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Recent Advancements Involving Immunoliposomes to Target Breast Cancer

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Abstract

Breast cancer is caused by genetic abnormalities resulting in uncontrolled growth of breast cells, and is the most commonly diagnosed cancer amongst women. The clinical use of liposomal-based drugs to treat solid tumors such as breast cancer has been shown to improve the overall pharmacological properties of otherwise "unencapsulated" cytotoxic agents. In this review, we discuss recent advancements reported in the literature involving liposomes surface-modified to include antibodies to form immunoliposomes, which are specifically intended to bind some of the more commonly targeted overexpressed cell surface receptors on breast cancer cells. Here, we focus on human epidermal growth factor receptor 2 (HER2), epidermal growth factor receptor (EGFR), as well as heparin-binding epidermal growth factor-like growth factor (HB-EGF) receptor.

Keywords: Immunoliposomes; Liposomes; Breast Cancer; Chemotherapy; Nanocarriers; Human Epidermal Growth Factor Receptor (HER2); Epidermal Growth Factor Receptor (EGFR); Heparin-Binding Epidermal Growth Factor-Like Growth Factor (HB-EGF)

Abbreviations: HER2: Human epidermal growth factor receptor; EGFR: Epidermal growth factor receptor; HB-EGF: Heparin-binding epidermal growth factor-like growth factor; EPR: Enhanced permeability and retention; FDA: Food and drug administration; PEG: Polyethylene glycol; PARP: Poly-ADP-ribose-polymerase

Introduction

Breast cancer is the worldwide second leading cause of cancer death amongst women and therefore new and more efficacious chemotherapeutics with fewer unintended deleterious side-effects to the patient are desperately needed [1]. Nanocarriers used as drug delivery systems have proven to be quite effective constructs for the delivery of cytotoxic agents to solid tumors such as breast cancer, and have therefore grown in popularity in recent decades [2,3]. This is primarily due to the fact that an effective dose of the drug can be delivered to the tumor-site in part due to a phenomenon first described by Matsumura and Maeda in 1986 known as the enhanced permeability and retention (EPR) effect [4]. The EPR effect arises not only due to the deregulated angiogenesis that occurs in and around tumors resulting in vascular gaps of approximately 200 nm or greater ("enhanced permeability"), but also from the lack of functional lymphatic vessels in tumor tissue resulting in poor lymphatic drainage ("enhanced retention") [5,6]. As a result, nanocarriers can somewhat selectively accumulate and are entrapped at the tumor-site in this process commonly referred to as "passive" drug delivery (Figure 1), while the nanocarrier itself shields healthy tissue from the cytotoxic effects of the encapsulated/incorporated drug while in circulation.

With respect to the various types of nanocarriers available, in theory there are many to choose from [7,8]. However, some of these have experienced more clinical success in the treatment of breast cancer than others. For instance, micelles, nanoparticles, and liposomes to name a few have all been successfully used clinically in the treatment of breast cancer (Table 1). For example, the drug Genexol-PM is produced by the Samyang Company in South Korea and is a micelle formulation containing paclitaxel, which is currently clinically approved to treat metastatic breast cancer in that country [9,10]. Abraxane is an albumin-bound nanoparticle containing the cytotoxic agent paclitaxel, and is also clinically approved to treat metastatic breast cancer [11,12]. In fact, the maximum tolerated dose of this protein bound-nanoparticle-based drug is significantly higher than its "free drug" counterpart Taxol, which is the commercialized formulation of paclitaxel containing the emulsifier Cremophor EL [11,13]. However, it should also be noted that it is somewhat unclear whether the added benefit of using this nanoparticle-based formulation with respect to lower toxicities compared to Taxol is solely attributed to the use of this particular nanocarrier or the removal of the Cremophor EL from the commercialized formulation which has toxicities of its own to include prolonged

