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Towards Clinical Translation of Ligand-Functionalized Liposomes in Targeted Cancer Therapy: Challenges and Opportunities

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Abstract

The development of therapeutic resistance to targeted anticancer therapies remains a significant clinical problem, with intratumoral heterogeneity playing a key role. In this context, improving the therapeutic outcome through simultaneous targeting of multiple tumor cell subtypes within a heterogeneous tumor is a promising approach. Liposomes have emerged as useful drug carriers that can reduce systemic toxicity and increase drug delivery to the tumor site. While clinically-used liposomal drug formulations show marked therapeutic advantages over free drug formulations, ligand-functionalized liposome drug formulations that can target multiple tumor cell subtypes may further improve the therapeutic efficacy by facilitating drug delivery to a broader population of tumor cells making up the heterogeneous tumor tissue. Ligand-directed liposomes enable the so-called active targeting of cell receptors via surface-attached ligands that direct drug uptake into tumor cells or tumor-associated stromal cells, and so can increase the selectivity of drug delivery. Despite promising preclinical results demonstrating improved targeting and anti-tumor effects of ligand-directed liposomes, there has been limited translation of this approach to the clinic. Key challenges for translation include the lack of established methods to scale up production and comprehensively characterize ligand-functionalized liposome formulations, and the inadequate recapitulation of *in vivo* tumors in the preclinical models currently used to evaluate their performance. Herein, we discuss the utility of recent ligand-directed liposome approaches, with a focus on dual-ligand liposomes, for the treatment of solid tumors and examine the drawbacks limiting their progression to clinical adoption.

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Keywords

ligand-functionalized liposomes, dual-functionalized liposomes, EPR effect, tumor heterogeneity, targeted therapy, nanomedicine

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Glossary of Key Terms

Non-ligand modified liposomes	Liposomes without surface-bound targeting ligands or modalities; efficacy is thought to be predominately achieved via the enhanced permeability and retention (EPR) effect
Passively-targeted liposomes	Non-functionalized liposomes that accumulate at the tumor site via the EPR effect
Actively-targeted liposomes	Liposomes with one or more surface-bound modalities (ligands) enabling binding to target cells to direct liposome uptake; encompasses single-ligand, dual-ligand and multi-ligand liposomes
Single-ligand liposomes	Liposomes with a single surface-bound targeting ligand or modality for targeting to a specific cell surface receptor
Dual-ligand liposomes	Liposomes with two different surface-bound ligands or modalities for targeting to two different cell surface receptors
Dual-functionalized liposomes	Liposomes with two different functions for cell targeting; may or may not include a ligand/modality
Enhanced permeability and retention (EPR) effect	The permeation and retention of particles less than 380-780 nm in size into the tumor interstitial space due to highly porous tumor vasculature and poor lymphatic drainage from the tumor site

In this review, the key aspects of both inter- and intratumoral heterogeneity, and the rationale for using ligand-directed liposomes in tumor targeting will be described, before highlighting current research using dual-ligand directed liposome approaches that aim to address tumor heterogeneity. This review will then explore some of the reasons why, despite clinical adoption of non-ligand directed liposomes and promising preclinical findings for ligand-directed liposomes, ligand-directed liposomes have not yet progressed to the clinic. Finally, this review will outline essential areas for future research that will allow for improved formulation and preclinical evaluation of therapeutic liposomes in order to facilitate the clinical translation of ligand-functionalized liposomes in the context of cancer therapy.

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Tumor Heterogeneity and Therapeutic Resistance

The molecular classification of tumors and the associated identification of tumor biomarkers are highly useful in both prognosis and determining the most appropriate treatment course. Many important biomarkers and cellular pathways involved in tumor progression and metastasis have been identified (for example, the estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) status in breast cancer) and assist in the prediction of patient responses to hormone, chemo-, immuno- and molecular targeted therapies, determination of mechanisms of therapeutic resistance (e.g. overexpression of *MDR1*), prediction of disease progression and likelihood of relapse [1, 2]. Overexpression of specific cell surface receptors by tumor cells may be exploited to directly target tumor cells using antibodies or smaller molecules, or to enable targeted delivery of cytotoxic compounds to tumor cells. Such targeted approaches enable more specific antitumor effects, potentially resulting in enhanced tumor cell kill and/or a reduction in off-target effects. Targeted therapies have been successfully used to treat some cancers – for example, the monoclonal antibodies trastuzumab and pertuzumab that target HER2 in the treatment of HER2-positive breast cancer [3].

Despite the therapeutic advantages of targeted therapies, the development of resistance to these therapies is now recognized as a significant clinical problem [4]. A leading example is the therapeutic resistance to imatinib (Gleevec), a tyrosine kinase inhibitor currently used as the standard of care in the treatment of chronic myeloid leukemia [5]. Resistance to targeted therapies can develop via a number of mechanisms and may be intrinsic or acquired. Intrinsic resistance can arise from a lack of expression of a drug target, a mutated drug target or via target-independent signaling mechanisms

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[6]. For example, some patients are intrinsically resistant to HER2-targeted therapies because of the ability of HER2 to form heterodimers with other HER receptors, allowing differential intracellular signaling [7]. In contrast, acquired (also known as pleiotropic or evasive) resistance can develop in patients that were once responsive to treatment, and can arise from de novo mutations or from clonal selection of intrinsically resistant clones [8]. The development of acquired resistance renders targeted therapies ineffective and subsequent cancer recurrence often results in death from metastatic disease. This phenomenon is observed in the use of anti-estrogen, anti-androgen and Herceptin therapies for breast cancer treatment, and vemurafenib therapy in the treatment of late-stage melanoma [8].

The genomic, functional and spatiotemporal heterogeneity that is characteristic of many solid tumors plays a key role in the development of resistance to targeted therapies (Figure 1) [9, 10]. Mechanisms of acquired resistance to molecular-targeted therapies have been extensively reviewed elsewhere [11, 12]. The intratumoral heterogeneity of tumors provides a template for the clonal selection and expansion of target-negative tumor cells [13] and is a known mechanism of acquired resistance to targeted therapies [14, 15]. Within a cancer subtype, individual tumors are comprised of a mixture of both target-positive and target-negative tumor cells [16]. The administration of a targeted therapy inevitably places a selection pressure on a genetically and functionally heterogeneous population of tumor cells, resulting in the selection of tumor cells that are no longer responsive to the targeted therapy [17]. With both time and the continuation of therapy, the negative tumor cell population is able to expand such that the tumor becomes predominately target-negative, at which point the patient no longer shows a response to the original targeted therapy [18]. In this way, the

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intratumoral heterogeneity of cancer can reduce the potential efficacy of targeted therapies and thus contribute to cancer recurrence and metastasis [19].

