Complex effects of tumor microenvironment on the tumor disposition of carrier-mediated agents

Major advances in carrier-mediated agents, including nanoparticle, conjugates and antibody–drug conjugates, have created revolutionary drug delivery systems in cancer over the past two decades. While these agents provide several advantages, such as greater duration of exposure and solubility, compared with their small-molecule counterparts, there is substantial variability in delivery of these agents to tissues and especially tumors. This review provides an overview of tumor microenvironment factors that affect the pharmacokinetics and pharmacodynamics of carrier-mediated agents observed in preclinical models and patients.

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The number of available carrier-based drug systems for the treatment of cancer and other diseases has seen exponential growth in the past three decades. As of 2013 there are more than 1600 nanotechnology-based products in the market, and almost 250 nanomedicine agents on the market or in clinical trials, with more emerging at a rapid pace [1]. While the number of agents used clinically is still limited, the plethora that are emerging as potential therapeutic agents warrants the need for detailed studies of their unique pharmacology in animal models and in humans. This review provides an overview of factors that affect the pharmacokinetics (PK) and pharmacodynamics (PD) of carrier-mediated agents (CMAs) in preclinical models and patients, especially in the treatment of cancer where tumor microenvironment factors alter and hinder the delivery and penetration of these agents in solid tumors.

Promises of CMAs

The theoretical advantages of CMAs in cancer treatment include increased solubility,

prolonged duration of exposure, selective delivery of entrapped drug to the tumor and an improved therapeutic index [2,3]. The primary types of anticancer CMAs are liposomes, nanoparticles (NPs) and conjugated agents. PEGylated liposomal doxorubicin (Doxil®; PLD), liposomal daunorubicin (DaunoXome®), paclitaxel albumin-bound particles (Abraxane®), paclitaxel polymeric micelles (Genexol® PM), liposomal irinotecan (Onivyde®) and liposomal vincristine (Marqibo®) are members of this relatively new class of drugs that are approved by the US FDA for the treatment of solid tumors [4]. However, the promise of these drugs is unfulfilled due to several factors including overall low tumor uptake [5,6].

Several reviews exist that summarize currently available and late stage development of chemotherapeutic CMAs [7,8]. Examples of various types of CMAs include liposomes, fullerenes, carbon tubes, quantum dots, nanoshells, polymers, dendrimers and conjugates, including antibody–drug

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conjugates (ADCs) [9]. Liposomes are vesicles that consist of a phospholipid bilayer with drug either contained in the aqueous center or embedded within the phospholipid bilayer. Liposomes may be either conventional or stabilized with polyethylene glycol (PEGylated) or another substance, which greatly increases their circulation time [10]. Nonliposomal NPs include solid lipid NPs and polymeric NPs. Solid lipid NPs consist of a microemulsion which has drug loaded in the lipid portion [11]. Polymeric NPs consist of a polymeric matrix, which can be molded into different shapes and sizes, with drug embedded within [10,12]. Conjugated and dendrimer agents consist of a small-molecule (SM) drug linked to a polymer, series of

polymers or another substance [10,13]. ADCs include highly cytotoxic or potent drugs linked to monoclonal antibodies designed to preferentially target cells expressing a certain cell-surface antigen [14]. **Table 1** summarizes currently available and late-stage development of chemotherapeutic CMAs for the treatment of cancer.

Pharmacokinetic characterization

The disposition of CMAs is dependent upon the carrier and not the therapeutic entity until the drug gets released [15]. This required the creation of nomenclature used to describe CMA PK, including: encapsulated or conjugated (the drug within or bound to the carrier),

Table 1. Approved and late phase chemotherapeutic carrier-mediated agents.

Antibody–drug conjugates		Liposomes	
Compound	Trial phase	Compound	Trial phase
Brentuximab vedotin (Adcetris®)	Approved	Liposomal daunorubicin (DaunoXome®)	Approved
Ado-trastuzumab emtansine (Kadcyla®)	Approved	Liposomal cytarabine (DepoCyt®)	Approved
Gemtuzumab ozogamicin (Mylotarg®)	Approved [†]	Liposomal vincristine sulfate (Marqibo®)	Approved
Inotuzumab ozogamicin	III	EndoTAG-1 (3)	III
Mirvetuximab soravtansine	III	ThermoDox® (3)	III
Sacituzumab govitecan	III	Vyxeos® (3)	III
Vadastuximab talirine	III	SGT53 (2)	II
ABT-414	II	PEGylated liposomes	
AGS-16C3F	II	Compound	Trial phase
Anetumab ravtansine	II	Liposomal doxorubicin (Doxil®)	Approved
Denintuzumab mafodotin	II	Liposomal irinotecan (Onivyde®)	Approved
Glembatumumab vedotin	II	IHL-305	II
Indusatumab vedotin	II	Micelles & polymer conjugates	
Lifastuzumab vedotin	II	Compound	Trial phase
Lorvotuzumab mertansine	II	Genexol® PM	III
SAR 566658	II	NK105	III
SC16LD6.5	II	Opaxio®	III
Albumin-stabilized nanoparticles		Paclical®	III
Compound	Trial phase	CRLX-101	II
Nab-paclitaxel (Abraxane®)	Approved		
ABI-009	II		

Criteria for inclusion: Listed carrier-mediated agents (CMAs) have either achieved US FDA approval (Approved) or reached late phase clinical trials. Late-phase agents classified by registration on ClinicalTrials.gov of any nonterminated Phase III trial (III), or >2 Phase II trials open or active, not recruiting (II) as of April 2017.

[†]Mylotarg® was voluntarily withdrawn in 2010; re-application for FDA approval submitted in 2017.

released (active drug that no longer associates with the carrier), and 'sum total' or 'total' (encapsulated/conjugated drug plus released drug) [15,16]. CMAs act as pro-drugs and are not active until the SM drug is released from the carrier. In theory, the PK disposition of the drug after release from the carrier should be the same as after administration of the SM or standard formulations [15]. Thus, the pharmacology and PK of CMAs are complex. As a result, analytical studies must be performed in order to assess the disposition of encapsulated/conjugated and released forms of the drug in plasma, tumor and tissues as part of PK and biodistribution studies in animals and patients. Other issues in estimating and calculating tumor PK of CMAs are discussed later in this review.

Interaction of CMAs with the immune system

Considerable inter-patient variability exists in the PK and PD of CMAs [17], and while the exact factors have not been finalized, it is hypothesized that the mononuclear phagocyte system (MPS) plays a key role [18]. Once a CMA enters the bloodstream, CMA uptake by immune cells can occur in circulating monocytes, leukocytes and dendritic cells in the bloodstream [19,20]. The interaction and subsequent effects of CMA therapeutics on the immune system have not been fully elucidated, but presently, are generally placed into one of two categories: responses to CMAs that are specifically modified to stimulate the immune system, and undesirable interactions and/or side-effects [19]. **Figure 1** illustrates the interaction and clearance of CMAs with these immune cells, collectively termed the MPS. Further discussion of the interaction with the MPS is discussed later in this review.

Tumor delivery & payload release

Influence of tumor barriers on delivery of CMAs

In theory, increased permeability of the tumor vasculature allows CMAs to enter the tumor interstitial space, while suppressed lymphatic filtration allows them to stay there. This phenomenon, termed the enhanced permeability and retention (EPR) effect, may be exploited by CMAs to deliver drugs to tumors [5–6,19]. However, progress in developing effective CMAs using this approach has been hampered by heterogeneity of the EPR effect in different tumors and the lack of information on factors that influence EPR [5–6,20]. Cancer cells in tumors are surrounded by a complex microenvironment comprised of endothelial cells of the blood and lymphatic circulation, stromal fibroblasts, collagen, cells of the MPS and other immune cells. Each of these components may be associated with the variability in EPR and are potential barriers to tumor delivery and

intratumoral distribution of CMAs (**Figure 2**) [5,20–23]. In addition, the potential barriers may be highly variable within and across tumors which leads to high variability in the EPR effect. Thus, it is important to understand potential barriers in the tumor microenvironment in order to improve the tumor delivery and drug release of CMAs in solid tumors.