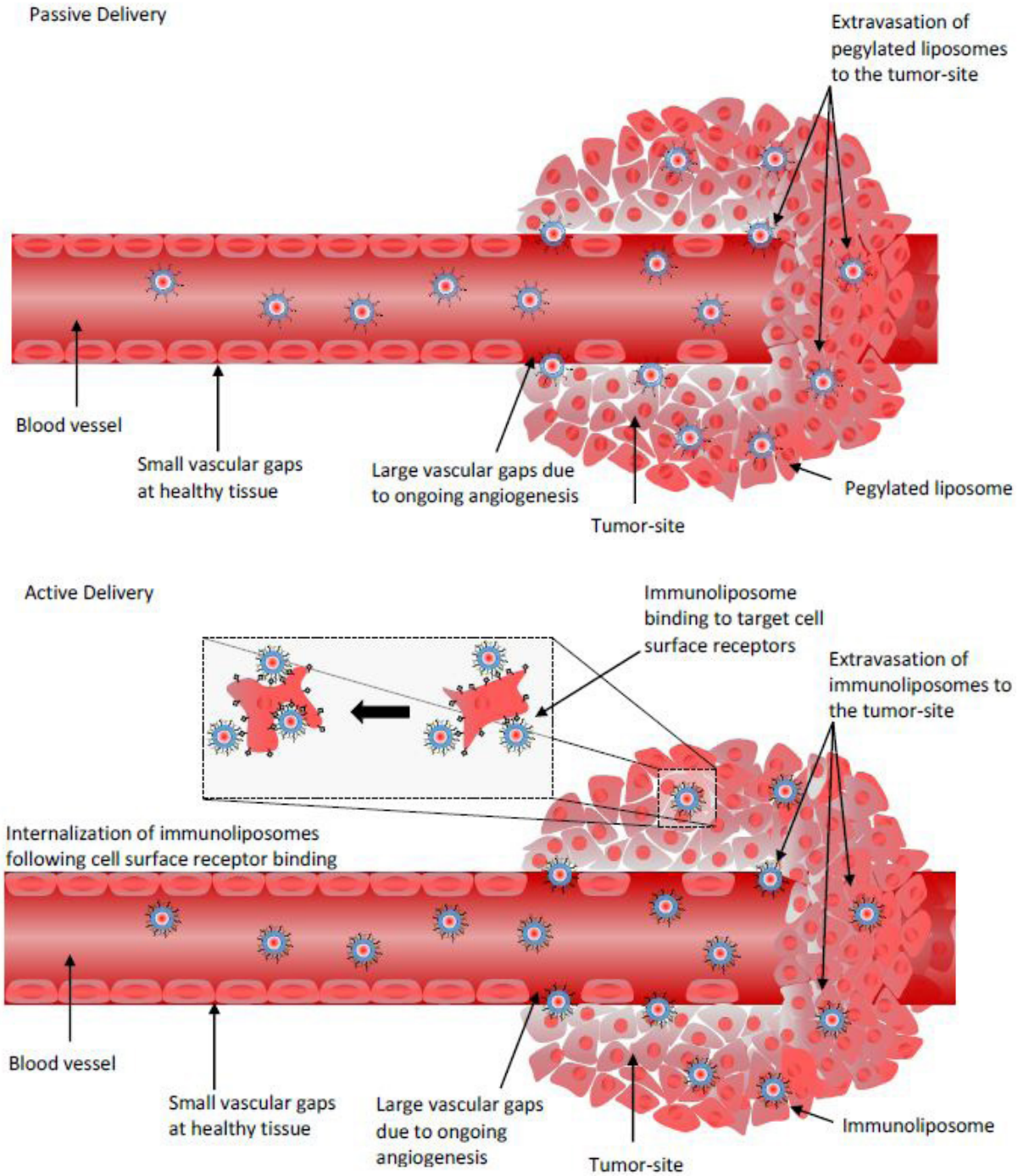

Figure 1: Depiction of passive delivery involving the EPR effect to include extravasation of pegylated non-targeted liposomes (top) and active delivery involving pegylated immunoliposomes (bottom) from circulation to the tumor-site through large vascular gaps

Nanocarrier	Trade Name	Drug	Status
Micelle	Genexol-PM	Paclitaxel	Approved(South Korea)
Nanoparticle	Abraxane	Paclitaxel	Approved
Liposome	Doxil (Caelix)	Doxorubicin	Approved
Liposome	Myocet	Doxorubicin	Approved
Liposome	Lipusu	Paclitaxel	Approved(China)
Liposome	PICN	Paclitaxel	Approved(India)
Liposome	Alocrest	Vinorelbine	PhaseI