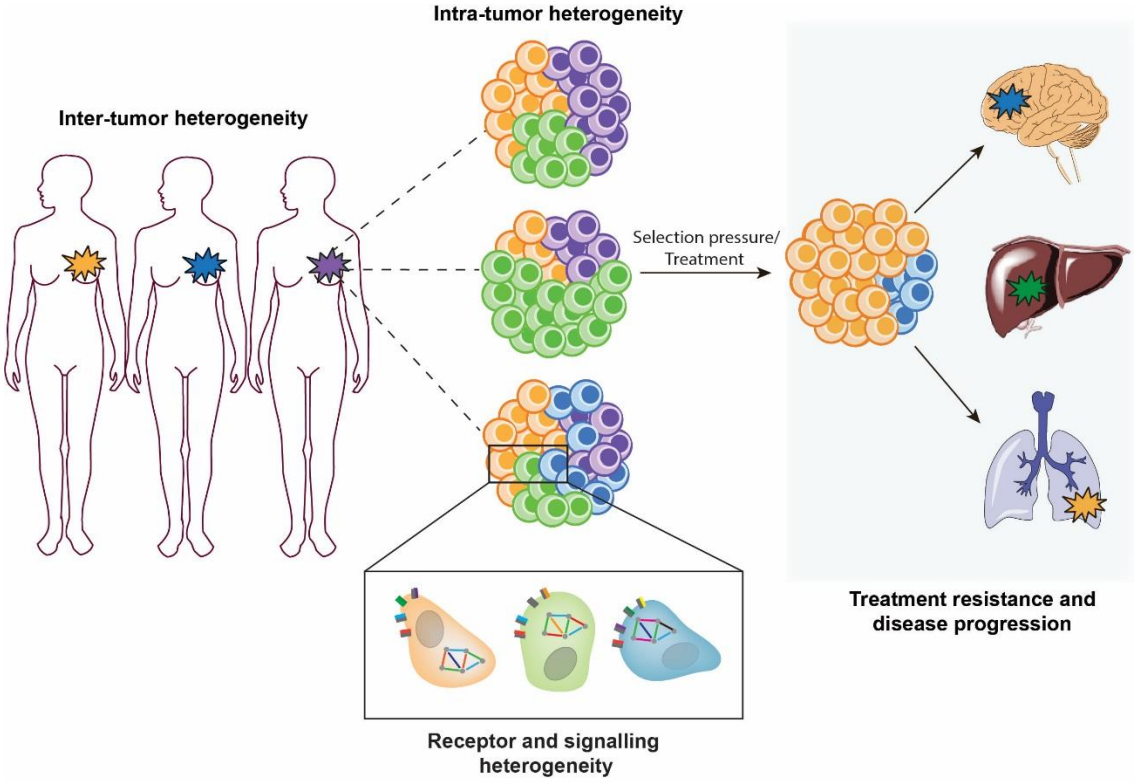

Figure 1: Schematic representation of inter-tumoral and intra-tumoral (biomarker) heterogeneity, including receptor and signaling heterogeneity.

The intratumoral heterogeneity characteristic of many tumor types suggests that a multiple-targeting strategy directed against a broader range of tumor cell (and tumor-associated immune cell) subtypes may be of benefit [20]. There is some evidence supporting the efficacy of targeting two or more different tumor cell receptors and/or populations using selective targeted therapies in order to improve the anti-tumor effect of mono-targeted therapies. Preclinical data supports the notion of combining two HER2-targeted therapies to achieve a synergistic anti-tumor effect in HER2-positive breast cancers [21]. The administration of antibodies trastuzumab and certuxumab,

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targeting HER2 and the human epidermal growth factor receptor (EGFR), respectively, in combination therapy has entered a phase I/II clinical trial in order to improve treatment efficacy of advanced pancreatic cancer [22]. The siRNA-mediated simultaneous knockdown of both HER2 and protein tyrosine kinase 6 in preclinical models of HER2-positive breast cancer reduced migration, invasion and cell proliferation of trastuzumab-resistant breast cancer cells *in vitro*, and a reduction of tumor growth *in vivo*, demonstrating a potential approach for treating breast cancer [23]. Additionally, recent evidence has shown that other cell types that support tumor cell growth and play key roles in facilitating metastasis, including endothelial cells, fibroblasts and immune cells, may too be potential targets for novel multi-targeted therapies [24]. For example, the superior efficacy of independently targeting both tumor and immune cells in various cancer types has been demonstrated previously [25, 26].

Several receptor-targeted molecular therapies have been developed to treat cancer, including a range of monoclonal antibodies and antibody fragments that derive an anti-tumor effect through binding to cell surface receptors in order to inhibit tumor cell proliferation [27]. Another tumor cell targeting approach involves the use of monoclonal antibodies, proteins or other ligands to facilitate target cell uptake of specific molecules to achieve an anti-tumor cell effect. For example, if the binding of a ligand to its target receptor results in the receptor-mediated endocytosis of the ligand-receptor complex, the targeting ligand – which may be a currently-used targeted molecular therapy – can be used for the intracellular delivery of covalently-attached cytotoxins or other molecules to tumor cells that express the ligand receptor [28, 29]. This tumor targeting approach may help to circumvent intrinsic resistance driven by alternative signaling mechanisms [30]. While the plasma half-life of most targeted

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molecular therapies tends to be relatively short, association of these molecules with larger nanostructures, such as lipid-based nanoparticles or liposomes, can significantly extend the plasma circulation time of the targeted therapy and increase the therapeutic payload delivered to the tumor site [31]. Such receptor-targeted nano-particulate therapies may incorporate currently-used targeting molecules, such as antibodies, onto the surface of the nanoparticle so that they can be used as targeting ligands to direct the nanoparticles to receptor-positive tumor cells and facilitate cellular uptake of the nanoparticle, achieving intracellular delivery of the nanoparticle cargo for anti-tumor effect. This is of particular importance for the targeted delivery of therapeutic macromolecules, including DNA, RNA and proteins, which otherwise would not be able to enter cells.

Liposomes for Tumor Targeting and Drug Delivery

Liposomes have emerged as a useful delivery system for the transport of drugs and other molecules to solid tumors [32]. Liposomes are spherical lipid-based vesicles, typically 100-200 nanometers in diameter, comprised of associating phospholipids that form a lipid bilayer surrounding an aqueous core (Figure 2) [33]. This unique structure allows for the encapsulation of hydrophobic or hydrophilic drugs, or other small molecules, in the lipid bilayer or aqueous core, respectively [34]. The circulation time of liposome particles is largely dependent on their lipid composition, size, surface charge, morphology and other physicochemical characteristics. The dominant mechanism by which liposomes are typically cleared from the bloodstream is based on interactions with the phagocytic cells the mononuclear phagocyte system (MPS). The inclusion of hydrophilic polymers, most commonly polyethylene glycol (PEG), at the

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outer surface of the liposomes, can increase the *in vivo* circulation time by reducing recognition and clearance by the MPS [35]. For this reason, PEGylated liposomes have long been considered a clinically useful nanoparticle for drug delivery applications. However, despite the general trend of improved circulation time of PEGylated liposomes, researchers have found that the circulation time is dependent not only on the liposome type, but also on the number of injections administered [36]. The Accelerated Blood Clearance (ABC) phenomenon describes how the first dose of a PEGylated nanoparticle may affect the pharmacokinetic properties of subsequent doses; specifically, an increased clearance rate of PEGylated nanoparticles from the blood was observed with second and subsequent intravenous injections of the formulation [37, 38]. In this context, reduced circulation time correlates with increased liver and spleen accumulation [38]. While the exact mechanism of the ABC phenomenon remains unknown, a key identified mechanism is the production of anti-PEG IgM following the first injection, which selectively binds to the surface of subsequently injected PEGylated particles and acts to accelerate clearance by substantial complement activation [39]. The ABC phenomenon has been described for PEGylated liposomes, polymeric nanoparticles and PEGylated solid lipid nanoparticles delivered intravenously [40]. In other reports, an initial subcutaneous injection of a PEGylated nanoparticle has similarly been shown to reduce the circulation time of subsequent intravenous injections of the nanoparticle [41]. To assess whether the FDA-approved PEGylated liposomal doxorubicin formulation, Doxil®, induces the ABC phenomenon, studies in rodents, dogs and non-human primates have demonstrated a dose dependent loss of long circulation of Doxil® upon multiple intravenous injections [42]. Importantly, amongst other factors, the occurrence of the ABC effect is dependent on

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the lipid dose administered (relatively high in the case of Doxil®) and duration of the administration interval (being much longer, i.e. 3-4 weeks, in case of Doxil®), making clinical Doxil® treatment insensitive to the ABC phenomenon [38, 43]. In a recent case study, Doxil® was found to activate the complement system in animals and humans, leading to a hypersensitivity reaction known as Complement Activation Related Pseudoallergy (CARPA), which would indeed impact upon the pharmacokinetics and pharmacodynamic properties of the drug [44]. Such research demonstrates that the ABC phenomenon is an important factor to consider in the design and development of PEGylated liposomes and other nanopharmaceuticals for repeat dosing therapeutic applications.

