrier retention[17–22] – it is very obvious when evaluating the liposome biodistribution and tumor accumulation patterns in Figure 4a,b, that nanomedicines do clearly accumulate at sites of malignancy, in patients with different types of cancer. Besides in tumors, nanomedicines also prominently accumulate in the liver and spleen, which are characterized by similar anatomical and physiological features, that is, high vascular density, leaky blood vessels, and prominent presence of phagocytic cells. Not surprisingly, quantification of radiolabeled liposome tumor accumulation shows significant variability in the amounts of nanomedicines concentrating at pathological sites (Figure 4c). In the 17 patients evaluated by Harrington et al, whole-body gamma-scintigraphy imaging was able to detect liposomes in 12 out of 17 patients, while based on more sensitive single-photon emission computed tomography (SPECT), even 15 out of 17 tumors were found to be positive for nanomedicine accumulation. This corresponds to proof of concept for nanomedicine tumor targeting in 88% of patients. In these 15 patients, however, the efficiency of tumor targeting varied substantially, from ≤ 5% of the injected dose per kilogram of tumor tissue for breast cancer to ≥ 50% ID $kg^{-1}$ for head and neck cancer (Figure 4c).

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| Pat. | Tumor Type | GS | SPECT | %ID/kg |
|---|---|---|---|---|
| 1 | Head & Neck | + | + | 53.0 |
| 2 | Head & Neck | + | + | 46.8 |
| 3 | Head & Neck | + | + | 32.0 |
| 4 | Lung | + | + | 25.4 |
| 5 | Head & Neck | + | + | 24.2 |
| 6 | Lung | + | + | 16.7 |
| 7 | Lung | + | + | 12.5 |
| 8 | Breast | + | + | 9.5 |
| 9 | Head & Neck | + | + | 9.0 |
| 10 | Breast | + | + | 5.2 |
| 11 | Breast | + | + | 3.9 |
| 12 | Breast | + | + | 2.7 |
| 13 | Cervix | - | + | N.A. |
| 14 | Glioma | - | + | N.A. |
| 15 | Glioma | - | + | N.A. |
| 16 | Lung | - | - | N.A. |
| 17 | Breast | - | - | N.A. |
| **All** | Accumulation in Tumors | **12/17 =71%** | **15/17 =88%** | |

e

| Pat. | Tumor Type | TD Scan | DD Scan |
|---|---|---|---|
| 1 | Endometrial | 2/2 | 2/2 |
| 2 | Colorectal | 6/10 | 5/10 |
| 3 | Adenocarcinoma | 5/9 | N.D. |
| 4 | Ovarian | 1/2 | 1/2 |
| 5 | Myoepithelial | 6/17 | 5/17 |
| 6 | Breast | 1/5 | N.D. |
| 7 | Esophageal | 0/1 | 0/1 |
| **All** | Accumulation in Metastases | **21/46 =46%** | **13/32 =41%** |

**Figure 4.** Nanomedicine tumor targeting in patients. a) Technetium-99m-labeled doxorubicin-loaded liposomes were i.v. administered to patients with different types of sarcomas and tumor accumulation were visualized using gamma scintigraphy. b,c) Indium-111-labeled liposomes were administered to patients with different types of solid tumors, and gamma scintigraphy (GS) and single-photon emission computed tomography (SPECT) were employed to visualize and quantify the extent of tumor targeting. To promote inter-lesion cross-comparison, concentrations in tumors were normalized to mass units and expressed as percentages of the injected dose per kilogram tumor (%ID $kg^{-1}$). In the biodistribution images, T denotes tumor, L is liver, S is spleen, CP is cardiac blood pool (representing systemic circulation), BM is bone marrow, and LN is a metastatic lymph node. d,e) Zirconium-89-labeled core-crosslinked PEG-b-pHPMAmLac$_n$ polymeric micelles were i.v. administered to patients with metastatic cancers and their biodistribution and target site accumulation was visualized and qualified using positron-emission tomography combined with computed tomography (PET-CT). Micelle tumor targeting was studied at the full therapeutic dose (TD: corresponding to 60 mg $m^{-2}$ docetaxel), as well as at an 80-fold lower companion diagnostic dose (DD). Together, the liposome and micelle imaging studies clearly show that nanomedicine tumor targeting does work in patients, but also that it is highly heterogeneous. Images and tables reproduced and adapted with permission.[93,94,97]

### 4.5. Nanomedicine Targeting to Metastases

Patients with non-hematological cancers typically die from metastases, not from primary tumors. In addition, patients with an isolated solid tumor are typically treated with surgery and radiotherapy, in order to remove the tumorous mass from the body. Chemotherapy can be added either prior to surgery and/or radiotherapy (i.e., neo-adjuvant), to pre-shrink the tumor and facilitate complete removal, or it can be given afterward (i.e., adjuvant), to prevent metastatic spread and/or to target tiny metastatic lesions not detected via routine diagnostic protocols. Together, these notions indicate that for solid malignancies, chemotherapy is mainly useful as an add-on to surgery and radiotherapy and that it is particularly needed in case of metastatic disease. Strikingly, however, the vast majority of nanomedicine studies focus on primary tumors, oftentimes inoculated under the skin, where they grow "in isolation", with generally no more than 20% or 30% of the tumor surface area contacting non-cutaneous host tissue. This is obviously suboptimal for studying how nanomedicine can and should help in real-life situations, where patients with metastases have cancerous lesions spreading from the primary tumor to various distant sites, typically to lymph nodes, lung, liver, bone, and brain.