Table 1: Current status of recently developed non-targeted nanocarrier-based chemotherapeutics used to treat breast cancer in the United States unless stated otherwise

peripheral neuropathy [13,14]. In any event, liposomes have probably been used with the most clinical success, particularly with respect to the treatment of breast cancer. In fact, Doxil (also known as Caelix in some countries) is a liposomal-based chemotherapeutic containing encapsulated doxorubicin, and was the first nanocarrier-based formulation clinically approved in the United States by the Food and Drug Administration (FDA) in 1995, which is now used to treat metastatic breast cancer [2,15-17]. The drug Myocet also encapsulates doxorubicin and is clinically approved to treat metastatic breast cancer in Europe and Canada [2,16]. Both Lipusu and PICN are liposomal formulations involving encapsulated paclitaxel which are clinically approved to treat breast cancer in China and India respectively, while Alocrest containing vinorelbine is currently in Phase I clinical trials for the treatment of breast cancer [16,18-20]. The ongoing clinical successes using liposomes as a nanocarrier for the delivery of cytotoxic agents to solid tumors can be explained by a number of reasons. For example, they are generally composed of phospholipids and are therefore biocompatible, can accommodate both hydrophilic and hydrophobic drugs (either in the internal aqueous core or phospholipid bilayer respectively), and can essentially be formulated to be of any desired size [21-23]. This last point is of particular importance as optimal liposomal size intended for this purpose range anywhere from 50-150 nm in diameter such that they are large enough to remain in circulation and not penetrate normal vessel walls of 10 nm in size or less (Figure 1), and yet small enough to extravasate out of circulation at the tumor-site based on the EPR effect as described above [24,25]. While there are many advantages associated with the use of liposomal-based drugs to treat solid tumors, their use also presents some obstacles to efficacious drug delivery. For example, low bioavailability of the drug can occur resulting from minimal accumulation within tumor tissue as these formulations are particularly subject to opsonization while in circulation resulting in low circulation times in vivo. While larger liposomes can in theory deliver more of the cytotoxic agent to the tumor-site compared to smaller liposomes, larger liposomes are removed from circulation much faster than their smaller counterparts. In fact, early studies by Woodle et al. demonstrated that liposomes 250 nm were removed from circulation more than twice as fast as liposomes 100 nm in diameter of similar compositions [26]. Therefore, many liposomal