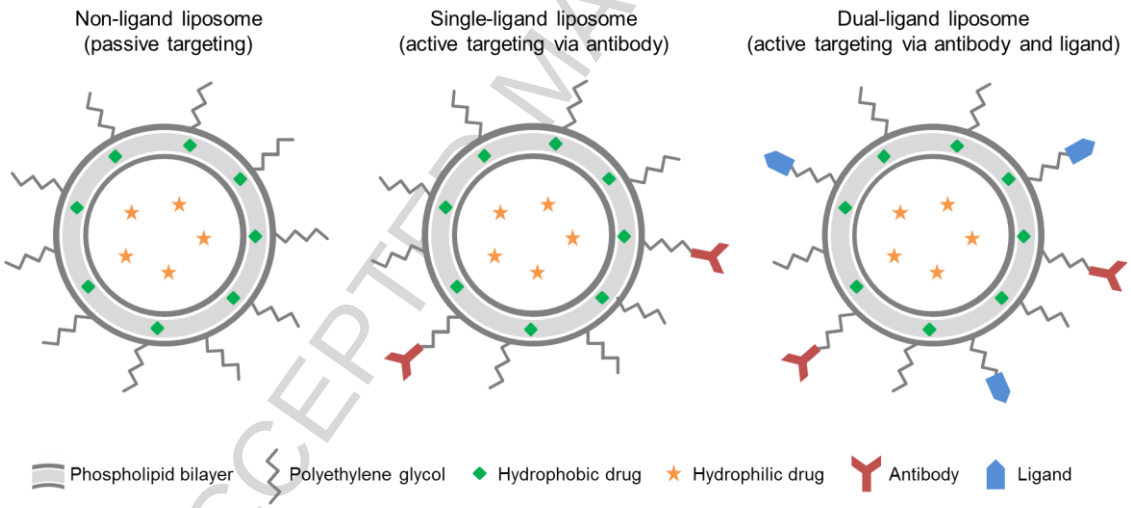

Figure 2: General structures of non-ligand (passively targeted), and single-ligand and dual-ligand (actively-targeted) drug-loaded liposomes.

Liposome-based drug formulations can offer several distinct advantages over free drug in addition to an increased *in vivo* circulation time, including improved stability and solubilization of encapsulated drug, reduction in systemic toxicity of the drug and

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increased drug delivery to the tumor site [45]. The superior activity of drug-loaded liposomes relies on a multi-step process involving both passive and active targeting mechanisms. Passive targeting is primarily mediated by the enhanced permeability and retention (EPR) effect, defined as the extravasation and retention of particles less than 380-780 nm in size into the tumor interstitial space due to highly porous tumor vasculature and poor lymphatic drainage from the tumor site [46, 47]. The encapsulated drug can be released from liposomes in the tumor interstitium and then taken up by the tumor cells, or the liposomes containing the drug are internalized by the tumor cells or other tumor-associated cells [48]. Therefore, in theory, passive targeting enables targeting to tumors via the EPR effect. In addition, liposome formulations reduce exposure of normal tissues to the drug as liposomes cannot pass through intact continuous endothelium, and so do not localize there (except for liver and spleen which have different anatomy of vasculature), minimizing associated off-target effects while simultaneously providing a mechanism for enhanced accumulation in the tumor site. The variability and limitations surrounding drug targeting via the EPR effect will be discussed in detail below.

In addition to their versatile drug encapsulation capabilities, liposomes permit the active targeting of specific cell types via the conjugation of ligands, such as monoclonal antibodies, antibody fragments, proteins, peptides, carbohydrates, glycoproteins, aptamers and small molecules, to the liposome surface for drug delivery to cells expressing the target surface receptor(s) of interest [49]. Active targeting using liposomes is achieved via conjugation of one or more ligands to the liposome surface to form liposomes that bind to a target receptor(s) expressed on the tumor cell surface. Following liposome extravasation into the tumor interstitial space, subsequent ligand-

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directed surface binding and internalization (usually via receptor-mediated endocytosis) promotes liposome and drug entry into specific cell types. As actively-targeted liposome formulations combine both passive and active drug delivery mechanisms, ligand-directed liposomes should show superior drug delivery compared to non-ligand liposomes, depending on tumor type [50].

Currently, all clinically-approved liposome drug formulations are non-ligand directed, with efficacies relying solely on passive targeting to achieve tumor accumulation. Despite extensive research into nanomedicine-based therapeutics, and the preclinical development of dozens of liposome drug formulations spanning several decades, less than a dozen liposomal drug formulations have been approved by the FDA for clinical use to date [51, 52]. Of these FDA-approved liposomes, only several distinct formulations have been approved for the treatment of cancer, including Kaposi's sarcoma, acute lymphoblastic leukemia, pancreatic cancer, ovarian cancer, multiple myeloma and metastatic breast cancer (Table 1). Evidently, there is a bottleneck in the translation of liposomes from preclinical development through to clinical utility, with many preclinical formulations never proceeding to clinical trials, and only a small percentage of those that do eventually making it onto the market. This bottleneck is even more profound for the development of ligand-directed liposomes, where there are currently no clinically-approved formulations available [53].

Active targeting strategies using ligand-directed liposomes have been explored extensively in the preclinical setting, showing improved efficacy over non-ligand liposomes in *in vitro* and *in vivo* models. For example, *in vitro* testing of doxorubicin-loaded liposomes (analogous to Doxil®) that were surface-functionalized with an anti-HER2 monoclonal antibody fragment demonstrated effective binding to breast cancer

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cells expressing HER2 and a 700-fold increase in drug uptake compared to non-ligand directed liposomes *in vivo* [54]. MM-302, a HER2-targeted liposomal formulation of doxorubicin, showed efficacy in xenograft models of breast cancer and proceeded through to clinical trials [55]. A phase II/III clinical trial comparing trastuzumab therapy in combination with either MM-302 or chemotherapy of physician's choice was recently terminated as the trastuzumab/MM-302 treatment did not show improved efficacy over the current standard of care for HER2-positive breast cancer [56]. This may be due to the current lack of understanding around how actively-targeted liposomes behave in immune-competent animals (i.e. humans). The development of actively-targeted liposomes to improve the efficacy of their passively-targeted predecessors has been explored preclinically, with many formulations progressing through clinical trials (Table 1). However, as indicated above there are currently no clinically-approved ligand-directed liposome formulations [53]. Given the long history of ligand-directed liposomes and the significant investment of research into this area, it is important to explore the reasons why there has been limited translation of actively-targeted liposomes in the field of cancer therapy. Following an overview of previous research in the field, we will highlight and discuss some of the likely reasons for this bottleneck in clinical progression.

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Table 1: Non-ligand, single-ligand and dual-ligand liposomes in clinical use, clinical trial and preclinical development for cancer treatment.

Type	Name	Cargo	Targeting ligand(s)	Indication	Status	Reference
Non-ligand	Doxil®/Caelyx™ (Janssen)	Doxorubicin	-	Kaposi's sarcoma Ovarian cancer Multiple myeloma Metastatic breast cancer	FDA approved (1995) FDA approved (2005) FDA approved (2008) FDA approved (2012)	[57] [58] [59] [60]
	DaunoXome® (Galen)	Daunorubicin	-	Kaposi's sarcoma	FDA approved (1996)	[61]
	Myocet® (Elan Pharmaceuticals)	Doxorubicin	-	Metastatic breast cancer	EMA approved (2000)	[62]
	Marqibo® (Onco TCS)	Vincristine	-	Acute lymphoblastic leukemia	FDA approved (2012)	[63]
	Onivyde® (Merrimack)	Irinotecan	-	Metastatic pancreatic cancer	FDA approved (2015)	[64]
	Vyxeos™ (Jazz Pharmaceuticals)	Daunorubicin, cytarabine	-	Acute myeloid leukemia	FDA approved (2017)	NCT02533115
	LEP-ETU	Paclitaxel	-	Lung squamous cell carcinoma	Phase IV clinical trials	NCT02996214
	EndoTAG-1	Paclitaxel	-	Breast cancer Pancreatic cancer	Phase III clinical trials Phase III clinical trials	NCT03002103 NCT03126435
	Liposomal cytarabine	Cytarabine	-	Breast cancer	Phase III clinical trials	NCT01645839
	ThermoDox	Doxorubicin	-	Hepatocellular carcinoma	Phase III clinical trials	NCT02112656 NCT02850