Many of the mouse models used to study nanomedicine targeting to tumors versus metastases are suboptimal. Cell line-derived xenograft (CDX) tumors only grow in immunodeficient mice, which will affect microenvironment composition and their metastatic spread behavior. CDX also do not properly reflect the heterogeneity observed in human tumors. Patient-derived xenografts (PDX) better reflect human tumor biology, the microenvironment in patients, and the heterogeneity that is typically observed in both of them. However, they also suffer from the fact that they only grow in immunodeficient mice. In addition, only very few robustly metastasizing PDX models have been described to date, which makes systematic studies on metastasis targeting difficult. As a result of these limitations, the majority of metastasis targeting experiments are done in syngeneic mouse models of mouse cancer, such as 4T1 triple-negative breast cancer in BALB/c mice or B16 melanoma in C57BL/6 mice. If properly induced (i.e., by waiting until orthotopically or subcutaneously inoculated cancer cells metastasize to host tissues, and thus not via tail vein injection of cancer cells), these have the advantage of really mimicking metastatic spread and growth, and thus targeting pattern, albeit in mouse cancer. The same holds true for genetically engineered mouse models (GEMM; like, e.g., the mouse mammary tumor virus overexpressing the polyomavirus middle T-antigen (MMTV-PyMT)), in which tumors and metastases develop spontaneously.

How different nanomedicine tumor targeting versus metastasis targeting is, is not well known, neither in mice nor in patients. This likely depends on the type and stage of the primary tumor, and on the location and size of the metastasis. It also seems reasonable to assume that metastases adopt a somewhat host-tissue-like phenotype in terms of vascularization and stromal composition, mixing features of the primary tumor with those of the organ affected by metastasis. This is something we are currently evaluating in the lab. In patients, we have evaluated how well metastases can be targeted with mPEG-b-pHPMAmLac$_n$-based core-crosslinked polymeric micelles (CCPMs). These CCPMs were co-loaded with docetaxel as a drug and $^{89}$Zr as a long-lived radioisotope, for positron emission tomography (PET) - based visualization and quantification (Figure 4d).[94] In seven patients with a total of 46 metastases (according to inclusion criteria, i.e., > 2 cm in diameter), we found that metastases successfully accumulated CCPMs in 46% of cases ($n$ = 21 lesions; Figure 4e). Importantly, besides administering CCPMs at their full therapeutic dose (corresponding to 60 mg m$^{-2}$ docetaxel)), they were also applied at an 80-fold lower diagnostic dose, to trial the possibility of using them purely for patient stratification purposes, without side effects, prior to the first therapeutic dose. In this companion diagnostic dose setup, CCPM accumulation could be visualized and quantified in 41% of cases (13 out of 32; note that only five patients were evaluated at this dose level). Interestingly, the absolute number of CCPM particles administered at this diagnostic dose (i.e., 2–4 × 10$^{14}$) was below the predicted number of nanoparticles needed for prolonged circulation (1.5 × 10$^{15}$;[93]). This study therefore shows that the dose threshold for long circulation and efficient nanoparticle tumor targeting does not seem to apply for these CCPMs because the circulation kinetics in blood were similar for the diagnostic versus the therapeutic dose.[94] This notion is further corroborated by our earlier finding that mPEG-b-pHPMAmLac$_n$-based CCPM show hardly any protein adsorption,[99] and thus are unlikely to have a protein corona in vivo, which is presumed to be responsible for more rapid nanoparticle clearance from the bloodstream at lower doses.[100] Actual patient stratification and prediction of therapeutic outcome was impossible in this CCPM clinical trial, because patients suffered from too different types and stages of cancer, and were all heavily pretreated. Nonetheless, these efforts nicely showcase that nanomedicine formulations are able to target metastases in patients, and they furthermore exemplify the feasibility of using noninvasive and quantitative imaging tools for patient stratification purposes.

### 4.6. Translational Challenges – Imaging versus Biopsy Biomarkers

While noninvasive imaging is undeniably highly suitable for cancer nanomedicine patient stratification, it is questionable whether it is practically achievable. The reason for this is that quantitative imaging using PET or SPECT requires access to radiochemistry laboratories and dedicated imaging equipment, which may not be widely available in community hospitals. In addition, given the prolonged circulation kinetics of nanomedicine formulations and the fact that optimal tumor targeting typically occurs at 1–4 days after i.v. administration, patients would either have to stay in the hospital or come back for a scan a couple of days after they have been injected. This complicates the use of imaging for patient stratification purposes and makes it pragmatically difficult to implement it in day-to-day clinical practice.