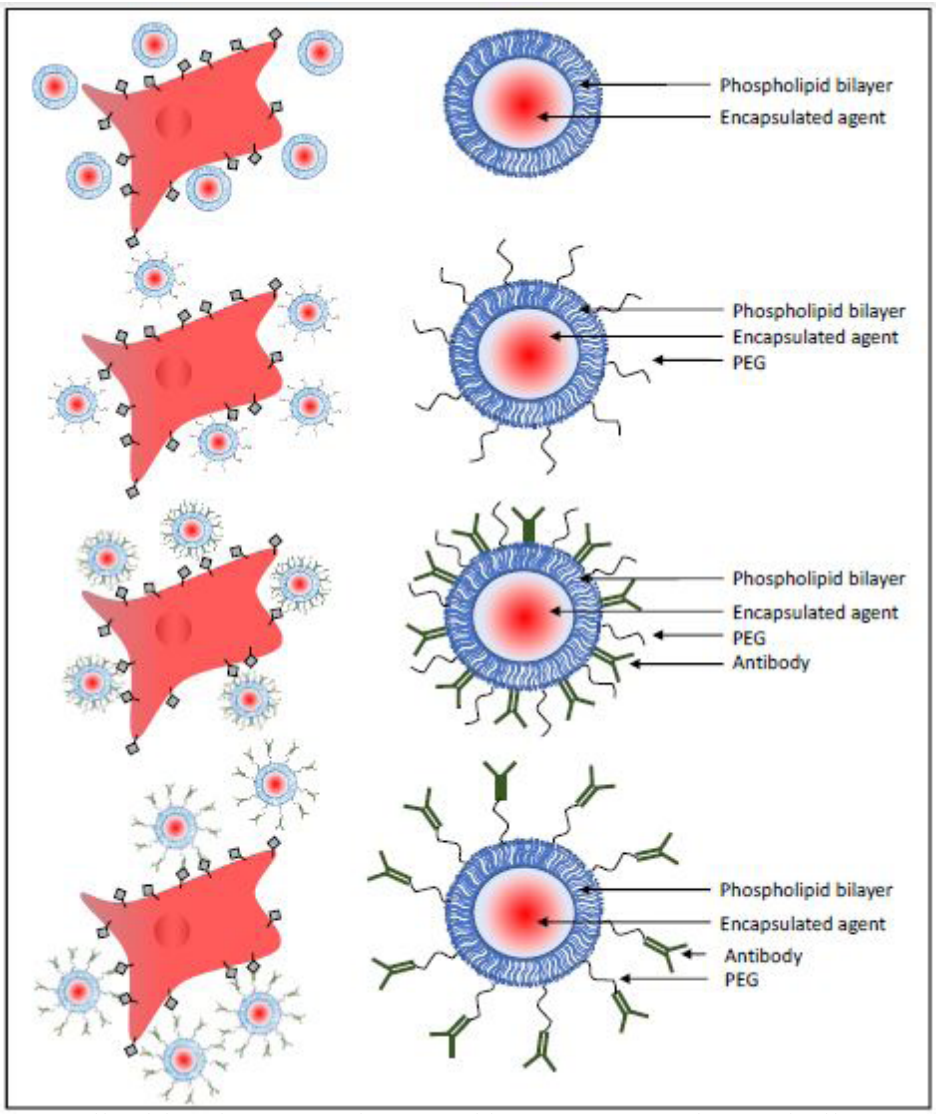

Figure 2: Liposomal based chemotherapeutics involving passive delivery using non-targeted non-pegylated liposomes (a.) or non targeted pegylated liposomes (b.), while active delivery involving liposomes and antibodies generates targeted immunoliposomes with the antibody/antibody binding fragment conjugated to either the liposomal surface (c.) or at the distal end of the PEG (d.)

formulations involve surface modification to include the addition of various polymers such as polyethylene glycol (PEG). This process, commonly referred to as pegylation, results in pegylated, liposomes which have dramatically increased circulation times *in vivo* compared to their non-pegylated counterparts, thereby improving tumor-site accumulation [27,28]. In fact, the already mentioned clinically approved drug Doxil is a pegylated liposomal-based formulation. However, the mere presence of the PEG moiety also presents a complication to effective drug delivery in that it becomes a steric barrier between the drug and tumors cells, thus cancer cellular uptake of the drug can be dramatically reduced [29]. Therefore, delivery of the encapsulated cytotoxic agent is somewhat dependent upon leakage in the tumor microenvironment and subsequent tumor cellular uptake of the free drug. This process is somewhat inefficient, particularly when you consider the fact that many cytotoxic agents such as doxorubicin have a high affinity for various components of the extracellular matrix, further limiting cellular uptake of the drug [30]. Therefore, while all of the liposomal-based formulations mentioned thus far deliver encapsulated cytotoxic agents to the tumor-site via a "passive" form of drug delivery, future work aims to replace this type of delivery with a more "active" one (Figure 1). Active drug delivery involves the incorporation of targeting ligands at the liposomal surface which are designed to specifically bind known overexpressed cancer cell surface receptors in order to improve overall delivery through enhanced colocalization between cancer cells and the drug. In fact, there have been numerous types of targeting ligands that have been used for such delivery and reported in the literature with varying levels of success to include peptides, proteins, carbohydrates, as well as vitamins [31-33]. However, the use of antibodies or antibody binding fragments have proven to be particularly effective targeting ligands in part due to their specificity and high binding affinity to the overexpressed cancer cell surface receptor for which they are intended to bind [33]. Furthermore, they can easily be added to either the liposomal surface or the tip of the PEG moiety for increased accessibility to the intended cell surface receptor (Figure 2). It should also be mentioned that antibody conjugation to the tip of the PEG moiety would also have the additional advantage of eliminating any potential masking effects that could occur with antibody addition directly to the surface of pegylated liposomes. In any event, this modification to pegylated liposomal-based chemotherapeutics in theory would potentially allow patients to receive much higher doses of the drug with far fewer negative side-effects, thereby allowing for more effective frequent treatments. Due to the fact that breast cancer is the most commonly diagnosed cancer amongst women, coupled with the recent clinical successes involving the use of liposomes as nanocarriers in order to treat, solid tumors, research involving the use of this new generation of targeted immunoliposomes (Figure 2) to treat breast cancer has grown significantly in recent years [34]. In this review, we discuss recent advancements reported in the literature using immunoliposomes to target metastatic breast cancer based on known overexpressed cell surface receptors commonly targeted using this type of strategy to include HER2, EGFR, as well as HB-EGF.