CMAs are able to deliver more drug cargo to tumor compared with SM drugs; however, the overall tumor delivery of CMAs is relatively low and inefficient. A recent meta-analysis of 117 studies suggests that only 0.7% of an administered CMA dose reaches the tumor [25]. Another study evaluating published concentration versus time curves of several SM and CMA agents showed that 82% (14/17) of SM agents evaluated showed a greater ratio of tumor AUC to plasma AUC ratios compared with their CMA counterpart [26]. This suggests that while SMs are cleared from plasma much faster than CMAs the tumor exposure relative to plasma exposure is greater for SMs compared with CMAs [26]. These studies also highlight the potential limitations of EPR-based CMA delivery to tumors and the need to study tumor barriers to drug delivery.

A recent workshop by the Alliance for Nanotechnology in Cancer concluded that there are major gaps in the understanding of factors that affect and inhibit EPR effect and CMA tumor delivery and new fundamental preclinical and clinical studies in this area are needed to effectively advance CMA drug delivery and efficacy in solid tumors [5]. So far, the advancement of CMA treatment of cancer has been focused primarily on modifying formulations to overcome PK, efficacy and toxicity issues. However, this approach alone may not be adequate as biologic issues, such as barriers within the tumor microenvironment, appear to play important roles in low and inefficient tumor delivery of CMAs. Thus, the development of novel methods to evaluate and overcome these barriers and increase the tumor delivery and efficacy of CMAs in a variety of solid tumors are desperately needed.

Tumor specific targets & active targeting of CMAs

Active targeting of CMAs may further improve tumor delivery and activity by allowing the CMA to bind to tumor cells using surface-attached ligands [19]. However, 'active targeting' has also been used to describe a specific binding target for therapeutic activity, such as ado-trastuzumab emtansine targeting HER2 receptors, and also 'trapping' CMAs by binding to targeted receptors to provide increased delivery efficiency to tumors. While antibody-mediated targeting has been the method of choice, other targeting strategies using nucleic acids, carbohydrates, peptides, aptamers and

Figure 1. Clearance of nanoparticle and carrier-mediated agents via the mononuclear phagocyte system. Small-molecule anticancer agents undergo a standard route of metabolism and elimination, including enterohepatic recycling and removal through the kidney. However, carrier-mediated agents, which are engulfed by phagocytes, are contained primarily in compartments such as the spleen, liver and peripheral blood mononuclear cells. Antibodies and antibody–drug conjugates are cleared in similar compartments, though through Fc-receptor mediated endocytosis. For full color figures, please see online at www.futuremedicine.com/doi/full/10.2217/nmm-2017-0101

vitamins are also being evaluated [19]. New methods of 'active targeting' of CMAs may further improve tumor delivery and activity by maximizing the specificity of binding of a targeting agent to specific cells within tumors [19]. However, in general the use of active targeting of NPs has not resulted in significant improvements in tumor delivery due to systemic clearance and tumor barrier issues [27,28]. The addition of targeting ligands on the surface of NPs results in higher rates of clearance of the targeted NPs compared with nontargeted NPs [28]. The targeting ligand may not overcome the inherent tumor barriers associated with the NP carrier. The next generation of targeted CMAs may be able to address these issues, but additional fundamental physiochemical and biological issues still remain to be solved in this field before actively-targeted CMAs can consistently improve the response in solid tumors.

The use of antibody technology as the targeting mechanism has been incorporated into other CMA formulations such as liposomes. MM-302, currently in early phase clinical studies, incorporates anti-HER2 scFv-PEG-DSPE conjugates to the outer surface of PLD [29]. In preclinical models, MM-302 has shown

superior antitumor activity to SM doxorubicin and PLD in HER2 over-expressing tumors [30].

While the addition of targeting antibodies may be advantageous for cell selectivity, antibody production can be expensive and significantly increase the hydrodynamic size (~20 nm) of a CMA. Smaller, easier to manufacture targeting molecules may provide benefits. BIND-014 is a targeted polymeric nanoparticle designed to target cancerous tissues expressing prostate-specific membrane antigen (PSMA) and release docetaxel at a controlled rate after accumulation within tumor tissue. The targeting function of BIND-014 is accomplished by incorporation of the SM ligand S,S-2-[3-[1-carboxy-5-amino-pentyl]-ureido]-pentanedioic acid in the polymer matrix encapsulating docetaxel. Pentanedioic acid selectively binds to PSMA, a cell-surface protein expressed on prostate tumor cells and neovasculature of most non-prostate solid tumors [31,32]. In human-xenograft bearing mice of breast, non-small-cell lung, and prostate cancers, as well as in rats and nonhuman primates, BIND-014 demonstrated prolonged circulation and controlled release of docetaxel, maintaining plasma

concentrations 100-fold higher than SM docetaxel [32]. To demonstrate the targeting capability of BIND-014, intratumoral concentrations of docetaxel were measured in mice bearing LNCaP prostate cancer xenografts. Drug levels were similar 2 h after administration of either SM docetaxel or BIND-014 but 12 h after dosing drug concentrations in tumor were sevenfold higher with BIND-014 [32]. PSMA-targeted BIND-014 administered to mice bearing MX-1 breast cancer xenografts demonstrated a 26% decreased tumor mass, whereas nontargeted BIND-014 resulted in a 75% increase in tumor mass ($p < 0.01$), demonstrating targeting efficacy [32]. However, while mice implanted with NCI-H460 non-small-cell lung cancer xenografts did not display a statistically significant difference, a similar trend was observed [32]. Targeted and nontargeted BIND-014 exhibited similar antitumor effects in non-PSMA expressing tumors, presumably due to increased drug accumulation in the tumors by means of the EPR effect. Taken together, these data demonstrate BIND-014's prolonged circulation time, ability to target tumors expressing PSMA and the controlled release of its chemotherapeutic payload. BIND-014 has now completed Phase II trials with positive efficacy results in the treatment of squamous non-small-cell lung cancer and metastatic castration-resistant prostate cancer [33].

Other targeting ligands have shown promising pre-clinical results. Cell surface lectins recognize and bind sugar moieties in glycoconjugates and have been found to be overexpressed on several cancer types [34]. The asialoglycoprotein receptor specifically binds lactoferrin and is present on hepatocellular carcinoma cells. Lactoferrin-modified PEGylated liposomes containing doxorubicin displayed significantly higher anti-tumor efficacy in a hepatocellular carcinoma mouse model and increased uptake into ASPGR-positive cells versus unmodified doxorubicin-loaded PEGylated liposomes [35]. Nucleic acid aptamers are able to fold, forming complex tertiary structures capable of binding targets with high specificity [36]. *In vivo* results in human prostate murine xenograft models using aptamer targeted CMAs loaded with docetaxel demonstrated significantly reduce tumor size after a single injection and 3-month survival compared with nontargeted docetaxel controls [37]. These examples represent alternative targeting strategies which maintain selectivity benefits while avoiding the use of mAbs.

The use of antibodies as the targeted carrier is a popular technique as demonstrated by the numerous ADCs in late-stage clinical trials. These immunoconjugates are designed to exploit the specificity of monoclonal antibodies to deliver potent cytotoxic drugs to tumors while limiting off-target exposure.

Figure 2. Complexity of the tumor microenvironment. The tumor microenvironment poses multi-faceted barriers to drugs' transport owing to the dense stromal tissue, which is composed of collagens, fibronectin and hyaluronan, an abundance of cancer-associated fibroblasts, and aberrant interactions between infiltrating tumor-associated immune cells, cancer cells, and cancer-associated fibroblasts. Adapted with permission from [24].