As an alternative, we set out to study whether biomarkers extracted from tumor tissue can be used to predict whether nanomedicines accumulate in tumors.[101] To this end, we started off by staining 23 different (patho)physiological features in three subcutaneous tumor models, with low, medium, and high levels of tumor accumulation. Gradient tree boosting-based machine learning was employed to extract the most important features, identifying six parameters as statistically meaningful. Of these, five were related to vasculature, while the other one was TAM density. When considering the fundamental principles of tumor-directed drug delivery, it is not surprising that these features surface, as blood vessels are needed to take nanomaterials to and into tumors, while TAM keep them there (and oftentimes also play a role in activating them, by mediating nanocarrier degradation and drug release). The identified biomarkers were subsequently validated in three orthotopic tumors in immunocompetent mice, as well as in 10 cell line-derived and patient-derived xenografts. In these experiments, liposomes were used instead of polymers, and we also switched from fluorescence staining and fluorescence microscopy to clinically relevant DAB staining and histopathologically standard light microscopy. Ten blinded observers, including 3 clinical pathologists, scored tumor blood vessel and TAM density in 30 sections from the 10 CDX and PDX tumors, and the resulting scores were correlated with liposomal doxorubicin accumulation in these models. Our newly conceived biomarker product score correctly identified three out of three tumor models with low liposome accumulation as true negatives, and 6 out of 7 tumor models with medium to high levels of nanomedicine accumulation as true positives (AUROC = 0.91). One tumor model was found to be an outlier, with very low vessel and TAM scores,

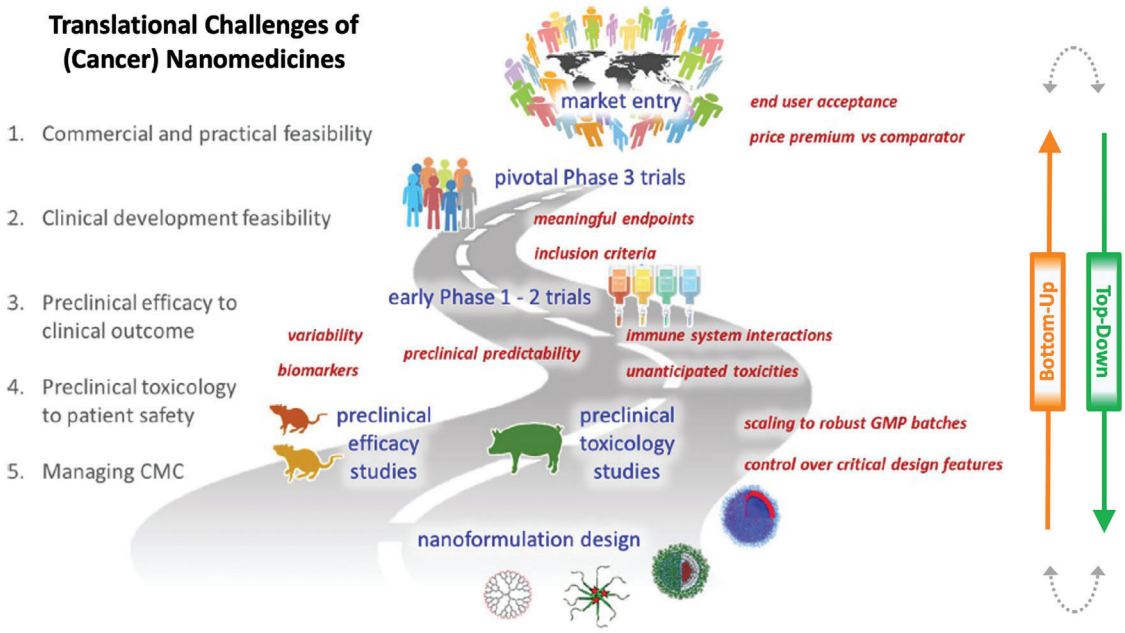


**Figure 5.** Challenges in nanomedicine clinical translation. Key translational and industrial aspects of nanomedicine product development are depicted. Challenges are traditionally approached in a bottom-up manner. However, also considering challenges in a top-down manner, from the vantage point of end-users, with commercial, practical, and clinical feasibility firmly in mind, is considered to be important for ensuring success. The top-down analysis allows for the identification – from the initiation of the clinical translation process onwards – of the most important issues that can be encountered along the way, triggering proactive thinking and planning to overcome potential challenges already at early stages. Image reproduced with permission.[102]

but – unexplainably – very high levels of liposome accumulation. Findings were confirmed in biobanked patient tumor tissues and biopsies, for lesions type- and stage-matched with the liposome tumor accumulation patterns reported by Harrington et al. (Figure 4b). Together, these efforts indicate that it is possible to use histopathological biomarkers based on tumor biopsies (NB, which are readily and easily available, for all patients, because they are used for disease diagnosis) for patient stratification, aiming to identify those individuals that should be excluded from clinical trials, because their tumors are unlikely to accumulate nanomedicines in significant amounts.