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				Breast cancer	Phase II clinical trials	19
	Liposomal Grb-2	Grb2 oligodeoxynucleotide	-	Acute myeloid leukemia	Phase II clinical trials	NCT02781883
	Vincristine sulfate liposome	Vincristine	-	Acute myeloid leukemia	Phase II clinical trials	NCT02337478
	Mitoxantrone hydrochloride liposome	Mitoxantrone	-	Metastatic breast cancer	Phase II clinical trials	NCT02596373
	SPI-077	Cisplatin	-	Advanced solid tumors	Phase I/II clinical trials	NCT01861496
	LiPlaCis	Cisplatin	-	Advanced solid tumors	Phase I/II clinical trials	NCT01861496
	Liposomal dexamethasone	Dexamethasone	-	Multiple myeloma	Phase I/II clinical trials	NCT03033316
	MM-398	Irinotecan	-	Recurrent solid tumors	Phase I clinical trials	NCT02013336
Single-ligand	Anti-EGFR immunoliposome	Doxorubicin	Cetuximab Fab fragment	Breast cancer	Phase II clinical trials	NCT02833766
Dual-ligand	Anti-CD19/CD20 liposomes	Doxorubicin	Anti-CD19 and anti-CD20 monoclonal antibodies	B cell lymphoma	Preclinical development	[65]
	T7/TAT-LP-PTX	Paclitaxel	Ligand peptide (HAIYPRH), cationic cell penetrating peptide (TAT)	Lung cancer	Preclinical development	[66]
	P-selectin/avβ3 integrin liposome	Fluorescent marker	Peptides targeting P-selectin and avβ3 integrin	Metastatic breast cancer	Preclinical development	[20]
	Integrin avβ3 peptide/[D]-H6L9	Paclitaxel	Integrin avβ3 peptide,	Colon cancer	Preclinical development	[67]

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	liposome		[D]-H6L9 peptide			
	RGD/TF-LP	Paclitaxel	Cyclic arginine-glycine-aspartic acid (RGD) and transferrin (TF)	Brain glioma	Preclinical development	[68]

EMA, European Medicines Agency; FDA, Food and Drug Administration. The ClinicalTrials.gov identifier is listed as the reference for liposomes in clinical trials.

Dual-Ligand Liposomes for Dual-Targeting Approaches in Cancer

Liposomes have been used for tumor targeting for several decades, and while no single-ligand or dual-ligand liposomes have yet been clinically adopted, such targeted liposome formulations have been reported extensively in the literature. The utilization of a dual-targeted approach has a range of reported purposes; most commonly, for overcoming intratumoral heterogeneity by targeting multiple tumor cell subtypes and targeting tumor-associated cells; for targeting tumor vasculature as a means to halt tumor growth; and for facilitating nanoparticle delivery across biological barriers, such as the blood-brain barrier, for drug delivery to the brain.

Dual-Ligand Liposomes for Targeting Two Tumor Cell Receptors

Given the demonstrated performance of non-ligand liposomes in drug delivery and the large number of studies describing the design of ligand-bearing liposomes to target tumor-associated receptors, the development of liposomes that can target more than one tumor cell subtype in a heterogeneous tumor may help to overcome therapeutic limitations of current therapies (Figure 3). Previous *in vitro* and *in vivo* studies have demonstrated that ligand-directed liposomes targeting two different cell surface receptors can increase the amount of total liposome binding to the cancer cells within a

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tumor, as the liposome is able to bind to any target cell expressing either receptor, which increases the breadth of targeting.

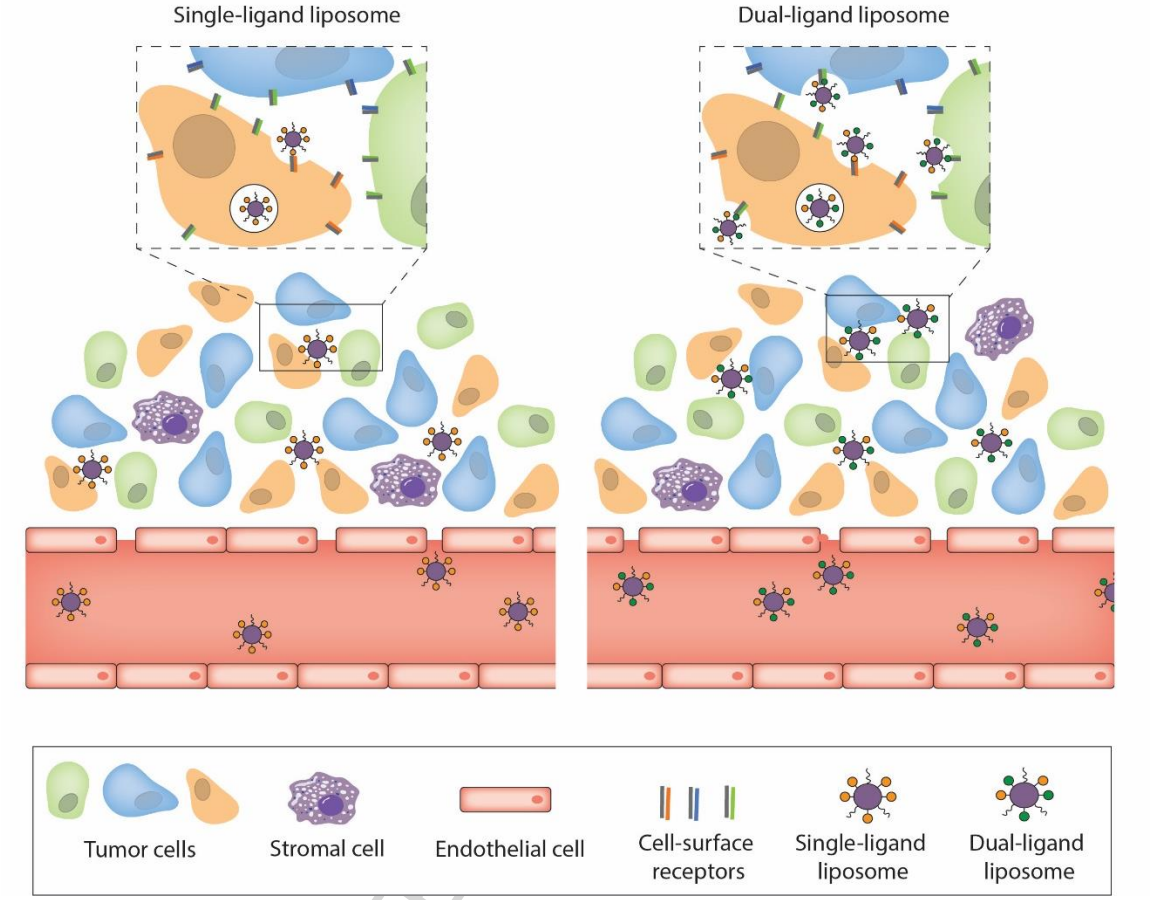

Figure 3: Targeting multiple tumor cell subtypes using dual-ligand directed liposomes may help overcome therapeutic limitations caused by inter-tumoral heterogeneity of cancer. Liposomes bearing two disparate ligands enable liposome uptake via receptor-mediated endocytosis by tumor cells bearing either (or both) target receptors, thus increasing the range of tumor cell targeting. Single-ligand liposomes only enable targeting of the tumor cells bearing the target receptor. Given the intratumoral heterogeneity of cancer, some tumor cells will not be targeted, and instead that population may be able to expand. Ligand-directed liposomes may also be designed to target stromal cells for an intended anti-tumor effect.