HER2 Targeted Immunoliposomes

Human epidermal growth factor receptor 2 (HER2) is a member of the HER family along with HER1, HER3, and HER4, and is an important biomarker overexpressed in approximately 25-30% of breast cancers, which increases the aggressiveness of the tumor resulting in a relatively poor prognosis [35,36]. HER2 activation causes alterations in gene expression which can influence a variety of cell functions to include cell proliferation, migration, as well as cell survival [37]. The monoclonal antibody trastuzumab is known to bind HER2, which has the downstream effect of increased p27 production, a protein known to stop cell proliferation [38]. However, due to its negative side-effects which include congestive heart failure, several groups have used this particular antibody to generate HER2 targeted immunoliposomes against breast cancer cells with the hopes of potentially reducing such unwanted side-effects (Table 2) [39]. For example, using a panel of human breast cancer cells varying in HER2 expression levels, Barrajon-Catalan et al. demonstrated that liposomes containing a cytotoxic agent and surface-modified to contain the anti-HER2 antibody trastuzumab decreased cancer cell viability in a manner that correlated with their HER2 expression levels [40]. Kullberg et al. have reported similar in vitro results using targeted liposomes conjugated to the antibody trastuzumab [41]. In this study, the targeted liposomes containing the encapsulated fluorophore calcein demonstrated specificity toward HER2 positive cells relative to HER2 negative cells using fluorescence microscopy. Furthermore, when the fluorophore was replaced with the cytotoxic agent bleomycin, the targeted liposomes significantly reduced cell viability of several HER2 positive cell lines when compared to the HER2 negative cell lines. Gao *et al.* obtained similar results with to respect to HER2 specificity using targeted immunoliposomes containing encapsulated siRNA and coated with the anti-HER2 antibody trastuzumab [42]. In another very interesting study, dual-targeted immunoliposomes have been generated to target both HER2 receptors on breast cancer cells using the antibody trastuzumab as a targeting ligand, as well as CD3 receptors on T-lymphocytes using the anti-CD3 antibody OKT-3 [43]. The *in vitro* results of this study demonstrated that the dual-targeted immunoliposomes containing doxorubicin exhibited a cytotoxic effect on HER2 overexpressing cells, and were superior to both the mono-targeted trastuzumab-bearing liposomes as well as non-targeted liposomes.

While very promising *in vitro* results have recently been reported in the literature, encouraging *in vivo* studies have also been described. For example, trastuzumab-bearing immunoliposomes co-loaded with both paclitaxel and rapamycin have not only been shown to exhibit selectivity in cytotoxicity experiments, but have also demonstrated the ability to better control tumor growth *in vivo* using human xenograft HER2 overexpressing tumors in mouse models [44]. Both scientific research groups, Kikumori *et al.* as well as Park *et al.*, have also reported similar results with respect to tumor growth suppression in either mouse

or rat models respectively using liposomes surface-modified to contain the anti-HER2 antibody trastuzumab [45,46]. Hare *et al.* reports liposomal formulations involving both trastuzumab-bearing liposomes containing encapsulated doxorubicin to target the breast cancer tumor cells, as well as NGR peptide-bearing liposomes containing encapsulated vincristine to target tumor vascular endothelial cells [47]. In this study, the combination of both drugs (order of administration did not matter) was therapeutically superior to either single agent when tested in mouse models. Immunoliposomes surface coated with anti-HER2 antibodies (scFv) loaded with either vincristine or doxorubicin have also proven to be quite successful when tested *in vivo*, with the later currently in phase II clinical trials (Table 2) [48,49].

Receptor(s)	Targeting Ligand(s)	Encapsulated Agent(s)	Status	Reference
HER2	Trastuzumab	Melittin	*In vitro*	Barrajon-Catalan *et al.*
HER2	Trastuzumab	Bleomycin	*In vitro*	Kullberg *et al.*
HER2	Trastuzumab	siRNA	*In vitro*	Gao *et al.*
HER2+CD3	Trastuzumab/OKT3	Doxorubicin	*In vitro*	Vaidya *et al.*
HER2	Trastuzumab	Paclitaxel/Rapamycin	*In vivo*	Eloy *et al.*
HER2	Trastuzumab	Magnetite Nanoparticle(HML)	*In vivo*	Kikumori *et al.*
HER2	Trastuzumab	Doxorubicin	Preclinical	Park et al.
HER2+CD13	Trastuzumab/NGR peptide	Doxorubicin/Vincristine	Preclinical	Hare *et al.*
HER2	Anti-HER2 Antibodies (scFV)F5	Vincristine	*In vivo*	Noble et al.
HER2	Anti-HER2 Antibodies (scFV)F5	Doxorubicin	PhaseII	Espelin *et al.*
EGFR	Cetuximab	Celecoxib	*In vitro*	Limasale *et al.*
EGFR	Cetuximab	Topotecan	*In vitro*	Drummond *et al.*
EGFR	Cetuximab	Doxorubicin/Epirubicin/Vinorelbine	*In vivo*	Mamot *et al.*
EGFR	Cetuximab/Mab EMD72000	Doxorubicin	*In vivo*	Mamot, Ritschard *et al.*
EGFR	Recombinant Murine EGF	Gemcitabine	*In vivo*	Sandoval *et al.*
HB-EGF	Anti-HB-EGF IgG3E9	siRNA	*In vitro*	Okamoto *et al.*
HB-EGF	Anti-HB-EGF IgG3E9	Doxorubicin	*In vivo*	Nishikawa *et al.*