### 4.7. Translational and Industrial Challenges

Cancer nanomedicine development and translation are traditionally approached in a bottom-up manner. Translational efforts typically start with nanoformulation design and CMC, that is, chemistry, manufacturing, and controls. CMC covers all relevant pharmaceutical production steps, as well as the physical and chemical characteristics of the starting materials, intermediates, and end-products, ensuring their purity, quality, and consistency. From a combined feasibility, efficiency, impact, and industrial point of view, we have argued that it would be wise and valuable to also look at (cancer) nanomedicine development and translation in a top-down manner.[102] As illustrated in **Figure 5**, this would entail taking the perspective of end-users, to single out those products and trajectories which really have clinical and commercial potential. To this end, key commercial and clinical feasibility questions have to be asked and answered very early on. This could include addressing the classical 5R principles in reversed order, starting from the end, 1) right commercial potential (market, clinical need, price premium); 2) right patient (biomarkers, inclusion criteria, endpoints); 3) right safety (use of known vs novel materials, standard vs immuno-toxicity); 4) right tissue (dedicated pharmacokinetic and biodistribution analysis, whole-body imaging as part of early clinical development, tumor vs non-tumor targeting); 5) right target (depending on the drug delivered and/or on the receptor addressed in case of active targeting).[12] In parallel, it is important that preclinical efficacy and toxicology experiments are planned in such a way that they proactively and dedicatedly address some of the key questions and uncertainties that may come up during clinical development. Identifying and addressing such key clinical challenges and industrial considerations as early on as possible, and ideally doing this in a top-down translational scenario, will help to implement and integrate adequate risk-mitigation strategies. This is something that investors and other commercial parties typically already want to see from the very early stages of development onwards.

## 5. Prospects

The prospects of generating clinical impact with cancer nanomedicines are bright. To realize these prospects, academic mindsets may have to change and translational trajectories will have to be revisited. There has been quite a bit of criticism on cancer nanomedicine in the last decade, and this is partially justified, because of the overly blunt, naive, and optimistic claims made in the literature. However, as for almost all technological developments, according to Gardner's hype cycle, after a trough of disillusionment, there will be a slope of enlightenment, and

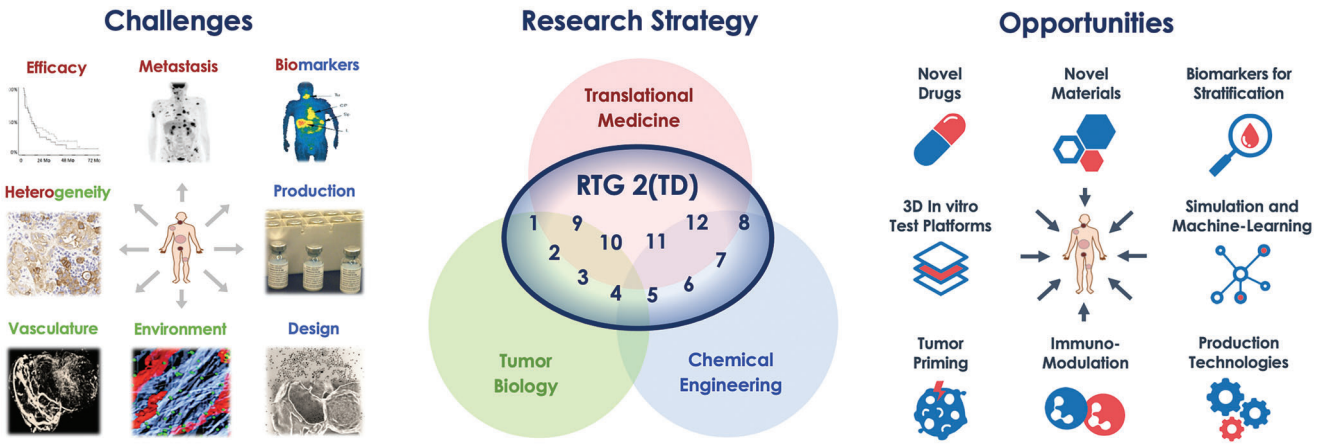


**Figure 6.** Challenges and opportunities in cancer nanomedicine and tumor-targeted drug delivery. As part of the German Research Foundation (DFG) - funded Research and Training Group 2375, entitled 2(TD) – Tumor-Targeted Drug Delivery, concerted efforts are being made to identify and address key challenges and opportunities. Challenges were mainly addressed in the first half of the 9-year funding period, with 12–24 doctoral researchers performing research at the interfaces of the 3 key contributing fields tumor biology, chemical engineering, and translational medicine. In the currently ongoing second funding phase, prospects and opportunities in cancer nanomedicine and tumor-targeted drug delivery are being explored. Altogether, research and training in RTG 2(TD) will contribute to a better biological and pathophysiological understanding of cancer, to enhanced nanoformulation design and production engineering, and to viable concepts to promote cancer nanomedicine clinical translation.

then a plateau of productivity. For cancer nanomedicine, we are currently on the slope of enlightenment, and productivity and impact are already growing. In the years to come, progress and productivity will undoubtedly continue to increase. This will likely be particularly prominent in the research directions alluded to below.