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Several preclinical studies have successfully modified liposomes with two surface-bound moieties to create ligand-directed, drug-loaded liposomes that show specific binding to receptor-bearing tumor cells, and a resultant higher tumor cell uptake and kill than non-targeted or single-ligand liposomes [69]. For example, the cellular uptake and cytotoxicity of dual-ligand liposomes targeting lymphoma biomarkers CD19 and CD20, or an equal combination of the two single-ligand liposomes at equal antibody amounts, was greater than for either single-ligand liposome alone [70]. Similarly, a pH-sensitive doxorubicin-loaded liposome formulated to promote intracellular drug release was surface-functionalized with folic acid and AS1411 aptamer (targeting the folate receptor and nucleolin, respectively) and showed increased cancer-targeting and efficacy relative to single-ligand and non-ligand liposomes [71]. Dual-ligand liposomes showed enhanced cellular uptake, higher intracellular delivery of doxorubicin and greater apoptosis in human breast and pancreatic cancer cell lines than single-ligand liposomes, and had no adverse doxorubicin-related effects on a non-cancerous human cell line. Using a murine model of human B-cell lymphoma, drug-loaded liposomes functionalized with antibodies targeting CD19 or CD20 showed improved outcome compared to non-ligand liposomes, with a trend of increased therapeutic efficacy for a combination of the two compared to each alone [70]. Liposomes containing paclitaxel and bearing both a cell ligand peptide and cell penetrating peptide to target lung cancer showed greater liposome internalization in lung cancer cells, greater accumulation of paclitaxel in tumor spheroids, and significantly greater inhibition of tumor growth in a mouse model of lung cancer than single-ligand and non-ligand liposomes [66]. Dual-ligand paclitaxel-loaded liposomes containing the integrin avβ3 peptide and an anti-microbial peptide showed increased

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cellular toxicity and improved tumor growth inhibition in a colon carcinoma mouse model relative to single-targeted liposomes [67]. This improved delivery effect of dual-ligand over single-ligand targeting was also demonstrated using a nanostructured lipid carrier containing plasmid DNA that was surface-functionalized with both transferrin and hyaluronic acid, which showed increased transfection efficiency than single-ligand or non-ligand carriers in a mouse model of lung cancer [72]. While the ligand density and stoichiometry were not quantified in any examples, we hypothesize that ligand-directed liposomes targeting two different cell surface receptors can increase the total amount of liposome binding to the tumor cell surface within a heterogeneous tumor, as the liposome is able to bind to any target cell expressing either receptor (Figure 3). This is likely to increase the breadth of cellular targeting beyond a single receptor/cell type, subsequently enhancing drug uptake, dose and hence the anti-tumor effect [65]. Furthermore, dual-ligand liposomes could act to unify the pharmacokinetic and biodistribution properties of different ligand-functionalized liposomes for precise delivery to target cells as compared to using two individual ligand-functionalized liposomes with disparate targeting moieties and pharmacological profiles.

Dual-Ligand Liposomes for Targeting the Tumor and its Microenvironment

The tumor microenvironment which consists of fibroblasts, immune cells, vasculature, and extracellular matrix (ECM) components such as collagen and fibrin, has increasingly been found to play a key role in tumor progression, metastasis and response to therapy. Treatment strategies that target aspects of the tumor microenvironment such as anti-angiogenic and immunostimulatory therapies show promising preclinical and clinical results; however, factors such as lack of drug penetration into the tumor, non-specific drug delivery, rapid clearance from serum, or

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toxic side effects contribute to the failure of many conventional therapies to completely eliminate the tumor. Dual-ligand liposomes offer a potential solution to some of the aforementioned problems, as many recent studies have shown encouraging results using nanomedicines to target the tumor vasculature, the ECM and cancer associated immune cells [73]. For example, Doolittle *et al*. described the creation of dual-ligand liposomes targeting two different angiogenesis-specific receptors overexpressed at different stages of metastatic disease. Given tumors display a dynamic, heterogeneous microenvironment, that undergoes spatiotemporal changes in the expression of cell-surface biomarkers during disease progression, the authors reasoned that targeting P-selectin and αvβ3 integrin would target the liposome towards blood vessels associated with metastases at different stages of disease progression. Here, a metastatic site transitions, after initial adhesion of circulating tumor cells onto endothelium, from P-selectin-dependent cell rolling on the endothelium to firm attachment that is αvβ3 integrin-mediated [74]. In a resectable mouse model of metastatic triple-negative breast cancer their dual-ligand strategy achieved complementary targeting of different tumor sites that was missed using two independent single-ligand liposomes. This was attributed to poor co-localisation of both single-ligand liposomes at metastatic sites at the same point in time [20]. This approach was similarly demonstrated by Kluza *et al* in the context of magnetic resonance imaging of angiogenesis [75].

Spatiotemporal changes in the expression of cell-surface molecular markers is also observed in cancer stem cells (CSCs), a small population of cells within a tumor with the ability to undergo both self-renewal and differentiation. CSCs are now recognized for their role in driving the initiation, invasion, metastasis, resistance and recurrence of a tumor and the development of targeted nanotherapies that disrupt the

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maintenance and survival of CSCs are the subject of intense research [76]. For example, a multi-functional nanoparticle conjugated to a ligand targeting a specific CSC marker; and a chemosensitizer (such as an ABC transporter inhibitor) to overcome drug resistance has been proposed [76]. Altogether these studies further support the potential advantage of a multiple receptor targeting strategy using dual-ligand liposomes to better target the spatiotemporal changes in receptor expression that occur during metastatic disease progression. Additional examples of potential target combinations for the design of dual-ligand liposomes are listed in Table 2.

Table 2: Potential target receptors for the design of dual-ligand liposomes with the ability to concomitantly target the tumor and its dynamic microenvironment.

Cancer type	Biomarker 1	Cell population targeted	Biomarker 2	Cell population targeted
Breast	HER2 ER EGFR	Tumor cells [77] Tumor cells [78] Tumor cells [79]	ALDH-1 CTLA-4 uPAR	CSC [80] CSC [81] Activated fibroblasts and tumor-associated macrophages [82], invasive tumor cells [83] and CSC [84]
Pancreatic	EGFR uPAR CD109	Tumor cells [85] Tumor cells [86] Tumor cells [87]	CD133 CD44 CD24	CSC [88] CSC [89] CSC [90]
Melanoma	AXL receptor tyrosine kinase	Tumor cells [91]	$CD20^{+}$ VEGFR	Tumor-associated B cells (in cutaneous melanoma) [92] Endothelial cells [93]
Prostate	PSMA	Tumor cells and new blood vessels [94]	CD44/CD133	CSC [95]
Colorectal	uPAR	Tumor cells and tumor-infiltrating macrophages [96]	VEGFR EpCAM	Endothelial cells [97] CSC [98]

HER2, human epidermal growth factor receptor 2; ER, estrogen receptor; EGFR, epidermal growth factor receptor; ALDH-1, aldehyde dehydrogenase 1; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; uPAR, urokinase plasminogen activator receptor; CSC, cancer stem cell; CD, cluster of differentiation; VEGFR, vascular endothelial growth factor receptor; PSMA, prostate-specific membrane antigen; EpCAM, epithelial cell adhesion molecule

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Dual-Ligand Liposomes for Overcoming Biological Barriers

In the context of glioma treatment, ligand-directed liposomal drug formulations may enhance drug transport across the blood-brain barrier (BBB) for drug delivery to the brain [99]. Dual-ligand liposomes containing daunorubicin and surface-functionalized with both transferrin and p-aminophenyl-α-D-manno-pyranoside showed increased transport across the BBB, increased cellular uptake and increased survival compared to treatment with free daunorubicin in a rat model of brain glioma [100]. Another study using doxorubicin-loaded liposomes surface-functionalized with transferrin and one of two different cell-penetrating peptides showed improved delivery of doxorubicin across the brain endothelial barrier (BEB) compared to single-ligand and non-ligand liposomes *in vitro*, and efficient translocation across the BEB in an *in vitro* brain tumor model [101]. Similarly, docetaxel-loaded nanoparticles that were surface-functionalized with IL-13 and RGD peptide to target both tumor cells and neovasculature showed greater uptake in a glioma cell line than single-ligand and non-ligand nanoparticles, and the dual-ligand nanoparticle induced higher apoptosis of cells in the glioma site *in vivo*, indicating an improvement in cell uptake and the anti-tumor effect by dual-targeting [102]. This was further supported by experiments using dual-ligand liposomes bearing both an aptamer and a peptide moiety to target glioma and the BBB in an *in vitro* glioma model designed to recapitulate the tumor microenvironment [103]. Collectively, the aforementioned studies demonstrate the potential utility of dual-ligand directed liposomal drug formulations for cancer therapy, with an increased degree of liposome uptake acting to improve the anti-tumor effect.