Table 2: Recently developed immunoliposomal-based chemotherapeutics used to treat breast cancer

EGFR Targeted Immunoliposomes

Epidermal growth factor receptor (EGFR) is another overexpressed protein reportedly found in 15-40% of breast cancers, and its overexpression is therefore a predictor of poor prognosis [50-52]. Thus, several research groups have also recently reported promising results targeting this particular receptor using various antibodies to generate immunoliposomal-based chemotherapeutics in order to treat breast cancer (Table 2). For example, the monolclonal antibody cetuximab is known to bind the extracellular domain of EGFR, which prevents normal downstream effects associated with the activation of this receptor, resulting in many antitumor effects which include cell-cycle arrest and induction of apoptosis [53,54]. Thus, cetuximab is in fact clinically approved to treat various types of cancers [55]. However, the clinical use of this antibody is similar to that of the already mentioned antibody trastuzumab in that undesired negative side-effects can occur. For example, cardiopulmanry arrest, interstitial lung disease, as well as pulmonary, embolus have all been associated with the use of cetuximab [56,57]. Therefore, in a similar fashion to trastuzumab-bearing immunoliposomes, several groups have successfully developed liposomal-based drugs surface modified to include the cetuximab antibody as a targeting ligand. For example, Limasale et al. reports a system involving cetuximab-bearing immunoliposomes, which are significantly more toxic to cancer cells with high EGFR expression than those with lower EGFR expression [58]. Interestingly, the inhibitor of the COX-2 pathway celecoxib was the encapsulated cargo within these liposomes, which is noteworthy as the COX-2 pathway has been shown to play a significant role in various biological processes throughout tumorigenesis [59]. Drummond et al. had similar results using anti-EGFR immunoliposomal formulations containing the highly active anticancer drug topotecan, which were much more toxic when tested with multiple breast cancer cell lines compared to the non-targeted liposomes [60]. Besides promising *in vitro* results, Mamot *et al.* reports encouraging in vivo data using cetuximab-bearing immunoliposomes containing either encapsulated doxorubicin, epirubicin, or vinorelbine tested against tumor xenograft models in mice [61,62]. Regardless of the encapsulated cytotoxic agent, all targeted liposomal formulations in this study demonstrated superior tumor accumulation and anti-tumor effects when compared to non-targeted liposomes. Interestingly, in the latter study Mamot *et al.* also reports promising data with respect to multidrug resistant cells. Besides cetuximab, recombinant murine EGF has also been used as a targeting ligand to successfully guide nanoparticles to breast cancer cells in both *in vitro* and *in vivo* trials in manner that correlated to the EGFR density of the cells [63].

HB-EGF Targeted Immunoliposomes

Heparin-binding epidermal growth factor-like growth factor (HB-EGF) is also overexpressed in many breast cancers, and has been shown to play an important role in mammary carcinoma progression, to include metastasis and invasion [64-66]. Thus, while not as prevalent in the literature as the two previously mentioned receptors, this overexpressed cell surface receptor on breast cancer cells has also been somewhat successfully targeted using HB-EGF-bearing liposomal-based chemotherapeutics (Table 2). For example, liposomes surface modified to include HB-EGF Fab' antibodies have been shown to selectively associate with cells expressing HB-EGF with high affinity *in vitro* [67]. In this study, effective gene silencing within breast cancer cells was reported using siRNA encapsulated within the HB-EGF-bearing liposomes. As the authors point out in their paper, this particular cell surface receptor was selected as the target for their targeted drug delivery formulation because the precursor of HB-EGF (proHB-EGF) is expressed at the cell surface and anchored to the cell membrane prior to being processed to the soluble form while mediating intracellular signaling. Thus, they concluded that HB-EGF is ideal for the delivery of siRNA to tumors. Also, promising *in vivo* results have been reported by Nishikawa et al. using mice bearing breast cancer cells known to overexpress HB-EGF [65]. In this study, HB-EGF immunoliposomes containing encapsulated doxorubicin demonstrated not only selectivity toward cells with high HB-EGF expression, but were also shown to suppress both tumor progression and tumor regression. The authors conclude by stating that this particular liposomal-based formulation could in fact be used to potentially treat various HB-EGF-expressing cancers.