### 5.1. From Challenges to Opportunities

In the 1980′s up until the 2000′s, there was a lot of positive attention for cancer nanomedicine and tumor-targeted drug delivery. In the 2010′s, several experts became increasingly critical, and righteously so. With the enormous advancements in nanomaterial conception and construction, there was a general tendency towards producing ever more nanoparticles and making ever stronger claims on their potential. Typically for drug delivery applications, and in 9 out of 10 cases for cancer therapy. Without realizing that in the case of cancer, drug therapies typically fail due to heterogeneity, as well as due to highly unfavorable pathophysiology, including very poor access to tumors and tumor cells. Beyond these key challenges, there are multiple additional biological, chemical, and clinical barriers hindering tumor-targeted drugs and drug delivery systems from making to the market. In a large Research and Training Group funded by the German Research Foundation (DFG; RTG2375; entitled 2(TD) – Tumor-Targeted Drug Delivery; coordinated by Fabian Kiessling and Twan Lammers; **Figure 6**), we set out to systematically address the most important barriers and challenges. These have included the hostile pathophysiological microenvironment in solid tumors and metastases, and the lack of biomarkers for cancer nanomedicine patient stratification. In the second funding phase of this project, we have also integrated recent advances and opportunities, such as the use of novel drugs (e.g., RNA), tools (e.g., machine learning), and technologies (e.g., 3D printing), the priming of tumors with pharmacological and physical co-treatments, and the combination of nanomedicine with immunotherapy (Figure 6).

### 5.2. Tumor Perfusion

Starting all the way at the beginning of tumor-targeted drug delivery, the process of tumor perfusion – that is, efficient blood flow through tumor blood vessels – has been emerging as a massively overlooked barrier. Presuming that a nano-drug is fully stable in circulation, one of its key advantages over a small molecule drug is prolonged presence in the bloodstream, thereby making it more likely that the nano-drug eventually accumulates in the cancerous lesions. Consequently, when critically contemplating the tumor-targeted drug delivery process, it is striking that efficient tumor perfusion is often taken for granted and that the deliberation typically starts at the level of vascular permeability and/or transcytosis, upon which follow-up processes such as perivascular transport, penetration, and intratumoral distribution are considered. However, it has remained largely neglected to date that tumor perfusion per se is highly heterogeneous and that it can be very poor, both in mouse models and in patients. In multiple conceptual, preclinical, and clinical papers focusing on tumor perfusion imaging, it has been found that tumor blood flow is inconsistent and suboptimal.[103–106] This notion has to date largely gone without considering what this means for tumor-directed drug delivery and drug therapy.[107,108] Among the most noteworthy efforts to center-stage tumor perfusion and come up with strategies to enhance tumor blood flow is the concept of vessel normalization.[109,110] This can, for example, be achieved by medium-dose anti-angiogenic therapy, as well as by targeting the stromal cells and stromal compartment which can mechanically compress tumor blood vessels. A number of pioneering (pre)clinical studies have shown that vascular normalization can enhance tumor perfusion, drug delivery, and the efficacy of anticancer (nano)therapy.[111–113] Since efficient tumor blood flow

is crucial for almost all cancer treatments, including chemotherapy, radiotherapy, hormone therapy, and immunotherapy, it is critically important to start identifying, exploring, and translating probes and protocols that are able to promote tumor perfusion.

### 5.3. Tumor Priming and Combination Therapies

Building upon and extending several of the above notions, tumor priming and nanomedicine-based combination therapies are predicted to play increasingly important roles in future medical practice. Tumors can be primed for improved delivery and therapeutic outcomes both pharmacologically and physically. Examples of pharmacological priming are vascular permeabilization, vascular promotion, and vascular normalization, as well as antistromal pharmacotherapies, aiming to promote drug and drug delivery system penetration, via reducing extracellular matrix deposition.[30,114] The key advantage of pharmacological priming is that it works systemically, thus affecting both the primary tumor as well as distant metastases. Moreover, pharmacological priming treatments can typically be administered relatively easily, without the need for hospitalization. The most important downside is that it is quite difficult to identify the optimal drug doses for inducing pharmacological priming in individual patients and tumors, taking the highly heterogeneous nature of tumors and metastases into account. Physical tumor priming, for example, via hyperthermia, ultrasound, or radiotherapy, can be performed in a more tailorable manner, particularly also because it is often done under imaging guidance. However, physical tumor priming is more labor- and cost-intensive and typically requires semi-elaborate clinical procedures. Key pros thus are an enhanced level of control, plus the ability to individually tailor interventions, via theranostic protocols. Key cons are the intrinsic locality of the approach, making it less suitable for treating systemic disease. Although one can confidently claim that priming primary tumors using local physical treatments holds the potential to promote systemic (nano)immunotherapy. Beyond pharmacological and physical priming, also certain physiological strategies can be considered. As an example, we recently showed that priming of the tumor microenvironment via intermittent fasting promotes vascularization and reduces extracellular matrix deposition, resulting in improved tumor-targeted drug delivery and enhanced antitumor activity, for both standard small molecule drugs and for nano-drugs.[115]