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Challenges and Outstanding Questions

Despite convincing preclinical research in the field of ligand-directed liposomes for the treatment of solid tumors and other diseases, there has been limited progression of targeted liposome formulations towards clinical application [104]. There are several important factors that may be responsible for this lack of clinical development of targeted liposomes past the preclinical stage.

Large-Scale Production of Ligand-Directed Liposomes

Most – if not all – of the current clinically approved nanotherapies are arguably quite simplistic in their composition and structure, a characteristic which is well regarded by the processes of large-scale manufacture – for example, Doxil. However, laboratory-based preparation and testing of ligand-directed liposomes is usually performed on a small scale, often in milliliter quantities. Volumes produced at this small scale are sufficient for *in vitro* and *in vivo* testing, but upscaling of ligand-directed liposome production – as required for clinical use – can be challenging, since currently-used lab-based liposome production methods are generally not amenable to scale up beyond the milliliter scale. For example, the formation of liposome thin films via use of rotary evaporation is limited by the size of the flask used to create the film, and flask overloading may increase liposome polydispersity and alter other physicochemical characteristics of the resultant sample [105]. The extrusion of liposomes through membranes as required to achieve a desired size distribution is another labor-intensive step in the production process, as preparations need to be passed repeatedly across a membrane and usually on a 1-20 milliliter scale. In the laboratory setting, preparation of multiple separate batches of ligand-directed liposomes can be used to overcome these

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issues. However, the current lack of established methods to quantify the ligand density on the surface of liposomes means that it is difficult to account for batch-to-batch variability of a ligand-directed liposome formulation, and even more so for dual-ligand or multi-ligand liposomes. Without robust methods to enable detection of ligand conjugation and quantification of surface ligands, variation between batches may lead to deviations in the physicochemical characteristics of the preparation, which would ultimately influence stability, *in vivo* circulation time, clearance properties, tumor uptake, therapeutic efficacy and toxicity of a targeted liposome formulation [106].

Characterization of Ligand-Directed Liposomes

Various methods for liposome characterization are well documented. Commonly measured characteristics include: liposome size and polydispersity by dynamic and static light scattering; surface charge by measuring zeta potential; degree of drug encapsulation by spectrophotometry or high performance liquid chromatography; and morphology and physical state by cryo-transmission electron microscopy and atomic-force microscopy [107]. The development of methods to characterize more complex liposomes, particularly ligand-directed liposomes, are lacking and this is a significant barrier to the feasible and practical development of actively targeted liposomes for clinical utility. Controlling for batch-to-batch variability is difficult without effective methods for characterization, and the inability to control or correct for variability in ligand attachment to liposomes will become an issue in the regulatory processes required for clinical translation of a novel formulation. Notably, adequate methods for the confirmation and quantification of ligand attachment to liposomes have not been reported [108]. The direct measurement of small amounts of protein in a targeted

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liposome formulation using biochemical assays is often problematic due to phospholipid interference, and if successful only provides a quantification of the total protein in a liposome sample, rather than a quantification of the average number of protein ligands bound to each liposome. Characterization of ligand-directed liposomes has been performed using indirect assays, such as flow cytometric methods that detect the insertion of fluorescently-labelled micelles (to which protein ligands are bound) into liposomes to confirm that ligand incorporation into the liposome has occurred, but these methods are only semi-quantitative at best [109]. Understandably, this poses a larger challenge for dual-ligand and multi-ligand liposomes, where the determination of stoichiometry of ligand attachment becomes an important step in the characterization process. Theoretical values of ligand conjugation and ligand ratios have been reported but this has not been demonstrated empirically for most liposome formulations, as the methods used to generate such data are technically challenging. Our group has recently developed a novel single-molecule fluorescence imaging technique that is able to quantify the density and stoichiometry of proteins attached to the surface of liposomes with high sensitivity. By removing ensemble averaging, single-molecule approaches allow the direct visualization of liposome population distributions and the precise characterization of sub-populations, and the ability to detect single-molecule changes therein (Belfiore *et al.*, under review at Journal of Controlled Release).

An important consideration in the characterization of ligand-directed liposomes concerns questions beyond the *in vitro* setting and in the context of the *in vivo* biological milieu. Specifically, the question of what happens to liposome integrity, ligand attachment, ligand function, and therefore the biophysical properties of a liposome formulation after intravenous administration, including *in vivo* circulation

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time and clearance properties of the liposomes. The well-documented propensity of biological molecules, especially proteins, present in the bloodstream to associate non-specifically with the surface of liposomes *in vivo*, and the subsequent formation of a protein 'corona' around the liposome may affect numerous biophysical properties of a liposome formulation. In the case of ligand-functionalized liposomes, the physical presence of a protein shield around the surface of the liposome, including association of plasma proteins with liposome ligands, may act to inhibit binding of the liposome targeting ligand with its target receptor, therefore reducing or masking the targeting ability of the liposome, which would affect the targeting success *in vivo* [51]. Such potential changes to the liposome are usually unaccounted for in the *in vitro* setting but could indeed affect the anticipated biodistribution, pharmacokinetics and efficacy profiles of a liposome formulation [110]. Therefore, it is important to consider these effects in biological testing systems, noting that attempting to recapitulate such effects *in vitro* comes with inherent limitations.

Another aspect for consideration is the potential negative effects that ligand-directed liposomes may have on healthy tissues. In order to minimize off-target effects, target receptors are usually chosen based on their very high expression on tumor cells relative to healthy cells [111]. To demonstrate this point, Park *et al.* reported that a receptor density of 10^5 HER2 molecules per cell was required for increased therapeutic effect of HER2-targeted liposomal doxorubicin over non-targeted liposomal doxorubicin in a metastatic breast cancer model [112]. Similarly, the differential expression of estrogen and progesterone receptors in hormone receptor-positive breast cancer, compared to healthy tissue, are useful indicators of response to therapy [113]. Indeed, many of the current FDA-approved molecular targeted therapies for cancer,

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including trastuzumab, lapatinib and pertuzumab in the case of breast cancer, involve targeting receptors with very high prevalence on tumor cells in order to attain a degree of targeting sufficient to achieve the therapeutic response.

While there are now comprehensive libraries that catalogue a variety of new potential ligands for nanotherapeutic applications [114], the basic principle of ligand-mediated targeting remains constant and is subject to two critical criteria; the accessibility of the target receptor for ligand binding, and whether receptor binding leads to cellular internalization. Targeting ligands need to be highly selective, but also relatively safe – and in the case of utilizing a ligand to direct a nanoparticle to a target cell, rather than using the ligand itself to exert an anti-tumor effect, the ligand need not be toxic. However, this can be difficult to assess, as even if the free ligand is studied for toxicity, the toxicity profile may be very different after coupling to the surface of liposomes. Commonly-used ligands, such as folate and transferrin, have been relatively well-characterized using *in vitro* and in *in vivo* models. Folate-targeted nanoparticles functionalized with folate ligands have shown low systemic toxicity in a mouse model of epidermoid carcinoma [115]. Liposomes surface-coated with hyaluronan have shown no measured cytokine induction after intravenous administration in mice, indicating no immune activation, despite the fact that administration of low-molecular weight hyaluronan itself has previously been shown to stimulate inflammatory responses [116]. Such studies highlight the importance of determining potential off-target effects of ligands for new ligand-directed liposome formulations. However, the effect of ligands on healthy cells and the immune system in the context of human diseases becomes difficult to ascertain without the development of models that enable accurate

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determination of these systemic effects. This topic is discussed in the following sections.