Discussion

The use of nanocarriers such as liposomes as drug delivery vehicles for the delivery of cytotoxic agents to solid tumors to include breast cancer has proven to be quite promising. Furthermore, these nanocarriers can easily be surface-modified to contain targeting ligands such as antibodies to generate immunoliposomes intended for active delivery of chemotherapeutics, and many recently reported formulations have been described here. However, with such a popular and rapidly growing field, it is not feasible to describe every recently reported immunoliposomal-based formulation intended to treat breast cancer. Rather, we have provided a general overview of some of the more commonly targeted overexpressed cell surface receptors on breast cancer cells using this type of strategy, as well as successful liposomal-based constructs currently being reported in the literature to target those receptors. While surface-modified liposomes to include PEG incorporation can serve to improve tumor-site accumulation of the drug, and antibody addition can facilitate more efficient drug transfer via improved colocalization between the drug and tumor cells, deep penetration within tumor tissue can still be somewhat challenging. This in part is attributed to the high interstitial pressures and the highly heterogeneous vascular supply present within human tumors, which can limit the benefits realized by the EPR effect [68-71]. Furthermore, stromal fibroblasts are known to undergo myofibroblastic differentiation in the tumor microenvironment in response to tumor growth in a process commonly referred to as tumor-induced mesenchymal stroma progression, resulting in a somewhat dense tumor microenvironment with increased deposition of various extracellular proteins [71-74]. This creates a difficult environment for which relatively large nanocarriers must not only accumulate, but also penetrate deep within. Thus, future strategies involving the use of immunoliposomal-based drugs to treat solid tumors such as breast cancer may in fact utilize a combinatorial approach in order to further maximize the benefits associated with the use of these types of drugs. For instance, it has been suggested that magnetized particles could be incorporated within the drug formulation, and with the aid of an external magnet placed near the tumor, one could potentially overcome the reduced EPR effect [70,75]. Alternatively, the coadministration of pegylated immunoliposomal-based drugs such as those described here along with stromal depleting drugs could also prove to be quite effective. For example, the antistromal effects associated with the use of the drug Cellax has been shown to effectively suppress breast cancer metastasis based on its significant stromal depletion abilities [76]. Yet another possibility may involve the use of pegylated immunoliposomal-based drugs intended to target the mitochondrion. For example, the outer mitochondrial membrane contains voltage-dependent anion channels known to play a key role in the activity of various proteins that participate in the rapid cell growth typically observed in cancer cells, as well as various apoptosis suppressive properties [7,77,78]. Thus, it has been suggested by some that targeting these voltage-dependent anion channels may in fact prove to be an effective strategy in the treatment of cancer. It should also be noted that approximately 15-20 % of all newly diagnosed cases of breast cancer are in fact triple negative, meaning that they lack estrogen and progesterone receptors, as well HER2 [79,80]. This lack of well-defined molecular targets, coupled with the fact that triple negative cancer is a particularly heterogeneous disease, makes this type of breast cancer rather difficult to treat. However, future combinatorial strategies involving immunoliposomes may also involve the targeting of DNA repair agents and/or poly-ADP-ribose-polymerase (PARP) inhibitors as current ongoing research using DNA-damaging agents such as these seems to be somewhat promising [79,81]. Regardless of the strategy selected, a combinatorial approach involving pegylated immunoliposomes would not only be a targeted approach, but could also have the effect of improved tumor-site accumulation and deep penetration due to longer circulation times associated with the use of PEG and also other methods that serve to either compensate for the poor EPR effect and/or facilitate stromal depletion. Furthermore, other targets can be considered when there is a lack of viable molecular targets. In any event, the use of immunoliposomes to treat breast cancer continues to be an ongoing, exciting, and promising strategy with, many possible constructs recently being reported in the literature with encouraging results, some of which have been described here.

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