In general, one needs to consider in this context that one of the key advantages of using nanomedicine formulations for anticancer therapy is that they make combination therapies more tolerable and more efficient. This is crucial, and often overlooked. The clinical use of classic cancer nanomedicines, such as Doxil and Abraxane, is most broadly justified by their ability to reduce the spectrum of side effects of multimodal combination therapy. If tolerability is improved, patient compliance typically goes up, benefiting overall therapeutic outcomes. Several combination therapy setups can be envisaged in which the use of nanoformulations is advantageous, including, for example, radio(chemo)therapy and nano-immunotherapy.[116–118] Examples of the former encompass carrier-based radiochemotherapy concepts, as well as intrinsically radiotherapy-promoting nanomedicines, such as hafnium oxide- and gadolinium-based nanoformulations, which are approved for clinical use and evaluated in clinical trials, respectively.[119–123] Examples of the latter, that is, nanomedicine-combination therapy and nano-immunotherapy, are separately discussed in the sections below.

### 5.4. Nanomedicine-Based Multi-Drug Delivery

A very attractive prospect of nanomedicine-based anticancer therapy is its (cap)ability to improve multi-drug treatment. Nanomedicines are – because of their size dimensions and design features – exquisitely useful for concerting the delivery of multiple active pharmaceutical ingredients (API). Pioneering and prominent clinical evidence for nanomedicine-based multi-drug delivery has been obtained using Vyxeos, a clinically used dual-drug liposome delivering cytarabine and daunorubicin to cancer cells in a fixed 5:1 ratio. This fixed ratio has been shown to be synergistic with respect to killing leukemia cells.[124,125] If both agents were administered intravenously in free form, they would spread throughout the body completely differently, because of their different physicochemical and pharmacokinetic properties. Consequently, even if co-administered at synergistic doses in free form, they would be very unlikely to act synergistically in vivo. When co-encapsulated in liposomes, the nanoformulation dictates the pharmacokinetic profile of the agents, and it ensures that both APIs reach the same compartments at the same time in the right ratio, thereby producing pharmacological synergy. Beyond Vyxeos, increasingly many nanoformulations are explored for multi-drug delivery.[126] Fifteen to twenty years ago, this already involved the co-loading of two chemotherapy drugs in liposomes, as well as the co-conjugation of two anticancer drugs to long-circulating polymers.[127–130] Somewhat more recently, efforts in this regard have opened up towards micelles and other nanoparticles, as well as towards formulations in which different drugs with different mechanisms of action are combined. Examples of the latter, for example, include a combination of chemotherapy drugs with siRNA molecules for knocking down drug efflux pumps to overcome resistance, as well as co-loading of chemotherapy drugs with agents that affect angiogenesis, modulate the tumor microenvironment, or activate the immune system.[131–136] Recently, also triple-drug nanomedicine formulations have been explored, showing that statistical mixtures of polymer-prodrugs are able to deliver synergistic ratios of three anti-multiple myeloma agents to tumors in vivo, producing potently enhanced therapeutic effects.[137] It is expected that these and other cancer nanomedicine-based co-formulations will play increasingly important roles in future targeted therapy and clinical practice.

### 5.5. Indirect Tumor Targeting

Not all nanomedicine-based anticancer therapies rely on tumor targeting, that is, on extravasation or transcytosis across the endothelial lining. Alternative methods of getting drugs delivered to cancer cells can entail concepts such as intravascular triggered release and immune cell hitchhiking. A key example of the former is hyperthermia-responsive drug release using temperature-triggerable liposomes. The most well-known formulation for this

specific application is Thermodox,[79,80] which was already introduced above as a promising product in clinical trials in Section 3. Thermodox contains lysolipids with single fatty acid tails in their its bilayer, which liquefy the liposome membrane when heated slightly above physiological temperature.[138] Besides Thermodox, multiple follow-up versions of temperature-response liposomes have been created, including non-lysolipid formulations, all aiming for optimally controlled triggerable drug release in the range of 39–42 °C.[81,82,139,140] Hyperthermia used to be performed exclusively with needles, in a radiofrequency ablation (RFA) setup, but it is nowadays more and more done completely non-invasively, using focused ultrasound, typically under magnetic resonance imaging guidance.[141] A fairly common misperception in this context is that temperature-responsive liposomes first accumulate in the tumor via EPR, and are then activated afterward to induce drug release. This is not the case, tumors are pre-heated just before liposome i.v. administration and heating is then continued for a certain period of time (typically up to max. 1 h). It has been mathematically and experimentally shown that this protocol is favorable over other administration protocols.[142,143] To date, several advanced-stage clinical trials have been performed in primary liver cancer using Thermodox combined with RFA. Thus far, however, without convincing clinical evidence for improved outcomes. There are multiple reasons why these studies have not worked out, including the fact that there was no proper methodological standardization, and also because liver cancer is – in general – very difficult to treat.[144,145] Multiple phase I-II trials have been completed or are ongoing in liver and pancreatic cancer in which RFA is replaced by focused ultrasound in combination with temperature-sensitive liposomes,[146–148] together indicating significant promise for future clinical impact.