The drugs conjugated to the antibody are highly potent ($IC_{50} < 10^{-9}$ M) and thus are unable to be safely dosed systemically without a carrier. Examples of approved ADCs include ado-trastuzumab emtansine (Kadcyla®), brentuximab vedotin (Adcetris®) and gemtuzumab ozogamicin (Mylotarg®). Examples of ADCs in clinical trials include inotuzumab ozogamicin, an anti-CD22-calicheamicin conjugate, and ABT-414, an anti-EGFR-auristatin conjugate [38].

Despite active targeting, one of the major challenges to efficacy is the heterogeneous distribution of mAbs and ADCs in tumors after systemic administration [39]. This allows large groups of untargeted cells to escape therapy, and thus inadvertently select for more resistant cells. Furthermore, tissue and blood vessel architecture will influence tumor targeting [40]. For many mAbs and ADCs, systemic clearance of the antibodies from plasma plays a key role in determining the penetration of targeting [39]. Although intact IgG is cleared relatively slowly from the blood, smaller fragments (e.g., Fab) are cleared rapidly via renal filtration, preventing saturation of tumor tissues [41]. Reducing the systemic clearance of mAbs/ADCs in theory should increase the amount of tumor uptake by maintaining a greater diffusive gradient of antibodies entering the tumor for a longer period of time [39]. Several different methods have demonstrated improved tumor accumulation of smaller antibodies after conjugation to large molecules, such as polyethylene glycol (PEG) [42] or albumin [43]. However, using larger proteins or polymers to reduce serum clearance of an antibody results in a corresponding decrease in the antibody's capillary permeability and ability to diffuse into and within tumors [44]. Decreased uptake and penetration of PEGylated IgGs compared with native IgGs observed within mice bearing colon cancer xenografts demonstrates this phenomenon [45]. Thus, the most effective size and makeup of the targeting and carrier components of ADCs are unclear and may vary by target or disease.

Rapid & extensive release of drug from CMAs in blood

CMAs alter both the PK and PD profiles of their associated drug(s). Ideally, the properties of the CMA will prolong circulation time, limit systemic exposure of the released drug, and increase tumor accumulation and targeting of drug cargo, thereby leading to decreased off target toxicities and increased efficacy of the active agent. To accomplish this, the encapsulated or conjugated drug must be stable in systemic circulation but efficiently released at the desired site of action. Achieving this balance is critical to favorably altering the therapeutic index of the active drug.

Mylotarg (gemtuzumab ozogamicin) was the first ADC to reach the market after gaining FDA approval in 2000. It is made up of an extremely potent antitumor antibiotic, calicheamicin, coupled to an anti-CD33 IgG via a pH-sensitive hydrolysable linker. Early trials showed promising remission rates in the high-risk population of older patients with relapsed acute myeloid leukemia [46]. However, confirmatory trials indicated a higher incidence of early fatality in patients receiving Mylotarg and it was voluntarily withdrawn from the market in 2010 [46]. *In vitro* studies showed that the linker was poorly thermostable, with rapid and extensive release of calicheamicin from the antibody, and toxicity to CD33-negative MOLT-16 cells [47]. This high systemic exposure to the released active drug is implicated in the significant toxicities associated with administration of Mylotarg in patients, including prolonged severe thrombocytopenia and sinusoidal obstructive syndrome [48]. This example highlights the need to perform detailed PK studies of both conjugated and released forms of drug after administration of an ADC.

Novel formulations of paclitaxel including Abraxane (paclitaxel protein-bound particles) and Genexol PM (paclitaxel polymeric micelles) have gained approval in the USA and abroad. In contrast to many other nano-formulations the goal of these formulations is generally to improve tolerability by avoiding the vehicle used to solubilize paclitaxel in the conventional formulation, Cremophor EL (CrEL). CrEL causes several toxicities including acute hypersensitivity reactions which can disrupt treatment [49]. Letchford and Burt investigated the *in vivo* pharmacokinetics of paclitaxel containing MePEG-*b*-PCL micelles and nanospheres in comparison to conventional paclitaxel with CrEL in CD1 mice. Their results show rapid dissociation of paclitaxel from both NPs leading to rapid clearance from the plasma. In fact, the micellar formulation of paclitaxel had lower AUCs and half-lives than the conventional paclitaxel formulation, likely due to the effects of CrEL on the distribution and clearance of paclitaxel and/or additional clearance mechanisms of the micelles, such as the MPS [50]. Decreased AUC and half-life has also been observed in patients treated with Genexol PM relative to conventional paclitaxel [51]. The clinical and regulatory success of novel paclitaxel formulations highlights the broad utility of NP-based delivery beyond reliance on EPR.

Inefficient drug release from CMAs in tumors

While encapsulation or conjugation stability in systemic circulation is desirable to limit toxicity, the released active drug must be available at the site of action in order to achieve efficacy. SPI-077, the first

liposomal cisplatin formulation to reach clinical trials, displayed promising preclinical results including enhanced tumor exposure of total (encapsulated + released) platinum in murine lung cancer models [52] and decreased toxicity in cynomolgus monkeys compared with cisplatin [53]. Unfortunately, SPI-077 had disappointingly low efficacy in clinical trials of lung [54], ovarian [55], and head and neck cancer [56]. Pharmacokinetic analysis revealed nondetectable released platinum in plasma and tumor extracellular fluid and low platinum-DNA adducts, indicating minimal release of cisplatin from the PEGylated liposomal carrier [57]. The results of this study emphasized the need to evaluate the encapsulated and released form of drugs in plasma and tumor and intracellular exposures of the cytotoxic form of the drug.

Following the clinical failure of SPI-077, Zisman *et al.* investigated the influence of drug release on antitumor activity in a murine lymphocytic leukemia model [58]. Pharmacokinetic, biodistribution and efficacy studies were completed for SM cisplatin and liposomal cisplatin formulations with varied lipid compositions. Their results indicate that intermediate rates of drug release produce superior efficacy compared with rates of drug release at either extreme. In addition, the slowest releasing formulation had increased accumulation in liver and spleen, the primary organs of the MPS, compared with the intermediate release rate formulation and SM cisplatin which could lead to increased off-target toxicity without efficacy benefit [58].

Release rate from drug conjugates has shown similar relationships with antitumor efficacy. Self-assembling micelle formulations of dextran-doxorubicin (DexDOX) conjugates were recently examined by Li *et al.* In B16F10 melanoma-bearing BALB/c mice both conjugates exhibited superior antitumor efficacy compared with SM doxorubicin. However, higher molecular weight dextran (500 kDa) resulted in increased drug release rate and decreased tumor volume compared with the lower molecular weight dextran (40 kDa) conjugate [59]. These studies highlight the need to optimize the release kinetics of each carrier-drug combination to maximize efficacy.

'Triggered' release of cytotoxic payload

Release of active drug from its carrier can be controlled or altered by numerous mechanisms. Various ADC linkers depend upon hydrolysis, enzymatic cleavage or proteolysis for liberation of the active drug. Other nanoformulations including polymeric NPs and liposomes may rely upon similar strategies such as pH- or temperature-responsive elements. In an effort to further improve the targeted release of active drug from CMAs, novel release triggers that take advantage of

known tumor properties or controllable stimuli are currently being investigated.