Models that Accurately Reflect Tumor Heterogeneity

A diverse range of cancer cell lines derived from tumor biopsies have been established in the laboratory and retain many – but not all – of the genotypic and phenotypic properties of the original tumor cells, making them useful representative models for testing targeted therapies [117, 118] and to study mechanisms of therapeutic resistance [119]. However, despite their widespread use, cells grown in a two-dimensional (2D) monolayer do not adequately recapitulate several key elements of *in vivo* tumors, including three-dimensional (3D) tumor architecture, tumor cell interactions, tumor-stroma interactions and the various proliferative and metabolic gradients that form when tumor cells exist as a 3D structure [120]. The absence of these features in cell monolayers is highlighted by differences in cell morphology and gene expression in 2D versus 3D cultures [121], and results in differences in responses to drug treatments. For example, the sensitivity of breast cancer cells to trastuzumab, pertuzumab and lapatinib changes depending on whether the cells are grown as 2D or 3D cultures [122], and the apparent differences in HER2 signaling observed between 2D and 3D cell culture models of breast cancer suggest that 3D models better recapitulate *in vivo* HER2 signaling pathways [123]. Given their closer similarity to *in vivo* tumors, 3D models are generally considered more informative in the translation of *in vitro* results to *in vivo* and clinical settings [124]. Multicellular tumor cell spheroid models are a commonly used 3D cell culture model in which cancer cells are grown as a spherical association resembling small tumors and micrometastases [125]. The ability of cancer cells to form

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spheroids is strongly related to the expression of several cell-cell adhesion molecules [126] and can be facilitated by culturing cells in conditions that prevent adherence to the cell culture plate [127]. Changes in spheroid morphology and diameter in response to a drug treatment can be measured using manual or automated imaging techniques [128], and end-point biochemical assays allow for determination of cell viability [129]. Multicellular tumor cell spheroids may also be grown with additional extracellular matrix components (i.e. fibronectin and collagen), or support cell types that are associated with *in vivo* tumors, including tumor-associated fibroblasts which have been shown to influence tumor growth, invasiveness and overall disease progression [130], and therefore targeting of both the tumor microenvironment and tumor cells may produce synergistic anticancer effects. Such aspects of spheroids allow for the creation of a more clinically relevant model to study the interactions between distinct cell types in the tumor microenvironment and test the effects of novel targeted therapies while improving the translation of results from the *in vitro* to the *in vivo* setting [131].

While 3D and *ex vivo* models are considered more physiologically relevant than 2D cell monolayers, most models still do not adequately capture the nature of tumor heterogeneity [132]. A single cancer cell line used in a spheroid model or even injected into an animal to create an *in vivo* model of disease fails to recapitulate the intratumoral heterogeneity that is observed in human tumors across many cancer types. As the cells are clonally similar, any treatment is expected to affect most if not all cells in that model in the same way. Therefore, using these models to develop and test novel therapies, especially targeted therapies that are designed to address intratumoral heterogeneity, is limited as they are not representative of the clinical situation and do not permit evaluation of therapeutic resistance. To address this, cell monolayers and spheroids can

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be grown as co-culture models, where distinctly different cell lines are cultured together to recapitulate some aspects of tumor heterogeneity, but with limitations [133]. For example, these static models rely solely on passive drug diffusion to permeate the tumor cells or spheroids, and do not account for transport across the vascular endothelium. Further, they do not reproduce the complex vascular network, hypoxia, interstitial fluid pressure and fluid shear observed in the *in vivo* tumor microenvironment. In order to better understand the impact of tumor heterogeneity and the complexity of the tumor microenvironment, Kiani and colleagues have recently developed a microfluidic-based platform for monitoring drug delivery in a 3D environment recapitulating circulation, extravasation and delivery to the tumors across the interstitial space [134].

In addition to intratumoral heterogeneity, the interpatient heterogeneity observed in cancer warrants the development and utilization of patient-derived xenografts and patient-derived cell lines to more accurately assess patient responses to novel therapies, particularly in cases where resistance to currently used therapies is frequently observed [135]. Additionally, given the effect of the immune system in tumor growth and metastasis [136], there is a need for tumor models in immunocompetent animals in addition to the often-used immunocompromised models that eliminate potential effects of the immune system in the evaluation of new anticancer therapies [137]. The increasing use of such models lends itself to the improved assessment of targeted therapies in the context of cancer treatment. However, *in vivo* models should be chosen with care given the high level of variability observed between different animal models and disease states [138].

Accounting for the Enhanced Permeability and Retention Effect in the Preclinical Setting

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The field of nanomedicine is founded on the central dogma of the enhanced permeability and retention (EPR) effect. Evidence for this phenomenon has been reviewed elsewhere [139] but the research to date collectively suggests that the EPR effect does appear to enable the passive accumulation of liposomes and nanoparticles to tumor sites. However, the EPR effect is reported to be highly variable between different tumor types and is not observed for all solid tumors [140, 141]. For solid tumors that are typically poorly vascularized, any significant accumulation of nanoparticles in the vicinity of the tumor via the EPR effect is unlikely [142]. In such cases, the application of nanoparticles in the treatment of some solid tumors may have greater potential for use in the adjuvant setting to target vascularized micrometastases, rather than (or in addition to) the primary tumor [143]. The nanoparticle targeting of hematological and lymphoid tumors, particularly for ligand-directed liposomes, has generally shown greater success in *in vivo* tumor models since tumor cells in circulation are more directly accessible to liposomes than large solid tumors immersed in complex microenvironments [144, 145].

The EPR effect is known to be highly variable between different animal models, different disease models and between animal models and the human patients [146], with the rate of animal model tumor growth and resultant angiogenesis much greater than the formation of a tumor in humans [147]. The EPR effect has been demonstrated in humans using CRLX101, a polymer-drug nanoparticle, which was shown to localize in patient tumors and not in adjacent tissues following administration [148]. In this experiment, the fluorescent nanoparticle signal observed was lower than that previously observed in mouse xenograft models. Given the observed differences in the EPR effect between animals and humans, the initially reported efficacies of many novel

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nanotherapies are often much higher in preclinical models than later reported in humans due to the former having a more pronounced EPR effect [139], and this may partially explain why many nanotherapies that show promise in *in vivo* studies fail in clinical trials. The development of animal models that recapitulate the EPR effect at a level more analogous to the human condition would be of benefit in the initial evaluation of novel targeted nanotherapies.

Whether via the EPR effect and/or via other mechanisms, it has been reported that only approximately 0.7% of the injected dose of nanoparticles administered intravenously accumulates in tumors in preclinical models [50]. However, it should be noted that the accumulation of nanoparticles in tumors via EPR is largely dependent on the *in vivo* circulation time of the nanoparticle formulation; for example, the tumor accumulation of Doxil® in humans has been reported as high as 10% of the injected dose, owing to the long circulation half-life of up to 45 hours [149]. While the percentage of injected dose accumulating in tumors may indeed be lower in humans due to noted differences in the EPR effect between species, previous studies have demonstrated a tangible effect of nanoparticle drug delivery to human tumors. For example, in a study of gastric cancer in humans, it was demonstrated that the degree of passive accumulation of nanoparticles in gastric tumors was sufficient to cause a down-regulation of two target enzymes in the tumor tissue [148]. Although the EPR effect has only been directly demonstrated in animal models, this study indirectly supports the notion of accumulation of nanoparticles in human tumors for therapeutic effect, which may be due, wholly or in part, to the EPR effect. Further research is required to better understand the EPR effect and elucidate the differences in this phenomenon between animal and human tumors, and between different tumor types, in order to increase

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translation of nanoparticle-based therapeutics into the clinic [150]. One way that this could be achieved is via imaging of radiolabeled liposomes to determine their fate in humans [151]. Single photon emission computed tomography (SPECT) and positron emission tomography (PET) have previously been used to quantify the *in vivo* distribution of nanoparticles, including accumulation of nanoparticles at the tumor site, in a non-invasive manner in locally advanced cancers of the head and neck, breast and cervix [152]. The use of nanoparticles in conjunction with such imaging techniques may also have theranostic applications, whereby both diagnostic and therapeutic agents are utilized in order to better guide and monitor treatment [153].