Another attractive, but clinically clearly less mature, method for indirect tumor targeting is via hitchhiking with circulating cells. This has been best explored for myeloid immune cells and can be exploited in both ex vivo and in vivo setups. Regarding the former, myeloid cells can be ex vivo incubated and endowed with phagocytosis-resistant and cytokine-loaded polymeric backpacks, to promote their in vivo performance.[149–151] Regarding the latter, cRGD-peptide-modified liposomes have been shown to be able to engage with neutrophils and macrophages in systemic circulation in vivo, and subsequently localize to tumors and sites of inflammation by exploiting myeloid cells' intrinsic propensity to target these lesions.[19,152]

A last key example of indirect tumor targeting is discussed in detail below, as part of the cancer nano-immunotherapy section, summarizing efforts to deliver drugs not to tumors, but instead to spleen and bone marrow, for systemic immunopriming. Altogether, indirect tumor targeting approaches are – among other reasons – useful and promising because they help to bypass limitations associated with heterogeneity in tumor vascularization and microenvironment make-up, thereby bypassing the need for efficient direct nanoparticle tumor targeting.

### 5.6. Nano-Immunotherapy

Immunotherapy probably is the most impactful recent addition to the cancer therapy armamentarium. It is in principle enormously powerful, employing the body's own adaptive and innate defense mechanisms against cancer cells. The primary concepts of cancer immunotherapy date back to the 19th century and arguably start with Rudolf Virchow's observation that leukocytes can often be found within solid tumors. At the end of the 19th century, William Coley started bottom-up exploring its therapeutic use, injecting killed bacteria into cancer patients, inspired by some of his previous observations of spontaneous regression or even disappearance of solid tumors in patients with infections. From this time onwards, it took almost a century before cancer immunotherapy really started taking off. In the 1990s, the first CAR (i.e., chimeric antigen receptor) -expressing T cells were generated, and the first immune checkpoints (i.e., CTLA-4 and PD-1) were discovered.[153–156] These events kick-started the immune-oncology revolution, with ever-increasing numbers of CAR T cell products and immune checkpoint-inhibiting antibodies gaining approval for clinical use in the 2–3 decades that followed.[157,158]

Immunotherapy has already substantially contributed to prolonged patient survival times. However, there is still significant room for improvement (Figure 6a). Nanomedicine formulations hold, for multiple reasons, enormous promise and potential to promote the performance of cancer immunotherapy. CAR T cells are typically prepared ex vivo, that is, the harvesting, isolation, and transfection of patients' own T cells takes place outside of their bodies, upon which they are then re-injected into the same patients for therapeutic purposes. Given their cellular nature, large size, relatively short circulation time, and relatively low volume of distribution, they are to date mainly used for non-solid hematological cancers, particularly for lymphomas. Using nanotechnology, however, it has recently been shown that CAR T cells can also be generated in vivo. Initial proof of concept for this has been provided by showing that T cells can be targeted and transfected in vivo in mice using CD5-targeted lipid nanoparticles loaded with mRNA encoding for the antigen receptor.[159]

Immune checkpoint-inhibiting antibodies are nowadays widely used for the treatment of many solid tumors.[160] They are particularly effective against cancers characterized by large amounts of genetic mutations, such as melanoma and lung cancer, as the resulting neo-epitopes expressed on the surface of cancer cells make them more readily recognizable to the immune system. However, patients with tumors with a low(er) mutational burden, and also a significant fraction of patients with melanoma and lung cancer, do not respond well to immune checkpoint blockade. This is oftentimes not only the result of too low mutational load, too few neo-epitopes, and too inefficient cancer cell recognition, but it can also arise from local and/or systemic immuno-suppression. The mechanisms responsible for locally and/or systemically suppressing responses to immune checkpoint-inhibiting antibodies can be manyfold, and they tend to be highly heterogeneous, both inter- and intra-individually.[161] They can, for example, include insufficient neo-antigen expression, impaired immune cell infiltration in tumors, impaired pro-inflammatory (particularly interferon-gamma [IFN$\gamma$]) signaling, presence of immunosuppressive cells (prominently M2-like tumor-associated macrophages (TAM), regulatory T cells ($T_{regs}$) and myeloid-derived suppressor cells [MDSC]), and T cells exhaustion and/or epigenetic modification.[162] Biomarker tools, such as the cancer immunogram or immunoscore, are