Matrix metallo-proteinase 2 (MMP2) is widely known to be overexpressed in a variety of cancers and has been associated with metastasis and invasion via hydrolysis of type IV collagen. Cantisani *et al.* developed a PLGA block copolymer NP composed of doxorubicin bound to PEG via an MMP2-cleavable peptide (PELGA-TAP) [60]. The action of MMP2 *in vitro* increased doxorubicin release from PELGA-TAP NPs by approximately twofold over 48 h versus incubation without MMP2. CMA penetration and release was assessed in tumor (U87-MG; human glioma) and healthy tissue (HDF; primary human dermal fibroblasts) spheroids incubated with PELGA-TAP or PELGA-Dox (control NPs without MMP2-sensitive function). U87-MG cells had 8.4-fold higher active MMP2 compared with HDF and exhibited enhanced doxorubicin accumulation following PELGA-TAP NP incubation. At 48 h, the U87-MG spheroids had 80% higher fluorescence intensity than HDF spheroids. At the same time point, PELGA-Dox NPs had only 25% higher fluorescence intensity in U87-MG compared with the HDF spheroids indicating that the PELGA-TAP NPs more effectively release doxorubicin into tumor in response to MMP2 action [60]. *In vivo* PK studies will need to be performed to validate and translate these results.

External stimuli may provide the ability to control the location and timing of drug release from CMAs. Potential triggers include thermal, pH, UV light, ultrasound, radiofrequency and application of magnetic fields [61–63]. One such example of this technology is ThermoDox[®], a thermosensitive PEGylated liposome, which is formulated to release its encapsulated drug by applying radiofrequency ablation to elevate local tissue to 39–42°C [64]. Another recent example of this technology focuses on the use of x-ray induced photosensitizers, which used x-rays to induce the release of drug from lanthanide micelles loaded with hypericin [65]. Another example of a triggered carrier is PEGylated tumor necrosis factor- α (TNF)-coated gold nanoparticles (i.e., CYT-6091), which releases its payload after a photothermal stimuli. The use of gold-nanoparticles provides additional imaging benefits via MRI or CT scans to image affected areas. CYT-6091 was able to demonstrate reduced tumor growth in a murine squamous head and neck tumor model and has recently been granted FDA approval to begin Phase I trials [66].

An alternating magnetic field (AMF) is a desirable external trigger due to its ability to deeply penetrate tissues (compared with light or radiation), safety and the availability of clinically approved biocompatible magnetic field-responsive elements. Ferriera *et al.* recently

prepared and characterized gemcitabine-loaded magnetoliposomes composed of magnetite, dipalmitoylphosphatidylcholine (DPPC) and cholesterol. Exposure to AMF at 356 kHz resulted in efficient release of gemcitabine from the magnetoliposomes. Approximately 40 and 70% of the encapsulated gemcitabine was released after 1 min and 5 min of AMF, respectively. In contrast, incubation of the magnetoliposomes at 37°C for 72 h without AMF exposure resulted in only 17% of the encapsulated gemcitabine being released. Drug release in the presence of AMF in this system is the result of magnetic hyperthermia causing lipid phase transition of the encapsulating liposome which allows for the gemcitabine to be released. AMF therefore has the potential for the dual application of local hyperthermia and drug delivery in cancer treatment [67].

Other advanced mechanisms of transport and release are being developed to combine triggered release of agents with macro-carriers. Polymeric CMAs have been attached to red blood cells (RBC) as a means to improve the *in vivo* circulation time of these agents, where the force due to circulation and cell–cell interactions causes their delayed release by becoming sheared from the surface of the RBC [68]. An improvement on this technique utilizes vitamin B12 as a light-sensitive releasing agent, allowing for site specific release of agents from RBCs when a particular wavelength of light is delivered through the skin [69].

The release of the active-chemotherapeutic agent from the carrier and into the tumor matrix or cancer cell is difficult to measure but appears to be very low in most cases. Moreover, the low release of drug from the carrier may even be a more critical issue for CMA treatment of solid tumors than the apparent low delivery of the carrier to the tumor. Thus, the development of novel carrier characteristics or external stimuli to significantly enhance the release of drug from the carrier is needed. The development of such nanoformulations that include ‘trigger’ technologies may enable unique advances as related to enhancing the release of active-drug from the carrier.

Tumor microenvironment factors

Many of the limitations of chemotherapy treatment were thought to be primarily due to mechanisms of drug resistance at the cellular level [70]. These individual gene mutations encode proteins that alter the uptake, metabolism, export of chemotherapy from individual tumor cells and the ability to interact with the cellular target. However, substantial evidence has recently been reported that suggests the tumor microenvironment also mediates a level of drug resistance in solid tumors via inhibiting or altering tumor deliv-

ery of drugs and immune changes. The delivery and distribution of chemotherapy in tumors is highly heterogeneous, resulting in only a fraction of tumor cells being exposed to cytotoxic levels of chemotherapy. These physical and biochemical changes caused by tumors are highly variable within and across tumors and can affect the PK and PD of CMAs. The following sections will review factors of the tumor microenvironment and how they can impact tumor delivery and disposition of anticancer agents.

Tumor vascularity, perfusion & permeability

The utility of CMAs depends on the successful delivery of the carrier and contained agent to individual tumor cells. This process includes several steps and complex factors such as transport from the systemic blood to tumors, extravasation from tumor vasculature and transport in the tumor interstitium. Compared to normal tissues, tumor tissues have poorly organized and defective vasculature with substantial location-dependent heterogeneity [71]. In general, the tumor vasculature is leakier and more permeable with larger pore sizes (ranging 100 to 780 nm) compared with healthy tissues (<6 nm; liver/spleen sinusoids 50 to 150 nm) [71]. These conditions are favorable for CMA extravasation (via diffusion and convection) into tumors and allow for passive targeting of larger CMAs (greater than 100 nm) into the tumor extracellular fluid. These factors were the theoretical basis for CMA delivery to tumor via the EPR effect.

However, the abnormally high leakiness of the vasculature in tumors is highly variable, and in fact, may hinder CMA drug delivery by inducing blood flow stasis and increases in interstitial fluid pressure within the tumors [71,72]. Furthermore, normalized vasculature becomes less leaky to macromolecules compared with abnormal vasculature [71,72]. Although permeability may be less in normalized vasculature in tumors compared with abnormal vasculature in tumors, the permeability of the normalized vasculature in tumors is still significantly higher than healthy tissues [72]. However, while tumor blood vessel normalization has been achieved, the benefits of normalization appear transient (2 to 5 days) [71,72]. Vascular regression is common after antiangiogenic therapies, where the blood vessel density will begin to drop due to cellular apoptosis [71,72]. Moreover, this leads to drug delivery to again become compromised.

Vascular normalization refers to the induced alteration of the vasculature to a more structurally and functionally normal state through the balance of pro- and antiangiogenic signals [73]. Tumor blood vessel normalization had first been achieved in 1972 using topoisomerase II inhibitors (ICRF-159), though antiangio-

genic agents have been used more recently to illicit the same effects [74]. DC101, a VEGF receptor-2 antibody, blocked VEGF signaling which lead to ‘normalization’ of the tumor vasculature as assessed by intravital microscopy in mice bearing various xenograft models [72]. It is believed that by pruning immature vessels and improving the integrity and function of remaining vessels, the basement membrane and pericyte coverage is enhanced. This leads to the formation of transvascular gradients that decrease tumor interstitial fluid pressure (IFP) and improve the penetration of macromolecules into tumors [72].

The transport of CMAs in systemic circulation is further regulated by the hydrodynamic forces and fluid–formulation interaction of the body’s blood flow. A CMA will experience several forces (e.g., tumbling and rolling dynamics) and interactions (e.g., particle–cell interactions) while within circulation, which can influence the ultimate biofate of the CMA. Therefore, the interaction of the CMA with the endothelial walls, through particle–cell and receptor–ligand interactions, as it travels through fenestrated tumor blood vessels is a very important design consideration and highly relevant to drug delivery [75]. Recent work has focused on utilizing drug-carrier mediated systems to improve retention and delivery of several therapeutics to hard-to-access tissues, such as tumors and the CNS [76–79]. Yet swift blood flow, such as in the cerebrum (~750 ml/min, or ~12% of an adult’s cardiac output), can create a barrier against particle-cell binding as shear forces simply drag the CMA off the neovasculature endothelium, rendering insufficient targeting [80]. Using traditional analytical methods, it would be very difficult to determine this effect *in vivo*. However, with recent advances using *in vivo* imaging and mass spec techniques, it could be possible to observe the effect of blood fluid dynamics and the interaction of CMAs with endothelial walls of the vasculature as well as nanoparticle penetration through the vasculature [81–84].