Emerging Trends and Future Directions

The development of new methods and technologies to prepare and characterize ligand-directed liposomes will enable a more comprehensive evaluation of ligand-directed – and importantly, dual-ligand – liposome formulations, to facilitate their clinical development. To meet the demands for large-scale preparation of liposomes as required for clinical use, microfluidic approaches have recently emerged as a way to produce large quantities of liposomes of a uniform size and consistent physicochemical properties, which may be a way forward for efficient and cost-effective liposome preparation [154]. With advances in technologies to prepare liposomes on a large scale and to create actively targeted liposomes using antibody engineering, the future for nanoparticle-based drug delivery strategies can permit multiple targeting of target cell types, including genetically distinct tumor cells, but also key cells of the tumor microenvironment that are known to play a role in supporting tumor growth and spread, including immune cells and cancer stem cells [155].

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The use of short chain antibody fragments as targeting ligands, as opposed to whole antibodies, is a promising strategy for creating actively targeted liposomes as the ligands can be engineered to optimize binding affinity and other physical properties for improved tumor cell targeting and uptake. As antibody fragments are smaller than whole antibodies, the immunogenicity may be lower and the *in vivo* circulation time of the resultant targeted liposomes more appropriate (i.e. more prolonged) for tumor targeting [156]. Recent technological developments have contributed to a shift away from conventional covalent coupling methods of attaching ligands to the surface of liposomes, and towards the specific engineering of antibodies and fragments for cellular targeting applications. Protocols to develop bispecific immunoliposome formulations using two different single-chain FV fragments on the liposome surface to target two different tumor cell populations have been reported and show a retention of binding activity of each ligand for its target receptor [109]. The creation of multivalent liposomal therapeutic antibody constructs to bind more than one antigen has been reported [157], as well as PEGylated hyper-branched polymers bearing two different targeting ligands [158]. The use of bispecific antibodies bound to the surface of liposomes potentially allows recognition of multiple antigens to achieve the same effect attained with conventional dual-ligand liposomes [159]. The successful development of a liposome with a single surface-attached bispecific antibody that can recognize and bind to both endoglin (CD105) and fibroblast activation protein demonstrates the feasibility of this approach in dual-targeting [160]. These approaches allow for more control in the stoichiometry of ligand targeting (i.e. always 1:1) compared to the traditional conjugation of two separate ligands, and for this reason may aid the

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production and regulatory processes required for clinical use of actively targeted liposomes.

The gradual movement away from simplistic monolayer and monoculture cell models and utilization of models that better recapitulate *in vivo* tumors, including computer simulated models [161], *ex vivo* multicellular tumor spheroid models, co-culture models, biomimetic microfluidic tumor microenvironment models and patient-derived xenografts, will allow for the inclusion of some aspects of tumoral heterogeneity and the contribution of the tumor microenvironment in the evaluation of novel nanotherapies. The use of other assessment approaches, such as comparative oncology in non-human patients with prostate or other spontaneous cancers that mimic the human disease are valuable models for assessing liposome efficacy [140]. These approaches are expected to help guide nanotherapy research in its early stages and provide a more accurate understanding of the expected efficacy should the formulation progress to clinical trials.

To better guide the movement of novel liposomes into clinical trials, liposomes and other nanoparticles can be used in a theranostic setting, combining both diagnostic and therapeutic capabilities in a cancer context. Theranostic nanoparticles may bear a ligand for tumor targeting and a second ligand or other molecule for imaging *in vivo*. Radiolabeled liposomes have been previously detected in humans *in vivo* using positron imaging tomography imaging techniques [151]. Liposomes bearing a folate ligand and containing a photothermal agent offer both therapeutic and diagnostic functions, respectively, in the treatment and imaging of cancer *in vivo* [162]. Dual-ligand micelles with surface-bound trastuzumab and FLAG peptide showed co-localization of the antibody and peptide in SKBR-3 cells by confocal microscopy, while non-

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functionalized micelles showed no uptake, indicating tumor cell targeting and a receptor-dependent effect [163]. The theranostic potential of liposomes can also be utilized for companion diagnostics in the preselection of patients for clinical trials and use in the clinic, and is an area of developing research in the field [153].

Finally, in addition to defining the precise liposome engineering conditions for optimal pharmacological profiles, there is a concomitant need to gain a thorough understanding of the aberrant biological processes driving disease in order to identify new molecular targets (and their ligands) for targeted drug delivery. Correlating the genotype of tumors to drug susceptibility will also help to establish guidelines for the use of targeted nanotherapies and to predict successful therapeutic outcomes. As reviewed in detail elsewhere [164, 165], many disease-specific ligands have been conjugated to liposomes in order to achieve site-specific drug delivery. Historically, these efforts have focused on the design of nano-delivery systems that utilize ligands (most commonly antibodies) to target breast, prostate and colorectal cancer; however, our ever increasing knowledge of the genetic or molecular alterations that underlie disease pathophysiology is providing novel targeting ligands. For example, thyroid-stimulating hormone (TSH) has been attached to the surface of PEGylated liposomes with the aim of targeting the TSH receptor (thyrotropin receptor). TSH receptor expression is maintained in most thyroid pathologies, including benign and malignant tumors [166], but more importantly, is also present in the majority of less differentiated and more aggressive tumors [167], making it a novel opportunity for targeted delivery of chemotherapeutics. Such insights offer new avenues for improving therapeutic efficacy of nanotherapeutics and is critical for the success of next generation targeted therapeutic approaches.

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Conclusions

The first clinically-approved liposome, Doxil®, has been in use for over 20 years and is still used as an effective treatment for several cancer types. Despite this, the liposome field has not evolved into translating effective actively-targeted analogues. Nonetheless, despite the many hurdles left to overcome in the production, evaluation and translation of ligand-directed liposomes towards clinical use in the context of cancer therapy, the utility of dual-ligand liposome technologies is promising. Ligand-directed liposomes have the potential to increase the selectivity of therapy, improving efficacy and reducing the potential for harmful side effects, and dual-ligand liposomes may additionally address intratumoral heterogeneity to overcome patient resistance to targeted therapies. The development of better methodologies and preclinical models to comprehensively characterize novel ligand-directed liposomes and better assess the likelihood of their performance in humans, including recapitulation of intratumoral heterogeneity, will likely improve translation of these nanotherapies from preclinical models through to the clinic.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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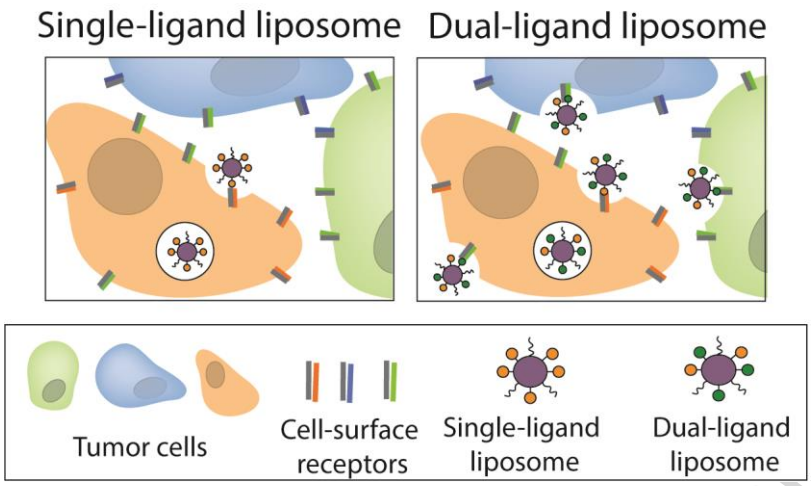

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