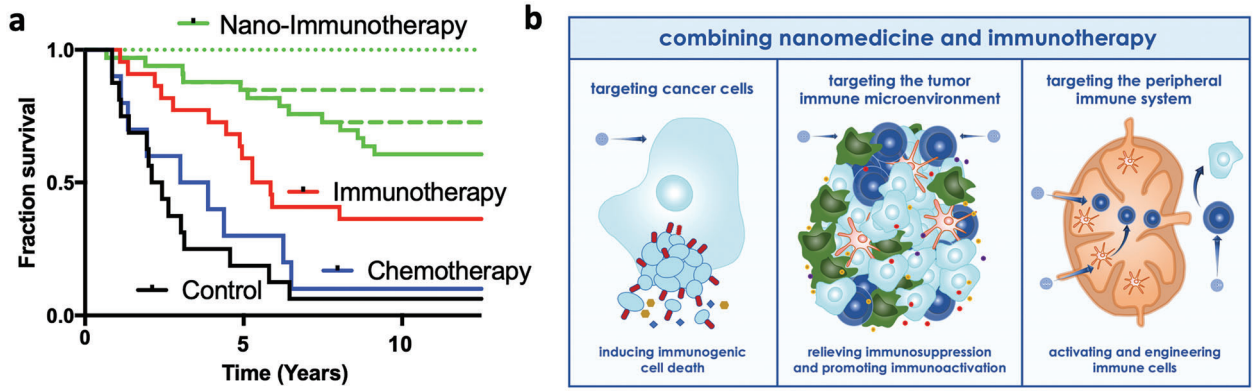


**Figure 7.** Promise and principles of nano-immunotherapy. a) Immunotherapy has revolutionized cancer therapy by substantially prolonging patient survival times. For an ever-increasing number of malignancies, it has been shown that treatment with CAR T cells or immune checkpoint-inhibiting antibodies can induce complete cures and significantly increase the proportion of long-term survivors. Combining immunotherapy with nanomedicine is expected to further boost response rates and long-term survival times. b) Nanomedicine can promote cancer immunotherapy at various levels and in different ways, including at the cancer cell, tumor microenvironment, and systemic level. Figure partially reproduced with permission.[155]

increasingly employed to get a grip on heterogeneity and treatment response.[163,164]

Nanomedicines can assist in some of the above situations and hold value for improving cancer immunotherapy.[165–167] As schematically sketched in **Figure 7b**, they can be employed to enhance the tumor-targeted delivery of (chemotherapeutic) agents that induce immunogenic cell death (ICD;[168]), they can contribute to beneficial priming of the tumor immune microenvironment (TIME;[169]), and they can target non-tumor tissues to promote systemic antitumor immunity. Regarding the former, using doxorubicin or oxaliplatin as potent ICD inducers in free form as well as in nanoformulations, especially also together in one nanoformulation with agents that can help to relieve immune suppression, has been shown to potently promote therapeutic outcomes in patients and mouse models, respectively.[170–173] With regard to TIME priming, given the key role of tumor-associated macrophages (TAM) and other myeloid suppressor cells (MDSC) in suppressing antitumor immunity, as well as the intrinsic propensity of nanoformulations to efficiently accumulate in such cell types, many efforts are being undertaken to target and repolarize them towards more immune-permissive phenotypes.[20,174] Lastly, to promote systemic antitumor immunity, nanomedicines hold promise for delivering cargo to non-tumorous immune-modulating organs and tissues. This has become widely appreciated in the COVID-19 pandemic, showcasing how intramuscularly injected lipid nanoparticles (LNP) can target lymph nodes, promote mRNA-mediated antigen expression, and induce strong and specific systemic immune responses. It is important to keep in mind in this regard that BioNTech already started exploring their mRNA-loaded lipid nanoparticles in cancer patients years before COVID-19, with formulations optimized for delivery of neo-epitope-encoding mRNA to antigen-presenting cells in the spleen, to induce patient-specific systemic antitumor immune responses.[175,176] Nanomedicines can also be designed to target myeloid progenitor or stem cells in the bone marrow, to help train the innate immune system for improved immunotherapy outcomes.[177–179] Such nano-immunotherapy strategies hold great promise for boosting the efficacy of anticancer therapy.

## 6. Conclusion

Nanomedicine is a very popular and productive research field. Nanomedicine formulations are widely explored to assist in improving tumor-targeted drug delivery and anticancer treatment efficacy. The clinical translation of cancer nanomedicines has been lagging behind expectations, though, for several reasons. Some of these reasons are more obvious than others. This manuscript describes the basic principles of tumor-targeted delivery, summarizes relevant recent progress, provides an overview of cancer nanomedicine products currently used in the clinic, outlines the most prominent problems and pertinent challenges hindering translation, and discusses the most promising future directions for the use of nanomedicine formulations for cancer therapy.

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## Conflict of Interest

The authors declare no conflict of interest.

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