Physiological factors related to the tumor vasculature, such as heterogeneous blood supply, uneven permeability, and larger transport distances in the interstitium, have been found to be responsible for the poor localization of NP and macromolecule delivery within tumors [5,20]. Such abnormalities in the tumor microvasculature can further create hostile microenvironments, hindering anticancer treatments (including chemotherapy and radiation) [5]. Unlike the necrotic core of a tumor, a tumor’s periphery is highly perfused but has lower vascular permeability compared with the necrotic core [85]. This has been demonstrated in adenocarcinoma xenograft models using window chambers to observe labeled liposomes [86]. While labeled liposomes

showed significant accumulation in xenografts compared with normal tissues, they also showed heterogeneous distribution in tumor, with greater accumulation in perivascular regions [86]. Similar heterogeneous distribution results were observed with ¹¹¹In labeled polymeric micelles using SPECT imaging in MCF7 and MDA-MB-468 tumors [87]. However, both studies showed CMA uptake primarily in the periphery of tumors, which suggests that the problem with penetration of CMAs into tumors is limited by perfusion (ability to migrate within the microenvironment) and not permeability (ability to cross vascular barriers). Dual contrast agent MRI assessments of a glioblastoma xenograft model revealed intratumoral regional differences in blood volume and vessel permeability. The tumor periphery was found to have increased blood volume relative to both normal tissue and the tumor center while displaying significantly decreased permeability in comparison to the tumor center [88]. However, how these results on perfusion and permeability extend to other tumor models remains unclear.

Studies evaluated the role of heterogeneity of tumor microenvironment, including the vasculature, and other factors at baseline and after treatment on the variability of tumor delivery and efficacy of CMA and standard SM anticancer agents. One such study evaluated the role of heterogeneity of the tumor microenvironment on the tumor delivery and efficacy of PLD and SM-doxorubicin in genetically-engineered mouse models (GEMMs) of triple negative breast cancer (TNBC) [15]. Basal-like (C3-Tag) and claudin-low (T11/TP53-/-) orthotopic syngeneic transplant GEMMs of human TNBC subtypes were evaluated [15]. Using immunohistochemistry, each tumor model was evaluated for TAM (F4/80), vascularity (CD31) and collagen (collagen IV) after PLD or SM-doxorubicin was administered at 6 mg/kg IV $\times$ 1. There was a substantial difference in tumor exposure of PLD, but not SM-doxorubicin, in the two GEMMs (Table 2). C3-Tag demonstrated greater antitumor response to PLD compared with T11. Changes in microvessel density (MVD) over time in C3-Tag and T11 GEMMs after administration of SM-doxorubicin and PLD administration are presented in Figure 3 & B. In C3-Tag tumors, the MVD remained relative stable after administration of SM-doxorubicin and PLD. However, in the T11 tumors there was a 30% decrease in MVD at 96 h after PLD administration compared with baseline ($p < 0.05$). Intratumoral concentrations of VEGF- α versus time profiles after PLD or SM-doxorubicin in the C3-Tag and the T11 tumors are presented in Figure 3C. T11 tumors had significantly higher levels of VEGF- α ($p = 0.003$) compared with C3-Tag tumors. PLD had greater impacts on VEGF- α

levels ($p = 0.02$) compared with SM-doxorubicin and the effects vary with tumor subtype. These results suggest that tumor-specific factors in TNBC may affect the delivery of CMAs but not SMs. The similar TAM, MVD and collagen at baseline in GEMMs suggest alternative tumor factors (e.g., pericytes or tumor perfusion) may affect the tumor delivery of CMAs but not SMs. Also changes in vascularity and VEGF- α over time may reduce tumor delivery of PLD in T11 tumors. The results of this study also highlight the bi-directional interaction between CMAs and the tumor microenvironment and how administration of a CMA may ultimately change the tumor microenvironment and reduce its own tumor delivery.

Tumor stroma

The stroma, a collective term for the basement membrane, fibroblasts, immune cells and extracellular matrix, makes up the majority of a tumor’s mass [89]. However, unlike normal tissue, the interaction between tumor cells and the stroma is characterized by increased inflammation and matrix building activity [90]. For instance, as fibroblasts in tumors are not regulated, this leads to continuous proliferation [90]. This buildup of extracellular matrix further forms a barrier to diffusive and convective transport, which inhibits the distribution of CMAs in tumor significantly more than SM drugs [91,92]. For instance, Jain and Stylianopoulos have reviewed how the dense collagen fibers decrease the intratumoral transport of PLD, DaunoXome and Abraxane [20]. A steady supply of nutrients is also required for cells to be able to replicate and nutrient deprivation can lead to cell cycle arrest. As a result, the rate of tumor proliferation tends to decrease the further away from the blood vessels [93]. This becomes problematic as most chemotherapeutic agents, and possibly biologics, are more effective against proliferat-

ing cells [93]. In addition, cells further away from the vasculature proliferate more slowly and are likely to be resistant to therapy.

There has been a growing interest in targeting the stroma using CMAs. Such strategies have specifically targeted fibroblasts as a means to debulk tumors and allow for greater perfusion of CMAs [94]. One such example is a docetaxel NP conjugate composed of PEGylated and acetylated carboxymethylcellulose that selectively targets fibroblasts [95]. This study showed that greater than 85% of the NPs were internalized by cancer-associated fibroblasts in an orthotopic murine breast cancer model, leading to a near complete depletion of fibroblasts, significant increase in tumor perfusion, and reduced IFP within a week of administration [95]. Additional studies need to be performed to determine if this CMA treatment can lead to increased delivery of other CMAs or macromolecules (i.e., antibodies).

Another study evaluated how CMAs might exploit the expression of secreted cytotoxic proteins from tumor-associated fibroblasts as an anticancer strategy [96]. Traditionally, the off-target distribution of anticancer CMAs to fibroblasts creates a barrier to the effective treatment of desmoplastic tumors [96]. Plasmids encoding sTRAIL, a secretable TNF-related factor, were loaded into lipid-coated protamine DNA complexes in culture and administered to a murine xenograft model of human desmoplastic bladder carcinoma. Administration of three doses of the lipid-coated complexes resulted in approximately 70% of tumor-associated fibroblasts to express sTRAIL and apoptosis in adjacent tumor cells [96]. This was confirmed *in vivo* in a separate orthotopic xenograft model of human pancreatic cancer, where the desmoplastic stroma is well known to be a major barrier to the delivery of therapeutic NPs [96]. In addition, residual fibroblasts were also reverted into a quiescent state,

Table 2. Summary of PEGylated liposomal doxorubicin and NL-doxo pharmacokinetics and tumor profiling at baseline (predose) in T11 and C3Tag triple negative breast cancer genetically-engineered mouse models (PEGylated liposomal doxorubicin and NL-doxo were administered IV × 1 alone).			
	T11 tumor	C3-TAg tumor	p-value
PK parameter			
Plasma PLD encap AUC (ng/ml•h)	1,449,359 ± 57,535	1,609,775 ± 111,180	p > 0.05
Plasma PLD release AUC (ng/ml•h)	26,957 ± 1634	30,817 ± 2839	p > 0.05
Tumor PLD total AUC (ng/ml•h)	209,622 ± 26,476	480,110 ± 71,419	p = 0.02
IHC tumor profiling			
Baseline tumor microvessel density (# × 10 ⁵ /area)	8.8 ± 2.5	6.1 ± 1.8	p > 0.05
Baseline tumor collagen (H-score)	54 ± 48	81 ± 26	p > 0.05
Baseline tumor macrophages (H-score)	113 ± 45	110 ± 50	p > 0.05
IHC: Immunohistochemistry; PK: Pharmacokinetic; PLD: PEGylated liposomal doxorubicin.

Figure 3. Microvessel density score and intratumoral VEGF- α concentration after SM-doxorubicin or PEGylated liposomal doxorubicin in C3-TAg and T11 tumors. (A) MVD score at baseline and at 96 h after SM-doxorubicin (NL-doxo) or PLD in C3-TAg and tumors. There was little change in the amount of the vasculature in the C3-TAg tumors after SM-doxorubicin or PLD. (B) MVD score at baseline and at 96 h after SM-doxorubicin or PLD in T11 tumors. There was a 30% decrease in the MVD score observed in the T11 tumors after PLD administration ($p < 0.05$). (C) Intratumoral concentrations of VEGF- α versus time profiles after PLD or SM-doxorubicin in the C3-TAg and the T11 tumors. T11 had significantly higher levels of VEGF- α ($p = 0.003$) compared with C3-TAg. PLD had greater impacts on the levels of VEGF- α ($p = 0.02$) compared with SM-doxorubicin and the effects appeared to vary with tumor subtypes.

MVD: Microvessel density; PLD: PEGylated liposomal doxorubicin.

effectively remodeling the tumor microenvironment to favor future CMA therapy [96].

Interstitial fluid pressure

While the theoretical basis of the EPR effect is that the tumor vasculature is highly permeable to CMAs, increased tumor penetration is also dependent on convective flow. One of the factors affecting this convective flow is the IFP, or the pressure of the fluid found between cells within a tissue (interstitial space). Traditionally, there is a net negative pressure between the blood vessel and interstitial space, drawing fluid into the interstitial space and lymphatic ducts [97]. However, the IFP is increased within tumors due to numerous factors [97]. Large molecules and CMAs rely on low IFP and convective flow for efficient transport into and throughout tumors [98]. For CMAs, high IFP will reduce the ability for CMA extravasation into tumors

and travel from sites farther away from the blood vessel. Because of this reduced extravasation, interest in targeting stromal cells to debulk tumors to reduce IFP, thus allowing for greater perfusion of CMAs, have been explored [99].

Tumor blood flow and IFP are intimately linked and display bidirectional interactions with leaky tumor blood vessels contributing to elevated IFP while IFP has a reciprocal negative effect on local blood flow patterns. This complex interaction was assessed in a mouse model of breast cancer by Stapleton *et al.* Strong correlations were identified between IFP and tumor perfusion (Spearman's $r = -0.88$ to -0.97 , $p < 0.0001$) or liposome accumulation in tumor (Spearman's $r = -0.64$, $p = 0.0029$) [99]. The relationship between IFP and liposome accumulation in tumor was weaker (Spearman's $r = -0.64$, $p = 0.0029$) and model dependent (subcutaneous vs orthotopic). These results sug-

gest that IFP may have an impact on NP accumulation via a complex relationship with tumor perfusion [99].

Mononuclear phagocyte system

The PK and PD variability of CMAs in animal models and in patients can be attributed to many factors including degree of interaction and overall activity of MPS components. Many CMAs have been developed for the purpose of avoiding rapid clearance from the bloodstream, thereby extending systemic circulation time. Uptake mechanisms of CMAs by the MPS may occur through different pathways and are often facilitated by the adsorption of opsonins to the CMA surface and subsequent phagocytosis [100]. In addition, the MPS affects the delivery of CMAs to tumors and tissues [16,101–105]. Although this interaction has been reported, the mechanisms by which interactions of CMAs with the MPS affect tumor and tissue distribution are unclear and may occur by different processes. CMA uptake by the MPS can occur locally in the tumor, liver and spleen after the CMA distributes from blood to the organ [106]. This would be termed ‘capture.’ On the other hand, the circulating MPS cells in blood may take up or ‘hijack’ the CMA and carry it to the liver, spleen or site of action, such as the tumor [106]. With current methods it is difficult to determine the degree of influence or occurrence of capture or hijacking but both appear to play a role in the PK of CMAs.

A recent study reported that the heterogeneous tumor microenvironment and/or tumor cell features was associated with differences in the tumor delivery and efficacy of PLD, but not SM-doxorubicin, in GEMM of TNBCs [15]. These findings implicate that profiling of the tumor microenvironment and selection of patients with tumors conducive to CMAs are required for the optimal delivery and therapeutic outcomes for CMA-based therapy. It is unclear why within a patient with solid tumors there can be a reduction in the size of some tumors; whereas, other tumors can progress during or after treatment, although the genetic composition of the tumors is similar. Similar effects may also be occurring in clinical studies and thus studies need to be performed in preclinical tumor models and in patients to determine factors that alter tumor delivery of CMAs and identify tumors with the highest propensity for tumor delivery of CMAs.

Up to 60% of cells in certain tumors consist of macrophages, also called tumor-associated macrophages (TAMs) [107,108]. The role of TAMs have been shown to influence the delivery and transport of CMAs to tumors and release of drug from CMAs into the tumor matrix [108–110]. In addition, TAMs have also been shown to play distinct roles in immune suppression and metastases [111–113]. Studies measuring radiolabeled

paclitaxel poliglumex, a polyglutamate-paclitaxel conjugate, demonstrated that drug metabolites were found primarily in TAMs at levels 100- to 1000-fold higher than tumor cells [109]. Similarly, the polymer formulation of paclitaxel poliglumex conjugated to gadolinium (an MRI contrast agent) displayed co-localization of gadolinium and TAMs within tumors [110]. This suggests that TAMs were taking up the polymer conjugate and transporting it throughout the tumor, especially within the necrotic areas of the tumor [109,110]. A similar finding was also found with a cyclodextrin-conjugate of camptothecin in a glioblastoma model [108].

The MPS also significantly affect the clearance, tumor delivery and efficacy of CMAs. Although animal models have successfully predicted the PK of SM drugs in humans, the ability to predict CMA PK in humans based on results in animal models appears to be significantly more complex. A recent study profiled the differences between macrophage presence in flank and orthotopic xenograft cancer models of several types of human cancer (breast, ovarian, endometrial cancers and melanoma) [114]. Significant differences in MPS presence existed between tumor types (e.g., ovarian vs endometrial tumors), cell lines within the same tumor type (e.g., SKOV3 vs ES-2 ovarian cancer), and location of tumor implantation (i.e., flank vs orthotopic xenografts) [114]. These findings suggest that profiling the presence of MPS cells within preclinical xenograft models and the use of orthotopic implantation is important in tumor model selection.

The influence of the MPS on the PK and PD disposition and efficacy of PLD and SM-doxorubicin was evaluated in ovarian and endometrial cancer xenograft models [114]. SKOV-3 ovarian cancer and HEC1A endometrial cancer orthotopic xenografts were selected for PK and efficacy studies due to a CMA dilemma unique to endometrial cancer treatment where SM-doxorubicin works better than PLD in endometrial cancer. After administration of PLD, the doxorubicin exposures were similar in the plasma, liver and spleen in the two models. However, the ratio of tumor to plasma AUC_{0-96h} of PLD was approximately twofold higher in the SKOV-3 ovarian cancer model compared with HEC1A endometrial cancer model [114]. Furthermore, an improved survival benefit was observed after PLD treatment in the SKOV-3 compared with the HEC1A model [114]. These results suggest that CMA-based therapies should be evaluated with respect to the heterogeneity of tumor matrix factors, especially the MPS, across preclinical tumor models.

CMAs can undergo catabolism by tissue-residing MPS cells (macrophages and dendritic cells), resulting in a release of drug from its carrier. This relationship between tumor dispositions and the MPS was

evaluated using S-CKD602, a liposomal formulation of CKD602, a camptothecin analog, in mice bearing A375 melanoma and SKOV-3 ovarian flank xenografts [115]. While the plasma disposition was similar within both models, the ratio of tumor total AUC to plasma total AUC was 1.7-fold higher in SKOV-3 than A375 bearing mice [115]. In addition, the tumor extracellular fluid AUC to tumor total AUC was two-fold higher in SKOV-3 xenografts compared with A375 [115]. Tumors were also stained for the presence of CD11b and CD11c as markers for macrophages and dendritic cells, respectively. The staining of CD11c was 4.5-fold higher in SKOV-3 compared with A375 ($p < 0.0001$) [115]. These data suggest that both the increased tumor delivery of S-CKD602 and release of CKD602 within ovarian xenografts, compared with melanoma xenografts, was consistent with increased staining of CD11c. This suggests that variability in tumor MPS can affect the tumor delivery of liposomal agents and release of drug from liposomal agents.

CMA, or its chemotherapeutic payloads, can also be metabolized by MPS cells within tumors, leading to variability in antitumor response. Previous studies of carboxylesterase (CES) activity were unable to predict the antitumor response of SM irinotecan in preclinical and clinical studies. However, there was a relationship between CES activity in tumor cells and the PK and efficacy of nanoliposomal formulation of irinotecan [116]. Tumor models with the highest CES activity and conversion of CPT-1 to SN-38 achieved the greatest antitumor response despite lower overall CPT-11 exposure in the tumors [117–119]. This suggests the importance of individual tumor CES expression in determining SN-38 exposure following nanoliposomal irinotecan administration [116]. However, while tumor cells express CES enzymes that play a role in the activation of CPT-11 to SN-38, other components of the tumor microenvironment, including TAMs, have also been shown to express these enzymes [116]. TAMs were also reported to hydrolyze CPT-11 to SN-38, confirming that MPS cell involvement within tumors plays a role in the activation and response after nanoliposomal irinotecan administration and potentially other CMAs [116].

Concomitant treatments to increase tumor delivery of CMAs & release of drug from the carrier

To tackle the low tumor delivery issue of CMAs a number of different methods have been evaluated. **Table 3** summarizes some of the strategies that have been used to improve drug penetration into tumors. The majority of these strategies involve pretreatment of tumors with some sort of therapy to impact one of the factors described previously in this review.

Jain and colleagues attempted to increase the penetration of CMAs, such as antibodies and viral NPs, into tumor by pretreating tumors with collagenase [120,135–136]. Collagenase resulted in a two- to threefold increase in the penetration of these agents into tumor over noncollagenase treated tumors. Similarly, pretreatment with the hormone relaxin, which induces the degradation and structural changes to collagen, has increased the delivery of antibodies and macromolecules to tumors by two- to threefold [120,135–137]. Pretreatment with losartan, which reduces stromal collagen and hyaluronan production and leads to increased vascular perfusion, produced only a 74% increase in 5-FU delivery to tumors compared with 5-FU alone [121]. The priming of tumors with low dose IV administration of traditional chemotherapy drugs prior to the administration of liposomal formulations has also been evaluated to increase liposomal tumor delivery but only increased the delivery of these agents by approximately twofold [123]. Moreover, the use of other chemotherapeutic agents to prime the tumor may have off target effects that inhibit or are toxic to the MPS and thus the increase in tumor delivery of the CMA is due to reduced systemic clearance and not enhanced tumor delivery. In addition, conventional radiation therapy has been reported to only moderately (0.2- to threefold) enhance drug delivery to tumors and is associated with toxicities [122]. Hyperthermia has been demonstrated to greatly increase intravascular drug release using thermally sensitive liposomes but this technique is limited to sites where mild hyperthermia can effectively be applied which limits its widespread adoption [133,134]. In summary, the current methods only increased the tumor delivery of CMAs from 0.5- to fivefold. Furthermore, the administration of concomitant therapies to increase the tumor delivery of other CMAs are all associated with additional toxicities. Thus, more effective and tumor specific methods are needed to increase the tumor delivery of CMAs.

Synergistic effects on CMAs have also been investigated by using a second agent to promote greater release of drug from the CMA. Previously we described how the antitumor efficacy of liposomal agents, such as PLD, is hindered by the poor release of drug from the liposome at the tumor sites. A recent study investigated the possibility to enhance drug release from PLD by administering Pluronic P85 block copolymers once the liposomal drug accumulates in the tumor sites [138]. The antitumor growth and PLD distribution were determined in A2780 ovarian xenografts when Pluronic P85 was injected 1, 48 and 96 h after PLD administration. The co-administration of Pluronic P85 resulted in both the increased release of doxorubicin from PLD as well as significantly increased

doxorubicin delivery in tumor cells *in vitro* [138]. This effect was replicated *in vivo* in a A2780 tumor model where mice administered Pluronic P85 48 h after PLD achieve the greatest antitumor effect compared with PLD alone ($p < 0.01$) [138]. These data demonstrate a simple approach where co-administration of a polymer with NP-like properties can produce synergistic effects by both increasing release of the cytotoxic agent from its carrier within tumors.

Problems estimating & calculating tumor PK of CMAs

Analytical methods to evaluate the tumor disposition of CMAs

Several methods have been used to study drug distribution of CMAs in tumor. Window chambers have classically been used to study agents with natural fluorescence or natural coloring, allowing the drug distribution to be photographed over a period of time after administration within a living animal. This same method has also been applied where a fluorophore has been conjugated

to the CMA carrier – though this may alter the properties of the carrier. Alternatively, naturally fluorescent drugs can also utilize computer models to quantify the amount of drug in relation to blood vessels *in vivo* in tissue segments, illuminating drug distribution similar to immunohistochemistry. Other techniques, including optical, x-ray, magnetic resonance, nuclear and ultrasound imaging techniques have been reviewed previously on their resolution and contrast agents for use in preclinical animal models [139].

Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) is the gold standard for quantifying anticancer drug exposure in plasma, tissues and tumor in drug development studies. This approach requires the homogenization of tissue followed by extraction of the drug from the matrix (i.e., protein precipitation, liquid-liquid extraction, solid-phase extraction) in order to measure an average drug concentration in tissue; however, no information can be gained on the spatial distribution or depth of penetration of the drug within the tissue. Further-

Table 3. Strategies utilized to improve carrier-mediated agent penetration into tumors.			
Strategy	Method	Mechanism of action	Ref.
Modify the ECM	Degrade ECM using collagenase or relaxin	Remodeling of ECM	[120]
	Co-administration with losartan	Increased vascular perfusion	[121]
	Conventional radiation therapy	Remodeling of ECM	[122]
Increase tumor blood flow	Neoangiogenesis inhibition	Normalize tumor vasculature	[71,72]
	Modify vasculature muscular tone	Inhibit contraction of tumor vasculature	[116]
Reduced IFP	Target VEGF (antiangiogenic therapy)	Decrease vessel permeability	[72–74]
	Induce apoptosis (pretreatment with small-molecule chemotherapy)	Reduce tumor density	[123]
	Bradykinin agonist	Increase tumor vasculature pore size	[124,125]
	Prostaglandin E1	Reduce stromal cell concentration	[126]
Increased blood permeability	Damage tumor endothelium	Alter endothelium barriers	[127,128]
Hypoxia targeting	Treatment with hypoxic cytotoxins (e.g., tirapazamine)	Compounds that specifically kill hypoxic cells	[129]
Modify drug uptake	Co-administration with folate	Alter pH to increase/decrease drug transport	[130]
	Co-administration with transport inhibitors	Block surface transported (e.g., P-glycoprotein) to increase uptake of substrates	[131,132]
	Hyperthermia	Selective release of thermo-sensitive carriers	[133,134]
ECM: Extracellular matrix; IFP: Interstitial fluid pressure.			

more, when evaluating the active drug concentrations for carrier-mediated agents in tissue and tumor, there is currently no way to determine if it is encapsulated or released by LC-MS/MS. Thus, the development of sample processing or analytical methods to evaluate the exposure of CMA encapsulated/conjugated and released drug are desperately needed. Knowledge about the relative distribution of the drug in a tissue is desirable in order to obtain a better understanding of how targeted drugs interact with tumor and tissue cells, and to determine if a drug, as well as its relevant entities (i.e., prodrug, metabolite, carrier), is reaching the appropriate target in order to exert its effect.

Recent advances in the field of mass spectrometry imaging (MSI) have utilized the speed, sensitivity and specificity of mass spectrometry to allow the interrogation of drug distribution and relative amounts in intact tissue. MSI offers a label-free approach to perform simultaneous analysis of the relative amount of drugs and metabolites as well as potential drug targets, which may include endogenous proteins, peptides, lipids or hormones [140]. It will also address important questions about drug exposure such as the ability of a CMAs to pass through the blood–brain barrier or the depth of penetration of a drug into the tumor, which will help to explain pharmacokinetics, efficacy and toxicity.

PK parameters used to describe CMA tumor disposition

A recent study suggests that only a small amount (mean of 0.7% of injected dose) of an administered CMA dose reaches the tumor [141]. However, as written in a follow-up perspectives article by McNeil [142], such evaluations may be flawed as the evaluation of the active-drug in plasma and tumor is more relevant to PK, PD and efficacy than the number of particles that enters a tumor. They provided the example of Doxil (i.e., PLD), where less than 1% of the administered dose is found in the tumor, though it provides greater and more prolonged tumor exposure compared with standard SM doxorubicin [142]. Calculations of the % injected dose in a tumor or tissue also heavily influenced by the time point used in the estimate as a single time point, especially early time points, do not reflect the overall PK disposition of a CMA. Whereas, the comparison of AUCs of CMAs in tumor to plasma over extended periods of time (0–96 h or 0–168 h) provide a more extensive and accurate estimate of overall tumor delivery. These highlights how various methods can be used to calculate the amount of a CMA that reaches the tumor and can affect our interpretations of an CMA and study outcomes. In addition, studies need to consider both the inactive-encapsulated/conjugated and active-released forms of the agents, as well as the ratio of CMA expo-

sure in tumor to plasma and other normal tissues, to fully assess the overall PK impact of CMAs.

Standard PK parameters and metrics used for SMs have been used to describe CMAs. However, standard PK parameters may not provide all of the important and detailed information that differentiates SMs and CMAs, especially as related to efficiency of tumor delivery. As such, new methods of evaluating the efficiency of the tumor delivery of CMAs and SMs agents are being developed. For instance, the relative distribution over time (RDI-OT) is a novel PK parameter used to evaluate the ability of CMAs and SMs to penetrate into tumor or tissue from the circulation (e.g., plasma) [26]. RDI-OT provides several additional unique results that AUC calculations do not provide. First, RDI-OT can assess the efficiency of SMs and CMAs to enter tissues or tumors from plasma, whereas the ratio of AUC in tumor to plasma is only a relative measure of the total drug exposure between plasma and tumor or tissues [26]. Second, RDI-OT values are calculated at each time point, making it possible to evaluate the efficiency of delivery of a SM or CMA drug at single or various time points, while AUC ratio can only measure the total exposure over the total period of time [26]. While RDI-OT is an example of a new PK parameter, it has yet to be correlated to PD effects and future studies are still needed to determine its effect on outcomes.

Conclusion

Over the past two decades, significant improvements have been made in altering the construction and characteristics of CMAs to improve their PK and PD in preclinical models and in some cases in patients. However, current research has begun to emphasize the importance of understanding the variability observed in the distribution and delivery of CMAs to tumor and tissues and how the tumor microenvironment, which contains critical and complex factors, affects these processes. However, methods capable of quantitative analysis of the interaction of CMA PK and tumor microenvironment are only beginning to be utilized (e.g., MSI) or need to be developed in order to fully evaluate the results. Furthermore, there is still limited evidence in clinical studies demonstrating enhanced tumor delivery of CMAs and interactions between the tumor microenvironment and CMAs. Instead, investigators have relied on animal models to predict effects in patients. More preclinical and clinical studies and development of quantitative imaging technologies are needed to study known and unprobed systems, such as the lymphatic architecture within tumors, to determine how individual variables impact CMA penetration, retention and antitumor effect. Various

approaches have been studied to increase the tumor penetration of CMAs with hopes of improved efficacy and survival. However, current approaches have had limited success or have off target effects that may make the translation to patients very difficult. In summary, the tumor microenvironment and its barriers to tumor delivery are highly complex and the interaction with CMA may be highly specific. Thus, detailed tumor profiling and pharmacology studies in preclinical models and patients are needed to address these issues in a wide variety of solid tumors.

Future perspective

As the interaction between the tumor microenvironment and CMAs is highly complex, the successful use of CMAs for the treatment of solid tumors will require the optimization of CMA characteristics (e.g., size, charge, surface ligands, etc.), profiling tumor barriers, development methods or concomitant treatments

to overcome these barriers, and understanding the complex translational issues between solid tumors in preclinical models and patients. The advancement of the clinical impact of CMAs may also require imaging or other methods to determine if the type of solid tumor or, ultimately, the individual patient's tumor is conducive to CMA delivery alone or in combination with a modulating agent.

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Executive summary

Promises of carrier-mediated agents

- The theoretical advantages of carrier-mediated agents (CMAs) in cancer treatment include increased solubility, prolonged duration of exposure, selective delivery of entrapped drug to the tumor and an improved therapeutic index.

Pharmacokinetic characterization

- The disposition of CMAs is dependent upon the carrier and not the therapeutic entity until the drug gets released.

Influence of tumor barriers on delivery of CMAs

- The delivery and distribution of chemotherapy in tumors is highly heterogeneous, resulting in only a fraction of tumor cells being exposed to cytotoxic levels of chemotherapy.
- It is important to understand potential barriers in the tumor microenvironment in order to improve the tumor delivery and drug release of CMAs in solid tumors.
- The development of novel methods to evaluate and to overcome these barriers and increase the tumor delivery and efficacy of CMAs in a variety of solid tumors are desperately needed.

Tumor microenvironment factors

- Physiological factors related to the tumor vasculature, such as heterogeneous blood supply, uneven permeability and larger transport distances in the interstitium, have been found to be responsible for the poor localization of nanoparticle and macromolecule delivery within tumors.
- The buildup of extracellular matrix forms a barrier to diffusive and convective transport, which inhibits the distribution of CMAs in tumor significantly more than small-molecule drug.
- Data suggest that interstitial fluid pressure may have an impact on nanoparticle accumulation via a complex relationship with tumor perfusion.
- The role of tumor-associated macrophages has been shown to influence the delivery and transport of CMAs to tumors and release of drug from CMAs into the tumor matrix.

Problems estimating & calculating tumor pharmacokinetic of CMAs

- The development of sample processing or analytical methods to evaluate the exposure of CMA encapsulated/conjugated and released drug is desperately needed.
- Studies need to consider both the inactive-encapsulated/conjugated and active-released forms of the agents, as well as the ratio of CMA exposure in tumor to plasma and other normal tissues, to fully assess overall pharmacokinetic impact of CMAs.

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